Synthesis of (22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids and structure of haemulcholic acid, a unique bile acid isolated from fish bile[1]

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Abstract (22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids were synthesized, starting from chenodeoxycholic acid, in order to establish the chemical structure of haemulcholic acid, which has been found in certain fish as the major bile component. Oxidative decarboxylation of diformoxylated chenodeoxycholic acid with lead tetraacetate yielded 24-nor-5β-chol-22-ene-3α,7α-diol, which was hydroxylated to form a mixture of (22*R* and 22*S*)-24-nor-5β-cholane-3α,7α,22,23-tetrols. Lead tetraacetate oxidation of the mixture yielded 3α,7α-dihydroxy-23,24-dinor-5β-cholan-22-al. A Reformatsky reaction of the dihydroxydinorcholanal with bromoacetate resulted in the formation of a mixture of (22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids. The bile acids epimeric at C-22 were resolved by silica gel column chromatography, and their configurations at C-22 were assigned by a modification of Horeau's method and ^{13}C-nuclear magnetic resonance spectroscopy. By direct comparison with synthetic bile acids, the naturally occurring haemulcholic acid was shown to be (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid.—**Kihira, K., Y. Morioka, and T. Hoshita.** Synthesis of (22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids and structure of haemulcholic acid, a unique bile acid isolated from fish bile. *J. Lipid Res.* 1981. **22:** 1181–1187.

Supplementary key words bile acids · absolute configuration

A unique bile acid, haemulcholic acid, has been isolated from the bile of a marine teleost, *Parapristipoma trilineatum*, by Hoshita, Hirofuji, and Kazuno (1). Recently, Anderson et al. (2) noticed the presence of haemulcholic acid in the bile of two species of freshwater fishes, *Polypterus senegalus* and *Mormyrus caballus*. The structure of this unique bile acid was determined as a 3α,7α,22-trihydroxy-5β-cholan-24-oic acid by chemical procedures (1). Tentative assignment of the 22α_F-configuration was made on the basis of optical rotation differences.[2] However, definite assignment to either the *R* or the *S* configuration remained to be established.

In order to confirm the structure of haemulcholic acid and to establish the absolute configuration at C-22, the chemical synthesis of (22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids was undertaken by a route outlined in **Fig. 1.** The configurations at C-22 of the synthetic bile acids were assigned by a modification of Horeau's method (5) and ^{13}C-nuclear magnetic resonance spectroscopy (6). By comparison with these epimeric bile acids, the naturally occurring haemulcholic acid was identified as (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid.

MATERIALS AND METHODS

General

Melting points were determined on a Kofler-hot stage apparatus, and are uncorrected.

Infrared spectra were obtained on a Shimadzu model

Abbreviations: IR, infrared; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; Hi–MS, high resolution mass spectrometry; ^{1}H nmr, proton nuclear magnetic resonance; ^{13}C nmr, carbon-13 nuclear magnetic resonance; GLC–MS, gas–liquid chromatography–mass spectrometry; RRT, relative retention time; TMS, trimethylsilyl.

[1] This study is part XIX of a series entitled "Comparative biochemical studies of bile acids and bile alcohols". (Part XVIII. T. Kuramoto, K. Kihira, N. Matsumoto, and T. Hoshita. 1981. *Chem. Pharm. Bull. (Tokyo).* **29:** 1136–1139.)

[2] Haemulcholic acid, the 22-hydroxylated derivative of chenodeoxycholic acid, was more levorotatory than chenodeoxycholic acid (1). It had been demonstrated from data on cholesterol and the 22-hydroxycholesterols (3) that the effect on M_D of 22α_F-hydroxylation is small, while 22β_F- hydroxycholesterol is much more levorotatory than the 22α_F-epimer and cholesterol. Thus haemulcholic acid was assigned the 22β_F-configuration in 1967 (1). However, previously assigned configurations of the epimeric 22-hydroxycholesterols have been reversed (4), the more levorotatory epimer now being termed cholest-5-ene-3β,22α_F(22*S*)-diol. In view of the revised configurational assignments of the model compounds, 22-hydroxycholesterols, the naturally occurring haemulcholic acid becomes 3α,7α,22α_F-trihydroxy-5β-cholan-24-oic acid.

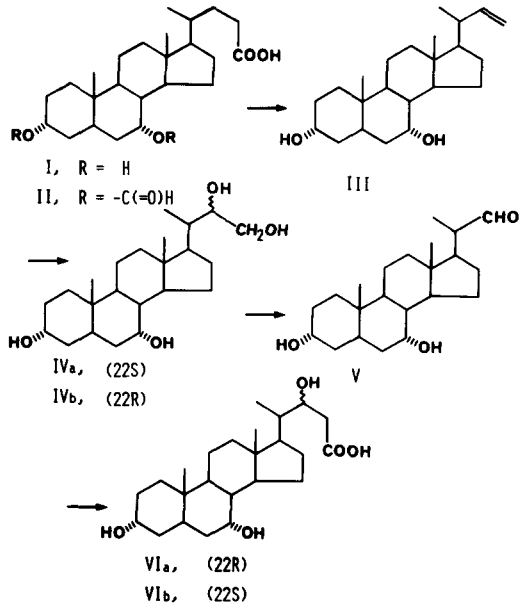

Fig. 1. I, Chenodeoxycholic acid; II, 3α,7α-diformoxy-5β-cholan-24-oic acid; III, 24-nor-5β-chol-22-ene-3α,7α-diol; IVa, (22*S*)-24-nor-5β-cholane-3α,7α,22,23-tetrol; IVb, (22*R*)-24-nor-5β-cholane-3α,7α,22,23-tetrol; V, 3α,7α-dihydroxy-23,24-dinor-5β-cholan-22-al; VIa, (22*R*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid; VIb, (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid.

IR-408 spectrophotometer as KBr discs. Absorption frequencies are quoted in reciprocal centimeters.

Optical rotations were measured in methanol on a Union Giken model PM-101 automatic polarimeter at 25°C.

Proton nuclear magnetic resonance spectra (^1H nmr), in δ ppm, were obtained in deuterated pyridine solution on a Hitachi model R-40 spectrometer using tetramethylsilane as internal standard.

Carbon-13 nuclear magnetic resonance spectra (^{13}C nmr), in δ ppm, were obtained in methanol solution or deuterated methanol solution on a JEOL JMN-PS-100 spectrometer equipped with Fourier transform capability using tetramethylsilane as internal standard.

Gas–liquid chromatography. The bile acid methyl esters and bile alcohols, as their trimethylsilyl derivatives, were run on a 2 m × 3 mm column packed with 3% OV-17, 2% OV-1, or 3% QF-1 on 80/100 mesh Gas Chrom Q. All retention times of the steroid samples are described relative to that of the TMS ether of methyl cholate (1.00).

Thin-layer chromatography. The samples were separated on silica gel G plates (Merck, 0.25 mm thickness). The spots were detected by spraying with phosphomolybdic acid (10% in ethanol) and heating at 110°C for 3 min.

High resolution mass spectra (Hi–MS) were measured on a JEOL D-300 mass spectrometer using the following operating conditions: ion source temperature, 250°C; ionizing voltage, 70 eV; and ionizing current 300 μA.

Gas–liquid chromatography–mass spectrometry was performed on a Shimadzu model GCMS-6020 gas chromatography–mass spectrometer using the following operating conditions: column, (2 m × 3 mm) 3% OV-17; column temperature, 270°C; ion source temperature, 300°C; ionizing voltage, 70 eV; ionizing current, 300 μA. The samples were analyzed as their TMS derivatives.

24-Nor-5β-chol-22-ene-3α,7α-diol (III)

3α,7α-Diformoxy-5β-cholan-24-oic acid (II, 14 g) prepared from chenodeoxycholic acid (I) according to the method previously reported (7) was dissolved in 100 ml of dry benzene containing 0.6 ml of pyridine, 20 g of $Pb(OAc)_4$, and 0.8 g of $CuSO_4$. The reaction mixture was refluxed for 12 hr under a nitrogen stream. The reaction mixture was extracted with two 500-ml portions of ether. The combined extracts were washed with 5% $NaHCO_3$ (300 ml × 2) to remove unreacted chenodeoxycholic acid and then with water to neutrality. The washed extract was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The resulting residue was refluxed with 5% methanolic KOH. After 2 hr the reaction mixture was diluted with five volumes of water. The resulting precipitate was collected by filtration and crystallized from methanol–water to give crystals (3.8 g). Recrystallization from methanol–water yielded colorless needles of 24-nor-5β-chol-22-ene-3α,7α-diol (III) with mp: 129.5–130°C; TLC (R_f): 0.84 (solvent system, ethyl acetate–acetone 7:3); GLC (RRT): 0.24 (OV-17); Hi–MS: M^+ = 346.2883 (Calcd. for $C_{23}H_{38}O_2$ = 346.2872); IR: 3400 (hydroxyl), 991 and 908 (end methylene); ^1H nmr (δ, ppm): 0.72 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.06 (3H, d, J = 6 Hz, 21-CH_3), 3.4–4.4 (2H, m, 3β-H and 7β-H), 4.90 (2H, m, 23-H_2), and 5.73 (1H, m, 22-H); GLC–MS of the TMS derivative (m/e, relative intensity): 400 (2, [M − 90]), 385 (3, [M − 15 − 90]), 345 (1, [M − side chain − 90]), 310 (100, [M − 2 × 90]), 295 (32, [M − 15 − 2 × 90]), and 255 (60, [M − side chain − 2 × 90]).

(22*R* and 22*S*)-24-nor-5β-cholane-3α,7α,22,23-tetrols (IVa and IVb)

The norcholenediol (III, 3.8 g) obtained above was dissolved in 40 ml of formic acid (Merck, 98%). After stirring for 2 hr at room temperature, 4 ml of 30% hydrogen peroxide was added and the reaction mixture was stirred an additional 12 hr at room temperature. After dilution of the reaction mixture with 250 ml of water, the resulting precipitate was collected by

filtration and refluxed with 5% methanolic KOH for 2 hr. The hydrolysate was diluted with five volumes of water and extracted with two 100-ml portions of ethyl acetate and then two 100-ml portions of n-butanol. The combined extracts were washed with saturated NaCl solution to neutrality. After drying over anhydrous Na_2SO_4, the solvents were evaporated to dryness. The residue was subjected to reversed phase column partition chromatography using isooctanol–chloroform 1:1 (v/v) as the stationary phase and 50% aqueous methanol as the moving phase. A glass column was packed with 360 g of Hostalene (polyethylene powder, Farbwerke Hoechst, Germany) supporting 240 ml of the stationary phase. The column effluents were monitored by TLC. The effluents from 1600 ml to 3400 ml were combined. Evaporation of the solvents gave 2.8 g of colorless solid consisting of a mixture of (22*R* and 22*S*)-24-nor-5β-cholane-3α,7α,22,23-tetrols (IVa and IVb). A portion (2.1 g) of the mixture was used for the next step without resolution. A 300-mg portion of the mixture (IVa and IVb) was then applied to a silica gel column (15 g) using a mixture of chloroform–acetone–methanol 25:25:1 (by volume) as eluting solvent. The isomer eluted first (92.6 mg) was collected and recrystallized from ethyl acetate to give colorless needles of (22*S*)-24-nor-5β-cholane-3α,-7α,22,23-tetrol (IVa) with mp: 138.5–140°C; $[\alpha]_D$: +8.2° (c = 1.6, methanol); TLC (R_f): 0.17 (solvent system, chloroform–acetone–methanol 70:50:5); GLC (RRT): 0.68, 1.00, and 0.53 (OV-17, OV-1, and QF-1, respectively); Hi–MS: M^+ = 380.2946 (Calcd. for $C_{23}H_{40}O_4$ = 380.2924); IR: 3400 (hydroxyl); ^{1}H nmr (δ, ppm): 0.71 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.24 (3H, d, J = 6 Hz, 21-CH_3), and 3.4–4.4 (5H, m, CH–OH's); GLC–MS (m/e, relative intensity): 565 (7, [M − 103]), 475 (36, [M − 103 − 90]), 385 (59, [M − 103 − 2 × 90]), 345 (5, [M − side chain − 90]), 295 (100, [M − 103 − 3 × 90]), and 255 (60, [M − side chain − 2 × 90]). The second isomer eluted (60 mg) was collected and recrystallized from ethyl acetate to give needles of (22*R*)-24-nor-5β-cholane-3α,7α,22,23-tetrol (IVb) with mp: 229–230°C; $[\alpha]_D$: +0.7° (c = 1.5, methanol); TLC (R_f): 0.11 (same solvent system as for IVa); GLC (RRT): 0.68, 1.00, and 0.54 (OV-17, OV-1, and QF-1, respectively); Hi–MS: M^+ = 380.2912 (Calcd. for $C_{23}H_{40}O_4$ = 380.2924); IR: 3400 (hydroxyl); ^{1}H nmr (δ, ppm): 0.77 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.21 (3H, d, J = 6 Hz, 21-CH_3), 3.4–4.4 (5H, m, CH–OH's); GLC–MS (m/e, relative intensity): 565 (7, [M − 103]), 475 (33, [M − 103 − 90]), 385 (67, [M − 103 − 2 × 90]), 345 (5, [M − side chain − 90]), 295 (100, [M − 103 − 3 × 90]), and 255 (59, [M − side chain − 2 × 90]).

3α,7α-Dihydroxy-23,24-dinor-5β-cholan-22-al (V)

The mixture of the norcholanetetrols (IVa and IVb, 2.1 g) was dissolved in a mixture of 5 ml of ethanol and 30 ml of benzene. To this solution, 2.4 g of $Pb(OAc)_4$ was added. The reaction mixture was kept at 60°C for 2 hr and then poured into 150 ml of water and extracted with two 200-ml portions of ethyl acetate. The combined extracts were washed with 3% $NaHCO_3$ solution and then with water to neutrality. After drying over anhydrous Na_2SO_4, the solvent was removed under reduced pressure. The residue (900 mg) was purified by silica gel (50 g) column chromatography. The fractions eluted with benzene–ethyl acetate 1:1 (v/v) were collected and the solvents were evaporated to give a colorless powder. Crystallization from methanol–water gave needles of 3α,7α-dihydroxy-23,24-dinor-5β-cholan-22-al (V) with mp: 82.5 – 83.5°C; TLC (R_f): 0.63 (solvent system, ethyl acetate–acetone 7:3 (v/v); GLC (RRT): 0.55, 0.50, and 0.68 (OV-17, OV-1, and QF-1, respectively); Hi–MS: M^+ = 348.2640 (Calcd. for $C_{22}H_{36}O_3$ = 348.2662); IR: 3400 (hydroxyl) and 1720 (carbonyl); ^{1}H nmr (δ, ppm): 0.68 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.08 (3H, d, J = 6 Hz, 21-CH_3), 3.4–4.4 (2H, m, 3β-H and 7β-H), and 9.73 (1H, d, J = 3 Hz, -CHO); GLC–MS (m/e, relative intensity): 492 (1, [M]), 402 (1, [M − 90]), 312 (100, [M − 2 × 90]), 292 (29, [M − ring A − side chain]), and 255 (22, [M − side chain − 2 × 90]).

(22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids (VIa and VIb)

A solution of 3α,7α-dihydroxy-23,24-dinor-5β-cholan-22-al (V, 500 mg) dissolved in 50 ml of dry benzene was added to a mixture of dry benzene (50 ml), ethyl bromoacetate (9 ml), granulated Zn (14.8 g), a few crystals of iodine, and a small amount of powdered Cu. The reaction mixture was refluxed for 3 hr. After cooling, 30 ml of 1 N HCl solution and 100 g of crushed ice were added to decompose Reformatsky products. The solution was extracted with three 150-ml portions of ethyl acetate. The combined extracts were washed with 3% $NaHCO_3$ solution and then with water to neutrality. After drying over anhydrous Na_2SO_4, the solvent was evaporated to dryness. The resulting residue was hydrolyzed with 5% methanolic KOH (20 ml). After refluxing for 2 hr, the reaction mixture was diluted with water (100 ml) and filtered to remove water-insoluble materials. The filtrate was acidified with 1 N HCl to precipitate the acidic products. The precipitate was extracted with ethyl acetate (100 ml × 3). The combined extracts were washed with saturated NaCl solution to neutrality, dried over

anhydrous Na_2SO_4, and evaporated to dryness. The residue (VIa and VIb) was dissolved in 20 ml of methanol containing three drops of conc. HCl. The reaction mixture was allowed to stand for 12 hr at room temperature, and diluted with water, and extracted with three 100-ml portions of ether. After washing with 3% $NaHCO_3$ solution and water, the extract was dried over anhydrous Na_2SO_4. The solvent was removed and the resulting residue (400 mg) consisting of methyl esters was chromatographed on a column of silica gel (20 g) made up in benzene. Elution with benzene–ethyl acetate 1:3 gave the methyl ester of VIa. Hydrolysis with 5% methanolic KOH (10 ml) and usual work-up afforded 51 mg of colorless powder. Recrystallization from methanol–water gave crystals of (22*R*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid (VIa) with mp: 141–142°C; $[\alpha]_D$: +1.0°C (c = 1.3, methanol); TLC (R_f): 0.21 and 0.69 (solvent system, isooctane–ethyl acetate–acetic acid 5:5:1 and chloroform–acetone–methanol–acetic acid 25:25:5:1); GLC (RRT): 1.19, 1.21, and 1.11 (OV-17, OV-1, and QF-1, respectively); Hi–MS (as methyl ester): M^+ = 422.3002 (Calcd. for $C_{25}H_{42}O_5$ = 422.3030), M − H_2O = 404.2945 (Calcd. for $C_{25}H_{40}O_4$ = 404.2925), and M − 2 × H_2O = 386.2729 (Calcd. for $C_{25}H_{38}O_3$ = 386.2818); IR: 3400 (hydroxyl) and 1720 (carboxyl); ^{1}H nmr (δ, ppm): 0.69 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.17 (3H, d, J = 6 Hz, 21-CH_3), 3.4–4.4 (3H, m, 3β-, 7β-, and 22-CH-OH's). Elution with ethyl acetate gave the methyl ester of VIb which was hydrolyzed as described above to yield semipurified VIb as a colorless solid. Recrystallization from methanol–water gave needles of (22*S*) − 3α,7α,22-trihydroxy-5β-cholan-24-oic acid (VIb) with mp: 247–250°C; $[\alpha]_D$: +2.4° (c = 1.3, methanol); TLC (R_f): 0.11 and 0.58 (same solvent systems for VIa); GLC (RRT): 1.19, 1.21, and 1.05 (OV-17, OV-1, and QF-1, respectively); Hi–MS (as methyl ester): M^+ = 422.3070 (Calcd. for $C_{25}H_{42}O_5$ = 422.3030), M − H_2O = 404.2944 (Calcd. for $C_{25}H_{40}O_4$ = 404.2925), M − 2 × H_2O = 386.2858 (Calcd. for $C_{25}H_{38}O_3$ = 386.2818); IR: 3400 (hydroxyl) and 1720 (carboxyl); ^{1}H nmr (δ, ppm): 0.74 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.16 (3H, d, J = 6 Hz, 21-CH_3), 3.4–4.4 (3H, m, 3β-, 7β-, and 22-CH-OH's).

Modified Horeau's method

Four mg of sample was dissolved in 12 μl of dry pyridine and 12 μl of (±)-α-phenylbutyric anhydride. The reaction mixture was kept at 40°C. After 2 hr, 12 μl of (+)-(*R*)-α-phenylethylamine was added and the reaction mixture was stirred. After standing at room temperature for 15 min, 400 μl of ethyl acetate was added and a 1-μl aliquot of the ethyl acetate extract was subjected to GLC analysis (column, OV-17, 2 m × 3 mm, 215°C). Measurements of the peak areas of (+)-(*R*)-α-phenylethylamides of (−)-(*R*)- and (+)-(*S*)-α-phenylbutyric acid (retention times, 7.8 min and 8.8 min, respectively) were accomplished with a Shimadzu model E1-A automatic integrator. This method was applied for the configurational analysis at C-22 of IVa, IVb, VIa, and VIb, and the results are listed in **Table 1.**

RESULTS AND DISCUSSION

This study describes the synthesis of (22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids. Chenodeoxycholic acid (I) was converted into the diformate (II) according to the reported method (7). Oxidative decarboxylation of the diformate (II) with lead tetraacetate yielded 24-nor-5β-chol-22-ene-3α,7α-diol (III). The synthesis of norcholenes by the same procedure has been reported by Carlson, Belobaba, and Hofmann (8). The norcholene (III) was converted into (22*R* and 22*S*)-24-nor-5β-cholane-3α,7α,22,23-tetrols (IVa and IVb). The tetrols epimeric at C-22 were resolved by silica gel chromatography. The mass spectra of the TMS derivatives of IVa and IVb were essentially identical. Each spectrum displayed a series of peaks at m/e 565, 475, 385, and 295. The fragment at m/e 565 resulted from the scission of the bond between C-22 and C-23 losing 103 amu (9). The fragments observed at m/e 345 and 255 are characteristic for the dihydroxysteroid nucleus such as chenodeoxycholic acid (10).

For the elucidation of the absolute configurations at C-22, measurement of ^{13}C nmr spectra and application of the modified Horeau's method of IVa and IVb were undertaken. In ^{13}C nmr spectra of IVa and IVb, the chemical shifts of the carbons on ring A, B, and C are essentially identical with those of chenodeoxycholic acid reported by Leibfritz and Roberts (11) as shown

TABLE 1. Modified Horeau's method

Compounds[a]	Peak Area (%) (+)-(R)-α-phenylethylamide of (−)-(*R*)-α-phenylbutyric acid	Peak Area (%) (+)-(R)-α-phenylethylamide of (+)-(*S*)-α-phenylbutyric acid	Configuration[b]
IVa	48	52	S
IVb	52	48	R
VIa	53	47	R
VIb	56	44	S

[a] IVa, (22*S*)-24-nor-5β-cholane-3α,7α,22,23-tetrol; IVb, (22*R*)-24-nor-5β-cholane-3α,7α,22,23-tetrol; VIa, (22*R*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid; VIb, (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid.

[b] See text.

TABLE 2. ^{13}C Chemical shifts

Carbon No.	III[a]	IVa	IVb	VIa	VIb	A	I	B	C
1	36.5	36.5	36.5	36.5	36.6	36.6	36.6		
2	31.2	31.4	31.2	31.4	31.5	31.5	31.3		
3	72.7	72.9	72.8	72.9	73.0	73.0	72.8		
4	40.3	40.5	40.4	40.6	40.6	40.6	40.4		
5	43.0	43.1	43.1	43.1	43.2	43.2	43.6		
6	35.8	35.9	35.8	35.9	36.0	36.0	35.8		
7	68.9	69.1	69.0	69.1	69.2	69.2	69.0		
8	40.6	40.8	40.8	40.8	40.9	40.9	40.8		
9	33.9	34.0	34.0	34.0	34.1	34.1	33.5		
10	36.0	36.1	36.1	36.1	36.2	36.2	36.1		
11	21.6	21.7	21.7	21.7	21.8	21.8	21.6		
12	40.6	42.0	41.1	40.8	41.2	41.1	40.9		
13	43.4	43.9	43.4	43.9	43.5	43.5	43.6		
14	51.4	51.4	51.4	51.2	51.4	51.5	51.4		
15	24.5	24.7	24.6	24.7	24.7	24.7	24.5		
16	29.4	28.6	28.7	28.4	28.7	28.7	29.1	(22R)	(22S)
17	56.7	54.3	53.6	54.6	53.8	53.8	57.2	53.2	52.6
18	12.4	11.8	12.0	12.1	12.1	12.1	12.1	11.9	11.8
19	23.4	23.4	23.4	23.4	23.4	23.4	23.4	—	—
20	42.4	43.1	38.8	43.1	42.0	42.0	36.6	42.6	40.3
21	20.7	13.5	12.4	12.8	12.4	12.4	18.7	12.5	11.6
22	146.1	75.3	74.6	71.2	70.9	71.0	32.2	74.0	73.8
23	112.0	63.3	65.6	36.5	41.5	41.5	31.9	27.5	33.3
24								36.1	35.7
25								27.9	27.8
26								22.5	22.6
27								22.9	22.6

[a] III, 24-nor-5β-chol-22-ene-3α,7α-diol; IVa, (22*S*)-24-nor-5β-cholane-3α,7α,22,23-tetrol; IVb, (22*R*)24-nor-5β-cholane-3α,7α,22,23-tetrol; VIa, (22*R*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid; VIb, (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid; A, haemulcholic acid from natural source; I, chenodeoxycholic acid; B, (22*R*)-22-hydroxycholesterol (4); C, (22*S*)-22-hydroxycholesterol (4).

in **Table 2.** The significant differences due to the C-22 configuration were observed for the chemical shifts due to the β-carbons, C-20 and C-23. They are at 43.1 ppm (C-20) and 63.3 ppm (C-23) for IVa and at 38.8 ppm (C-20) and 65.5 ppm (C-23) for IVb. In the modified Horeau's method (5) as shown in Table 1, in the GLC analysis of amide derivatives of (+)-(*R*)-α-phenylethylamine, the peak area for (−)-(*R*)-α-phenylbutyric acid (retention time 7.8 min) with respect to that for (+)-(*S*)-α-phenylbutyric acid (retention time 8.8 min) is much larger with IVb than IVa. In the case of IVa and IVb, the assignments of the configuration at C-22 should be reversed compared with the case of 22-hydroxycholesterol (6) because of the precedence of the hydroxyl groups at C-23, according to the Sequence Rule. Consequently, less polar isomer, IVa, was assigned the 22S configuration and its counterpart, IVb, the 22R configuration from both ^{13}C nmr spectroscopy and the modified Horeau's method.

The mixture of norcholanetetrols (IVa and IVb) was treated with lead tetraacetate to afford 3α,7α-dihydroxy-23,24-dinor-5β-cholan-22-al (V). Reformatsky reaction of the aldehyde (V) with ethyl bromoacetate followed by alkaline hydrolysis provided a mixture of (22*R* and 22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acids (VIa and VIb). The mixture was methylated and separated on silica gel column. Alkaline hydrolysis of the more rapidly eluted methyl ester gave the less polar bile acid (VIa) and the same treatment of the more slowly eluted methyl ester gave the more polar bile acid (VIb).

The mass spectra of VIa and VIb as their methyl ester TMS derivatives exhibited almost identical fragmentation patterns. Also the molecular ion was not seen in either spectrum; there were two series of fragments, one at m/e 623, 533, 443, and 353, and a second at m/e 548, 458, and 368. The former series results from the loss of methyl group (15 amu) followed by successive loss of one, two, and three TMS groups as trimethylsilanol (90 amu). The latter series results from consecutive loss of one, two, and three trimethylsilanols from the molecular ion. The fragments at 374 and 284 are attributed to the rupture of the bond between C-20 and C-22 (loss of 175 amu) together with the transfer of a hydrogen atom to the steroid nucleus and the loss of one or two nuclear TMS groups. However, the fragment at m/e 255, which is charac-

teristic for the dihydroxysteroids (10), was seen in both spectra of VIa and VIb; the fragment at m/e 254 was rather intense. Another characteristic fragment at m/e 345 was very weak and the fragment at m/e 344 was observed instead of it. These fragments with loss of one hydrogen atom arose from the loss of the side chain along with the capture of one hydrogen atom from the steroid nucleus, followed by subsequent loss of one and two trimethylsilanols. For the elucidation of the absolute configurations at C-22 of synthetic bile acids (VIa and VIb), the modified Horeau's method (5) was carried out for the methyl esters. As shown in Table 1, in the GLC analysis of the amide derivatives of (+)-(*R*)-α-phenylethylamine, the peak area for (−)-(*R*)-α-phenylbutyric acid (retention time 7.8 min) compared to that for (+)-(*S*)-α-phenylbutyric acid (retention time 8.8 min) was much larger with VIb than VIa. This result indicated that VIb, the more polar epimer, can be assigned the 22*S* configuration and its counterpart VIa, the less polar epimer, the 22*R* configuration. This assignment is further supported by measurement of the ^{13}C nmr spectra of VIa and VIb. The chemical shifts of the carbons on ring A, B, and C were essentially identical with those of chenodeoxycholic acid (11) as shown in Table 2.

The chemical shift differences were seen in the carbons α, β, and γ to the 22-hydroxyl group. The signals due to C-20 and C-23 disclose the configuration at C-22 especially. Those signals were at 43.1 ppm (C-20) and 36.6 ppm (C-23) for VIa and at 42.0 ppm (C-20) and 41.5 ppm (C-23) for VIb. According to the report by Letourneux, et al. (6), the chemical shifts of C-20 and C-23 due to the 22*R* configuration in 22-hydroxycholesterol are at 42.6 ppm (C-20) and 27.5 ppm (C-23); on the other hand, for the 22*S* configuration they are at 40.3 ppm (C-20) and 33.3 ppm (C-23). Therefore less polar acid (VIa), in which the C-20 is more deshielded and C-23 is more shielded, can be assigned the 22*R* configuration and its counterpart VIb the 22*S*. ^{1}H nmr spectra also gave important information concerning the configuration at C-22. The chemical shifts of C-18 and C-21 methyl groups exhibited significant differences in two pairs of 22-epimeric steroids, norcholanetetrols (IVa and IVb), and tri-

TABLE 3. Chromatographic data for synthetic bile acids (VIa and VIb) and naturally occurring haemulcholic acid

Compounds[a]	TLC Solvent System[b]			GLC Column		
	A	B	C	OV-17	OV-1	QF-1
	R_f value			*RRT*		
VIa	0.21	0.69	0.27	1.19	1.21	1.11
VIb	0.11	0.58	0.14	1.19	1.21	1.05
Haemulcholic acid	0.11	0.58	0.14	1.19	1.21	1.05

[a] VIa, (22*R*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid; VIb, (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid; haemulcholic acid, naturally occurring.

[b] A, Isooctane–ethyl acetate–acetic acid 5:5:1; B, chloroform–acetone–methanol–acetic acid 25:25:5:1; C, ethyl acetate–acetone 7:3 (developed as methyl ester).

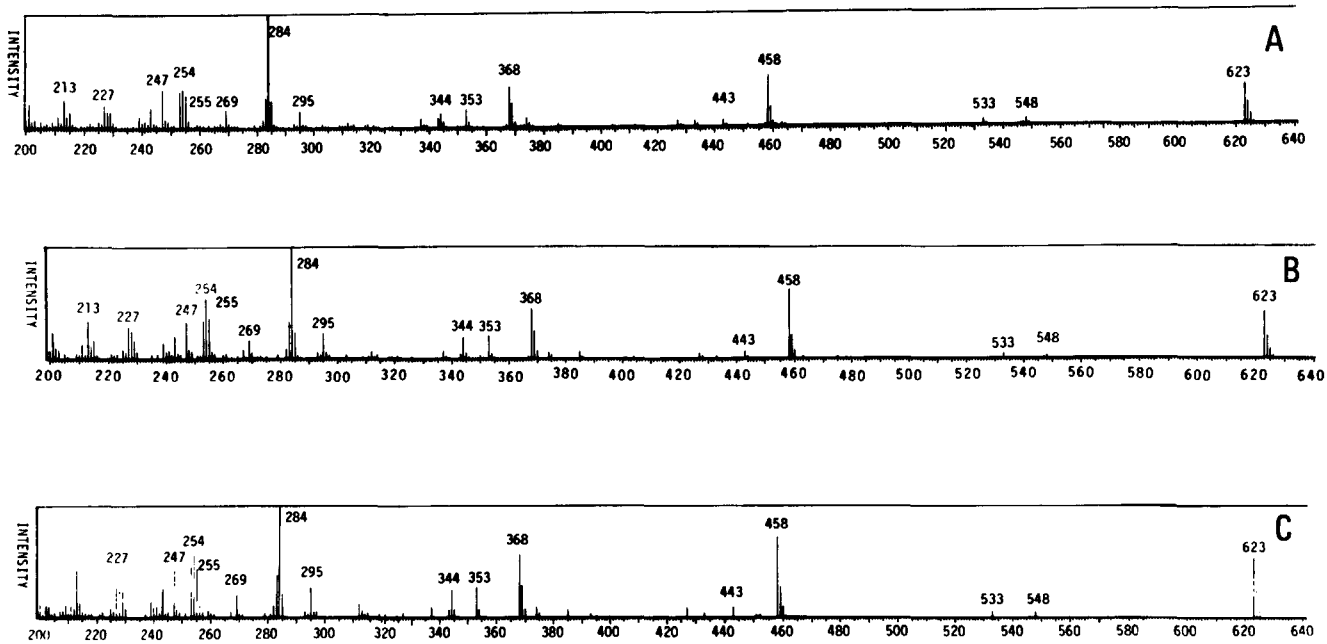

Fig. 2. Mass spectra of (22*R*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid (A), (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid (B), and naturally occurring haemulcholic acid (C), as their methyl ester-TMS derivatives.

hydroxycholanoic acids (VIa and VIb). The signals assignable to the C-18 methyl groups exhibited significant differences. In compound IVa (22*S*), the chemical shift of the C-18 methyl group was at 0.71 ppm and in IVb at 0.77 ppm. In compounds VIa and VIb, those were at 0.69 ppm and 0.74 ppm, respectively. The compounds having the same environments around C-22 showed the same effects on the chemical shift of the C-18 methyl group due to the C-22 asymmetric hydroxyls. These are shielded in IVa and VIa compared to those of IVb and VIb. Similar observations have been reported for (22*R* and 22*S*)-5β-cholestane-3α,7α,12α,22-tetrols (12), (22*R* and 22*S*)-5β-cholestane-3α,7α,12α,22,25-pentols (13), and other 22-hydroxylated steroids (14).

The naturally occurring haemulcholic acid had the same melting point, chromatographic properties (**Table 3**), infrared spectrum, ^{1}H nmr spectrum, ^{13}C nmr spectrum (Table 2), and mass spectrum (**Fig. 2**) as the (22*S*)-bile acid (VIb) by direct comparison with the specimen synthesized in this work. Consequently, the naturally occurring compound was shown to be (22*S*)-3α,7α,22-trihydroxy-5β-cholan-24-oic acid.

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