Bile salts of the West Indian manatee, *Trichechus manatus latirostris:* novel bile alcohol sulfates and absence of bile acids

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Abstract The bile salts present in gallbladder bile of the West Indian manatee, *Trichechus manatus latirostris,* an herbivorous marine mammal of the tropical and subtropical margins of the Atlantic Ocean, were found to consist of a mixture of bile alcohol sulfates. Bile acids, previously believed to be present in all mammals, were not detected. Using chromatography, mass spectrometry, and ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy, the major bile alcohol was identified as 5β-cholestane-3α,6β,7α-25,26-pentol; that is, it had the nuclear structure of α-muricholic acid and the side chain structure of bufol. This compound has not been described previously and the trivial name "α-trichechol" is proposed. The second most abundant compound was 5β-cholestane-3α,7α,25,26-tetrol. Other bile alcohols were tentatively identified as 5β-cholestane-3α,6β,7β,25,26-pentol (named β-trichechol), 3α,6α,7β,25,26-pentol (named ω-trichechol) and 5β-cholestane-3α,6β,7α,26-tetrol. The ^{1}H and ^{13}C NMR spectra of the four 6,7 epimers of 3,6,7 trihydroxy bile acids are described and discussed. All bile alcohols were present as ester sulfates, the sulfate group being tentatively assigned to the 26-hydroxy group. 12-Hydroxy compounds were not detected. The manatee is the first mammal found to lack bile acids, presumably because it lacks the enzymes required for oxidation of the 26-hydroxy group to a carboxylic acid. Trichechols, like other bile salts, are water-soluble end products of cholesterol metabolism; whether they also function as biological surfactants in promoting biliary cholesterol secretion or lipid digestion is unknown. —**Kuroki, S., C. D. Schteingart, L. R. Hagey, B. I. Cohen, E. H. Mosbach, S. S. Rossi, A. F. Hofmann, N. Matoba, M. Une, T. Hoshita, and D. K. Odell.** Bile salts of the West Indian manatee, *Trichechus manatus latirostris:* novel bile alcohol sulfates and absence of bile acids. *J. Lipid Res.* 1988. **29:** 509–522.

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Bile salts are the water-soluble end products of cholesterol metabolism and the major organic constituents of vertebrate bile. In higher vertebrates, bile salts have a number of functions such as stimulation of bile flow and solubilization of lipid digestion products (1). Whether bile salts have such functions in primitive vertebrates is not known.

The tradition of isolating bile salts from natural sources and determining their chemical structure began at least 150 years ago, long before elucidation of the steroid chemical structure by modern chemical methods (2, 3). Work on the comparative biochemistry of bile salts has continued largely in England (4), Japan (cf. 5), and the United States (6–8). Previous studies have shown that the structure of bile salts varies according to the evolutionary level of the animal (4). In the hagfish (an example of a primitive vertebrate) cholesterol, after loss of the 5–6 double bond (which occurs in the formation of all bile salts) is modified by only a single hydroxylation on the C_8 side chain to form the diol myxinol. In higher, albeit still primitive, vertebrates such as the shark, additional hydroxylations occur on both the nucleus and the side

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; RRT, relative retention time; GASPE, gated spin echo experiments; TMS, trimethylsilyl; MS, mass spectrometry; SIMS, secondary ion mass spectrometry.

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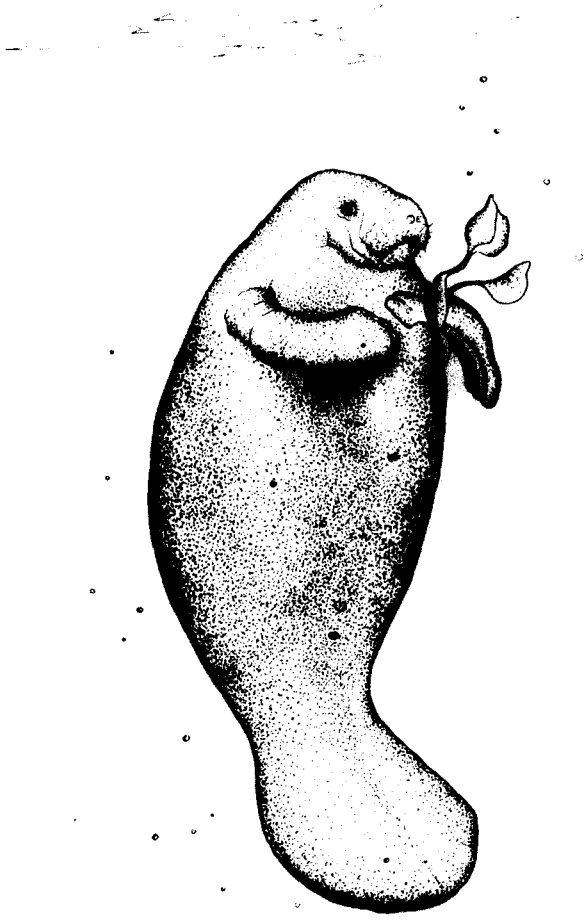
chain, and the A/B ring junction undergoes structural isomerization to form A/B *cis* compounds, as in modern bile acids. In reptiles and amphibians, the side chain terminal hydroxyl group is oxidized to form C_{27} or C_{26} carboxylic (bile) acids. In mammals, which are considered to be the "highest" vertebrates, the side chain undergoes further oxidation with loss of a 3-carbon fragment; the resultant C_{24} bile acids are considered "modern." However, small amounts of bile alcohols occur normally in human bile and urine (9, 10).

As part of a research program whose aim is to correlate chemical structure and physicochemical properties with the biological functions of bile salts (11), we have been examining the bile salts of a large number of vertebrates. In this study, we report the chemical structure of the bile salts of the West Indian manatee, *Trichechus manatus latirostris*. This animal, which is known to be of ancient evolutionary origin (12), was found to have bile that was devoid of bile acids and contained instead bile alcohol sulfates of a structure not previously described as its major bile salts. We propose the names α-, β-, and ω-trichechol for three of the newly discovered pentahydroxy bile alcohols.



The West Indian manatee, *Trichechus manatus latirostris*. Drawing by Lynn Rathbun.

MATERIALS AND METHODS

Reference compounds

5β-Cholestane-3α,7α,12α,26-tetrol (13) and 5β-cholestane-3α,7α,25,26-tetrol (14) were synthesized as reported previously. 5β-Bufol (5β-cholestane-3α,7α,12α,25,26-pentol) was isolated from the solvolyzed bile of the toad, *Bufo vulgaris formosus* (15). Lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) was purchased from Aldrich Chemical Corp., Milwaukee, WI. Chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid, Canada Packers Ltd., Toronto, Canada), cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid, Inolex, Park Forest South, IL), hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid, Canada Packers Ltd.), ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid, Diamalt AG, Raubling, West Germany), and hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-oic acid, Canada Packers Ltd.) were obtained from commercial sources as indicated. Nor-chenodeoxycholic acid (3α,7α-dihydroxy-24-nor-5β-cholan-23-oic acid) was prepared in the usual manner (16). "Murideoxycholic acid" (3α,6β-dihydroxy-5β-cholan-24-oic acid) was synthesized by borohydride reduction of the 6-oxo-3-cathylate, as described (17). α-Muricholic acid (3α,6β,7α-trihydroxy-5β-cholanoic acid) was synthesized as described previously (17). β-Muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid) and ω-muricholic acid (3α,6α,7β-trihydroxy-5β-cholanoic acid) were generous gifts from Dr. W. H. Elliott and Dr. K. Uchida, respectively.

Preparation of manatee bile

Bile samples were obtained at necropsy from manatees collected by the Florida Department of Natural Resources Manatee Carcass Salvage Program under the U.S. Fish and Wildlife Service Permit PRT-684532. Bile was aspirated from the intact gallbladder using a syringe and immediately injected into sealed vials containing 2-propanol. To make our data available for research on endangered species, individual identification of the donor animals is provided in **Table 1.** Bile samples were pooled and evaporated prior to analysis, which was performed on whole as well as solvolyzed sample material.

TABLE 1. Specification for *Trichechus manatus latirostris* samples

Specimen	Sex	Length	Field Number	Collection Date
		cm		
1	M	237	MSW-67	12-05-85
2	F	350	M-85-25	12-11-85
3	M	282	M-85-26	1-01-86
4	F	332	M-85-27	1-01-86
5	M	206	M-86-01	1-03-86
6	M	305	M-86-05	1-20-86
7	F	223	M-86-11	3-05-86

Thin-layer chromatography (TLC) was performed on silica gel F254 precoated TLC aluminum sheets (0.2 mm thickness, Merck, Darmstadt, West Germany). For two-dimensional TLC of non-solvolyzed bile, the alcohol extract was applied as a single spot and was developed in n-butanol–acetic acid–water 17:2:1 (v/v). After development (15 cm), the plate was air-dried and then developed in the other direction in chloroform–methanol–acetic acid–water 13:4:2:1 (v/v) (18). Solvolyzed bile alcohols were analyzed in solvent system A, chloroform–ethanol 4:1 (v/v), and solvent system B, ethyl acetate–acetone 3:2 (v/v). After development, bile alcohols were detected by spraying with phosphomolybdic acid (10 g/100 ml ethanol) followed by heating at 120°C for 2 min.

Preparative TLC was done on precoated silica gel G plates (20 × 20 cm, 0.5 mm thickness, Analtech, Inc., Newark, DE). A methanolic solution of the solvolyzed bile alcohols was applied as a line on the plates and developed in solvent system A. After development, the plates were air-dried, the bile alcohol bands were visualized by exposing the plates to iodine vapor, iodine was removed by sublimation, and the pertinent bands were scraped off. Purified bile alcohols were recovered by eluting with 95% ethanol.

High-performance liquid chromatography (HPLC) was performed on a C_{18} octadecylsilane column eluted with methanol–phosphate buffer, pH 5.35, as mobile phase as described (19). Bile acid amidates and bile alcohol sulfates were detected by ultraviolet spectrophotometry at multiple wavelengths between 200 and 220 nm, as well as by refractive index monitoring of column eluates.

Solvolysis (18) was performed as follows. Lyophilized bile solids were dissolved in water and the pH was adjusted to 1.0 by adding 1 N HCl solution (total volume 20 ml). NaCl (4 g) and 40 ml of ethyl acetate were added and the mixture was kept at 37°C for 3 days. After addition of 2.0 ml of 1 N NaOH solution, the solvolyzed bile alcohols were extracted three times with 40 ml of ethyl acetate. The combined extracts were washed with 5% $NaHCO_3$ solution (10 ml, three times until the washings became alkaline) and twice with water. Ethyl acetate was removed under a stream of N_2. To remove acetyl groups, the residue was hydrolyzed in 1 N NaOH in 90% ethanol (4 ml) at 75°C for 1 hr (20). Ethanol was removed under N_2 and replaced with water. The solution was cooled and the pH was adjusted to 8–9 with 2 N HCl (1.8 ml). The bile alcohols were extracted three times with n-butanol–ethyl acetate 1:1 (v/v) (4 ml each). The extract was washed with water (2 ml, four times) and the solvents were evaporated under N_2 at 40°C.

Gas–liquid chromatography (GLC) was carried out on a Hewlett-Packard 5830A gas chromatograph using a 3% SP-2250 (phenyl) column (6 ft × 2 mm i.d., Supelco, Inc., Bellefonte, PA) at 270°C and a 3% SE-30 (silicone) column (6 ft × 2 mm i.d., Alltech Associates, Deerfield, IL) at 250°C. The bile alcohols, as well as methyl esters of reference bile acids, were analyzed as their trimethylsilyl (TMS) ether derivatives and acetonide-TMS ether derivatives. For preparation of the acetonides, the samples were treated with dimethoxypropane (DMP, 1 ml) and 50 μl of 0.1 N HCl at 37°C for 30 min (21). Fifty μl of 0.1 N NH_4OH was added to neutralize the acid and the solvent was removed under a stream of N_2. Sil-Prep (Alltech) was used for silylation (100 μl, 37°C, 30 min). The acetonide-TMS derivatives of the bile alcohols decomposed on SP-2250 columns, necessitating the use of SE-30 columns for GLC and GLC–MS of acetonide derivatives.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was carried out on a Hewlett-Packard 5992B spectrometer under the following conditions: column, 1% SE-30 (2 ft × 2 mm i.d.); oven temperature, 260°C; ion source temperature, 140°C; source pressure, 2×10^{-6} torr; ionization energy, 70 eV.

HPLC–secondary ion mass spectrometry (HPLC–SIMS) was performed on lyophilized HPLC fractions with a Kratos MS-50 mass spectrometer equipped with a cesium ion source and operated at approximately 100 μamps/cm^2 beam flux in both positive and negative ion modes. Scan rate was 100 sec/decade and glycerol (1 μl) was used as liquid matrix on a copper probe tip. To determine the number of hydroxyl groups present in unknowns, samples were also analyzed in a matrix of glycerol–1 N HCl 1:0.5 (v/v). These analyses were performed at the UCSF Biomedical Mass Spectrometry Resource (Director, A. L. Burlingame).

Proton and ^{13}C nuclear magnetic resonance (^1H- and ^{13}C-NMR) spectra of 5β-bufol and α-trichechol were recorded on a JEOL GX-270 spectrometer at 270 MHz and 67.8 MHz, respectively. ^1H-NMR spectra of all other compounds were recorded on a 360 MHz instrument equipped with a modified Varian HR-220 console, an Oxford magnet, and a Nicolet 1180-E computer system; ^{13}C-NMR spectra were taken on a Nicolet NT-200 wide-bore spectrometer (with an Oxford magnet) operating at 50.31 MHz. ^1H-NMR assignments were confirmed by decoupling experiments whenever possible. To help in the assignment of ^{13}C-NMR spectra of all standard compounds, gated spin echo experiments (GASPE) (22) were performed with T = 7 msec, to distinguish CH_3 and CH resonances from CH_2 and quaternary carbons, and with T = 4 msec for selective detection of quaternary carbons. ^{13}C-NMR (proton broad band decoupled) of α-trichechol was obtained from a 5-mg sample (36,000 scans, ^{13}C pulse angle 30°, interval between pulses 1.5 sec). All spectra were recorded in d_5-pyridine; chemical shifts are given in ppm relative to tetramethylsilane. For ^{13}C-NMR, the central peak of the central triplet of d_5-pyridine was used as reference, δ 135.500 ppm.

RESULTS

Identification of bile salt class

When the bile salt composition of manatee bile was examined by TLC, the R_f values of the spots obtained were different from any of the common taurine- or glycine-conjugated bile acids. Using a system for common amidated bile acids (23), R_f values were 0.19 and 0.27. Similarly, when the bile was analyzed by HPLC, there appeared no peaks corresponding to known glycine- or taurine-amidated bile acids. Using a system useful for separating the major common natural bile acids (19), RRT values were 0.21, 0.27–0.29, and 0.34 relative to the glycine conjugate of chenodeoxycholic acid. Two-dimensional TLC (18) revealed two major spots whose chromatographic properties were consistent with those of bile alcohol sulfates. The most abundant bile salt co-migrated with cholestane-pentol sulfate, and the next most abundant one, with cholestane-tetrol sulfate. There were at least two fainter spots which had mobilities intermediate between those of the two major bile salts.

SIMS of the bile sample was performed to obtain the molecular weights of the intact conjugates. Major peaks were observed at m/e 531 and 515, which were consistent with (mono) sulfate esters of a cholestane-pentol and a cholestane-tetrol, respectively. On the basis of these results, as well as the known biological origins of the manatee (12), it was thought likely that bile acid amidates were not present and that the major components of the bile of the manatee were bile alcohol sulfates.

Solvolysis and chromatography of solvolysis products

Solvolysis of the bile samples released neutral compounds that were less polar than the original bile salts. The presence of the sulfuric acid moiety in the original bile salts was confirmed by the precipitation of barium salts (18) when the aqueous layer remaining after solvolysis was treated with barium chloride solution.

TLC analyses of the solvolyzed bile with two different solvent systems gave results that were consistent with the presence of bile alcohols. The most abundant bile alcohol had an R_f value similar to that of a cholestane-pentol (5β-bufol) in solvent system A and an R_f value greater than 5β-bufol in solvent system B. The second most abundant compound co-migrated with 5β-cholestane-3α,7α,25,26-tetrol. There were several other spots between these two which were not characterized further by TLC analysis.

The results of GLC analyses of the solvolyzed bile samples as their TMS ethers are depicted in **Fig. 1.** At least six distinct peaks were observed. The first peak was identified as the TMS ether of cholesterol. Other peaks were designated provisionally as bile alcohols 1–5 in order of increasing retention time on the SP-2250 column. The

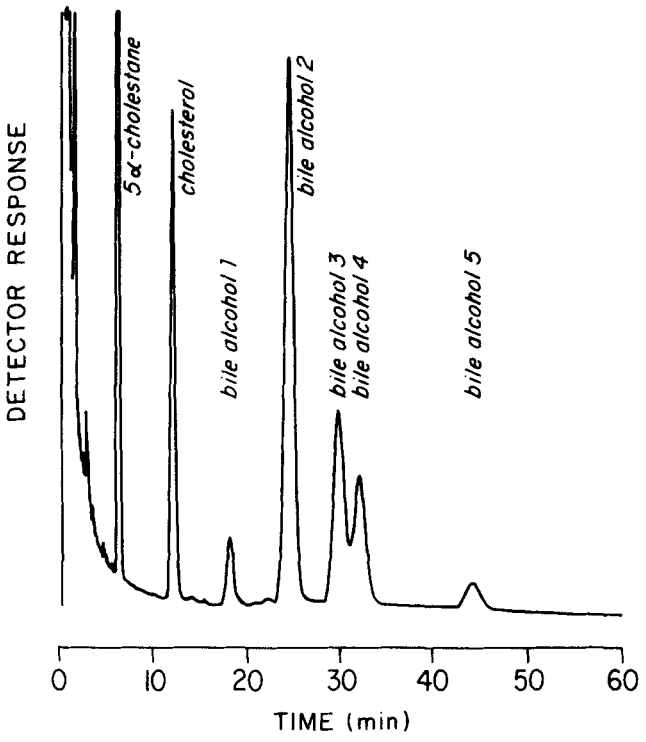


Fig. 1. Gas chromatogram of TMS ether derivatives of bile alcohols obtained by solvolysis of manatee bile. Column, 3% SP-2250 (6 ft × 2 mm i.d.); oven temperature, 270°C. Each bile alcohol was subsequently identified as follows: bile alcohol 1, 5β-cholestane-3α,6β,7α,26-tetrol; bile alcohol 2, 5β-cholestane-3α,6β,7α,25,26-pentol; bile alcohol 3, 5β-cholestane-3α,7α,25,26-tetrol; bile alcohol 4, 5β-cholestane-3α,6β,7β,25,26-pentol; bile alcohol 5, 5β-cholestane-3α,6α,7β,25,26-pentol. 5α-Cholestane was added as internal standard.

TMS ether of bile alcohol 3 had the same retention time as that of 5β-cholestane-3α,7α,25,26-tetrol TMS ether. However, the retention times of the other bile alcohols did not match those of known bile alcohols (6, 9, 10).

Mass spectroscopy of bile alcohols

The mass spectra of the TMS ether derivatives of bile alcohols 1–5 are shown in **Fig. 2.** The mass spectrum of bile alcohol 1 TMS ether (Fig. 2A) resembled that of the TMS ether of 5β-cholestane-3α,7α,12α,26-tetrol (14) with respect to a series of ions (m/e 634, 544, 454, and 364) arising by the successive losses of trimethylsilanols (TMS-OH, 90 mass units each) from the molecular ion (m/e 724). However, the intensity of the ion at m/e 253 (loss of the entire side chain and three TMS-OHs on the nucleus) in the mass spectrum of bile alcohol 1 TMS ether was significantly smaller than that of the mass spectrum of authentic 5β-cholestane-3α,7α,12α,26-tetrol TMS ether (base peak). Instead, there were additional ions at m/e 195 and 285, which are known to be characteristic of methyl ester-TMS ether derivatives of muricholic acids (24). The presence of these fragment ions suggested that the bile alcohol had three hydroxyl groups, at the 3, 6, and 7 posi-

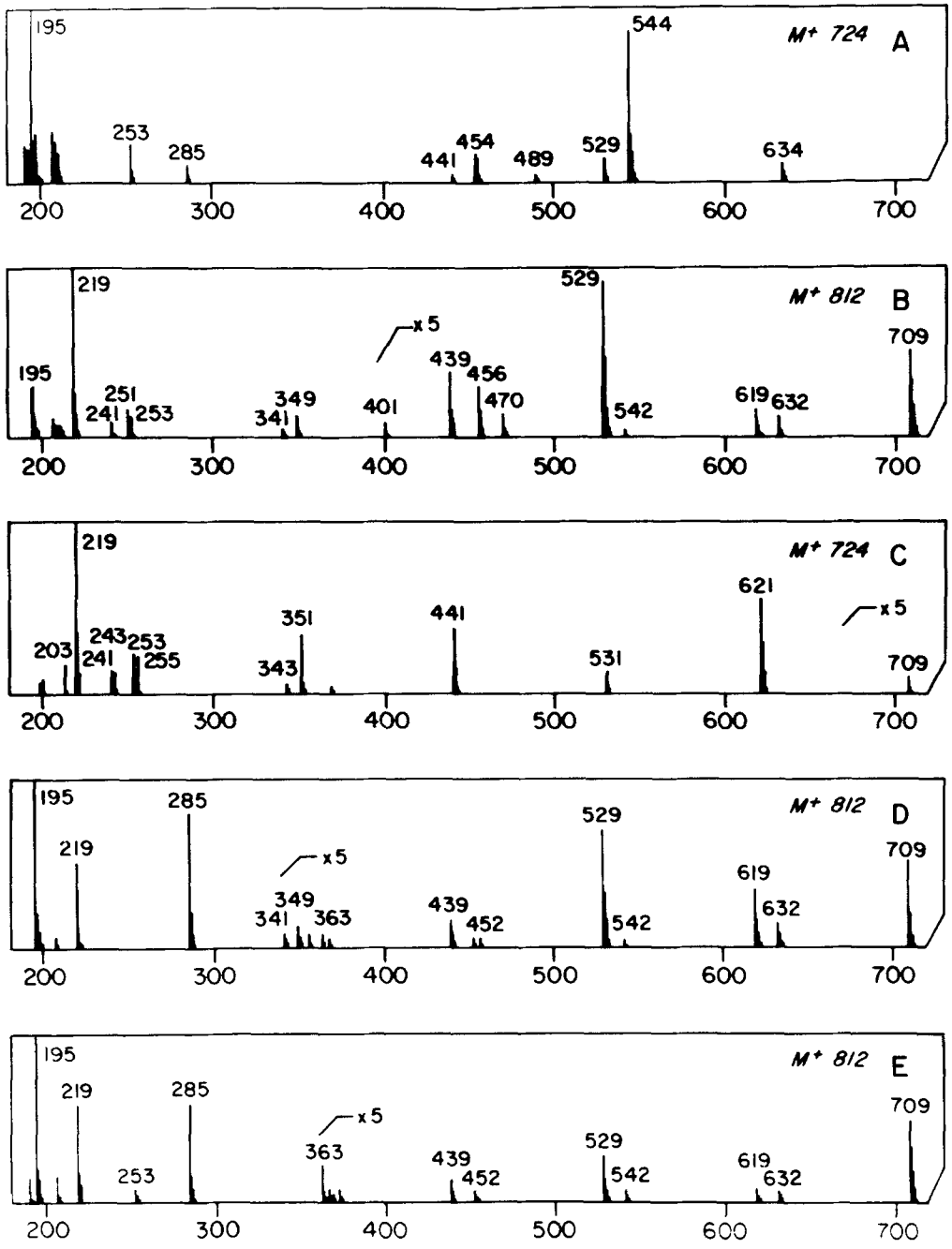


Fig. 2. Mass spectra (MS) of TMS ethers of bile alcohols of the manatee. MS A, bile alcohol 1 (5β-cholestane-3α,6β,7α,26-tetrol); MS B, bile alcohol 2 (5β-cholestane-3α,6β,7α,25,26-pentol); MS C, bile alcohol 3 (5β-cholestane-3α,7α,25,26-tetrol); MS D, bile alcohol 4 (5β-cholestane-3α,6β,7β,25,26-pentol); MS E, bile alcohol 5 (5β-cholestane-3α,6α,7β,25,26-pentol).

tions, and one hydroxyl group in the side chain at C-26.

The mass spectrum of bile alcohol 2 TMS ether (Fig. 2B) was quite similar to that of 5β-bufol TMS ether (9) except for the presence of the ion at m/e 195. The series of ions at m/e 709, 619, 529, 439, and 349 arises by scission of the bond between C-25 and C-26 and the successive losses of TMS-OH. The side chain fragment at m/e 219 (9) also indicates that there are two hydroxyl groups, at C-25 and C-26. However, as described above, the retention time of bile alcohol 2 was different from that of the TMS ethers of 5α- and 5β-bufol. Thus, it was considered likely that the compound was an isomer of bufol and that the nuclear hydroxyl groups were located at positions 3, 6, and 7.

The mass spectrum of the TMS ether of bile alcohol 3 (Fig. 2C) was identical with that of chemically synthesized

TABLE 2. Relative retention times (RRT) of bile alcohols and reference bile acids

Compounds[a]	Positions of Hydroxyl Groups	RRT[b] 3% SP-2250[c] TMS	3% SE-30[d] TMS	3% SE-30[d] Acet-TMS
Bile alcohol 1	3α,6β,7α,26[e]	0.66	0.65	0.65[f]
27-Deoxy-5β-cyprinol	3α,7α,12α,26	0.72	0.63	0.63[f]
Bile alcohol 2	3α,6β,7α,25,26[g]	0.89	1.01	0.77[h]
5β-Bufol	3α,7α,12α,25,26	1.00	1.00	0.74[h]
Bile alcohol 3	3α,7α,25,26[i]	1.08	0.98	0.73[h]
Bile alcohol 4	3α,6β,7β,25,26[e]	1.16	1.25	1.02[j]
Bile alcohol 5	3α,6α,7β,25,26[e]	1.59	1.60	1.29[h]
α-Muricholic acid	3α,6β,7α	0.92	1.02	1.02[f]
Cholic acid	3α,7α,12α	1.00	1.00	1.00[f]
Chenodeoxycholic acid	3α,7α	1.10	0.97	0.97[f]
β-Muricholic acid	3α,6β,7β	1.26	1.33	1.32[h]
ω-Muricholic acid	3α,6α,7β	1.78	1.75	1.75[f]

[a]Bile alcohols 1–5 correspond to the GLC peaks of the unknown bile alcohol TMS ethers as depicted in Fig. 1. The compounds are listed in order of increasing RRT on SP-2250 columns.

[b]Bile alcohols and bile acid methyl esters were chromatographed as their TMS ether or acetonide-TMS ether (Acet-TMS) derivatives. The RRT values were calculated relative to 5β-bufol TMS ether (RRT = 1.00, absolute retention times; 28.71 min on Sp-2250 and 31.63 min on SE-30) for bile alcohols and relative to methyl cholate TMS ether (RRT = 1.00, absolute retention times; 15.72 min on SP-2250 and 10.52 min on SE-30) for bile acids.

[c]Packed column, 6 ft, 2 mm i.d., oven temperature 270°C.

[d]Packed column, 6 ft, 2 mm i.d., oven temperature 250°C. Peaks corresponding to the TMS ethers of bile alcohols 2 and 3 of the solvolyzed bile samples could not be resolved on this column, and these data were obtained by purification of the mixture by preparative TLC followed by GLC analysis.

[e]Positions and orientations of hydroxyl groups are tentative.

[f]Compound did not form acetonide.

[g]Assignment of position of hydroxyl groups was based on subsequent MS and NMR studies (see text).

[h]Compound formed a mono-acetonide.

[i]Identical to synthetic standard.

[j]Compound formed a di-acetonide.

5β-cholestane-3α,7α,25,26-tetrol TMS ether. The series of ions at m/e 621, 531, 441, and 351 and the side chain fragment at m/e 219 were formed by the same mechanisms as those of 5β-bufol TMS ether described above. However, this compound did not have the ions at m/e 195 and 285. Because of complete identity by TLC, GLC, and MS, bile alcohol 3 (the second most abundant bile alcohol) was tentatively identified as 5β-cholestane-3α,7α,25,26-tetrol.

The mass spectra of the TMS ethers of bile alcohols 4 (Fig. 2D) and 5 (Fig. 2E) resembled each other and also revealed ions characteristic of the bufol structure. However, these ions were relatively small compared with the high intensities of the fragments of m/e 195 and 285. These mass spectra strongly suggested that these new bile alcohols had hydroxyl groups at positions 3, 6, and 7. If this were the case, bile alcohols 2, 4, and 5 were epimers at positions 6 and 7, just as in the case of α-, β-, and ω-muricholic acids. On the basis of the intensities of the ions at m/e 195 and 285, it was judged that bile alcohols 1 and 2 had a 7α-hydroxyl group and that bile alcohols 4 and 5 had a 7β-hydroxyl group. The mass spectra of the methyl ester-TMS ether derivatives of β- and ω-muricholic acids (7β-OH) have strong fragment ions at m/e 195 and 285 and those of α-muricholic acid and hyocholic acid (7α-OH) reveal very weak fragments at those molecular weights (24).

The relative retention times (RRTs) of the TMS ether derivatives of the new bile alcohols on SP-2250 and SE-30 are listed in **Table 2.** RRTs of authentic bile acids (methyl ester-TMS ether derivatives) obtained under the same conditions are also presented in Table 2. The changes of the RRTs of the TMS ethers of 5β-bufol and bile alcohols 2–5 were similar to those of cholic acid and α-muricholic acid, chenodeoxycholic acid, β-muricholic acid, and ω-muricholic acid, respectively. The relationship of the RRTs of bile alcohol 1 and bile alcohol 2 was also similar to that of 27-deoxy-5β-cyprinol (3α,7α,12α,26-OH) and 5β-bufol (3α,7α,12α,25,26-OH). The chromatographic behavior of the new bile alcohols was compared with that of known bile acids and was also used to identify the bile alcohols with results obtained as follows: bile alcohol 1, 5β-cholestane-3α,6β,7α,26-tetrol; bile alcohol 2, 5β-cholestane-3α,6β,7α,25,26-pentol; bile alcohol 4, 5β-cholestane-3α,6β,7β,25,26-pentol; bile alcohol 5, 5β-cholestane-3α,6α,7β,25,26-pentol. These structures are consistent with those suggested by the MS data above.

The orientations of the 6- and 7-hydroxy groups were also supported by the properties of the bile alcohol

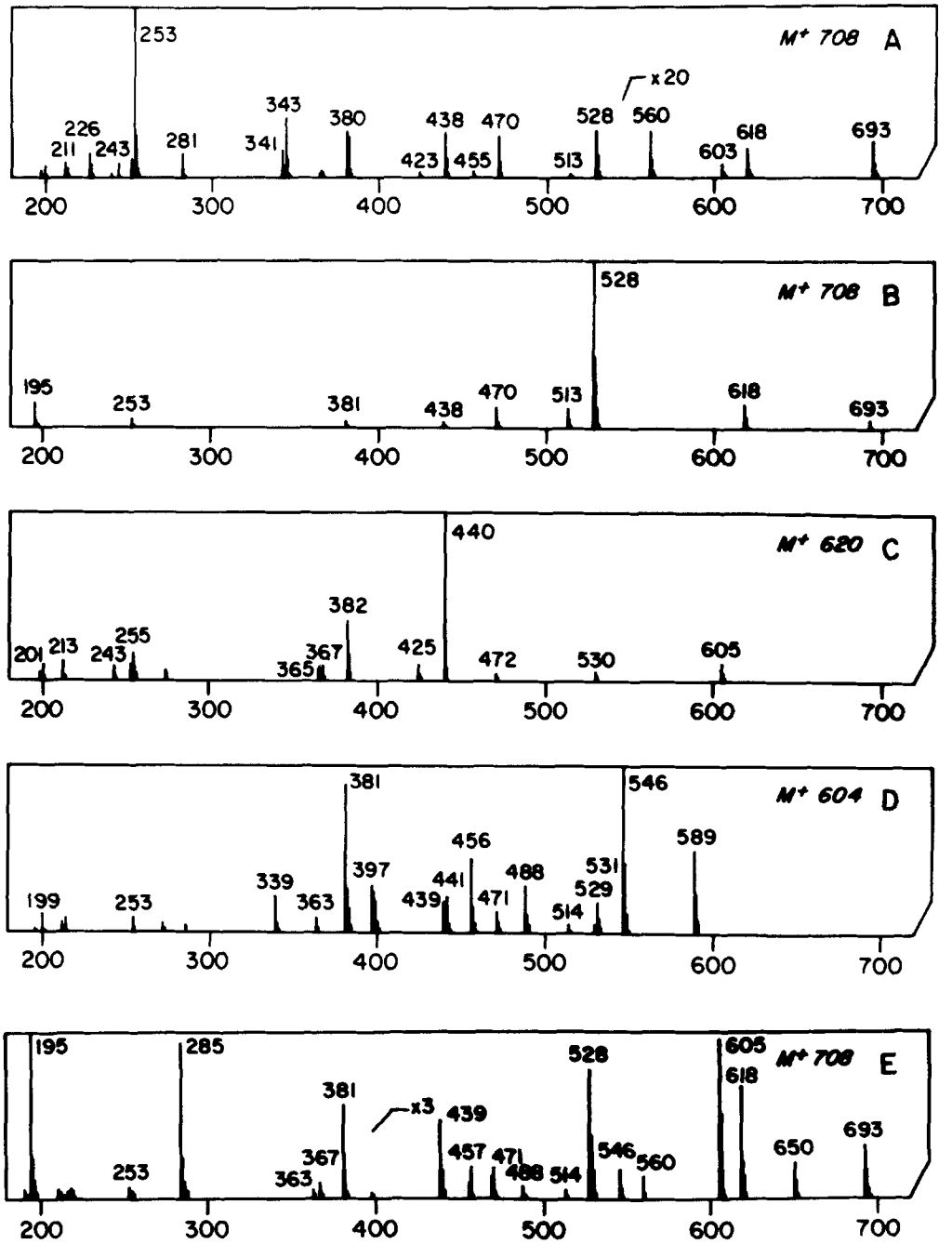


Fig. 3. Mass spectra (MS) of acetonide-TMS derivatives of bile alcohols. MS A, 5β-bufol (5β-cholestane-3α,7α,12α,25,26-pentol); MS B, bile alcohol 2 (5β-cholestane-3α,6β,7α,25,26-pentol); MS C, bile alcohol 3 (5β-cholestane-3α,7α,25,26-tetrol); MS D, bile alcohol 4 (5β-cholestane-3α,6β,7β,25,26-pentol); MS E, bile alcohol 5 (5β-cholestane-3α,6α,7β,25,26-pentol). All of the above bile alcohols formed one acetonide in the side chain (between C25 and C26). In addition, bile alcohol 4 (MS D) formed a second acetonide between C-6 and C-7. Bile alcohol 1 did not form an acetonide under the conditions employed (see Materials and Methods).

acetonides. The bile alcohols were treated with DMP and HCl, followed by derivatization to the TMS ether and GLC-MS. The RRTs of the acetonide-TMS ether derivatives of the bile alcohols and of the reference bile acids are listed in Table 2. The mass spectra of the bile alcohol derivatives are shown in **Fig. 3.** Under the conditions employed, the 6,7-*cis* alcohols of β-muricholic acid (6β,7β), hyocholic acid (6α,7α), and the vicinal alcohols of 5β-bufol (25 and 26 hydroxyls) formed acetonides (Fig. 3A). However, the 6,7-*trans* glycols of α-muricholic acid (6β,7α) and ω-muricholic acid (6α,7β) did not form acetonides; the RRTs and MS of bile alcohol 1 were iden-

tical before and after DMP treatment, suggesting a 6,7-*trans* glycol structure.

Bile alcohol 2 formed one acetonide in the side chain (Fig. 3B). The mass spectrum showed a series of fragment ions of m/e 618, 528, and 438 arising by successive losses of TMS-OH from the molecular ion (M^+, 708). Disappearance of the side chain fragment (m/e 219) indicated that the acetonide was formed between C-25 and C-26. Other major fragment ions [693 (M – 15), 513 (M – 2 × TMS – 15), 470 (M – acetone – 2 × TMS), 195 (C-3 to C-6)] were consistent with the structure of a molecule having 6,7-*trans* and 25,26 glycols.

Major fragment ions of bile alcohol 3 acetonide-TMS (Fig. 3C) were analogous to those of the bile alcohol 2 derivative (Fig. 3B) but with 88 mass units less. The side chain fragment (m/e 219) also disappeared. The RRT and MS of the acetonide-TMS ether derivative of chemically synthesized 5β-cholestane-3α,7α,25,26-tetrol were completely identical with those of natural bile alcohol 3.

Although bile alcohols 2 and 4 were considered to be epimers, the MS of bile alcohol 4 acetonide-TMS (Fig. 3D) was very different from that of bile alcohol 2 acetonide-TMS (Fig. 3B). The absence of the fragment ions at m/e 195 and 285 as well as of the ion at m/e 219 indicated that bile alcohol 4 formed two acetonides per molecule and that the orientation of the nuclear glycol is *cis* (6β,7β). Major fragment ions were assigned as follows (M^+, 604): 589, M – 15; 546, M – 58 (loss of acetonide group as acetone); 531, M – (58 + 15); 529, M – 75 (loss of acetonide group as 2,2-propanediol); 514, M – 90; 488, M – 116 (scission between C-24 and C-25; 471, M – (58 + 75); 456, M – (58 + 90); 441, M – (58 + 90 + 15); 439, M – (75 + 90); 397, M – (116 + 90 + 1); 381, M – (58 + 75 + 90).

The MS of the acetonide-TMS ether derivative of bile alcohol 5 (Fig. 3E) revealed intense ions at m/e 195 and 285 without the side chain fragment (m/e 219). A series of ions at m/e 618, 528, and 439 (M – TMS-OH groups) and the ion at m/e 693 (M – 15) are seen in the mass spectra of the derivative of bile alcohol 2 (Fig. 3B). Another series of fragment ions at m/e 650, 560, 471, and 381 arises by the loss of an acetonide group (m/e 58) and

TABLE 3. ^{13}C-NMR data of bile acids and alcohols in d_5-pyridine

	LC 3α	CDC 3α,7α	UDC 3α,7β	HDC 3α,6α	MDC 3α,6β	MeMDC3c 3αcath,6β	HC 3α,6α,7α	ωMC 3α,6α,7β	βMC 3α,6β,7β	αMC 3α,6β,7α	α-Trichechol	5β Bufol	C 3α,7α,12α
1	35.9	35.8[a]	35.5	36.3[b]	36.7	35.8	36.4	36.1	36.8[a]	37.1	37.1	35.9[a]	35.9[a]
2	31.4	31.7	31.0	31.4	31.1	26.7	31.7	31.9	31.1	31.6	31.6	31.7	31.2
3	71.1	71.7	70.7[a]	71.3	70.9	77.9	71.7	71.2	70.7	71.6	71.6	71.9	71.6
4	37.2	40.9	38.4[b]	30.4	37.6	32.8	33.8	31.5	36.5[a]	38.0	38.0	41.1	40.5
5	42.4	42.5	43.0	49.6	49.0	48.5	49.2	49.2	48.8	49.1	49.2	42.6[b]	42.4[b]
6	27.6[b]	36.2[a]	37.9[b]	67.2	72.5	72.0	69.6[a]	73.4	76.4	77.4	77.5	36.3[a]	35.5[a]
7	26.7[b]	67.5	70.3[a]	35.8[b]	35.5	35.4	72.2[a]	75.7	73.3	73.0	73.1	67.8	67.4
8	35.9	40.1	43.7	35.1	31.3	31.4	39.2	42.3	39.2[b]	36.0	36.1	40.8	40.3
9	40.7	33.2	39.5	40.3	41.1	41.0	33.2	40.1	40.2[b]	33.4	33.5	27.5	27.1
10	34.7	35.5	34.2	36.1	34.9	34.8	36.3	35.7	34.4	35.7	35.7	35.4	35.0
11	21.1	21.0	21.4	21.1	21.0	20.9	21.0	21.7	21.3	21.0	21.1	29.7	29.3
12	40.3	40.0	40.3	40.2	40.3	40.2	39.9	40.4	40.4	40.1	40.2	72.5	72.1
13	42.8	42.6	43.6	42.9	42.9	42.9	42.7	43.9	43.8	42.8	42.8	46.9	46.6
14	56.2[a]	50.7	55.2	56.1[a]	56.4[a]	56.1[a]	50.6	55.4	55.5	50.5	50.6	42.8[b]	42.2[b]
15	24.3	24.0	27.3	24.4	24.5	24.4	23.8	27.4	27.8	24.2	24.2	23.9	23.5
16	28.4	28.5	28.8	28.3	28.4	28.3	28.4	29.0	29.0	28.6	28.8	28.3	27.8
17	56.4[a]	56.3	56.3	56.2[a]	56.3[a]	56.3[a]	56.2	56.5	56.3	56.4	56.7	47.6	46.9
18	12.2	12.1	12.2	12.2	12.2	12.1	12.0	12.5	12.4	12.1	12.1	13.1	12.7
19	23.6	23.2	23.5	23.9	26.2	25.9	23.5	24.1	26.3	26.2	26.2	23.3	22.9
20	35.6	35.7	35.5	35.6	35.7	35.6	35.6	35.7	35.8	35.8	36.3	36.5	35.7
21	18.5	18.6	18.5	18.5	18.5	18.4	18.5	18.8	18.8	18.6	19.0	18.0	17.3
22	31.7	31.7	31.6	31.7	31.7	31.2[b]	31.7	31.9	31.9	31.8	37.2	37.4	31.6
23	31.7	31.7	31.6	31.7	31.7	31.1[b]	31.7	31.9	31.9	31.8	20.7	20.8	31.6
24	176.4	176.4	176.2	176.4	176.4	174.3	176.4	176.5	176.5	176.5	40.2	40.3	176.2
25											72.7	72.7	
26											70.4	70.6	
27											24.8	24.6	
					-O<u>C</u>O$_2$-	155.1							
					-<u>C</u>H$_2$O-	63.6							
					<u>C</u>H$_3$-	14.4							
					-COO<u>C</u>H$_3$	51.3							

Abbreviations: LC, lithocholic acid; CDC, chenodeoxycholic acid; UDC, ursodeoxycholic acid; HDC, hyodeoxycholic acid; MDC, murideoxycholic acid; MeMDC3c, methyl 3α-cathyloxy-6β-hydroxy-5β-cholan-24-oate; HC, hyocholic acid; MC, muricholic acid; C, cholic acid.

[a,b] Assignments bearing the same superscript can be exchanged in each column.

successive losses of one to three TMS-OH groups. These fragments are consistent with the structure proposed above, namely an ω-muricholic acid type nucleus and a bufol type side chain.

Confirmation of chemical structure by NMR

The structure of the most abundant bile alcohol of the manatee (bile alcohol 2) was confirmed by comparison of its ^{1}H and ^{13}C-NMR spectra with 5β-bufol and the four muricholic acids. Because the signals of d_4-methanol interfered greatly with those of the bile acids, especially when only a small amount of material was available, d_5-pyridine was chosen as solvent for ^{1}H and ^{13}C-NMR. In order to assign the ^{13}C-NMR spectra of the muricholic acids, the spectra (in d_5-pyridine) of lithocholic acid and 3α,6α-, 3α,6β-, 3α,7α-, and 3α,7β-dihydroxy-5β-cholan-24-oic acids were obtained (**Table 3**). In all cases, the multiplicity tests (GASPE) were used to assist in the assignment of spectra (see Methods).

Assignment of the signals of lithocholic acid, chenodeoxycholic acid, and ursodeoxycholic acid could be done by direct comparison with the values reported in d_4-methanol (25) and d-chloroform (26). As expected, the most important variations were found for the carbons in the vicinity of the hydroxyl groups, due to different hydrogen bonding with the solvent.

The spectra of 3α,6α- and 3α,6β-dihydroxy-5β-cholan-24-oic acids were assigned by comparison with the above compounds, and consideration of α, β, γ, and δ effects, as defined by Beierbeck, Saunders, and ApSimon (27). The assignment of the signals of methylenes 1, 4, and 7 in the 3α,6β isomer was difficult because they have similar chemical shifts. Cathylation, which selectively protects equatorial hydroxyl groups (28), afforded a way to prepare the 3-ester of this compound. The acylation shifts obtained, which agreed with those observed for the acetate of methyl chenodeoxycholate (8), permitted the unequivocal assignment of the signals: C-7 was not changed, C-1 was protected by 0.9 ppm, C-2 and C-4 were shielded by 4.4 and 4.8 ppm, respectively.

In the 3α,6α-isomer, C-4 is protected by 6.8 ppm (relative to lithocholic acid) because a new protective hydroxyl γ-*gauche* interaction is introduced on C-4, and the HC interaction between $H_{4\beta}$ and $H_{6\alpha}$ is eliminated (27). In the 3α,6β-isomer it is C-8 which is protected by 4.6 ppm (relative to lithocholic acid) by elimination of the HC interaction between $H_{6\beta}$ and H_8 and addition of a γ-*gauche* interaction. This effect is somewhat smaller than that usually observed for carbons γ to an axial hydroxy group (ca. −6 ppm). A similar situation is found in 5α steroids, where introduction of an axial hydroxyl group 1,3-*syn*-diaxial to the 19 methyl group causes shielding of γ carbons of only about 4.5 ppm. Deformation of the valence angle of the two groups to relieve steric strain could in turn diminish the γ interaction between the γ carbon and the hydroxyl group (29).

Calculations, according to Beierbeck et al. (27), indicate that the chemical shift of C-5 should be the same in the 3α,6α and 3α,6β isomers: a CO interaction involving C-4 and the 6α hydroxyl is replaced by another one between C-10 and the 6β hydroxyl group, and no other changes occur. Only in the 3α,6β isomer is C-19 deshielded by 2.6 ppm (relative to lithocholic acid) due to the δ interaction (27) with the 6β hydroxyl; this is a useful diagnostic feature if a 6-substituted bile acid is suspected.

Based on the model compounds already discussed, the assignment of the spectra of the muricholic acids is straightforward (Table 3). The changes in carbon 9, 14, and 15 are analogous to those in cheno- and ursodeoxycholic acids. Relative to the compounds with a 7α hydroxyl group, the 3α,6β,7β and 3α,6α,7β isomers show C-15 3.6 ppm downfield on account of a δ interaction with the 7β hydroxyl group. Also, changing the 7 hydroxyl group from the axial to the equatorial position eliminates a hydroxyl γ-*gauche* interaction with C-9 and 14 and adds an HC interaction with each one of them, the net result being deprotection of approximately 6.8 and 6 ppm, respectively (See Table 3). The effects on carbons 8 and 19 are similar to those already described for the 3α,6α and 3α,6β dihydroxy bile acids. The chemical shifts of C-4 exhibit a more complex pattern. Introduction of the 6α hydroxyl in chenodeoxycholic acid shields C-4 by 7.1 ppm and by 6.9 ppm in ursodeoxycholic acid, as already found in the case of 3α,6α-dihydroxy-5β-cholan-24-oic acid. Introduction of the 6β hydroxyl group in chenodeoxycholic acid shields C-4 by 2.9 ppm and by 1.9 ppm in ursodeoxycholic acid. It is interesting to note that the similar introduction of a 6β hydroxyl group in lithocholic acid causes deprotection of C-4 (0.4 ppm), more in line with the effect observed for axial hydroxyl groups on γ-*anti* carbons (30), thus illustrating the dependency of this parameter on conformational changes.

The assignment of carbons 6 and 7 is complicated because substituent effects are not additive for vicinal diols. However, applying the corrections derived from steroidal diols (31), a good agreement was found with the calculated values and a tentative assignment was possible for three of the muricholic acids (**Table 4**). The substituent effect of a hydroxyl group on a β carbon was obtained subtracting its chemical shift in lithocholic acid from the shift of the same carbon in the appropriate dihydroxy bile acid. The correction terms are those of VanAntwerp et al. (31), except for the case of equatorial-axials diols where the terms were estimated from the secondary hydroxyl cases only (Table 4). The assignments for hyocholic acid were derived from C-6 (72.2 ppm) and C-7 (71.3 ppm) in 5α-cholestane-6α,7α-diol (31) considering that a change of A/B junction from *trans* to *cis* shifts C-6 by approxi-

TABLE 4. ^{13}C Resonance assignments for C-6 and C-7 of the muricholic acids (ppm)

Carbon	δ in Dihydroxy Bile Acids	Substituent Shift	Correction	Calculated	Observed
	3α,6α,7β (ec-ec)				
6	67.2	+10.3	−4.0	73.5	73.4
7	70.3	+9.1	−4.0	75.4	75.7
	3α,6β,7α (ax-ax)				
6	72.5	+8.6	−3.0	78.1	77.4
7	67.5	+8.8	−3.0	73.3	73.0
	3α,6β,7β (ax-ec)				
6	72.5	+10.3	−6.4	76.4	76.4
7	70.3	+8.8	−5.4	73.7	73.3
	3α,6α,7α (ec-ax)				
6	67.2	+8.6	−5.4	70.4	69.6[a]
7	67.5	+9.1	−6.4	70.2	72.2[a]

Correction factors are ± 0.5 ppm. Contents of parentheses indicate the orientation of 6 and 7 hydroxyls.
[a]Assignments can be exchanged.

mately −3 ppm [67.2 in hyodeoxycholic acid (Table 3) and 70.0 in 6α-cholestanol (29)]. A similar selection was done in the case of methyl 3α,6α,7α,12α-tetrahydroxy-(5β)-cholan-24-oate (isolated from urine) (32).

The ^{13}C-NMR spectrum of 5β-bufol in pyridine could be assigned by comparison with cholic acid and the values already reported in methanol (15).

The ^{1}H-NMR data of the muricholic acids and the necessary model compounds in d_5-pyridine are presented in **Table 5.** It is interesting to note that in the 3,6 dihydroxy compounds the axial hydrogen ($H_{6\beta}$) appears at lower field than the equatorial ($H_{6\alpha}$), as happens in the parent hydrocarbon (25). The four muricholic acids give sufficiently different patterns for H-6 and H-7 to allow identification of each particular isomer on the basis of a ^{1}H-NMR only. The bile acids with a 6β hydroxyl group show the signal of 19-CH_3 highly deshielded (1.4–1.5 ppm; Table 5), while in the spectra of α- and β-muricholic acids in d_4-methanol this group appears at 1.07 and 1.1 ppm, respectively (data kindly supplied by Dr. Shoichi Ishihara and Dr. K. Uchida). Hydrogen binding of pyridine to the axial 6β hydroxyl group could cause the hydrogens of the methyl group to fall in the descreening volume of the aromatic ring.

Bile alcohol 2 (10.7 mg) was isolated by preparative TLC of the solvolyzed bile of seven animals. Its ^{13}C-NMR showed excellent agreement with C-21 and C-1 through C-19 of α-muricholic acid, while the rest of the side chain matched the corresponding carbons of 5β-bufol (Table 3). Comparison of the ^{1}H-NMR spectrum also showed close agreement with the model compounds, except for CH_3-21 which is sensitive to changes in both the nucleus and the side chain and is found in α-trichechol in an intermediate position (Table 5).

On the basis of these spectroscopic data, we concluded that bile alcohol 2 is 5β-cholestane-3α,6β,7α,25,26-pentol. The configuration of C-25 is not known.

TABLE 5. ^{1}H-NMR data of bile acids and bile alcohols in d_5-pyridine

Compound	H-18	H-19	H-21	H-3	H-6	H-7	H-12	Other
UDC (3α,7β)	0.64	0.90	0.96 (d,6.1)	3.73 (m)		3.73 (m)		
Me norCDC (3α,7α)	0.69	0.96	1.07 (d,5.8)	3.81 (m)		4.03 (bs)		$H_{4\alpha}$ 3.10 (dt,11.7,13.0) -$COOCH_3$ 3.68
C (3α,7α,12α)	0.74	0.93	1.16 (d,5.4)	3.65 (m)		4.00 (bs)	4.16 (bs)	
HDC (3α,6α)	0.56	0.91	0.95 (d,5.4)	3.91 (m)	4.32 (bs)			
MDC (3α,6β)	0.60	1.41	0.96 (d,6.1)	3.87 (m)	4.02 (bs)			
α-MC (3α,6β,7α)	0.76	1.53	1.02 (d)	3.88 (m)	4.30 (m)[a]	4.34 (m)[a]		
β-MC (3α,6β,7β)	0.70	1.43	1.03 (d,6.1)	3.88 (m)	4.06 (bs)	3.79 (dd,10.0,4.3)		
ω-MC (3α,6α,7β)	0.70	0.99	1.02 (d,6.5)	3.93 (m)	4.23 (dd,9.4,5.0)	3.76 (t,9.4)		
HC (3α,6α,7α)	0.67	0.98	0.99 (d)[b]	3.78 (m)	4.17 (m)	4.17 (m)		
5β-Bufol	0.84	1.03	1.26 (d)	3.77 (m)		4.12 (m)	4.29 (m)	H-26, 3.89; H-27, 1.50
α-Trichechol	0.77	1.55	1.13 (d)	3.89 (m)	4.32 (m)[a]	4.35 (m)[a]		H-26, 3.92; H-27, 1.54

δ are in ppm from TMS (internal standard). Contents in parentheses are multiplicities and coupling constants in Hz; singlets are not indicated.
[a]Assignments can be exchanged in each line.
[b]Partially overlaps H-19 signal.

TABLE 6. Bile alcohol composition of the bile of the manatee[a]

Bile Alcohol Number	Position of Hydroxyl Groups	Proposed Name	Animal Number 1	2	3	4–7	Mean
1	3α,6β,7α,26		2.7	1.0	Tr[b]	4.7	3.2
2	3α,6β,7α,25,26	α-trichechol	53.8	34.8	84.5	53.6	55.0
3	3α,7α,25,26		35.0	41.4	9.6	21.8	24.7
4	3α,6β,7β,25,26	β-trichechol	5.5	15.8	5.9	16.0	13.0
5	3α,6α,7β,25,26	ω-trichechol	3.0	7.0	Tr	4.7	4.1

[a] Bile samples from seven manatees were solvolyzed and analyzed by GLC as their TMS-ether derivatives. Animals 1–3 were analyzed individually and bile samples from animals 4–7 were combined (5 ml each) and analyzed. The values represent percent composition in each sample.
[b] Tr, trace amount.

Biliary bile salt composition of the manatee

The quantitative bile alcohol composition of three individual manatee bile samples and a combined sample from four other manatees is shown in **Table 6.** The bile alcohol composition varied somewhat from sample to sample; however, in general, the most abundant bile alcohol was 5β-cholestane-3α,6β,7α,25,26-pentol and the second most abundant bile alcohol was 5β-cholestane-3α,7α,25,26-tetrol. The hydroxyl group esterified with sulfuric acid was assumed to be at C-26 as is the case with most bile alcohol sulfates found in the lower vertebrates (5), but direct evidence was not obtained in the present study.

Proposed trivial names

We wish to propose trivial names for the newly discovered bile alcohols. Since they were first identified in the bile of the West Indian manatee, *Trichechus manatus latirostris,* the name trichechol is proposed for the group of pentols with hydroxy groups at the 3, 6, 7, 25, and 26 carbons. Since the nuclear structures are identical to those of the muricholic acids, we have named the bile alcohols as follows: α-trichechol (5β-cholestane-3α,6β,7α,25,26-pentol), β-trichechol (5β-cholestane-3α,6β,7β,25,26-pentol), and ω-trichechol (5β-cholestane-3α,6α,7β,25,26-pentol). It is recognized that the chemical structures of the bile salts in other members of the family Trichechidae (*T. inuguis* and *T. senegalensis*) are unknown, but the name trichechol follows established tradition.

DISCUSSION

Bile alcohols, as their sulfate esters, are major constituents of the bile of more primitive vertebrates such as some fishes and amphibians (4). As far as we are aware, extensive investigations dealing with the bile salts of the vertebrates (4, 6) have indicated that mammals do not usually have bile alcohols as their major biliary constituents. Thus, the present study constitutes the first demonstration of a mammalian bile which contains exclusively bile alcohol sulfates and no cholanoic acids. The Trichechidae are known to be a very ancient family, with fossils from the Eocene period having been described.

The order Sirenia includes 20 genera, 18 of which are extinct. Only two genera survive, *Trichechus* and *Dugong,* which are also endangered species. The West Indian manatee, *Trichechus manatus latirostris,* is a large aquatic herbivorous animal which eats primarily sea grasses. It requires water temperature higher than 68°F (20°C) and is very susceptible to cold because of its low metabolic rate (12). Because the manatee does not eat fish, the bile alcohols found in its bile must have been produced by hepatic synthesis. However, it is possible that the tetrahydroxy derivatives lacking a 6 hydroxy or 25 hydroxy group resulted from bacterial biotransformation and were either absorbed from the intestine or excreted in stool and then ingested orally; the manatee is known to be coprophagic (33). Some years ago, Caldwell et al. (34), using TLC and color reactions, reported that the bile of *Dugong dugon* contained cholic, chenodeoxycholic, and deoxycholic acids, a bile acid pattern similar to that of many mammals; the presence of bile alcohols was not assessed.

The ^{1}H and ^{13}C-NMR spectra of the isomeric muricholic acids, hyocholic acid, and the other model compounds which are reported here, make possible the unequivocal determination of the nuclear structure of 5β steroid metabolites with a 3α,6,7 trihydroxy substitution pattern. These should include the two minor bile alcohols also present in manatee bile whose structure was tentatively identified by MS and GLC in this paper.

A possible pathway of bile alcohol synthesis in the manatee is depicted in **Fig. 4.** Considering the known routes of synthesis (5, 35, 36), it is probable that all the bile alcohols found in this study were formed from cholesterol (I) via 5β-cholestane-3α,7α,26-triol (V). The latter is an intermediate of chenodeoxycholic acid (VII)

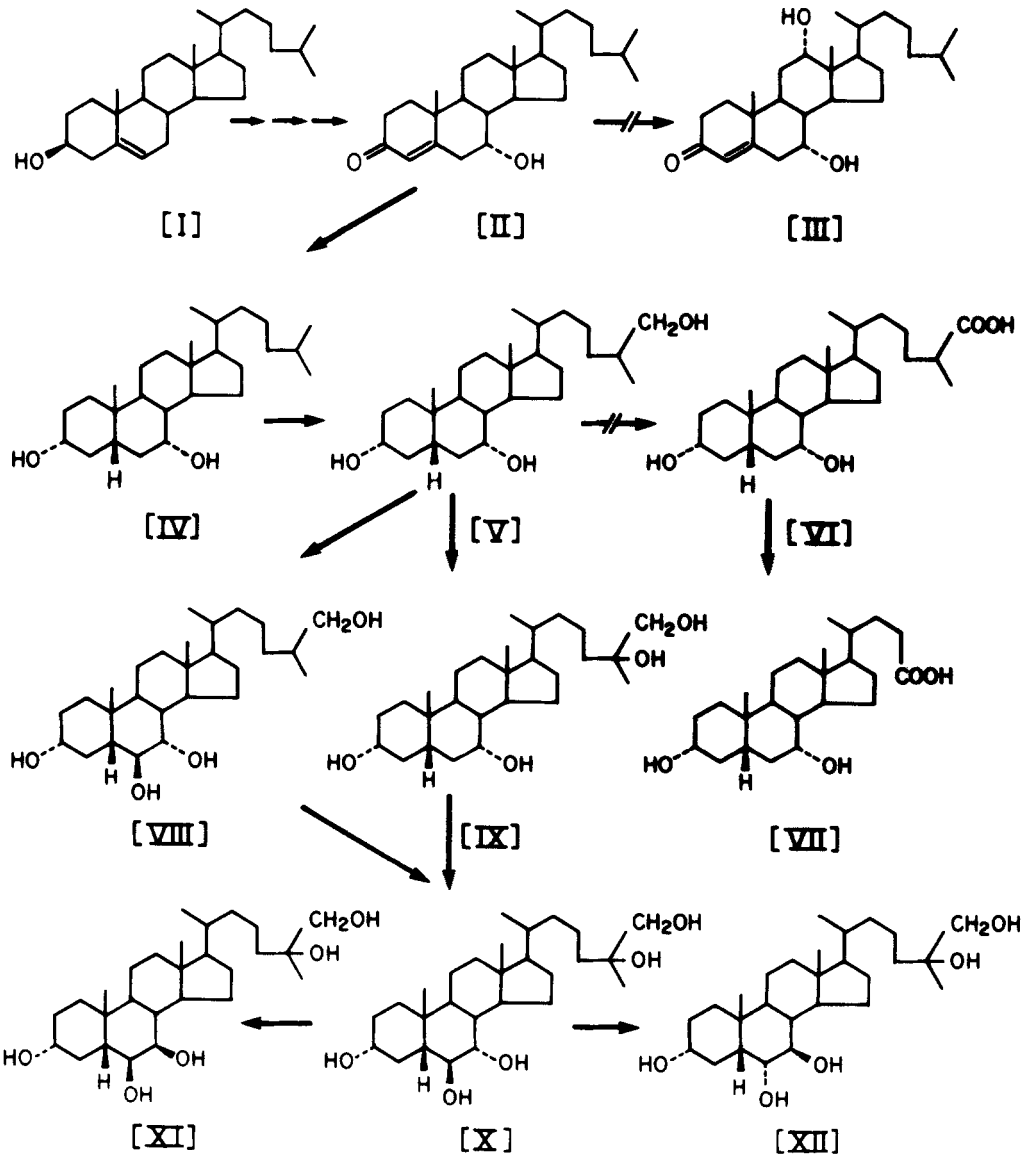


Fig. 4. Possible biosynthetic pathways of the new bile alcohols found in manatee bile. I, cholesterol; II, 7α-hydroxycholest-4-en-3-one; III, 7α,12α-dihydroxycholest-4-en-3-one; IV, 5β-cholestane-3α,7α-diol; V, 5β-cholestane-3α,7α,26-triol; VI, 3α,7α-dihydroxy-5β-cholestan-26-oic acid; VII, chenodeoxycholic acid; VIII, 5β-cholestane-3α,6β,7α,26-tetrol (25-deoxytrichechol); IX, 5β-cholestane-3α,7α,25,26-tetrol (6-deoxytrichechol); X, 5β-cholestane-3α,6β,7α,25,26-pentol (α-trichechol); XI, 5β-cholestane-3α,6β,7β,25,26-pentol (β-trichechol); XII, 5β-cholestane-3α,6α,7β,25,26-pentol (ω-trichechol).

biosynthesis and was identified recently in human bile (9). Usually, the 12α-hydroxyl group is introduced early during the biosynthesis (II→III) and most bile alcohols found in lower vertebrates (4, 6) and also in man (1, 4) have 12α-hydroxyl groups. Presumably, the manatee did not acquire the 12α-hydroxylase enzyme system or has a very low enzyme activity similar to the guinea pig (37). In any case, none of the new bile alcohols identified in this study possessed a 12α-hydroxyl group.

In most mammals, the 26-hydroxyl group of V is oxidized to 3α,7α-dihydroxy-5β-cholestan-26-oic acid (VI), which in turn is transformed into chenodeoxycholic acid (VII) (35). It seems likely that the manatee is not able to oxidize V to VI, whereas most other mammalian species apparently can do so. Instead, the manatee must possess efficient 6β and 25-hydroxylases. Hydroxylations of V at the 6β- or 25-position would yield 25-deoxy-α-trichechol (VIII, bile alcohol 1) or 6-deoxy-trichechol (IX, bile alcohol 3). The 25- or 6β-hydroxylation of VIII or IX, respectively, would lead to α-trichechol (X, bile alcohol 2), the most abundant bile alcohol in the manatee. The formation of β- and ω-trichechols (XI and XII) may be analogous to those of β- and ω-muricholic acids in the rat (17), but at present we have no information about the entero-

hepatic circulation, bacterial, or hepatic transformations of bile alcohols in the manatee. Nor is it known whether the trichechol sulfates function as biological surfactants to promote biliary excretion of cholesterol or to solubilize lipid products during digestion, as is known to be the case for the salts of the common bile acids in mammals (38).

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