Synthesis of new bile acid analogues and their metabolism in the hamster: 3α, 6α-dihydroxy-6β-methyl-5β-cholanoic acid and 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid

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Abstract This paper reports the chemical synthesis of two new bile acid analogues, namely, 3α, 6α-dihydroxy-6β-methyl-5β-cholanoic acid and 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid from 3α-hydroxy-6-oxo-5β-cholanoic acid and describes their metabolism in the hamster. A Grignard reaction of the oxo acid with methyl magnesium iodide in tetrahydrofuran gave two epimeric dihydroxy-6-methyl-cholanoic acids which were separated as the methyl esters by silica gel column chromatography. The configuration of the 6-methyl groups was assigned by proton nuclear magnetic resonance spectroscopy and was supported by the chromatographic properties of the new compounds. The metabolism of the two new bile acid analogues was studied in the hamster. After intraduodenal administration of the ^{14}C-labeled analogues into bile fistula hamsters, both compounds were absorbed rapidly from the intestine and secreted into bile. Intravenous infusion studies revealed that these compounds were efficiently extracted by the liver; the administered analogues became major biliary bile acids, present as either the glycine or taurine conjugates. These compounds are useful to study the effect of methyl-substituted bile acids on cholesterol and bile acid metabolism and may possibly possess cholelitholytic properties. —**Matoba, N., E. H. Mosbach, B. I. Cohen, M. Une, and C. K. McSherry.** Synthesis of new bile acid analogues and their metabolism in the hamster: 3α, 6α-dihydroxy-6β-methyl-5β-cholanoic acid and 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid. *J. Lipid Res.* 1989. **30:** 1005–1014.

Supplementary key words bile acids • Grignard reaction • 6-methyl bile acids • intestinal absorption

Certain dihydroxy bile acids are known to prevent the formation of cholesterol gallstones in animal models (1) and to dissolve gallstones both in animals and in humans (2–4). In humans, the oral administration of the two dihydroxy bile acids, chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA), has been found to cause the dissolution of cholesterol gallstones, but success rates of the therapy have not been uniformly high (although dissolution rates as high as 60% have been observed in groups of carefully selected patients) (5). Both CDCA and UDCA give rise to the potentially hepatotoxic lithocholic acid (LCA) in the large intestine and therefore may not be ideal for extended medical therapy of cholesterol cholelithiasis (6). However, in humans the toxicity of CDCA and, particularly, of UDCA has been very low. Our laboratory has studied the metabolism and cholelitholytic properties of several 7-methyl bile acid analogues in animal models (7, 8) in order to point the way toward the development of new gallstone-dissolving agents with greater efficacy and reduced potential for hepatotoxicity.

Several reports showed that in animal models a 3,6-dihydroxy bile acid, namely the hydrophilic hyodeoxycholic acid (HDCA), was effective in preventing the formation of cholesterol gallstones and showed some promise in gallstone dissolution (9–12). In the hamster and prairie dog, as opposed to humans (13), HDCA participates in the enterohepatic circulation. Gallstone prevention by this bile acid is believed to take place via a phase transition mechanism (formation of a liquid crystalline phase), as has been observed with UDCA in man (14).

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; 6β-Me-HDCA, 3α, 6α-dihydroxy-6β-methyl-5β-cholanoic acid; 6α-Me-MDCA, 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid; PMR, proton nuclear magnetic resonance; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high pressure liquid chromatography; RRT, relative retention time.

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HDCA was identified in urine of patients with malabsorption, liver disease, and in normal individuals (15, 16). In human subjects, following oral administration, HDCA is largely glucuronidated at the 6-position, sulfated, and excreted in the urine (13, 16, 17); thus, structurally unmodified HDCA is not suitable for use in man. The 6β-hydroxy isomer of HDCA (3α, 6β-dihydroxy-5β-cholanoic acid, murideoxycholic acid, MDCA) is also highly hydrophilic and prevents gallstone formation in the prairie dog (18). The metabolic fate of this epimer in humans has apparently not been studied; however, it is probably unsuitable for gallstone dissolution since oral administration to prairie dogs promotes the transformation of previously established cholesterol gallstones into bile salt stones which were radio-opaque when examined by low energy X-ray, and contained small proportions of calcium (19).

This report deals with the synthesis of methyl-substituted 3, 6-dihydroxy cholanoic acids, namely 3α, 6α-dihydroxy-6β-methyl-5β-cholanoic acid (6β-Me-HDCA) and 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid (6α-Me-MDCA). Certain biological properties of these new compounds, particularly their intestinal absorption and biliary secretion, were studied in the hamster.

MATERIALS AND METHODS

General

Melting points were determined on a Thermolyne melting point apparatus and are not corrected. Proton nuclear magnetic resonance (PMR) spectra, given in δ ppm, were measured in pyridine-d_5 solution on a Hitachi model R-40 spectrometer at 90 MHz using tetramethylsilane (0.00 ppm) as an internal standard. Radioactivity was measured using Aquasol-2 (NEN Products, Boston, MA) in a Beckman LS 3801 liquid scintillation system (Beckman Instruments, Fullerton, CA) with automatic quench correction.

Chromatography

Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} plates (0.2 mm thickness, EM Science, Darmstadt, West Germany) using a 10% ethanolic solution of phosphomolybdic acid to visualize the spots. Preparative TLC was done on precoated silica gel G plates (20 × 20 cm, 0.5 mm thickness, Analtech, Inc., Newark, DE). Silica gel 60 (35–70 mesh) from EM Science was used for column chromatography. Gas–liquid chromatography (GLC) was carried out on a Hewlett-Packard 5830A gas chromatograph using 3% SP-2250 and/or 3% SE-30 and/or 3% Poly I-110 columns on 80/100 gas chrom Q (Supelco, Bellefonte, PA). The bile acids were analyzed as their methyl ester-trimethylsilyl (TMS) ether derivatives. All retention times are reported relative to that of the TMS ether of methyl cholate (1.00). Gas-liquid chromatography-mass spectrometry (GLC-MS) was carried out on a Hewlett-Packard 5992B spectrometer under the following conditions: column, 3% SP-2250; column temperature, 260°C; injection port temperature, 265°C; source pressure, 2×10^{-6} torr; electron energy, 70 eV. For high pressure liquid chromatography (HPLC) we employed a Varian model 5000 liquid chromatograph equipped with a .variable wavelength UV detector (203 nm, Waters 481 spectrometer, Waters Associates, Milford, MA). The bile acid analogues were analyzed as their methyl ester derivatives. The analytical conditions were Radial Pak C_{18} cartridge (Waters Associates) in a Waters Z-module; solvent, methanol–water 8:2 (v/v); flow rate, 2 ml/min.

Synthesis of methyl 3α-hydroxy-6-oxo-5β-cholanoate

Methyl 3α-hydroxy-6-oxo-5β-cholanoate was prepared according to the method of Hoehn, Linsk, and Moffett (20). Methyl hyodeoxycholate (I, **Fig. 1**) (50 g) was dissolved in 70% acetic acid (400 ml), cooled to 0°C in an ice bath, and CrO_3 (12.5 g) in 70% acetic acid (100 ml) was added dropwise, into the solution. The reaction mixture was warmed to room temperature, stirred for 3 h, then poured into ice-cold water with stirring. A crystalline precipitate appeared when stirring was continued overnight. The precipitate was filtered and washed thoroughly with water until neutral. Repeated crystallization from benzene–hexane 1:1 (v/v) gave methyl 3α-hydroxy-6-oxo-5β-cholanoate (II) as colorless needles; 29.3 g, mp 142.0–143.0°C [lit. mp 144–146°C (20), 143–144°C (21), 141–142°C (22)], PMR 0.55 (3H, s, 18–CH_3), 0.81 (3H, s, 19–CH_3), 0.89(3H, d, J = 6Hz, 21–CH_3), 3.65 (3H, s, $COOCH_3$), 3.75 (1H, m, 3β–H).

Synthesis of 3α, 6α-dihydroxy-6β-methyl-5β-cholanoic acid (III) and 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid (IV)

The synthesis of the 6-methyl compounds from the oxo acid was carried out by the method of Une et al. (23): 6 g of methyl 3α-hydroxy-6-oxo-5β-cholanoate (II) was dissolved in 300 ml of THF and 30 ml of a 3.0 M ethereal solution of methyl magnesium iodide was added. After stirring for 1 min at room temperature, the reaction mixture was rapidly poured into acidified ice-cooled water (700 ml) and extracted with ether (500 ml × 2). The organic layer was washed with water, 10% $Na_2S_2O_3$, 5% $NaHCO_3$, and water until neutral. After drying over anhydrous Na_2SO_4, the solvents were evaporated in vacuo. The residue was dissolved in 50 ml of 7% methanolic KOH and refluxed for 2 h. Following dilution

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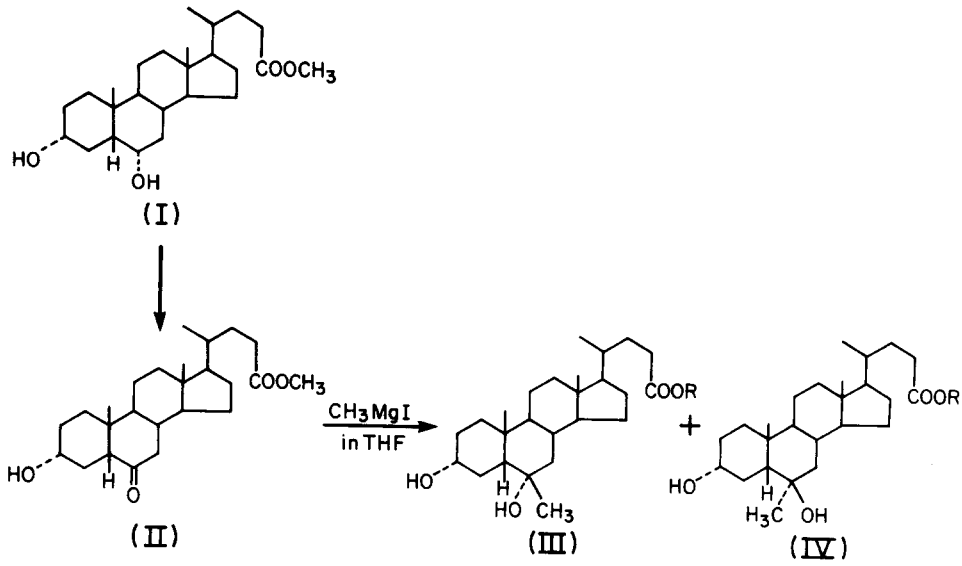

Fig. 1. Synthesis of bile acid analogues: I, hyodeoxycholic acid (HDCA); II, 3α-hydroxy-6-oxo-5β-cholanoic acid; III, 3α,6α-dihydroxy-6β-methyl-5β-cholanoic acid (6β-Me-HDCA); IV, 3α,6β-dihydroxy-6α-methyl-5β-cholanoic acid (6α-Me-MDCA).

with 10 vol of water, the neutral compounds were removed by extraction with ether. The acidic compounds were extracted with ethyl acetate after careful acidification with 1 N HCl to pH 2. The organic layer was washed with water until neutral, dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo. Treatment with freshly prepared diazomethane gave an oily residue of bile acid methyl esters (about 2 g) which was chromatographed on a silica gel column (200 g) using increasing proportions of acetone in benzene. Fifteen percent acetone in benzene eluted 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid as methyl ester (IV, 850 mg); the 6α-hydroxy isomer (III, 600 mg) was eluted with 30% acetone in benzene. Alkaline hydrolysis of the two isomers with 7% methanolic KOH, followed by standard extraction procedures (see above), afforded the corresponding free acids: 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid (IV, 6α-Me-MDCA), 710 mg, white solid from ethyl acetate, PMR 0.64 (3H, s, 18-CH_3), 0.92 (3H, d, J = 6Hz, 21-CH_3), 1.32 (3H, s, 19-CH_3), 1.42 (3H, s, 6α-CH_3), 3.50–3.98 (1H, m, 3β-H); 3α, 6α-dihydroxy-6β-methyl-5β-cholanoic acid (III, 6β-Me-HDCA), white crystals from ethyl acetate, 490 mg, mp 186.5–188.0°C, PMR 0.65 (3H, s, 18-CH_3), 0.93 (3H, d, J = 6Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 1.65 (3H, s, 6β-CH_3), 3.70–4.10 (1H, m, 3β-H).

Labeled compounds

[6β-Methyl-^{14}C]6β-Me-HDCA (sp act 7.8×10^5 dpm/mg) and [6α-methyl-[^{14}C]6α-Me-MDCA (sp act 8.0×10^5 dpm/mg) were prepared as described above using [^{14}C]CH_3MgI (prepared from [^{14}C]methyl iodide, 1.0 mCi, sp act 13.2 μCi/μmol, NEN Products). Radiochemical purity of the labeled compounds was better than 96% determined by radio-TLC. Each labeled compound was dissolved in an aqueous 1% $NaHCO_3$ solution (concentration 3 mg/ml).

Animal experiments

Male golden Syrian hamsters (Charles River Breeding Labs, Wilmington, MA) weighing 124 ± 6 g were maintained for at least 2 weeks on a commercial hamster chow. The animals were given food and water ad libitum, and kept under a controlled light/dark cycle. All animals were operated on between 9 and 10 AM. The animals were anesthetized by intramuscular injection of ketamine (Ketaset, Bristol Labs, Syracuse, NY) with a dose of 10 mg/100 g body weight, and diazepam (LyphoMed Inc., Rosemont, IL) at a dose of 1 mg/animal (24). Diethyl ether was used to maintain anesthesia in combination with intramuscular injections of ketamine (5–10 mg/dose) as required.

A polyethylene catheter (PE-10, 0.28 mm ID and 0.61 mm OD, Clay-Adams, Parsippany, NJ) was inserted into the left femoral vein and 0.9% NaCl solution was infused at a rate of 1.1 ml/h using a Harvard syringe pump (Harvard Apparatus, Millis, MA). The abdomen was opened by a midline incision, the cystic duct was ligated with a plastic clip (Absolok MCA, Ethicon Inc., Somerville, NJ), and an external biliary fistula was constructed using PE-10 polyethylene tubing. The urethra was ligated to allow urine to accumulate in the bladder.

In the intravenous infusion studies, saline was infused into the femoral vein for 40 min prior to the administration of labeled compounds. Each labeled compound was

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then infused for 20 min at a dose of 50 μg/min (total amount administered, 1 mg) (25); saline was then again infused until the end of the experiment. Bile samples were collected in weighed tubes every 20 min for a total period of 3.5 h. At the end of the infusion, blood and urine were obtained by cardiac puncture and by aspiration of the urinary bladder, respectively, and the liver was excised. All biological specimens were stored at −20°C. In control experiments, saline was infused throughout the experimental period. In intraduodenal injection experiments, after cannulation of both the bile duct and femoral vein as described above, the labeled compound (500 μg) was injected as a bolus into the duodenum. Bile samples were collected every 20 min for 2.5 h.

Analytical techniques

In both metabolic experiments, bile samples were collected every 20 min. Four aliquots were taken from each bile sample to determine the metabolic fate of the administered labeled compounds as follows: *1*) The radioactivity of the first set of aliquots was determined by liquid scintillation counting. *2*) The second set of aliquots was analyzed by TLC to check the conjugation pattern using solvent system C (ethyl acetate–acetic acid–water 7:2:1, v/v/v). Four μl of bile was applied directly on a TLC plate along with reference compounds. After development (17 cm) and detection of the spots, each TLC plate was cut into 5-mm segments from the origin to the solvent front and the radioactivity of each segment was measured as described previously (25). *3*) The third set of aliquots was taken from bile collected during the fourth collection period (60 to 80 min) and contained the highest radioactivity in each animal experiment; these samples were hydrolyzed enzymatically (25). The bile salts were extracted and analyzed by TLC using solvent system D (chloroform–ethyl acetate–acetic acid 9:9:1, v/v/v). Radioactivity was determined as described above. To confirm biliary bile acid composition, enzymatically hydrolyzed bile salts were also applied on preparative silica gel G plates and developed in solvent system D. After development, the plates were air-dried, exposed to iodine vapor, and the pertinent bands that contained radioactivity were scraped off. Bile acids were recovered by extraction with methanol and analyzed by GLC. *4*) A fourth aliquot (10 μl from each bile sample) was analyzed for bile acids by GLC (as methyl ester-TMS ether derivatives after enzymatic hydrolysis). Relative retention times were deoxycholic acid (DCA) 1.07; CDCA 1.07; cholic acid (CA) 1.00; 6α-Me-MDCA 1.31; 6β-Me-HDCA 1.42 on SP-2250; and DCA 1.28; CDCA 1.42; CA 1.00; 6α-Me-MDCA 2.01; 6β-Me-HDCA 2.02 on Poly I-110.

Aliquots of urine and serum were checked for radioactivity. Bile salts in the liver were analyzed according to the method of Yanagisawa et al. (26) and the radioactivity was measured as described above.

Calculations

The numerical data are expressed as mean ± SEM.

RESULTS AND DISCUSSION

This report describes the synthesis and metabolism of two new bile acids: 3α,6α-dihydroxy-6β-methyl-5β-cholanoic acid and 3α, 6β-dihydroxy-6α-methyl-5β-cholanoic acid. These compounds were synthesized to investigate their biological properties, especially their effect on cholesterol and bile acid metabolism, intestinal absorption, and participation in the enterohepatic circulation.

Synthesis of 6β-Me-HDCA and 6α-Me-MDCA

Methyl 3α-hydroxy-6-oxo-5β-cholanoate was obtained according to the method of Hoehn et al. (20). This compound is known to isomerize to the allo (5α) acid with relative ease (27, 28). To confirm the 5β-configuration of the oxo-acid, a portion of this compound was treated under alkaline conditions using 7% methanolic KOH to obtain its 5α isomer (22). The methyl esters of the two isomers were separable by TLC (5β isomer, R_f 0.36, 5α isomer, R_f 0.40, solvent system: benzene–acetone 7:3) and by GLC (5β isomer, RRT 1.87, 5α isomer RRT 2.00; methyl ester-TMS ethers, column SP-2250). After treatment with $NaBH_4$ in methanol, compound II (Fig. 1), the 5β isomer, gave two compounds which were identified as HDCA and MDCA, using TLC, GLC, and GLC-MS. This established that the correct starting material was available for the Grignard reaction (see below).

We employed the "direct" Grignard method to introduce a methyl group at the 6-position of 3α-hydroxy-6-oxo-5β-cholanoate without protecting the carboxyl ester at C-24. In our earlier studies, these esters were protected via formation of the oxazoline derivatives, as was reported for the synthesis of 7-alkyl bile acid analogues (29, 30). The use of the direct Grignard reaction offers several advantages. First, formation of oxazoline derivatives requires several synthetic steps, some of which result in only moderate yields (20–30%). Second, the conditions used for the synthetic reactions to form the oxazoline ring system (30) might conceivably isomerize the 6-oxo-5β-cholanoic acid to the 5α-acid (27, 28). Consequently, we considered it undesirable to use the oxazoline synthetic sequence in the synthesis of the 6-methyl bile acid analogues. We used tetrahydrofuran as a solvent instead of ether, as reported previously (23). When the Grignard reaction was performed in ether, only one isomer, namely the 6α-methyl compound, was obtained and the yield was less than 5%. However, using tetrahydrofuran, both the 6α and 6β isomers could be obtained and the yields were greater than those obtained with ether as the solvent. One

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undesirable side reaction of the "direct" Grignard reaction was the formation of several neutral products derived from the reaction between Grignard reagent and carboxylic ester (23). These products were formed even though the reaction was carried out for only 1 min at room temperature. When the reaction time was extended, the neutral bile alcohols were formed in larger amounts. This phenomenon might be ascribed to the reactivity difference between the carboxylic ester at C-24 and the keto function at C-6. The neutral reaction products, probably 6α-hydroxy-methyl-24-nor-5β-cholestane-3α, 6β, 25-triol, its 6α-isomer, and 3α, 25-dihdroxy-24-nor-5β-cholestan-6-one, were separated from the bile acid analogues by solvent extraction after mild alkaline hydrolysis. The ratio of acidic compounds and neutral compounds was about 1:2 in the case of a 1-min reaction. We considered, however, that the direct Grignard reaction was useful for the synthesis of 6-methyl compounds because of its simplicity and the reduced possibility of allomerization compared with the conventional oxazoline method. 6β-Me-HDCA and 6α-Me-MDCA were subsequently purified as their methyl ester derivatives on silica gel columns after esterification of the free acids with diazomethane.

The configurations of the new bile acid analogues at C-6 were determined by PMR. Additional characterization was made using TLC, GLC, HPLC (**Table 1**), and GLC–MS (**Table 2**). The chemical shift of the methyl protons at C-19 of 6α-Me-MDCA (1.32 ppm) shifted to a lower magnetic field compared with that of the 6β-Me-HDCA (1.02 ppm) (see Materials and Methods); a similar shift was observed in the spectra of MDCA (1.36 ppm) and HDCA (0.92 ppm) obtained under the same conditions.

TLC showed that 6β-Me-HDCA (containing a 6α-hydroxyl group) had R_f values similar to those of HDCA using two different solvent systems. In contrast, 6α-Me-MDCA had R_f values similar to but not identical with MDCA. GLC analysis showed that the methyl ester-TMS ether derivatives of these 6-methyl compounds had longer retention times on two different columns than the corresponding C_{24} bile acids (Table 1).

TABLE 1. R_f values (TLC) and relative retention times (GLC and HPLC) of bile acid analogues

	Compound			
	HDCA	III	MDCA	IV
TLC[a]				
A	0.19	0.19	0.25	0.35
B	0.11	0.10	0.21	0.31
GLC[b]				
SP-2250	1.12	1.42	1.04	1.31
SE-30	1.02	1.34	0.99	1.29
HPLC[c]				
RP-C_{18}	1.03	0.69	0.72	0.69

For experimental conditions see text. The roman numerals (III, IV) refer to bile acids: III, 6β-Me-HDCA; IV, 6α-Me-MDCA (see Fig. 1).

[a]Solvent systems: A (free acids), isooctane–ethyl acetate–acetic acid 5:5:1 (v/v/v); B (methyl esters), benzene–acetone 7:3 (v/v).

[b]Retention times relative to methyl ester-TMS ether derivative of cholic acid (RRT 1.00).

[c]Retention times relative to methyl cholate (RRT 1.00, RT 8.56 min).

The mass spectra of the 6-methyl compounds (Table 2) were almost identical and showed molecular ions at *m/z* 564. Both spectra exhibited base peaks at *m/z* 337. Elliott (31) reported that the ion at *m/z* 323 is common to HDCA and MDCA. A probable origin of ion *m/z* 323 in the spectra of 3,6-bis-TMS ethers was believed to arise by cleavage of the C-5, 6, C-8, 9, and C-12, 13 bonds (32). The ion at *m/z* 323 contains the side chain plus D-ring with the attached C-18, C-8, C-7, and C-6 with the trimethylsiloxy group (32). The presence of the ion at *m/z* 337 in the 6-methylated bile acids (323 + methyl group) supports the fragmentation described above. All other fragment ions were consistent with the structures of dihydroxy cholanoic acids possessing a methyl group in the nucleus.

HPLC, using a reversed phase μBondapak radial compression column, showed that both bile acid analogues had similar elution volumes. It should be mentioned that both compounds had lower elution volumes than either HDCA or MDCA, and therefore seem to be more hydrophilic than the corresponding compounds without a 6-methyl grroup. A similar observation was made in the case of 7-methyl-ursocholic acid (30). Both 6-methyl bile acids seem to be more hydrophilic than UDCA (RRT of methyl ester = 0.74).

To confirm that both 6-methyl bile acid analogues have the 5β configuration, we prepared the 5α(allo) 6-methyl bile acid analogues for comparison. 3α-Hydroxy-6-oxo-allocholanoic acid was obtained as described above (22) and treated with the Grignard reagent in the same manner as described for the 5β isomers. In the allo series, we obtained only one isomer, as was observed when 7-alkylated 5β-cholanoic acids were synthesized by the direct Grignard method (23). Purification by silica gel column chromatography afforded the methyl ester of a dihydroxy 6-methyl allo bile acid, PMR 0.66 (3H, s, 18-CH_3), 0.92(3H, d, J = 6Hz, 21-CH_3), 1.33(3H, s, 19-CH_3), 1.35(3H, s, 6-CH_3), 3.64(3H, s, $COOCH_3$), 4.38(1H, m, 3β-H). Chromatographic data for the allo compound were as follows: TLC, R_f value = 0.30, methyl ester, solvent system B, R_f value of free acid 0.35, solvent system A; GLC, RRT = 1.24 on SP-2250, RRT = 1.22 on SE-30 as the methyl ester-TMS ether derivative. The mass spectrum of the methyl ester-TMS ether derivative of this compound exhibited fragments at *m/z* 564, M^+ [2.3]; 549, M–15 [71.2]; 474, M–90 [6.4]; 459, M–(90 + 15) [100.0]; 443, M–(90 + 31) [3.6]; 384, M–2 × 90 [9.9]; 369, M–(2 × 90 + 15) [4.4]; 359, M–(90 + side chain) [0.3]; 353, M–(2 × 90 + 31) [3.1]; 337, fragment A [60.3]; 269, M–(2 × 90 + side chain) [2.1]; 263, M–(ring A + C6) [1.4].

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TABLE 2. Relative intensities of fragmentation ions of synthetic bile acid analogues and corresponding naturally occurring bile acids[a]

Ion	m/z	HDCA	MDCA	m/z	III	IV
M^+	550	1.9	0.7	564	13.3	19.3
M-15	535	1.1	2.4	549	1.6	6.2
M-90	460	14.9	100.0	474	0.5	1.1
M-(90 + 15)	445	5.9	12.1	459	1.1	4.3
M-(90 + 31)	429	2.4	5.1	443	0.4	0.8
M-Cl-4	405	10.1	79.8	419	0.1	0.3
M-2 × 90	370	100.0	82.2	384	2.5	5.0
M-(2 × 90 + 15)	355	25.0	22.6	369	1.3	1.7
M-(90 + side chain)	345	7.9	3.4	359	0.1	0.3
M-(2 × 90 + 31)	339	5.4	6.4	353	1.5	1.9
Fragment A	323	14.4	33.3	337	100.0	100.0
M-(2 × 90 + side chain)	255	37.6	41.1	269	0.9	1.4
M-(ring A + C6)	263	16.1	8.1	263	1.2	1.4
M-(2 × 90 + side chain + C15-17 + H) and/or M-(2 × 90 + side chain + C16-17 + methyl)	213	26.6	48.8	227	1.0	1.1

[a]Analyzed as their methyl ester-TMS ether derivatives. The roman numerals (III, IV) refer to footnote to Table 1. Fragment A: side chain + D-ring + C18, C6-8 with the O-TMS group; see text.

The relatively low intensity of the fragment ion at *m/z* 337, which was the base peak in both 6-methyl compounds with the 5β configuration, suggested that the cleavage between C-5 and C-6 was less intense in the 6-methyl compound with the allo configuration.

Metabolism of 6β-Me-HDCA and 6α-Me-MDCA in the hamster

Radioactive 6-methyl compounds were prepared using [^{14}C]CH_3MgI in order to estimate whether the newly synthesized bile acid analogues are extracted efficiently by the liver and secreted into the bile of the hamster. The recovery of the labeled compounds administered intravenously is summarized in **Table 3.** In the case of 6β-Me-HDCA, 85.0% was recovered in bile; for 6α-Me-MDCA, recovery was as much as 96.9%. Little radioactivity was excreted into the urine with either compound. **Fig. 2** shows the cumulative biliary excretion of radioactivity after the intravenous infusion of the labeled analogues. Both 6-methyl compounds rapidly appeared in bile; 6β-Me-HDCA was secreted into bile somewhat faster than 6α-Me-MDCA. Both analogues were conjugated with both glycine and taurine during a single pass through the liver (**Fig. 3**, upper panel). While 6β-Me-HDCA was almost completely amidated, 16.7% of infused 6α-Me-MDCA was secreted in the unconjugated form (**Table 4**).

Cholylglycine hydrolase treatment of the bile samples followed by radio-TLC revealed that both analogues gave a single peak of radioactivity with R_f values identical with those of unconjugated 6β-Me-HDCA or 6α-Me-MDCA (Fig. 3, lower panel). Preparative TLC followed by the GLC analysis confirmed that both analogues gave a single peak with RRT values identical with those of the infused 6-methyl compounds. These data indicate that after a single pass through the liver there was no label associated with compounds having the mobility of glucuronides or sulfates.

The biliary bile acid composition of fistula bile is shown in **Table 5**. During the infusion of 6β-Me-HDCA (time 40–60 min) and the following 20-min period (time 60–80 min), the proportions of the infused compound in bile increased to about 40–50% of total bile acids and exceeded that of CA. The rate of appearance of 6α-Me-MDCA in bile was somewhat different. At the start of bile acid infu-

TABLE 3. Recovery of radiolabel after intravenous infusion of ^{14}C-labeled bile acid analogues

Bile Acid Infused	No. of Animals	Isotopic Recovery[a] (%)				
		Bile	Urine	Liver	Blood	Total
6β-Me-HDCA	3	85.0 ± 4.0	0.0 ± 0.0	1.9 ± 1.2	1.1 ± 0.3	88.0 ± 2.9
6α-Me-MDCA	3	96.9 ± 1.1	0.1 ± 0.0	0.8 ± 0.3	0.2 ± 0.0	98.0 ± 0.8

^{14}C-Labeled compounds were infused intravenously at a dose of 50 μg/min for 20 min. Bile was collected for 3.5 h, and at the end of the experiment urine, liver, and blood samples were obtained to determine their radioactivity.
[a]The values are expressed as mean ± SEM.

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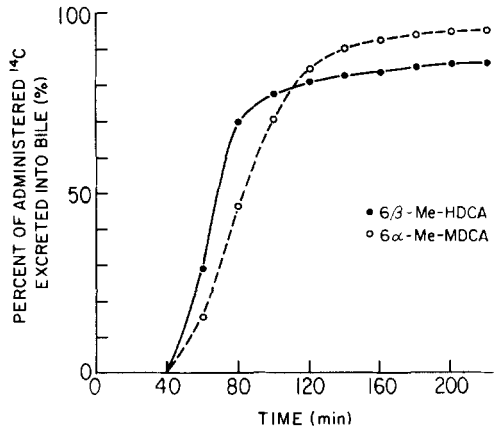

Fig. 2. Biliary secretion of radioactivity in bile fistula hamsters after intravenous infusion of ^{14}C-labeled 6β-Me-HDCA and 6α-Me-MDCA. After a 40-min control period, the bile acids were infused at a dose of 50 μg/min for 20 min. Each value represents the average of three male hamsters.

sion, 6α-Me-MDCA accounted for 18.1% of total bile acid during the first period (time 40–60 min). During the following 60 min this analogue continued to be a major biliary component but cholic acid predominated. This difference between the α-methyl and β-methyl isomers may be ascribed to differences in hepatic uptake, transport, or secretion. Though dose-response experiments and long-term feeding studies were not carried out, it seems probable that the bile acid analogues have the properties to become major biliary constituents. **Fig. 4** shows the biliary bile acid concentration during bile collection. Administration of 6β-Me-HDCA seemed to increase the total bile acid concentration when compared with the controls. On the other hand, no significant change was observed in the case of 6α-Me-MDCA. However, it cannot be concluded that the isomeric bile acids have significantly different effects on bile acid secretion, because of the small number of animals used in this study.

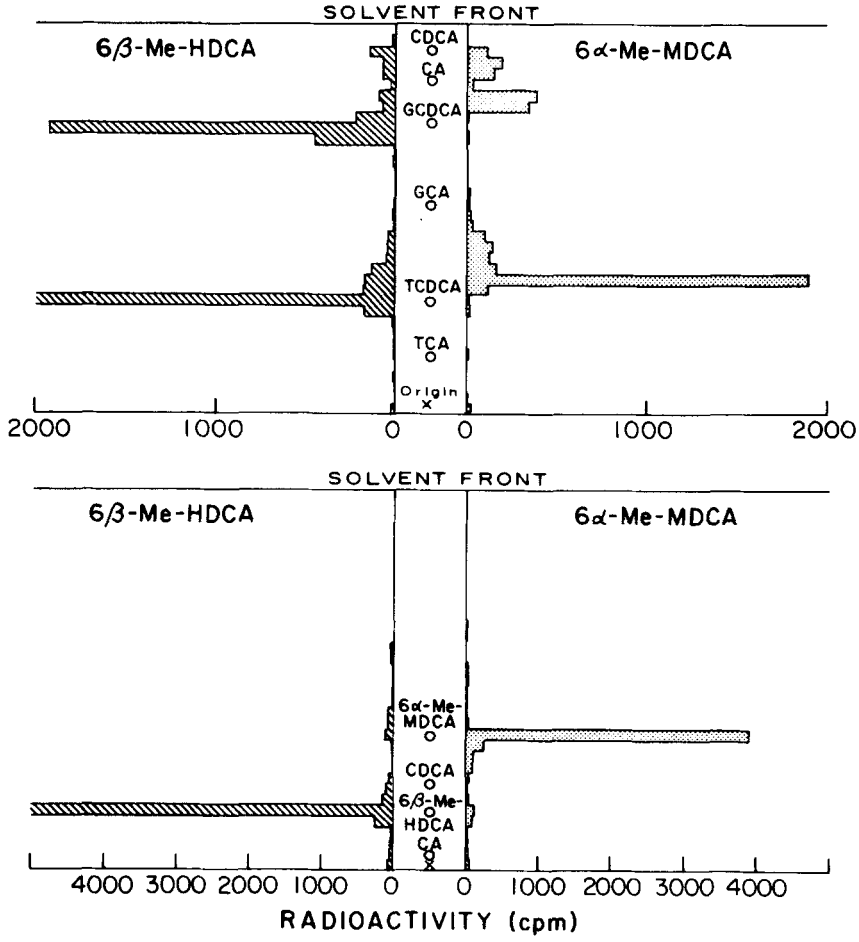

Fig. 3. Thin-layer chromatographic analysis of radioactivity in bile samples from bile fistula hamsters infused with labeled 6β-Me-HDCA (left) and 6α-Me-MDCA (right). Bile samples were analyzed by solvent system A (ethyl acetate–acetic acid–water 7:2:1, v/v/v) before hydrolysis (upper panel), or by solvent system B (chloroform–ethyl acetate–acetic acid 9:9:1, v/v/v) after enzymatic hydrolysis (lower panel). Reference compounds were as follows: taurocholate (TCA), taurochenodeoxycholate (TCDCA), glycocholate (GCA), glycochenodeoxycholate (GCDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), 6β-methyl-hyodeoxycholic acid (6β-Me-HDCA), and 6α-methyl-murideoxycholic acid (6α-Me-MDCA).

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TABLE 4. Conjugation profiles of intravenously infused bile acid analogues in bile fistula hamsters

Bile Acid Infused	No. of Animals	Conjugation Profile[a] (%)			
		Unconjugated	Glycine	Taurine	G/T Ratio
6β-Me-HDCA	3	3.6 ± 0.2	30.7 ± 6.8	57.7 ± 7.4	0.57 ± 0.18
6α-Me-MDCA	3	16.7 ± 5.1	15.9 ± 2.1	48.1 ± 5.8	0.33 ± 0.01

^{14}C-Labeled bile acid analogues were administered intravenously at a dose of 50 μg/min. Hepatic bile samples were analyzed by thin-layer chromatography using the solvent system: ethyl acetate–acetic acid–water 7:2:1 (v/v/v), followed by liquid scintillation counting.

[a]The values are expressed as mean ± SEM.

The intestinal absorption of the 6-methyl compounds was examined after intraduodenal administration of 500 μg of the labeled analogues. As shown in **Fig. 5**, both compounds appeared rapidly in bile and more than 85% was recovered within 2 h. This suggests that these compounds were absorbed efficiently from the intestine and secreted into the bile by the liver. The bile samples were analyzed by radio-TLC before and after enzymatic hydrolysis. Both of the administered compounds were amidated with glycine and taurine, as observed also in the intravenous infusion experiment depicted in Fig. 3; further biotransformations were not observed.

In man, HDCA is not likely to have cholelitholytic properties because it is glucuronidated (33), probably in the

TABLE 5. Biliary bile acid composition of bile fistula hamsters infused intravenously with labeled compounds

Bile Acid Infused	Collection Time	Bile Acid Composition[a] (%)				
		CA	CDCA	DCA	6β-Me-HDCA	6α-Me-MDCA
	min					
Control	0–20	62.1 ± 6.4	27.3 ± 4.5	9.7 ± 9.4		
	20–40	67.7 ± 9.8	23.9 ± 0.4	8.5 ± 9.5		
	40–60	68.2 ± 9.6	25.1 ± 2.8	6.7 ± 6.8		
	60–80	64.6 ± 7.8	26.4 ± 1.5	9.1 ± 9.3		
	80–100	64.5 ± 6.4	26.9 ± 2.2	8.7 ± 8.6		
	100–120	66.3 ± 4.6	25.5 ± 3.5	8.3 ± 8.1		
	120–140	67.7 ± 5.4	26.5 ± 0.0	5.8 ± 5.4		
	140–180	67.1 ± 8.1	26.3 ± 1.6	6.7 ± 6.6		
	180–220	73.4 ± 5.2	24.7 ± 4.5	1.9 ± 0.7		
6β-Me-HDCA	0–20	61.7 ± 3.6	36.7 ± 4.8	1.6 ± 2.0		
	20–40	62.0 ± 4.9	36.8 ± 6.2	1.3 ± 1.6		
	40–60	37.4 ± 8.8	22.4 ± 1.7	0.5 ± 0.6	39.8 ± 9.9	
	60–80	32.6 ± 5.0	19.7 ± 0.5	0.5 ± 0.6	47.2 ± 5.1	
	80–100	56.9 ± 3.1	30.0 ± 4.2	0.9 ± 1.1	12.2 ± 0.4	
	100–120	65.2 ± 4.5	29.1 ± 5.0	0.9 ± 1.1	4.9 ± 1.1	
	120–140	69.7 ± 4.0	27.3 ± 4.1	0.1 ± 0.2	2.9 ± 0.7	
	140–180	74.9 ± 1.6	22.3 ± 1.9	0.2 ± 0.3	2.7 ± 0.7	
	180–220[b]	70.2	28.4	0.0	1.3	
6α-Me-MDCA	0–20	64.6 ± 3.4	31.1 ± 4.6	4.3 ± 1.3		
	20–40	69.9 ± 3.1	26.9 ± 3.7	3.2 ± 0.9		
	40–60	60.5 ± 2.2	19.7 ± 2.0	1.8 ± 0.8		18.1 ± 1.9
	60–80	50.3 ± 1.6	15.1 ± 3.2	0.9 ± 0.6		33.8 ± 4.3
	80–100	49.9 ± 0.8	14.6 ± 3.6	0.6 ± 0.4		34.9 ± 4.3
	100–120	55.6 ± 1.7	16.3 ± 2.6	1.4 ± 0.8		26.8 ± 3.0
	120–140	65.6 ± 2.0	21.2 ± 2.9	0.6 ± 0.3		12.5 ± 2.9
	140–180	71.4 ± 1.9	21.7 ± 3.4	1.1 ± 0.9		5.8 ± 2.3
	180–220	75.6 ± 0.9	22.3 ± 1.1	0.5 ± 0.4		1.7 ± 0.7

After a 40-min control period (time 0–40 min), the ^{14}C-labeled bile acid analogues were infused at a dose of 50 μg/min for 20 min. In the control animals, saline was infused throughout the experiment. The bile acids were extracted after enzymatic hydrolysis, and were analyzed by GLC.

[a]The values are expressed as mean ± SEM.

[b]Bile samples obtained from one animal were analyzed.

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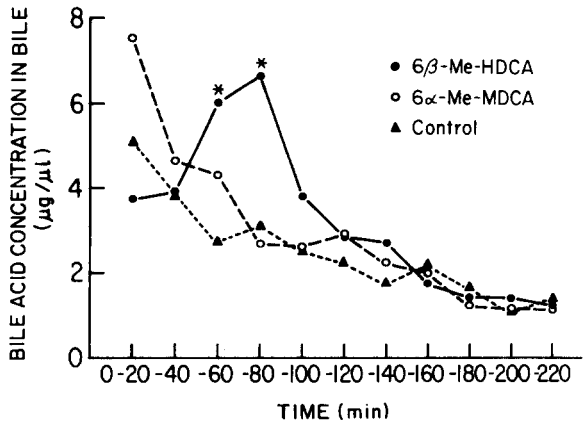

Fig. 4. Biliary bile acid concentration in bile fistula hamsters with or without intravenous infusion of 6β-Me-HDCA or 6α-Me-MDCA. After a 40-min control period, the bile acids were infused at a dose of 50 μg/min for 20 min. In the control animals, saline was infused throughout the experiment. Each value represents the average of three hamsters. *Differs from control group by *t*-test, $P<0.05$.

6α-position and rapidly excreted in the urine (34). Introduction of a methyl group at the 6-position of HDCA and MDCA will result in steric hindrance so that glucuronidation of the 6-hydroxyl may be prevented. We hypothesize that in humans (as in the hamster) 6-methyl-substituted HDCA and MDCA will be retained in the enterohepatic circulation and may then exhibit cholelitholytic properties.

In summary, we synthesized and characterized the 6-methyl derivatives of HDCA and MDCA, namely 6β-Me-HDCA and 6α-Me-MDCA. These compounds were absorbed from the intestine, extracted effectively by the liver, and secreted into the bile. It seems likely that these

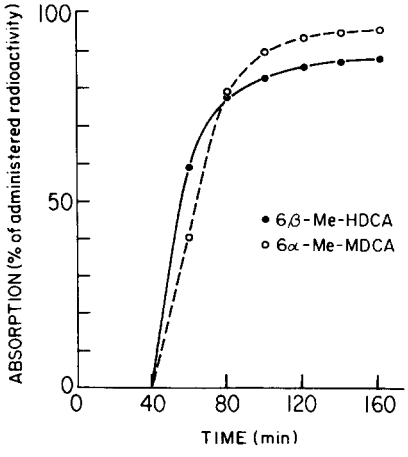

Fig. 5. Biliary secretion of radioactivity in bile fistula hamsters after intraduodenal administration of ^{14}C-labeled 6β-Me-HDCA and 6α-Me-MDCA. A bolus (0.5 mg) of each compound was given after a 40-min control period. Each value represents the average of three male hamsters.

bile acid analogues will undergo efficient enterohepatic cycling. Therefore, the new compounds are of interest as potential cholelitholytic agents, and their effects on cholelithiasis and cholesterol/bile acid metabolism in animal models seem indicated.

This work was supported in part by U.S. Public Health Service grant HL-24061 from the National Heart, Lung, and Blood Institute. We wish to thank Dr. Khalida Hakam for her expert technical assistance and helpful advice.

Manuscript received 28 September 1988 and in revised form 7 February 1989.

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