New bile acid analogs: 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid, 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid, and 3α-hydroxy-7ξ-methyl-5β-cholanoic acid

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Abstract Methods are described for the chemical synthesis of three new bile acid analogs, namely, 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid, 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid and 3α-hydroxy-7ξ-methyl-5β-cholanoic acid. The starting material, 2-(3α,7ξ-dihydroxy-7ξ-methyl-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline upon mild hydrolysis in aqueous HCl yields the two epimeric 3α,7-dihydroxy-7-methyl-5β-cholanoic acids which can be separated as the methyl esters by silica gel column chromatography. More rigorous hydrolysis in boiling methanolic HCl yields a mixture of unsaturated compounds which can be separated as their methyl esters into three fractions by silica gel-$AgNO_3$ column chromatography. The fractions containing 3α-hydroxy-7-methyl-5β-chol-6-enoic acid and 3α-hydroxy-7-methylene-5β-cholanoic acid when subjected to catalytic hydrogenation yield 3α-hydroxy-7ξ-methyl-5β-cholanoic acid.—**Une, M., B. I. Cohen, and E. H. Mosbach.** New bile acid analogs: 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid, 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid, and 3α-hydroxy-7ξ-methyl-5β-cholanoic acid. *J. Lipid Res.* 1984. **25:** 407–410.

Supplementary key words 2(-3α,7ξ-dihydroxy-7ξ-methyl-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline • 3α-hydroxy-7-methyl-5β-chol-6-enoic acid • 3α-hydroxy-7-methylene-5β-cholanoic acid

Certain dihydroxy bile acids, such as chenodeoxycholic acid (CDA) and ursodeoxycholic acid (UDA), have shown promise as gallstone-dissolving drugs in man (1, 2). However, there exists a need to produce drugs with greater efficacy and a reduced potential for hepatotoxicity (3). It is assumed that CDA is more toxic than UDA because the former is modified to a greater extent than the latter by intestinal bacteria (7-dehydroxylation), resulting in the formation of lithocholic acid (LA) (4). This monohydroxy bile acid is more toxic than the polyhydroxy bile acids, probably because it is hydrophobic, resembles cholesterol structurally and interacts unfavorably with hepatic membranes (5).

We postulated that analogs of CDA or UDA, which possess an alkyl group in the 7-position [3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid (7-Me-CDA) and 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid (7-Me-UDA)] for example, might be more resistant to modification by intestinal bacteria yet dissolve gallstones more effectively (3, 6). In addition, it seemed possible that LA with a methyl group in the 7-position [3α-hydroxy-7ξ-methyl-5β-cholanoic acid (7-Me-LA)] might also be active, at least as a 7-dehydroxylation inhibitor, yet might be less toxic to the hepatocyte than LA.

To investigate this hypothesis, 7-Me-CDA, 7-Me-UDA, and 7-Me-LA were prepared, thus making possible future studies of their effects on sterol and bile acid metabolism in animal models of cholesterol cholelithiasis and to estimate their hepatotoxicity.

METHODS

Melting points were determined on a Thermolyne melting point apparatus and are uncorrected. ^{1}H-Nuclear magnetic resonance (PMR) was measured at 100 MHz on a Varian XL-100. Chemical shifts (δ) are given in ppm downfield relative to tetramethylsilane as internal standard. Thin-layer chromatography (TLC) was done on 250-μm silica gel G plates obtained from Analtech, Inc., Newark, DE. The plates were sprayed with phosphomolybdic acid (Brinkmann Instruments, Inc., Westbury, NY) and heated at 150°C for 5 min to visualize the organic

Abbreviations: CDA, chenodeoxycholic acid; UDA, ursodeoxycholic acid; LA, lithocholic acid; 7-Me-CDA, 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid; 7-Me-UDA, 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid; 7-Me-LA, 3α-hydroxy-7ξ-methyl-5β-cholanoic acid; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; GLC, gas–liquid chromatography; GLC-MS, gas–liquid chromatography–mass spectrometry

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compounds. Silica gel 60 (35–70 mesh ASTM) from E. Merck, Darmstadt, Germany, was used for column chromatography. High-pressure liquid chromatography (HPLC) was carried out with a model 5000 liquid chromatograph (Varian Associates, Palo Alto, CA). The analytical conditions were: μBondapak/C_{18} column in a Waters Z module; solvents, methanol–water 9:1 or 8:2 (v/v); flow rate, 3 ml/min; detector, Varichrom 50 (Varian Associates, Palo Alto, CA), 195 nm.

Gas chromatography (GLC) was performed on a Hewlett-Packard 5830A gas chromatograph using an SE-30 column. Gas–liquid chromatography–mass spectrometry (GLC–MS) was carried out on a Hewlett-Packard 5992B spectrometer (column 3% SP2250, oven temp 260°C; injector temp 265°C; source pressure 2×10^{-6} torr). Trimethylsilyl ether derivatives of the bile acid methyl esters were prepared using Sil-Prep (Applied Science Laboratories, State College, PA).

RESULTS

2-(3α,7ξ-Dihydroxy-7ξ-methyl-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline (III, ***Fig. 1****)* was prepared from the 7-keto-lithocholic acid derivative (II) by the method previously reported from this laboratory (7).

3α,7β-Dihydroxy-7α-methyl-5β-cholanoic acid (IV) and 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid (V, Fig. 1). 2-(3α,7ξ-Dihydroxy-7ξ-methyl-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline (III) (2 g) was dissolved in 0.1 N HCl (500 ml/g) and allowed to stand at 37°C for 4 days. The solution was filtered and the precipitate obtained was dissolved in ethyl acetate. The ethyl acetate was washed with water until neutral and then it was evaporated. The residue (700 mg) was esterified with diazomethane and chromatographed on a 300-g silica gel column using increasing proportions of acetone in benzene. The 7β-hydroxy isomer was eluted with 12.5% acetone in benzene, and 15% acetone in benzene yielded the 7α-hydroxy isomer. Alkaline hydrolysis of each isomer provided the free acids, *3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid* (IV), 150 mg, mp 102.5–105°C from methanol–water; PMR (δ ppm): 0.77 (3H, s, 18-CH_3), 1.06 (3H, d, J = 6Hz, 21-CH_3), 1.09 (3H, s, 19-CH_3), 1.40 (3H, s, 7α-CH_3), 3.62 (1H, m, 3β-H); and *3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid* (V), 350 mg, mp 95–98°C from methanol–water; PMR (δ ppm): 0.79 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.05 (3H, d, J = 6Hz, 21-CH_3), 1.32 (3H, s, 7β-CH_3), 3.56 (1H, m, 3β-H).

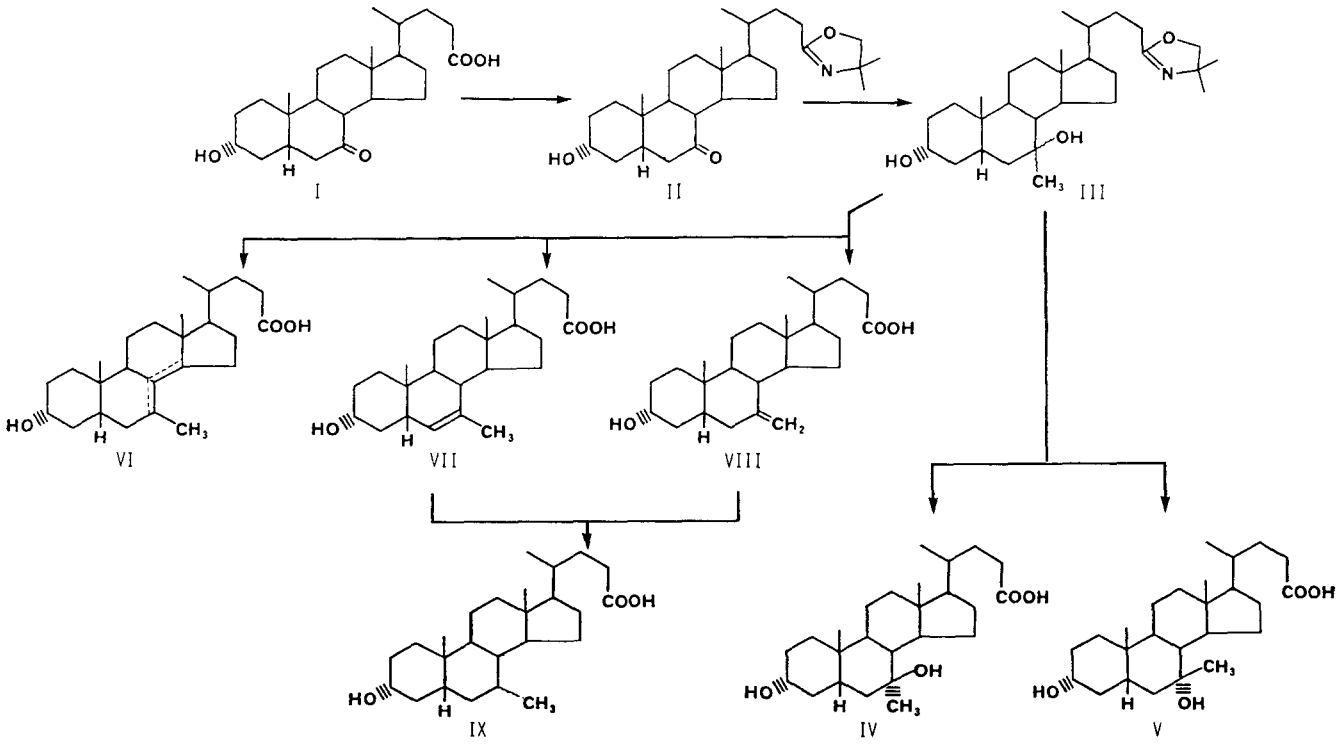

Fig. 1. Synthesis of bile acid analogs. I, 3α-Hydroxy-7-keto-5β-cholanoic acid; II, 2-(3α-hydroxy-7-keto-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline; III, 2-(3α,7ξ-dihydroxy-7ξ-methyl-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline; IV, 3α,7β-dihydroxy-7α-methyl-5β-cholanoic acid; V, 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid; VI, unidentified, possibly a mixture of 3α-hydroxy-7-methyl-5β-chol-7-enoic and 3α-hydroxy-7ξ-methyl-5β-chol-8(14)-enoic acid; VII, 3α-hydroxy-7-methyl-5β-chol-6-enoic acid; VIII, 3α-hydroxy-7-methylene-5β-cholanoic acid; IX, 3α-hydroxy-7ξ-methyl-5β-cholanoic acid.

3α-Hydroxy-7ξ-methyl-5β-cholanoic acid (IX, Fig. 1). 2-(3α,7ξ-Dihydroxy-7ξ-methyl-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline (III), 10 g, was refluxed in 200 ml of 3 N methanolic HCl and 1 ml of water for 6 hr. The reaction mixture was poured into water and extracted with ethyl ether. Evaporation of the solvent left 6 g of a mixture containing unsaturated products. These dehydration products were converted to the methyl esters with diazomethane and chromatographed on a 350-g silica gel column impregnated with 35 g of $AgNO_3$. Elution with increasing proportions of acetone in chloroform yielded three major fractions. Fraction 1, eluted with 1% acetone in chloroform, gave 2.1 g of an amorphous white solid. It was not possible to obtain a definitive PMR spectrum of this material; it apparently consisted of a mixture of cholanoic acids, predominantly unsaturated at the 7 and 8(14) positions. Since it was possible neither to separate this mixture into pure compounds nor to hydrogenate the double bonds to completion, this fraction was discarded.

Fraction 2, eluted with 2% acetone in chloroform, after alkaline hydrolysis, yielded 3 g of 3α-hydroxy-7-methyl-5β-chol-6-enoic acid (VII), mp 121–122°C from methanol. PMR (δ ppm), 0.82 (3H, s, 18-CH_3), 0.89 (3H, s, 19-CH_3), 1.06 (3H, d, J = 6Hz, 21-CH_3), 1.77 (3H, s, 7-CH_3), 3.64 (1H, m, 3β-H), 5.42 (1H, d, J = 4Hz, 6-H).

Fraction 3, likewise eluted with 2% acetone in chloroform, after alkaline hydrolysis, yielded 1.1 g of 3α-hydroxy-7-methylene-5β-cholanoic acid (VIII), mp 104–105°C from methanol. PMR (δ ppm), 0.77 (3H, s, 18-CH_3), 1.05 (3H, d, J = 6Hz, 21-CH_3), 1.14 (3H, s, 19-CH_3), 3.70 (1H, m, 3β-H), 4.72 and 4.76 (2H, m, C=CH_2).

Compounds VII and VIII (2 g) were dissolved in ethyl acetate and subjected to catalytic hydrogenation with 200 mg of PtO_2, yielding 2 g of 3α-hydroxy-7ξ-methyl-5β-cholanoic acid (IX) with a ratio of 7α- to 7β-methyl isomer approximately 1:1 (see **Table 1**). Crystallization from methanol yielded a white solid, mp 98–103°C. Recrystallization produced changes of mp and of the epimeric ratio. PMR (δ ppm), 0.75 (3H, s, 18-CH_3), 0.98, 1.00 (3H, d, J = 6Hz, 7α- and 7β-CH_3), 1.03 (3H, s, 19-CH_3), 1.03 (3H, d, J = 6Hz, 21-CH_3), 3.60 (1H, m, 3β-H).

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

Compound	IV	V	VI	VII	VIII	IX
TLC[a]						
A-1	0.33[b]	0.40				0.52
N-1	0.35[c]	0.29	0.73	0.73	0.73	0.73
N-2			0.73	0.60	0.50	0.75
GLC[d]						
(SE-30)	1.30[e]	1.13	0.82	0.84	0.92	0.90[f]

For experimental conditions see text. The Roman numerals (IV–IX) refer to the bile acid analogs described in Fig. 1.

[a] Solvent systems: A-1 (free acids), isooctane–isopropanol–acetic acid 30:10:1 (v/v/v); N-1 (methyl esters), benzene–acetone 80:20 (v/v); N-2 (methyl esters), chloroform–acetone 90:10 (v/v) (silica gel G impregnated with 10% $AgNO_3$).

[b] In solvent system A-1: R_f of UDA = 0.31; CDA = 0.38.

[c] In solvent system N-1: R_f of UDA = 0.33; CDA = 0.28.

[d] Relative to methyl cholate-TMS derivative (1.00).

[e] Relative retention times of UDA = 1.14; CDA = 0.97.

[f] Occasionally two peaks (RRT 0.86 and 0.94) were observed.

DISCUSSION

These experiments were carried out because the introduction of a 7-methyl group in CDA or UDA may produce compounds with improved stability to bacterial dehydroxylation. 7-Methyl-LA possesses no 7-hydroxy group and would be expected to be unaffected by bacterial 7-dehydroxylases. Availability of these compounds now makes it possible to evaluate their biological stability and hepatotoxicity, and to find out whether the molecular modification of the bile acid molecule at C-7 produces changes in its cholelitholytic potential.

In order to introduce a 7-methyl group into CDA or UDA, the carboxyl group of the starting material 7-keto-LA (I) must first be protected by formation of the oxazoline derivative (II). A Grignard reaction with methylmagnesium iodide then introduces the methyl group into the 7-position. Apparently, the formation of the 7β-methyl derivative is favored stereochemically but both 7α- and 7β-methyl epimers are produced. By selecting appropriate conditions, the intermediate 2-[3α,7ξ-dihydroxy-7ξ-methyl-24-nor-5β-cholanyl]-4,4-dimethyl-2-oxazoline (III) can be used for the production of the 7-methyl-3,7-dihydroxy bile acids (IV and V) and of 7-methyl-LA (IX). Thus, hydrolysis in dilute aqueous acid at 37°C produces 7α-methyl-UDA (IV) and 7β-methyl-CDA (V) which can be separated as their methyl esters by column chromatography. More rigorous acid hydrolysis in methanol under reflux removed the oxazoline moiety and simultaneously produced a mixture of unsaturated compounds (VI–VIII) which could be used to obtain 7ξ-methyl-lithocholic acid (IX).

The behavior of the bile acid analogs upon TLC, GLC, and GLC–MS is illustrated in Table 1 and **Table 2.** Upon TLC (Table 1) the two 7-methyl bile acids had R_f values similar to the corresponding dihydroxy bile acids (CDA and UDA) using two different solvent systems. The mass spectra of the 7-methyl compounds (Table 2) were almost identical and showed a major fragment ion at m/z 257 (see Table 2), strongly suggesting the presence of the 3,7-dihydroxyl configuration plus the 7-methyl group. This is analogous to the fragment m/z 243 observed for CDA and UDA (8). It is well known that UDA, during

TABLE 2. Relative intensities of fragmentation ions of synthetic bile acid analogs

Ion	m/z	IV	V	m/z	VI	VII	VIII	m/z	IX
M^+	564	8	8	474	7	1	1	476	15
M-15	549	7	13	459	2	2	3	461	7
M-90	474	2	3	384	100	100	100	386	100
M-(90 + 15)	459	2	5	369	38	9	41	371	33
M-180	384	4	12						
M-(Side chain + 90)	359			269	39	5	31	271	49
Fragment A[a]	257	100	100						

The methyl esters of the bile acid analogs were analyzed as the TMS ethers (see Methods section). The Roman numerals (IV–IX) refer to the bile acid analogs described in Fig. 1.

[a] Fragment A (m/z = 257) = C3–C7 + 2-OTMS + 7-CH_3 (see text).

HPLC with a reversed phase system, is eluted more rapidly than CDA and even precedes some trihydroxy bile acids such as cholic acid (9). This can be ascribed to the presence of the equatorial 7β-hydroxyl group in UDA (9). As might have been predicted, the mobility of compound IV (7β-OH) was greater than that of compound V (7α-OH): under the conditions employed (methanol–water 8:2), the 7α-OH isomer (V) was eluted at 9.83 min; the 7β-OH isomer (IV) was eluted at 4.24 min. These results confirm that compound IV possesses a 7β-hydroxyl group and its counterpart V, a 7α-hydroxyl group.

7ξ-Methyl lithocholic acid (IX) was obtained by hydrogenation of the two unsaturated compounds VII and VIII. The products of the reaction of compound III with 3 N methanolic HCl under reflux provided three unsaturated fractions which were postulated to be the Δ^7, Δ^6, and 7-methylene compounds. The separation of these three products was achieved by silica gel columns impregnated with $AgNO_3$. The mass fragmentation patterns of these compounds were very similar and exhibited a major fragment ion at m/z 384, suggesting the loss of one TMS group from the molecular ion. These three isomeric methyl esters were well resolved by HPLC with a reversed phase column (methanol–water 9:1), the compounds emerging in the order of VIII, VII, and VI, as well as by GLC and TLC (Table 1). The NMR spectrum of VII gave a signal for an olefinic proton at 5.42 ppm and a methyl group at 1.77 ppm, suggesting that the double bond was located at C6-C7. Similarly, it is suggested that compound VIII is 7-methylene-lithocholic acid on the basis of its NMR spectrum which gave signals for methylene at 4.72 ppm and 4.76 ppm. These two unsaturated compounds were easily hydrogenated with PtO_2 as catalysts.

The NMR spectrum of compound VI should have no olefinic proton signal but should show a methyl signal near 1.77 ppm like compound VII. However, it was not possible to obtain a definitive NMR spectrum of this fraction; apparently, it consisted of a mixture of unsaturated compounds, possibly the Δ^7 and $\Delta^{8,14}$ isomers (VI).

So far, attempts to separate the two 7-methyl isomers of the saturated compound were not successful although partial separation was obtained by GLC on SE-30 columns (Table 1). However, for preliminary biological studies it may be possible to employ this isomeric mixture.

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