Stereospecific synthesis and two-dimensional ^{1}H-NMR investigation of isoursocholic acid[1]

B. Dayal[2] and G. Salen

Department of Medicine, UMDNJ-New Jersey Medical School, Newark, NJ 07103, and Medical Service, Veterans Administration Medical Center, East Orange, NJ 07019

Summary This report describes the chemical synthesis of isoursocholic acid (3β,7β,12α-trihydroxy-5β-cholanoic acid) from its corresponding 3α-analog. The method consists of refluxing a mixture of ursocholic acid, triphenylphosphine, and diethyl azodicarboxylate in benzene solution with an acid such as formic acid. The sterically pure ester (3β-formate) so formed after saponification with LiOH–aqueous methanol then allowed an easy access to the epimer of the starting acid. Large scale preparative separation and purification of the final product and synthetic intermediates were accomplished by flash column chromatography of their methyl ester derivatives. Structural assignment of the isoursocholic acid molecule was confirmed by complete analysis of proton NMR spectra using 2-D NMR correlation experiments which rigorously established the (3β/3α) and (7β/7α) hydroxyl configurations in the isoursocholic and ursocholic acids. It is suggested that the isoursocholic acid will be useful as a reference compound and as a substrate in studies dealing with the hepatic inversion of the 3β-hydroxy group.—**Dayal, B., and G. Salen.** Stereospecific synthesis and two-dimensional ^{1}H-NMR investigation of isoursocholic acid. *J. Lipid Res.* 1991. **32:** 1381–1387.

Supplementary key words ursocholic acid • iso-bile acids

Iso-bile acids, the 3β-epimers of 5β-cholanoic acids, are formed in the intestine through the action of the intestinal bacteria (1, 2). Although substantial quantities may be produced, little is known about their metabolism (3–6). Recently, Shefer et al. (7) have shown that isochenodeoxycholic (3β,7α-dihydroxy-5β-cholanoic acid) and isoursodeoxycholic acid (3β,7β-dihydroxy-5β-cholanoic acid) are absorbed from the intestine, transported as the 3β-hydroxylated bile acids in the portal blood, and are converted to the naturally occurring 3α-hydroxy epimers during hepatic transit. In contrast, 7β-hydroxylated bile acids, namely ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid) and ursocholic acid (3α,7β,12α-trihydroxy-5β-cholanoic acid) are absorbed from the intestine and are excreted unchanged (as the ursodeoxycholic and ursocholic acid) by the liver (8–13). In order to study this phenomenon of hepatic (3β-hydroxy) inversion, a suitable 3β-hydroxy bile acid substrate needed to be synthesized and characterized. In this report, we describe a stereospecific synthesis and a large scale preparative separation and purification of 3β,7β,12α-trihydroxy-5β-cholan-24-oic acid (**Fig. 1**), and first application of two-dimensional ^{1}H-^{1}H, ^{1}H-^{13}C chemical shift correlation spectroscopy experiments affording full ^{1}H-line assignments in these isomeric bile acids.

MATERIALS AND METHODS

General procedures

Melting points were determined on a Thermolyne apparatus (Thermolyne Corp. Dubuque, IA), model MP-12600, and are uncorrected.

TLC. The bile acids were separated on silica gel G plates (Brinkmann, Westbury, NJ; 0.25-mm thickness) and the spots were visualized either with iodine or phosphomolybdic acid (3.5% in isopropanol).

Flash chromatography. Columns and the flow controller valves were purchased from ACE glass company, Vineland, NJ. The flow controller valve was a simple variable bleed device for precise regulation of the elution rate made from a glass/Teflon needle (ACE Glass Co. No. 8193-04) and a standard 24/40 joint. Flash chromatography was performed as described previously (14, 15) with E. Merck silica gel (400–230 mesh) (E. Merck No. 9385). Silica gel 40 μ average particle diameter, (Lot 527331, J. T. Baker Chemical Co.) was also used for flash chromatography.

^{1}H- and ^{13}C-NMR spectroscopy. Nuclear magnetic resonance spectra (^{1}H and ^{13}C) were recorded on a Varian XL-400 (400 MHz) spectrometer equipped with Fourier Transform mode. All NMR spectra were taken in $CDCl_3$ solution (unless otherwise indicated) with Me_4Si as the internal standard and the degree of substitution at each carbon was determined by experiments in the single-frequency off-resonance decoupled mode. The nature of each carbon in the isoursocholic methyl ester was deduced through Distortionless Enhancement by Polarization Transfer (DEPT) experiment (16, 17) performed by using polarization pulses of 90° and 135°, respectively, obtaining in the first case, only signals for CH groups and in the second case, positive signals for CH and CH_3 and negative signals for CH_2 groups.

The two-dimensional ^{1}H-^{1}H chemical shift correlated (2 D NMR, COSY-45) data for isomeric ursocholic and isoursocholic acid methyl esters were acquired at a sweep width of 3200 Hz using a standard pulse sequence (collection of 256 free induction decays, FIDs) (18). A transform size of 2K × 2K data points was obtained after zero-filling.

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; RRT, relative retention time; EI-MS, electron impact-mass spectrum; RT, retention time; DEPT, distortionless enhancement polarization transfer; 2D COSY NMR, two-dimensional homonuclear ^{1}H-^{1}H correlation spectroscopy; 2D HETCOR, two-dimensional heteronuclear ^{1}H-^{13}C correlation spectroscopy.

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[2]To whom reprint requests should be addressed at: G.I. Research Laboratory, Veterans Administration Medical Center, East Orange, NJ 07019

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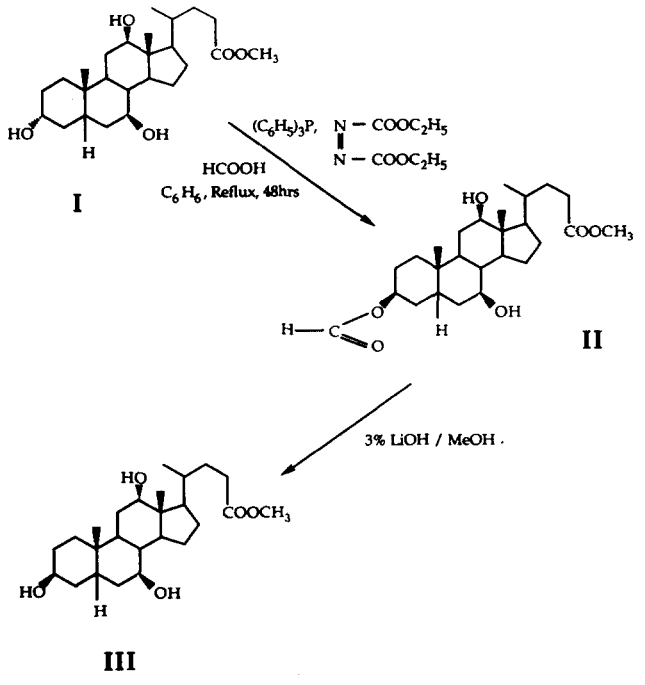

Fig. 1. Stereospecific synthesis of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate; I, methyl 3α,7β,12α-trihydroxy-5β-cholan-24-oate; II, methyl 3β-formyloxy, 7β,12α-dihydroxy-5β-cholan-24-oate; III, methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate.

The heteronuclear ^{1}H-^{13}C chemical shift correlated (HETCOR, 2D NMR) experiments were acquired (18–20) with sweep widths dictated by the appearance of the ^{13}C and ^{1}H spectra by using 2K data points in the 256 free induction decays (FIDs) collected on a 3200 Hz spectral width.

Optical rotation measurements were determined in $CHCl_3$ on a Perkin-Elmer 241 polarimeter.

GLC. Capillary GLC analysis of bile acid methyl esters (as their trimethyl silyl derivatives) was performed as described previously (21). The instrument used was a Hewlett-Packard model No. 4890 (equipped with a flame ionization detector) and a split column injector using a CP Sil 5 (CB) WCOT capillary column (25 m × 0.22 mm with 0.13 mm film thickness). Helium was used as a carrier gas at a flow rate of 20.2 ml/min (135 kPa).

Mass spectra of the epimeric bile acids and bile alcohols were obtained on a Varian MAT CH-5 spectrometer by electron impact at 10–70 eV as described previously (21, 22).

All reactions were carried out in a dry nitrogen atmosphere, unless water was used in the reaction, by using standard techniques for the exclusion of moisture.

Preparation of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate (Fig. 1, III)

This was prepared in one step using an adaptation of our previously published reaction with a modification (22). This method consisted of treating at reflux temperature a mixture of methyl 3α,7β,12α-trihydroxy-5β-cholan-24-oate 10.0 g (24 mmol) (Fig. 1, I), triphenylphosphine 31.59 (120 mmol), and diethylazodicarboxylate (DEADCAT, 19 ml, 120 mmol) in benzene (80 ml, freshly distilled over sodium) solution with formic acid (6.0 ml, 6.5-equiv.) and 1.5 g 4-Å molecular sieves. In 24 h a sterically pure ester (3β-formate Fig. 1, II) was formed in almost quantitative yield that corresponded to inversion of configuration as monitored by TLC, R_f 0.74, solvent system: $CHCl_3$:$(CH_3)_2CO$:CH_3OH 70:25:3.5 (v/v/v). The formulation of 3β-formate (small amount of crude reaction mixture purified by preparative TLC) was supported by ^{1}H-NMR and also by mass spectral data. NMR, δ ppm ($CDCl_3$) 8.07 (s, 1H, -OCOH), 4.02 (br.s, 1H, C-12βH), 3.6 (br.s, 1H, C-7αH), 5.18 (s, 1H, C-3αH), 1.00 (s, 3H, 19-CH_3), 0.73 (s, 1H, 18-CH_3), 0.99 (d, J=7Hz, 3H, 21-CH_3), 3.69 (s, 3H, $COOCH_3$). Mass spectrum: *m/z* 450 (M^+), 432 (2%,

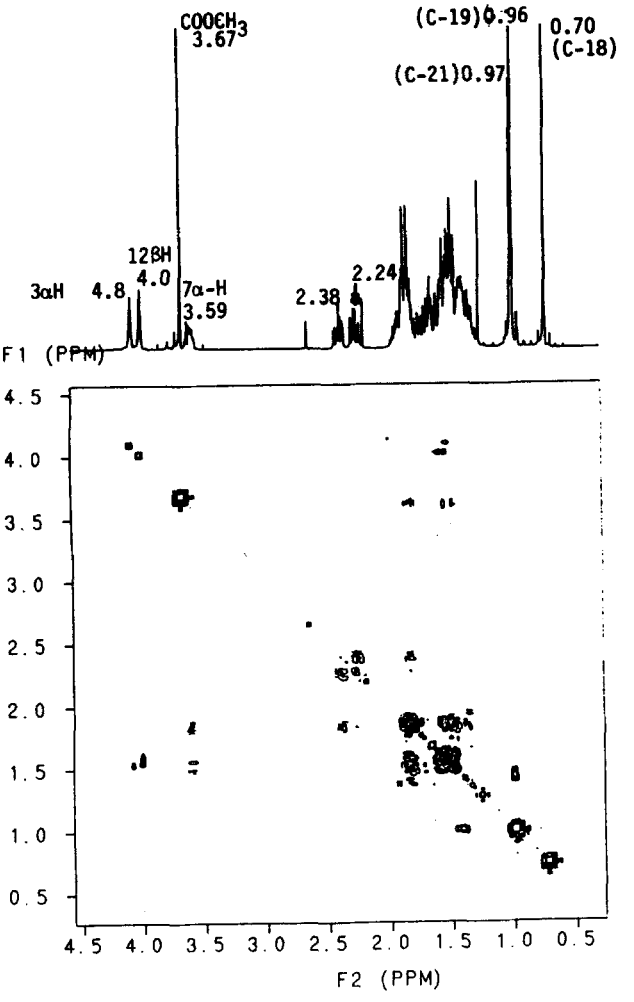

Fig. 2. A contour plot of 400 MHz homonuclear-correlated 2D ^{1}H-NMR spectrum of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate. The ^{1}H-NMR spectrum along the vertical axis is a standard one-dimensional spectrum for this 3β-epimer.

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M-H_2O), 414 (25% M-$2H_2O$), 404 (52%, M-HCOOH), 386 (16%, M-HCOOH-H_2O), 368 (23%, M-$2H_2O$-HCOOH), 355 (14%, M-H_2O-HCOOH-OCH_3), 317 (43%, M-H_2O-S.C.), 299 (97%, M-$2H_2O$-S.C.), 253 (100%, M-$2H_2O$-S.C.-HCOOH).

After evaporation of the solvent in vacuo, the residual material was dissolved in anhydrous diethyl ether which precipitated large excess quantities of triphenylphosphine oxide and diethylhydrazodicarboxylate ($C_2H_5O_2C$-NH-NH-$CO_2C_2H_5$) from the crude reaction mixture. This process to remove residual triphenyl phosphine oxide was repeated 2 to 3 times as needed using CH_2Cl_2-$(C_2H_5)_2O$ 1:1. After filtering the precipitates, removal of ether under reduced pressure and saponification of the methyl 3β-(formyloxy),7β,12α-dihydroxycholanate with LiOH/aq. MeOH (21) left a crude residue consisting of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate and methyl 3α,7β,12α-trihydroxy-5β-cholan-24-oate in a ratio of approx. 95:5 and other impurities (as indicated by ^{1}H-NMR and TLC). This was purified by silica gel column chromatography as described below.

Large scale preparative separation and purification of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate (isoursocholic acid methylester) (Fig. 2) by flash chromatography

A solvent system [chloroform–acetone–methanol 70:25:5.5 (v/v/v)] was chosen that gave good separation and moved the desired compound (Fig. 1, III), to R_f 0.36 on an analytical silica gel G TLC plate (Brinkmann Instruments, Westbury, NJ, 0.25 mm thickness); the corresponding 3α-hydroxy epimer (Fig. 1, I) had R_f = 0.33, (ΔR_f = 0.03). A column of the appropriate diameter (70 mm) and 45 cm long was selected and filled with 15 inches of dry 40–63 mm silica gel (302 g) (E. Merck No. 9385). Next a ½-inch layer of sand was carefully placed on the flat top of the dry silica gel bed and then filled with solvent [1200 ml chloroform–acetone 3:1 (v/v); the R_f values of methylursocholate and its 3β-epimer (Fig. 1, I, III) in this solvent mixture were 0.14 and 0.16 (with ΔR_f = 0.02), respectively]. During the process of elution, the first two or three fractions (200–300 ml each) were discarded and later fractions were saved and recycled in the same column. The crude reaction mixture of 3β-methylursocholate (see Materials and Methods for the diethylazodicarboxylate procedure) having impurities at $\Delta R_f \geq 0.05$ to 0.44 in chloroform–acetone 75:25 (v/v) was then applied by pipette as a 40% solution in the eluant to the top of the adsorbent bed and the flow controller was briefly placed on top of the column to push all of the sample into the silica gel. Then the column was refilled with the solvent [800 ml, chloroform–acetone 3:1 (v/v)] and eluted at a flow rate of 1 in/min. Smaller fractions (200 ml) were collected early in the elution, with larger ones (400 ml) being collected toward the end of the chromatography. Separated components, after monitoring by TLC, gave 8.5 g (85%) of the pure crystalline 3β-methylursocholate in less than 2 h, without contamination of the 3α-OH epimer (Fig. 1). Its corresponding acid was prepared by the usual method with 5% methanolic KOH and found to be identical according to melting point, TLC, and ^{1}H-NMR comparisons as described previously (23).

Physical properties: mp 60–63°C, $[\alpha]^{25}D$ +60.7°C (C 1.50, $CHCl_3$); RRT=1.61 (RT 5α cholestane=12.20 min). The 400 MHz homonuclear-correlated 2D NMR (^{1}H-^{1}H COSY) contour plot of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate is illustrated in **Fig. 2.** The ^{1}H-NMR spectrum along the vertical axis is a standard one-dimensional spectrum for this compound. For comparison, 2D NMR ^{1}H-^{1}H COSY spectrum for the 3α-epimer is illustrated in Fig. 3; DEPT spectra (3β-epimer Fig. 4); and 2D NMR ^{1}H-^{13}C HETCOR (3β-epimer Fig. 5).

Mass spectrum: *m/z* 404 (3%, M-H_2O), 386 (25%, M-$2H_2O$), 368 (1%, M-$3H_2O$), 289 (70%, M-S.C.-$2H_2O$), 253 (32%, M-S.C.-$3H_2O$).

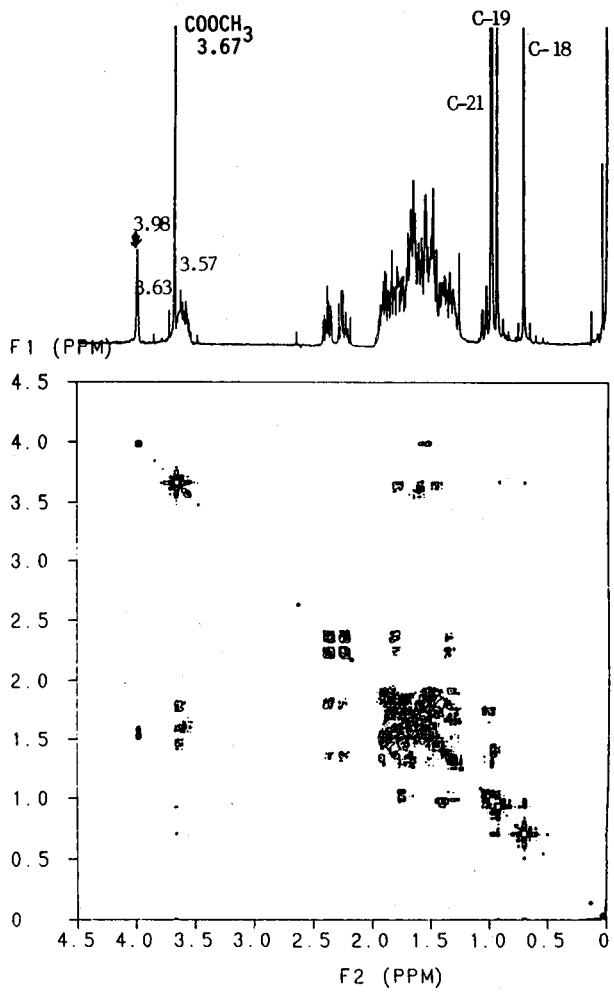

Fig. 3. 400 MHz ^{1}H homonuclear chemical-shift correlated (^{1}H-^{1}H 2D NMR COSY) spectrum of methyl 3α,7β,12α-trihydroxy-5β-cholan-24-oate. The ^{1}H-NMR spectrum along the vertical axis is a standard 1D-NMR spectrum for the 3α-epimer.

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Further elution of the flash chromatography column chloroform–acetone 3:1 (v/v) provided 312.0 mg of the pure 3α,7β,12α-trihydroxy-5β-cholan-24-oate, mp 73–76°C, $[\alpha]^{25}D$ +59.79°C, (C=1.0002, $CHCl_3$). The ^1H NMR data agreed with literature values (23). 2D NMR ^1H-^1H COSY (**Fig. 3**). Mass spectrum: *m/z* 404 (3%, M–H_2O), 386 (25%, M–2H_2O), 368 (25%, M–2H_2O), 368 (14% M–3H_2O), 355 (11%, M–2H_2O–OCH_3), 289 (87%, M–S.C.–H_2O), 271 (90%, S.C.–2H_2O), 253 (100%, M–S.C.–3H_2O).

The solvent system described above, dichloromethane–acetone or chloroform–acetone 3:1 (v/v), was a particularly good general solvent system for trihydroxy bile acid methyl esters (10 g impure methyl cholate under identical conditions was purified in 2.5 h). But for dihydroxy bile acids, chloroform–acetone 6:1 (v/v) was used (5.0 g methylursodeoxycholate was purified in 2 h with this proportion of solvents).

RESULTS AND DISCUSSION

Replacement of a hydroxyl group in a stereospecific fashion is an important transformation for steroids, carbohydrates, and some other classes of compounds. The reaction described here (Fig. 1) because of its high stereospecificity, mild reaction conditions, and high yields (see Materials and Methods) promises to be a useful synthetic method, specifically for polyfunctional molecules. So far, three chemical methods have mainly been used for the α/β inversion of C-3 hydroxyl of bile acids. The first method of Danielsson et al. (3) is based on the selective oxidation of the 3α-hydroxyl group followed by the catalytic reduction (four steps plus two chromatographic separations). The second method described by Iida and Chang (23) is based on the tosylation of the 3α-hydroxyl group followed by DMF treatment (four steps plus one chromatographic separation). The third method as reported from our laboratory (22) and others (24–26) is based on the Mitsunobu reaction (27) which utilizes azodicarboxylate and triphenylphosphine (two steps plus one chromatographic separation). We have compared these methods and found that the yields are 20% for the first method, 45–50% for the second, and 80% for the third method.

Furthermore, large scale chromatographic separation of the desired compound III (Fig. 1) was achieved by the method of Still, Kahn, and Mitra (15) who recently reported a simple and rapid technique for the preparative separation and purification of organic compounds. Since the product separations are quick and recoveries are high (14, 15, 28–31) we have replaced gravity flow alumina column (neutral Al_2O_3) chromatography [i.e., tedious long and short column chromatography (32, 33)] for the routine preparative separation and purification of impure mixtures of epimeric bile acids and bile alcohols from biological specimens as well as from synthetic organic mixtures.

Clean separation of compounds having $\Delta R_f \geq 0.12$ and in many cases separations at $\Delta R_f \geq 0.05$ were achieved in less than 2 h and a single silica gel column was used repeatedly up to four cycles without losing resolution. The preparative separation and purification of isomeric pairs of bile acids (methyl 3α,7β,12α-trihydroxy-5β-cholan-24-oate from methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate) and other structurally related bile acids of biological interest have been presented in Materials and Methods.

Also, during the course of synthetic and biosynthetic studies of iso-bile acids (7), it became clear that complete assignment of the ^1H and ^{13}C spectra of isoursocholic acid (compd. III, Fig. 1) would be highly desirable. Although partial attribution of the ^1H and ^{13}C resonances in isoursocholic acid had been made (23, 34), this spectral analysis combined with the two-dimensional (2D) NMR data described in the present studies provided a confident identification of synthetic intermediates for the accurate interpretation of precursor-product relationships.

An advantage of two-dimensional NMR spectroscopy over conventional one-dimensional (1D) NMR is that NMR parameters (namely, chemical shifts and spin-spin coupling constants) can be separately observed in the two-dimensional domain with consequent simplification of spectral analysis (Figs. 2 and 3). Therefore, overlapping signals in a 1D spectrum are well resolved. With the use of this correlation spectroscopy, the connectivity of signals among coupled spins can also be traced out. In homonuclear two-dimensional J-resolved spectroscopy (HOM2DJ) and J-correlated spectroscopy (COSY) NMR techniques (35, 36) one generally observes the separation of proton chemical shifts and spin-spin coupling constants along perpendicular axes. When suitably plotted, the data can often be interpreted unequivocally, in comparison to the normal one-dimensional spectrum where it may be necessary to make some assumptions for the interpretation of the data. Thus homonuclear-correlated (COSY) spectra are used mainly to determine which protons are scalarly coupled and heteronuclear-correlated (HETCOR) spectra generate a carbon-proton chemical shift correlation chart (Figs. 2, 3, and 5) (35–39). By making use of the above 2D NMR concepts, the extensive overlap of the methine (particularly for protons absorbing downfield from 2.5 ppm) and methylene proton resonances in bile acids may be simplified.

Fig. 2 illustrates the (^1H-^1H COSY) 2 D NMR diagram, whose results (**Table 1**) confirm the structure of isoursocholic acid methyl ester. The signals at δ 3.59, 4.0, and 4.08 in the ^1H-NMR spectrum were unambiguously assigned to the spin systems containing the hydroxy functions at C-7, C-12, and C-3. These signals showed cross peaks at δ 1.46 and 1.79 for the signal at δ 3.59, 1.53 for

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TABLE 1. Chemical shifts and ^1H-^1H correlation of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate and its 3α-epimer from COSY 45 experiments

Connectivities observed in the COSY spectrum of (3β-epimer)				
δ	0.99	⟷	δ	1.41
	2.24			1.33, 1.79 and 2.37
	2.37			1.33, 1.79 and 2.24
	3.59			1.46 and 1.79
	4.00			1.53
	4.08			1.49
Connectivities observed in the COSY spectrum of (3α-epimer)				
δ	0.98	⟷	δ	1.41
	1.03			1.73
	2.24			1.36, 1.81 and 2.38
	2.38			1.36, 1.81 and 2.24
	3.57 (H-7)			1.58
	3.63 (H-3)			1.42, 1.58 and 1.76
	3.98 (H-12)			1.52

the δ 4.0 signal, and a cross peak at δ 1.49 for the signal at 4.06. For comparison, 2 D ^1H-^1H COSY experiments of the closely related ursocholic acid methyl ester (Fig. 3) have also been performed. These assignments were derived by drawing rectangles between the off-diagonal peaks in the contour plots. Although connectivity between protons in the 3, 7, and 12 positions of the bile acid molecules may be difficult to determine, it was nevertheless possible to identify these protons by examination of the columns and rows of the two-dimensional matrix. A summary of all chemical shift assignments and ^1H-^1H connectivities on isomeric isoursocholic acid and ursocholic acid pair is presented in Table 1, and is in full agreement with the postulated stereochemistry.

With the proton NMR spectrum assigned, ^1H-^{13}C HETCOR (correlated spectroscopy) was used to identify the ^1H nuclei directly attached to the individual ^{13}C nuclei. A standard DEPT pulse sequence at 400 MHz was used (16, 17) to delineate the multiplicity of each carbon resonance. A complete analysis with respect to quaternary carbons and CH, CH_2, and CH_3 groups using spectral editing with the DEPT pulse sequence is shown in **Fig. 4.** Thus, with the previously deduced ^1H assignments, a heteronuclear ^1H-^{13}C shift correlation (HETCOR, Fig. 5) then permitted a detailed analysis of the ^{13}C spectrum.

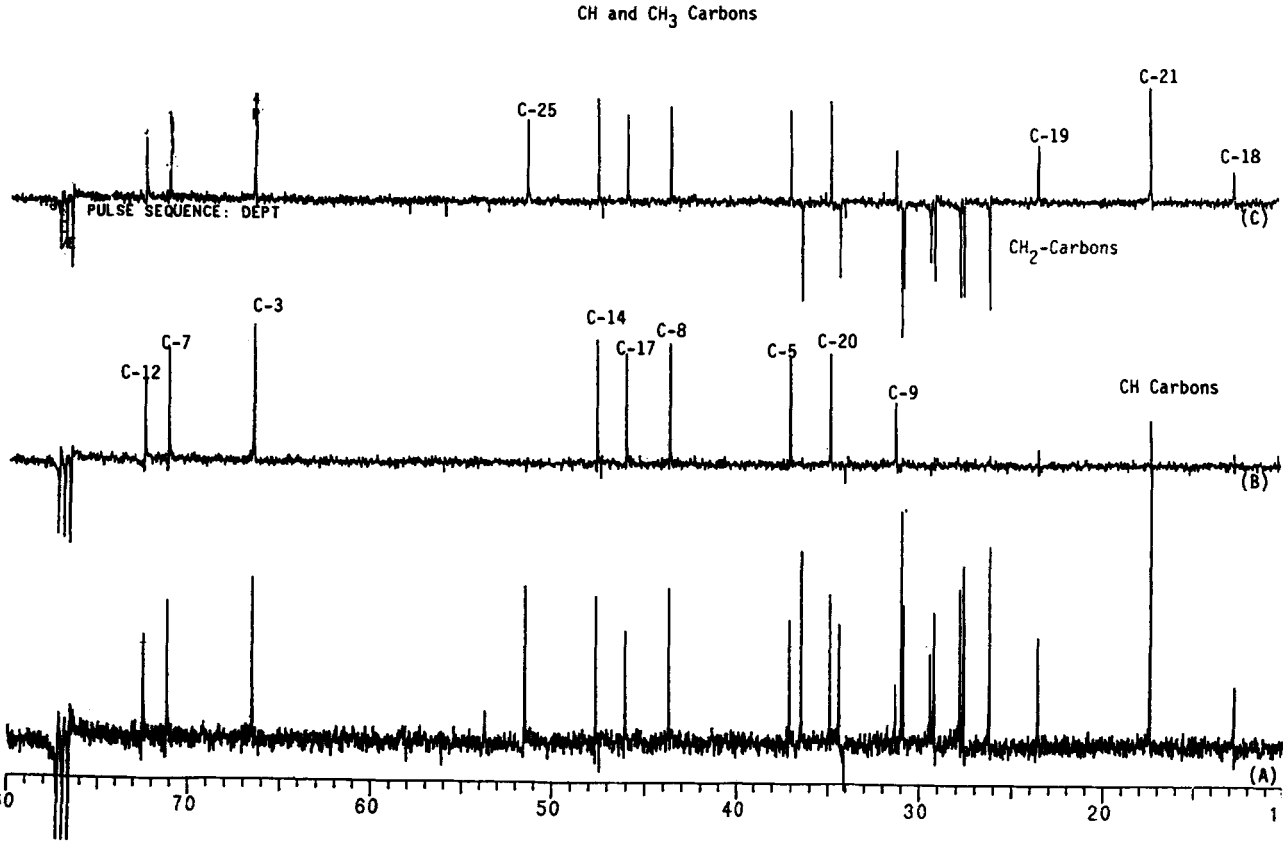

Fig. 4. Proton decoupled ^{13}C-NMR DEPT spectra of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate in CD_3OD at 75.4 MHz: (A) DEPT spectrum (lower, with CH, CH_2, and CH_3 carbons, quaternary carbons absent (B) DEPT 90, middle, CH carbons only (C) DEPT 135 spectra (upper), with inverted methylene resonances and the methyl and methine carbon resonances normally phased.

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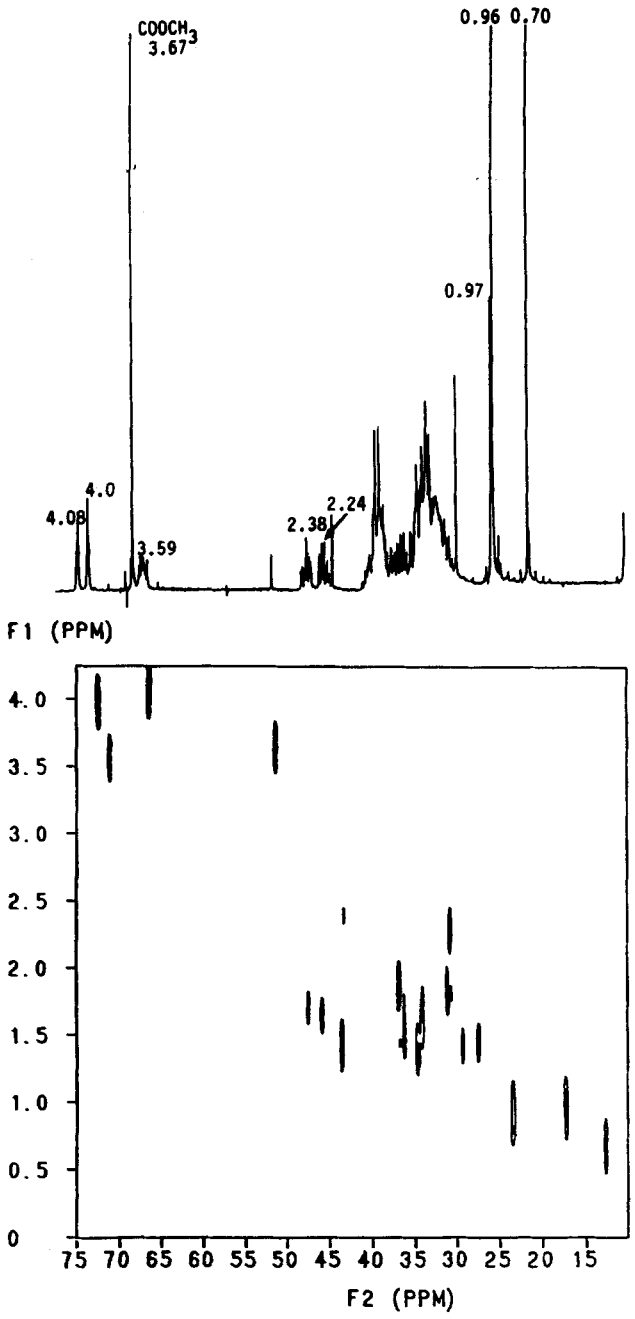

Fig. 5. 400 MHz Heteronuclear-correlated 2D NMR (HETCOR) contour plot of methyl 3β,7β,12α-trihydroxy-5β-cholan-24-oate. The ^{1}H-NMR spectrum along the horizontal axis is a standard 1D-NMR spectrum for this 3β-epimer.

The HETCOR spectrum in **Fig. 5** clearly illustrates that the methyl protons that resonate at 0.12, 0.98, 0.99, and 3.67 ppm in the proton spectrum are attached to the carbons that resonate at 13.0, 17.5, 23.8, and 51.5 ppm in the carbon spectrum. The three methine protons attached to 3, 7, and 12 carbons resonate at 4.08, 3.59, and 4.00 ppm, respectively. These methine resonances are attached to the carbons that resonate at 66.59, 71.28, and 72.59 ppm in the carbon spectrum. Thus, the corresponding correlation of the protons with the protonated carbons makes the assignments of the protonated carbons unambiguous.

Similarly, the assignments of methylene protons were confirmed by the use of HETCOR and COSY experiments and matching the observed values with reported assignments for close analogs (40, 41).

In summary, we have described a facile synthesis and full characterization of isoursocholic acid. Simultaneous measurements of 2D and 1D NMR experiments of isoursocholic/ursocholic acids described in this paper will be helpful in the assignment of structures to unknown epimeric or isomeric metabolites of other unusual bile acids. In addition, these studies will be of interest in establishing a relationship between physical-chemical properties of isobile acids and biliary lipid secretion and composition.

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