# Nuclear magnetic resonance spectroscopy of bile acids. Development of two-dimensional NMR methods for the elucidation of proton resonance assignments for five common hydroxylated bile acids, and their parent bile acid, 5β-cholanoic acid

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**Abstract** The complete $^1$H nuclear magnetic resonance assignments have been made for the common mono-, di-, and trihydroxy 5β-cholanoic acids; lithocholic acid, chenodeoxycholic acid, ursodeoxycholic acid, deoxycholic acid, cholic acid, and the unsubstituted parent compound, 5β-cholanoic acid, by heteronuclear-correlated two-dimensional NMR. The known $^{13}$C chemical shifts of these compounds were used to make the proton resonance assignments, and consistency of the carbon and proton assignments was verified by expected changes due to substituent effects. This has led to clarification of previously published $^{13}$C NMR resonance assignments. Addition of the 3α, 7α, and 12α hydroxyl substituent effects derived from the mono- and dihydroxycholanoic acids yielded predicted values for proton chemical shifts of the trihydroxy-substituted 5β-cholanoic acid, cholic acid, that agreed well with experimental values. It is suggested that the individual substituent effects can be used to predict proton chemical shifts for hydroxycholanic acids containing other combinations of 3α, 7α, 7β, and 12α hydroxyl groups. —**Waterhous, D. V., S. Barnes, and D. D. Muccio.** Nuclear magnetic resonance spectroscopy of bile acids. Development of two-dimensional NMR methods for the elucidation of proton resonance assignments for five common hydroxylated bile acids, and their parent bile acid, 5β-cholanoic acid. *J. Lipid Res.* 1985. **26:** 1068–1078.

**Supplementary key words** proton/carbon shifts • substituent effects

Proton nuclear magnetic resonance ($^1$H NMR) utilizes the most sensitive nucleus of biological importance. Thus it potentially offers the opportunity to study the conformation and dynamics of systems such as bile salt complexes where only small amounts of material are available. $^1$H NMR spectroscopy has not been systematically applied to bile acids because most of the 25 or more bile acid proton resonances, apart from the protons geminal to the hydroxyl groups and the methyl group protons, are in a limited chemical shift range of 1.0 to 2.3 ppm. The extensive overlap of the methine and methylene proton resonance patterns in this region has until recently (1) prevented the full assignment of proton resonances for a bile acid, even though partial assignments for some 300 bile acids have been reported (see reference 2 for a review of these data). In the previous study from this laboratory (1), a series of one-dimensional NMR experiments at high fields were used to fully assign the proton resonances for sodium cholate in dilute $D_2O$ solution. A similar approach has been used by Back, Fritz, and Populoh (3) to assign the proton resonances of a 1-hydroxylated derivative of cholic acid. However, application of these same techniques to the less substituted hydroxycholanoic acids becomes increasingly tedious, with proton assignments of the parent bile acid, 5β-cholanoic acid (**Fig. 1**), being virtually intractable by such methods.

The extensive series of experiments undertaken in previous investigations (1, 3) suggest that a more efficient approach to the systematic study of bile acid proton resonances is necessary. In the present study advantage has been taken of the previously reported $^{13}$C NMR resonance assignments of all the isomers of the mono-, di-, and trihydroxy bile acids substituted at the 3, 7, and 12 positions (4), by carrying out a homonuclear-decoupled-heteronuclear-correlated two-dimensional NMR experiment (hetcor 2D NMR) (5) to assign the bile acid proton

Abbreviations: NMR, nuclear magnetic resonance; nOe, nuclear Overhauser enhancement; hetcor 2D NMR, heteronuclear-correlated two-dimensional NMR; TMS, tetramethylsilane; MHz, megahertz; $J_{CH}$, carbon-proton coupling constant; APT, attached proton test; $T_1$, spin lattice relaxation time; ppm, parts per million; LEF, linear electric field effect. The following bile acid trivial names were used: lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid.

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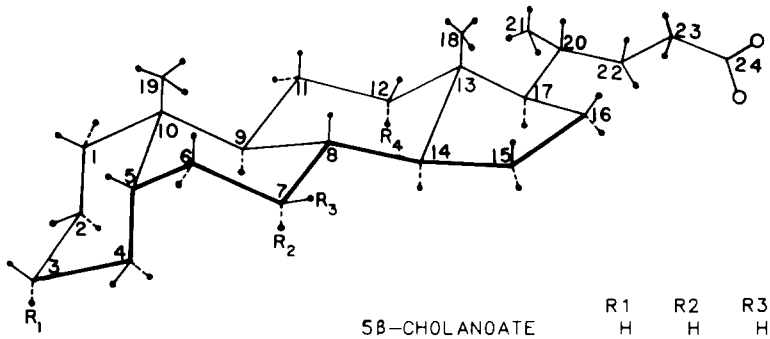

| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| 5β-CHOLANOATE | H | H | H | H |
| LITHOCHOLATE | OH | H | H | H |
| CHENODEOXYCHOLATE | OH | OH | H | H |
| URSODEOXYCHOLATE | OH | H | OH | H |
| DEOXYCHOLATE | OH | H | H | OH |
| CHOLATE | OH | OH | H | OH |

**Fig. 1.** Molecular structure of the 5β-cholanoic acids. The α and β protons are denoted by the dotted and solid lines, respectively.

resonances. Specifically, this pulse sequence gives a two-dimensional plot with the $^{13}C$ NMR chemical shifts displayed along one axis correlated to their respective protons dispersed according to their chemical shift along the other axis. Therefore, one gains the advantage of the carbon chemical shift dispersion in the assignments of the proton resonances. This particular sequence also eliminates the vicinal proton-proton coupling, giving an increase in resolution along the proton axis of the map.

In this study, the proton chemical shift assignments of five common hydroxylated bile acids (Fig. 1), lithocholic acid (3α-hydroxy-5β-cholanoic acid), chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid), ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid), deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid), and cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), and their parent, unsubstituted bile acid, 5β-cholanoic acid, were obtained by these 2D NMR techniques. From these data we have determined the effect of hydroxyl substitution at the 3α, 7α, 7β, and 12α positions and can make predictions of chemical shifts of other related bile acids. Some resolution and clarification of the carbon assignments made by Iida et al. (4) have also been made as a result of the work presented in this report.

## MATERIALS AND METHODS

The bile acids were purchased from Sigma Chemical Co. (St. Louis, MO). Chenodeoxycholic acid, lithocholic acid, and ursodeoxycholic acid were used without further purification. Cholic acid was recrystallized from ethanol–hexane, deoxycholic acid from acetone–water, and 5β-cholanoic acid from ethanol. Each was stored in vacuo over phosphorus pentoxide to remove residual solvents and moisture. The purity of each bile acid was greater than 98% as judged by thin-layer chromatography on silica gel G (solvent system: hexane–ethyl acetate–acetic acid–2-propanol 50:25:25:0.5 (by volume)) and by gas-liquid chromatography of their methyl ester O-trimethylsilyl ether derivatives and of their permethylated derivatives on a 50-meter fused silica capillary column wall coated with SP-2100 (6).

Deuterated solvents, dimethyl sulfoxide-$d_6$, deuteromethanol-$d_4$, and deuterochloroform, were obtained from Aldrich Chemical Co. (Milwaukee, WI). Cholic acid, chenodeoxycholic acid, and deoxycholic acid were prepared in deuteromethanol. Lithocholic acid was dissolved in deuterochloroform–dimethylsulfoxide-$d_6$ 4:1 (v/v) and 5β-cholanoic acid in deuterochloroform. Solutions containing 150–200 mg of each bile acid in a volume of 1–3 ml were filtered through a 0.2-micron filter (Gelman) to remove any particulate matter prior to NMR experiments.

Experiments were performed on a GE 300 wide bore spectrometer (NT series) equipped with an 1180e processor and a 293c pulse programmer providing resonance frequencies of 300.1 megahertz (MHz) and 75.4 MHz for $^1H$ and $^{13}C$ nuclei, respectively. Samples were placed in either 5-mm or 12-mm tubes and spectra were referenced internally to tetramethylsilane (TMS) at 0.00 ppm. Standard $^1H$ NMR spectra were obtained with 16K data points, 2500 Hz sweep width, 4.2-sec repetition rate, and a 70° pulse angle. Standard $^{13}C$ NMR spectra were obtained using 16K data points, 15,000 Hz sweep width, a 2.5-sec turnaround time, and a 70° pulse angle. The proton coupled and decoupled $^{13}C$ NMR spectra were run with nOe.

The attached proton test (APT) experiment (7) was carried out under two different conditions. An APT experiment using an 8-msec delay (corresponding to $1/J_{CH}$ where J is the C–H coupling constant of about 125 Hz for the cholanoic acids) gave a spectrum with methyl and

methine carbons having negative intensities and methylene and nonprotonated carbons with positive intensities. A second APT experiment using a delay of 4 msec, $1/2J_{CH}$, suppressed all peaks except the quaternary carbons. In both cases a 70°C pulse angle was used with a 2.5-sec repetition rate.

The hetcor 2D NMR pulse sequence is shown in **Fig. 2** with delay and pulse values given in a standard notation (5). In this notation, $t_1$ is the evolution time of spin vectors which is regularly incremented and $t_2$ is the detection period. All other values correspond to various evolution and recycling delay times.

This pulse sequence is a modification of the experiment described by Maudsley, Muller and Ernst (8) by the introduction of the $90_x(^1H)\ -1/(2J)\ -180_x(^1H)\ -1/(2J)\ -90_{-x}(^1H)$ sequence in the center of the proton evolution time. These pulses eliminate the vicinal proton-proton coupling and consequently increase the resolution of the proton resonances. In these experiments this is of considerable importance because of the near degeneracy and the high degree of coupling of the saturated polycyclic ring system.

The setup and execution of the hetcor 2D NMR experiment involved the following steps. *1*) The 90° and 180° $^{13}C$ pulses were determined with a 10% ethanol sample in the solvent used for the respective bile acid sample. *2*) The 90° $^1H$ decoupler pulse may be determined by a variety of methods (9, 10). An accurate 90° $^1H$ pulse is essential to optimize the hetcor 2D NMR experiment. The method chosen here was the use of the EDIT sequence (11) without the composite 90°C $^{13}C$ pulse. A correct 90° $^1H$ pulse results in the nulling of all carbon resonances with attached protons. A pulse angle less than 90° results in all peaks upright yet attenuated, whereas a pulse angle greater than 90° results in the methine and methyl peaks having negative intensity. The methine resonances are the most sensitive to the determination of a 90° pulse angle (11). A delay value of 4 msec was used for $1/2J_{CH}$. *3*) A recycle delay of 2 sec, a value at least three times the proton $T_1$s, was used for return of the spins to equilibrium. The $\Delta_1$ delay, corresponding to $1/2J_{CH}$, was 4 msec. The $\Delta_2$ delay, which can be varied from $1/2J_{CH}$ to $1/4J_{CH}$, was set at 2 msec (12). *4*) The $t_1$ increment values determine the sweep width in the $^1H$ domain. This value was set to 200 μsec corresponding to a 2500 Hz (±4.02 ppm) sweep width. The size of the sweep width is necessary since no quadrature phase detection was used in the $^1H$ domain, thus requiring the decoupler ($^1H$) transmitter to be placed at one side of the desired proton frequency range. The digital resolution in the proton domain is determined by the increment value and the number of accumulated spectra. Acquiring 128 successive iterations yielded spectra with a digital resolution of 19.5 Hz before zero filling. *5*) A sweep width of 6000 Hz with 2K data points (digital resolution of 5.88 Hz) was used in the $^{13}C$ spectra with the transmitter centered at 50 ppm. This excluded the carboxyl peak which exhibits no heteronuclear correlation. The initial 4 pulses were deleted and 256 transients were accumulated in each acquisition, requiring almost 20 hr for each experiment.

Optimal processing involved an exponential multiplication in the first domain (the carbon domain) with a line broadening of 1 Hz. A sine multiplication was used in the second domain (the proton domain) with zero filling (digital resolution of 10 Hz, ±0.03 ppm). An absolute value calculation was used to phase all resonances. All spectra were displayed as contour plots with standard one-dimensional spectra along each axis in order to facilitate correlation between the $^{13}C$ and $^1H$ resonances of each compound.

## RESULTS

From a comparison of the one-dimensional $^1H$ NMR spectrum of the parent bile acid, 5β-cholanoic acid (**Fig. 3A**), to that of cholic acid and of other biologically significant bile acids (Fig. 3), it is obvious that the resonances of the more than 25 methylene and methine protons that occur in the region from 1.0 to 2.5 ppm extensively over-

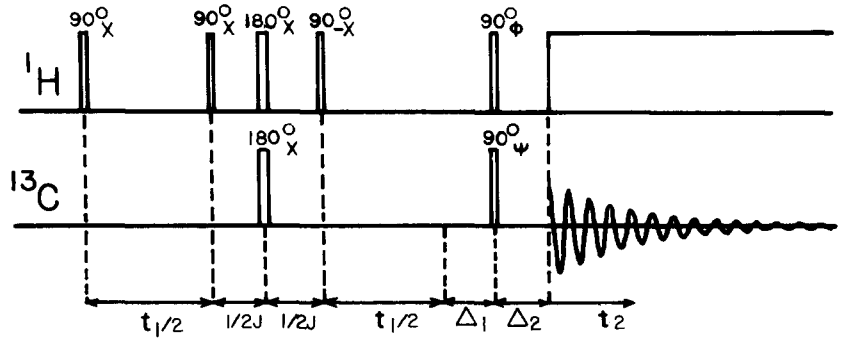


**Fig. 2.** Pulse sequence used for the hetcor 2D NMR experiment, as a modification by Bax (5) of a sequence originally described by Maudsley et al. (8).

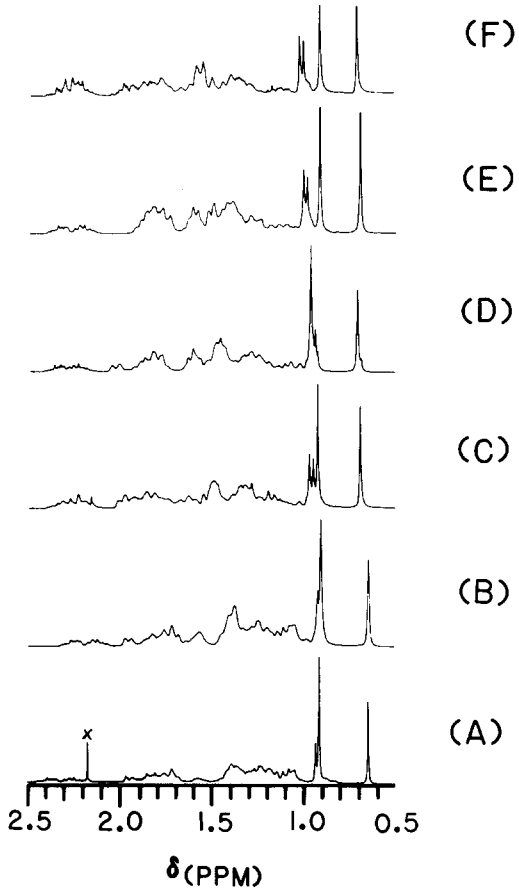


**Fig. 3.** $^{1}H$ NMR spectra of cholanoic acids at 300.1 MHz from 0.5 to 2.5 ppm: (A) 5$\beta$-cholanoic acid in $CDCl_3$, the resonance marked with an X is an impurity; (B) lithocholic acid in DMSO–$CDCl_3$ 1:4 (v/v); (C) chenodeoxycholic acid in $CD_3OD$; (D) ursodeoxycholic acid in $CD_3OD$; (E) deoxycholic acid in $CD_3OD$; (F) cholic acid in $CD_3OD$.

lap. Obtaining specific information is further complicated by the homonuclear coupling patterns that occur for each proton. These spectra, however, do indicate some general differences between each bile acid, reflecting changes in resonances and/or coupling patterns of the protons as a consequence of the number and position of hydroxyl substituents.

Relative to the $^{1}H$ NMR spectra, the $^{13}C$ NMR spectra of these compounds exhibit good resolution of almost all the 23 carbon resonances between 10–80 ppm (**Table 1**). The initial $^{13}C$ NMR assignments of those compounds have been reported by Iida et al. (4) who assigned the chemical shifts based on work by Leibfritz and Roberts (13), as well as utilization of single frequency off-resonance decoupling experiments and substituent effects. The substituent effects of such saturated carbon systems are well established and empirical rules describing these can be found in the literature (14, 15). However, these techniques, even though quite reliable, are not absolute means of carbon assignment. Many times closely lying resonances will be interchanged. The carbon assignments in Table 1 are those of Iida et al. (4), as modified by the results described in this communication.

The APT experiment, which has not been previously used in the assignment of $^{13}C$ resonances for bile acids, is a simple method to confirm the initial assignments made as to the carbon type (methyl, methylene, methine, and quaternary). The APT results for cholic acid (**Fig. 4C**) show the methine $^{13}C$ and methyl $^{13}C$ resonances as inverted peaks when the quaternary and the methylene $^{13}C$ resonances are phased upright. The three $^{13}C$ methyl resonances can be distinguished from the methine $^{13}C$ resonances by identifying the three corresponding quartets in the coupled spectra (Fig. 4B) centered around 13.0, 17.7, and 23.2 ppm (Table 1). However, due to the complexity of the coupled $^{13}C$ spectrum, it cannot be used as readily to distinguish between methine and methylene $^{13}C$ resonances (Fig. 4B), whereas the APT experiment clearly distinguishes between nearly degenerate methylene and methine $^{13}C$ resonance pairs (note $C_1$ and $C_{20}$ in Fig. 4C). The quaternary $^{13}C$ resonances were identified by using the APT sequence with a 4-msec delay.

### Cholic acid

The results of the hetcor 2D NMR experiment of cholic acid (**Fig. 5**) allow a straightforward assignment of the $^{1}H$ chemical shifts obtained from this single experiment. The most downfield resonance in the $^{13}C$ spectrum (excluding those for the carboxyl carbon and the hydroxylated carbons which are further downfield and are not shown), previously assigned to methine $C_{17}$ (4), shows a single peak on the contour plot centered at 1.86 ppm on the $^{1}H$ chemical shift scale, corresponding to the resonance of the attached methine proton, $H_{17\alpha}$. Similarly, each of the other $^{13}C$ resonances can be associated with its respective $^{1}H$ resonance(s). The $^{1}H$ chemical shift assignments resulting from this experiment (Table 1) agree well with the recent NMR results of cholic acid in the same solvent obtained by strictly one-dimensional techniques (3). However, the two-dimensional NMR approach offers a dramatic time savings by allowing for unambiguous assignments of the $^{1}H$ resonances as long as the $^{13}C$ resonances are known. Even $^{13}C$ resonances with nearly identical chemical shifts ($C_4$ at 40.5 ppm and $C_8$ at 41.0 ppm) can be used for assignment of their $^{1}H$ resonances by this technique.

The quaternary $^{13}C$ resonances are not seen in the contour plots; the lack of directly attached protons eliminates the polarization transfer that occurs between carbon and proton spins. Similarly, the resonances seen for the deuterated solvent in the standard $^{13}C$ spectrum are not observed. This is an advantage in these experiments since the $CD_3OD$ $^{13}C$ resonance obscures the $C_{17}$ peak position in the one-dimensional $^{13}C$ NMR spectrum (Fig. 4).

TABLE 1. $^{13}C$ and $^{1}H$ resonance assignments for bile acids[a]

| | 5β-Cholanoic Acid | | | | Lithocholic Acid | | | | Chenodeoxycholic Acid | | | | Ursodeoxycholic Acid | | | | Deoxycholic Acid | | | | Cholic Acid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Proton | | | | Proton | | | | Proton | | | | Proton | | | | Proton | | | | Proton | |
| # | Type | Carbon | α | β | Type | Carbon | α | β | Type | Carbon | α | β | Type | Carbon | α | β | Type | Carbon | α | β | Type | Carbon | α | β |
| 1 | $CH_2$ | 37.6 | 1.74, | 0.88 | $CH_2$ | 35.3 | 1.75, | 0.94 | $CH_2$ | 36.5 | 1.83, | 0.99 | $CH_2$ | 36.1 | 1.81, | 1.03 | $CH_2$ | 36.3 | 1.77, | 0.98 | $CH_2$ | 36.5 | 1.81, | 0.99 |
| 2 | $CH_2$ | 21.4 | 1.34(2) | | $CH_2$ | 30.3 | 1.29, | 1.60 | $CH_2$ | 31.3 | 1.36, | 1.59 | $CH_2$ | 31.0 | 1.28, | 1.62 | $CH_2$ | 30.9 | 1.44, | 1.59 | $CH_2$ | 31.2 | 1.45, | 1.59 |
| 3 | $CH_2$ | 27.1 | 1.74, | 1.18 | CH | 70.5 | — | 3.51 | CH | 72.9 | — | 3.37 | CH | 71.9 | — | 3.47 | CH | 72.5 | — | 3.54 | CH | 72.9 | — | 3.37 |
| 4 | $CH_2$ | 27.3 | 1.72, | 1.23 | $CH_2$ | 36.3 | 1.71, | 1.45 | $CH_2$ | 40.4 | 2.25, | 1.66 | $CH_2$ | 38.6 | 1.81, | 1.55 | $CH_2$ | 37.0 | 1.79, | 1.48 | $CH_2$ | 40.5 | 2.29, | 1.66 |
| 5 | CH | 43.8 | — | 1.27 | CH | 41.9 | — | 1.35 | CH | 43.1 | — | 1.36 | CH | 42.4 | — | 1.47 | CH | 43.5 | — | 1.39 | CH | 43.2 | — | 1.38 |
| 6 | $CH_2$ | 27.6 | 1.20, | 1.86 | $CH_2$ | 27.1 | 1.23, | 1.83 | $CH_2$ | 35.9 | 1.52, | 1.98 | $CH_2$ | 38.0 | 1.60(2) | | $CH_2$ | 28.3 | 1.26, | 1.89 | $CH_2$ | 35.9 | 1.53, | 1.95 |
| 7 | $CH_2$ | 26.6 | 1.08, | 1.37 | $CH_2$ | 26.3 | 1.09, | 1.39 | CH | 69.1 | — | 3.80 | CH | 72.1 | 3.49 | — | $CH_2$ | 27.3 | 1.19, | 1.42 | CH | 69.1 | — | 3.80 |
| 8 | CH | 36.0 | — | 1.39 | CH | 35.6 | — | 1.38 | CH | 40.2 | — | 1.50 | CH | 43.4 | — | 1.45 | CH | 37.3 | — | 1.46 | CH | 41.0 | — | 1.55 |
| 9 | CH | 40.6 | 1.39 | — | CH | 40.2 | 1.41 | — | CH | 34.0 | 1.87 | — | CH | 40.7 | 1.48 | — | CH | 34.6 | 1.89 | — | CH | 27.9 | 2.25 | — |
| 10 | C | 35.3 | — | — | C | 34.2 | — | — | C | 36.2 | — | — | C | 35.1 | — | — | C | 35.3 | — | — | C | 35.9 | — | — |
| 11 | $CH_2$ | 20.9 | 1.39, | 1.24 | $CH_2$ | 20.6 | 1.38, | 1.23 | $CH_2$ | 21.8 | 1.48, | 1.35 | $CH_2$ | 22.4 | 1.47, | 1.34 | $CH_2$ | 29.8 | 1.53(2) | | $CH_2$ | 29.6 | 1.58(2) | |
| 12 | $CH_2$ | 40.3 | 1.12, | 1.92 | $CH_2$ | 40.0 | 1.14, | 1.96 | $CH_2$ | 41.0 | 1.21, | 2.00 | $CH_2$ | 41.5 | 1.19, | 2.03 | CH | 74.0 | — | 3.98 | CH | 74.0 | — | 3.97 |
| 13 | C | 42.8 | — | — | C | 42.4 | — | | C | 43.7 | — | — | C | 44.8 | — | — | C | 44.8 | — | — | C | 47.6 | — | — |
| 14 | CH | 56.7 | 1.07 | — | CH | 56.3 | 1.05 | — | CH | 51.5 | 1.48 | — | CH | 56.5 | 1.09 | — | CH | 49.1 | 1.62 | — | CH | 43.0 | 2.00 | — |
| 15 | $CH_2$ | 24.3 | 1.02, | 1.59 | $CH_2$ | 24.0 | 1.04, | 1.56 | $CH_2$ | 24.6 | 1.09, | 1.74 | $CH_2$ | 27.9 | 1.46, | 1.90 | $CH_2$ | 24.8 | 1.09, | 1.62 | $CH_2$ | 24.2 | 1.12, | 1.76 |
| 16 | $CH_2$ | 28.2 | 1.88, | 1.29 | $CH_2$ | 28.0 | 1.85, | 1.27 | $CH_2$ | 29.2 | 1.90, | 1.32 | $CH_2$ | 29.6 | 1.86, | 1.30 | $CH_2$ | 28.5 | 1.87, | 1.29 | $CH_2$ | 28.7 | 1.90, | 1.32 |
| 17 | CH | 56.1 | 1.10 | — | CH | 55.8 | 1.10 | — | CH | 57.2 | 1.18 | — | CH | 57.4 | 1.25 | — | CH | 48.0 | 1.83 | — | CH | 48.1 | 1.86 | — |
| 18 | $CH_3$ | 12.1 | 0.65 | | $CH_3$ | 11.9 | 0.64 | | $CH_3$ | 12.2 | 0.69 | | $CH_3$ | 12.7 | 0.71 | | $CH_3$ | 13.2 | 0.71 | | $CH_3$ | 13.0 | 0.72 | |
| 19 | $CH_3$ | 24.3 | 0.91 | | $CH_3$ | 23.3 | 0.91 | | $CH_3$ | 24.6 | 0.93 | | $CH_3$ | 24.0 | 0.94 | | $CH_3$ | 23.7 | 0.93 | | $CH_3$ | 23.2 | 0.92 | |
| 20 | CH | 35.4 | 1.43 | | CH | 35.1 | 1.41 | | CH | 36.7 | 1.45 | | CH | 36.6 | 1.44 | | CH | 36.6 | 1.42 | | CH | 37.8 | 1.43 | |
| 21 | $CH_3$ | 18.3 | 0.95 | | $CH_3$ | 18.2 | 0.92 | | $CH_3$ | 18.8 | 0.96 | | $CH_3$ | 19.0 | 0.96 | | $CH_3$ | 17.5 | 1.01 | | $CH_3$ | 17.7 | 1.02 | |
| 22 | $CH_2$ | 30.9 | 1.79, | 1.34 | $CH_2$ | 30.9 | 1.75, | 1.29 | $CH_2$ | 32.4 | 1.79, | 1.31 | $CH_2$ | 32.3 | 1.80, | 1.32 | $CH_2$ | 32.2 | 1.78, | 1.35 | $CH_2$ | 32.4 | 1.79, | 1.35 |
| 23 | $CH_2$ | 31.1 | 2.38, | 2.24 | $CH_2$ | 30.9 | 2.32, | 2.12 | $CH_2$ | 32.3 | 2.31, | 2.24 | $CH_2$ | 32.0 | 2.35, | 2.21 | $CH_2$ | 32.0 | 2.38, | 2.23 | $CH_2$ | 32.0 | 2.37, | 2.21 |
| 24 | C | 180.2 | — | — | C | 178.1 | — | | C | 178.2 | — | — | C | 178.2 | — | — | C | 178.0 | — | — | C | 178.3 | — | — |

[a]The hetcor 2D NMR experiment does not distinguish between α and β methylene proton resonances. Assignments of the α or β $^{1}H$ resonances of cholic acid were made from previous studies (ref. 1). In the case of the $H_{11}$ and $H_{16}$ protons, arbitrary assignments have been made. Assignments of the α and β methylene proton resonances for the other bile acids were made from cholic acid.

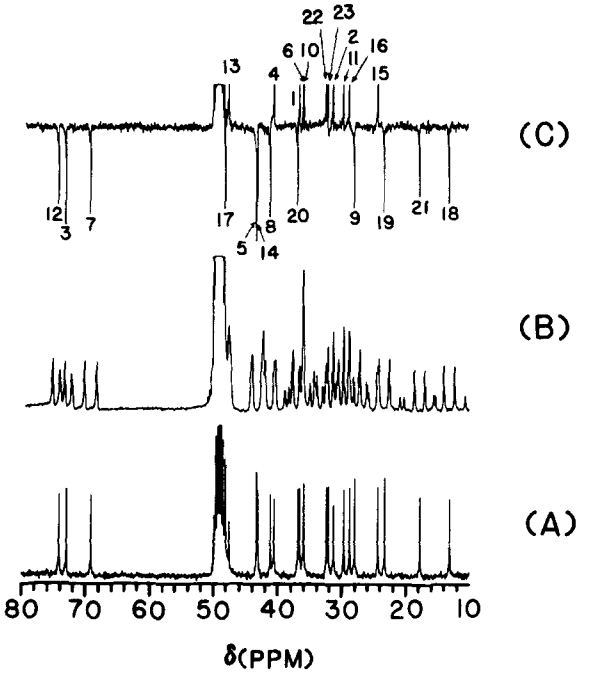


**Fig. 4.** $^{13}C$ NMR spectra of cholic acid in $CD_3OD$ at 75.4 MHz: (A) proton decoupled spectrum, (B) proton coupled spectrum, (C) an APT spectrum, with inverted methyl and methine resonances and the methylene and quarternary resonances normally phased.

The $^1H$ spectrum corresponding to any individual $^{13}C$ resonance can be viewed as an individual slice (**Fig. 6**). In general, there are two $^1H$ resonances observable for each methylene carbon and one resonance for the methine carbons. The pair of $^1H$ resonances for the $C_2$ and $C_{23}$ methylene protons are resolved in this experiment, even though the resonances are nearly degenerate (Fig. 6). Use of the hetcor 2D NMR pulse sequence described originally by Maudsley et al. (8) without homonuclear proton decoupling shows these two $^1H$ resonance pairs as single broad resonances (data not shown). However, the pulse sequence modification by Bax (5) gives the resolution needed to make these assignments. The two $^1H$ resonances for $C_{11}$ are degenerate and yield only a single peak (Fig. 6). It should be noted that the apparent resonance near 4.0 ppm arises from pulse imperfections; no resonance is observed at this position in the one-dimensional $^1H$ NMR spectrum.

### Chenodeoxycholic acid

The assignment of the $^1H$ resonances of chenodeoxycholic acid, which differs in structure from cholic acid by the removal of the 12$\alpha$ hydroxy group, is made from the hetcor 2D NMR experiment as described above for cholic acid. The major effect on the $^1H$ chemical shifts of chenodeoxycholic acid is the 1.97 ppm upfield chemical shift of the geminal $H_{12\beta}$ resonance from the removal of the hydroxyl group. The other significant changes in the $^1H$ chemical shifts are seen for $H_{9\alpha}$ (0.38 ppm), $H_{14\alpha}$ (0.52 ppm), and $H_{17\alpha}$ (0.68 ppm) (Table 1) which correlate to the large chemical shift changes for the $^{13}C$ resonances.

Several $^{13}C$ resonance assignments have been improved from those previously published (4). The $^{13}C$ chemical shifts for $C_1$ and $C_{20}$ which were previously unresolved (4), due to the use of a lower field strength (25 MHz versus 75 MHz in the present study for $^{13}C$), have been assigned by an APT experiment. The results of the hetcor 2D NMR experiment for the $^1H$ resonance assignments for $H_1$, 1.83 ($\alpha$) and 0.99 ppm ($\beta$), and for $H_{20}$, 1.45 ppm, reinforces these $^{13}C$ assignments from the APT experiment, since similar $^1H$ chemical shifts are seen for these protons in cholic acid ($H_1$, 1.81 ($\alpha$) and 0.99 ($\beta$) ppm, and $H_{20}$, 1.43 ppm). However, the APT experiment does not differentiate between the methylene carbons at $C_4$ and $C_{12}$ which had been previously reported as degenerate (4); in this study two distinct $^{13}C$ resonances are indicated near 40 ppm. The assignment of the $^{13}C$ resonance at 40.4 ppm to $C_4$, and the $^1H$ resonances at 2.25 ($\alpha$) and 1.66 ($\beta$) ppm for the attached protons, have been made by comparing the resonances for $C_4$ and $H_4$ in cholic acid (40.5 ppm, and 2.29 ($\alpha$) and 1.66 ($\beta$) ppm, respectively). This is consistent with expectations of small chemical shift changes at $C_4$ for chenodeoxycholic acid due to the removal of the distant 12$\alpha$ hydroxyl group. A similar strategy has been used to assign the $C_{22}$ and $C_{23}$ methylene carbon resonances. Resonances for the $H_{22}$ and $H_{23}$ protons occur at 1.79 and 1.31 ppm, and at 2.31 and 2.24 ppm, respectively. These chemical shifts are nearly identical to those observed for cholic acid (Table 1). The revised $^{13}C$ chemical shifts agree with those which were reported by Baillet-Guffroy et al. (16) during the course of this study.

### Ursodeoxycholic acid

Ursodeoxycholic acid is an epimer of chenodeoxycholic acid where the 7$\alpha$ hydroxyl group has been moved to the $\beta$-position. A comparison of the $^1H$ NMR resonances of ursodeoxycholic acid to those of chenodeoxycholic acid shows significant upfield changes for the $H_{4\alpha}$ (0.44 ppm), $H_{6\beta}$ (0.38 ppm), $H_{9\alpha}$ (0.39 ppm), and $H_{14\alpha}$ (0.39 ppm) chemical shifts (Table 1). Significant downfield chemical shifts are observed for the $H_{15}$ protons, $H_{15\alpha}$ (0.37 ppm) and $H_{15\beta}$ (0.16 ppm), due to the spatial proximity of the 7$\beta$ hydroxyl substituent. The $^{13}C$ resonances are in good agreement with those of Baillet-Guffroy et al. (16) in $CD_3OD$.

### Deoxycholic acid

Deoxycholic acid is related to cholic acid by the removal of the 7$\alpha$ hydroxyl group. The $H_{7\beta}$ proton, which is geminal to the hydroxyl group in cholic acid, resonates 2.38 ppm upfield in deoxycholic acid. Other upfield $^1H$

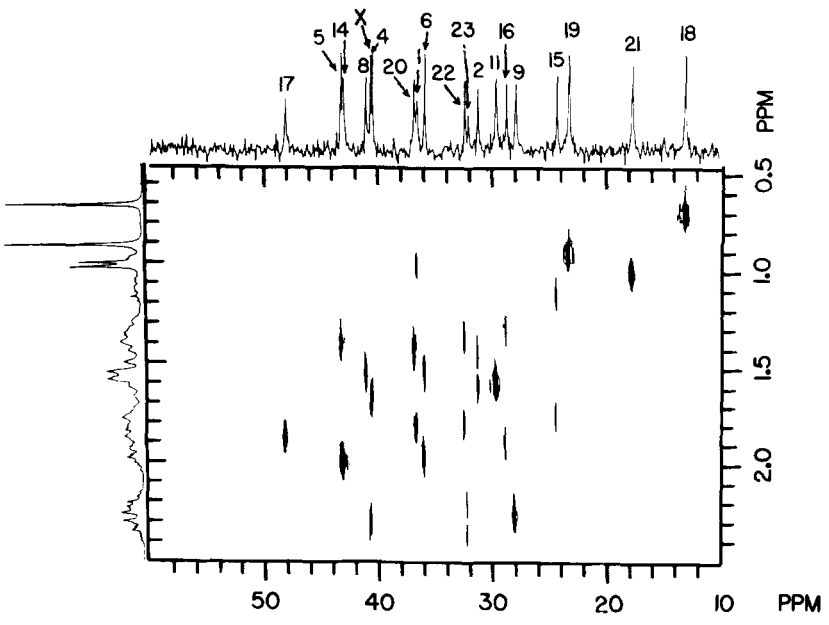


**Fig. 5.** Heteronuclear-correlated 2D NMR contour plot of cholic acid. See text for details of the procedure. The $^{13}C$ spectrum on the horizontal axis is a projection of the peaks in the contour plot and does not contain the quaternary $^{13}C$ resonances. The $^{1}H$ spectrum along the vertical axis is the standard one-dimensional spectrum shown in Fig. 3(f). $^{1}H$ resonances are assigned by identification of the $^{13}C$ resonance (horizontal axis) in the contour plot, determination of the center of the peak, and then measurement of its position in the vertical axis. For greater clarity the region of the plot containing the $^{13}C$ resonances for carbon atoms attached to hydroxyl groups has been omitted.

chemical shifts occur for the two $H_4$ protons (0.50 ($\alpha$) and 0.18 ($\beta$) ppm), $H_{9\alpha}$ (0.36 ppm), and $H_{14\alpha}$ (0.38 ppm). Unlike chenodeoxycholic acid, the $H_{17\alpha}$ resonance was not dramatically changed (0.03 ppm). The $^{13}C$ resonances listed in Table 1 are in agreement with those made by Iida et al. (4) except for two sets of changes. The $^{13}C$ chemical shift assignments of $C_1$ and $C_{20}$, which were not previously resolved (4), have been obtained from the APT experiment which distinguishes the methylene $C_1$ carbon from the methine $C_{20}$ carbon. Furthermore, the $^{1}H$ resonances for these carbons ($H_1$ at 1.77 ($\alpha$) and 0.98 ($\beta$) ppm, and $H_{20}$ at 1.42 ppm), assigned from the hetcor 2D NMR experiment, are near to those in cholic acid ($H_1$ at 1.81 ($\alpha$) and 0.99 ($\beta$) ppm and $H_{20}$ at 1.43 ppm), thus supporting the $^{13}C$ assignments. The $^{13}C$ chemical shifts for $C_4$ and $C_8$ are in the opposite order to those previously reported (4). These assignments are also made by the APT experiment since $C_4$ is a methylene carbon, while $C_8$ is a methine carbon. The $^{1}H$ chemical shifts for these two carbons assigned by the hetcor 2D NMR experiment ($H_4$, 1.79 ($\alpha$) and 1.48 ($\beta$) ppm, and $H_{8\beta}$, 1.46 ppm) cannot, by analogy to cholic acid, be used to reinforce these carbon assignments. This is because of the close spatial relation between the 7$\alpha$ hydroxyl group and the $H_4$ and $H_{8\beta}$ protons.

### Lithocholic acid

Lithocholic acid, 3$\alpha$-hydroxy-5$\beta$-cholanoic acid, lacks both 7$\alpha$ and 12$\alpha$ hydroxyl groups found in cholic acid. The $^{1}H$ resonances of lithocholic acid contain the combined trends for the removal of the 7$\alpha$ and 12$\alpha$ hydroxyl substituents in going from cholic acid to deoxycholic acid and from cholic acid to chenodeoxycholic acid, respectively. $^{1}H$ resonances solely affected by the 12$\alpha$ hydroxyl group, e.g., $H_{17\alpha}$, have a similar chemical shift in lithocholic acid (1.10 ppm) as in chenodeoxycholic acid (1.18 ppm), due to removal of this substituent in both compounds. This is substantially different from deoxycholic acid and cholic acid where the 12$\alpha$ hydroxyl group is present ($H_{17\alpha}$ at 1.83 ppm and at 1.86 ppm for deoxycholic acid and cholic acid, respectively). Similarly, the $^{1}H$ resonances for $H_4$ at 1.71 ($\alpha$) and 1.45 ($\beta$) ppm in lithocholic acid are near to those in deoxycholic acid at 1.79 ($\alpha$) and 1.48 ($\beta$) ppm, due to the removal of the 7$\alpha$ hydroxyl group. Other protons which are perturbed by both the 7$\alpha$ hydroxyl and 12$\alpha$ hydroxyl groups show combined effects in their removal. For example, $H_{9\alpha}$ and $H_{14\alpha}$ protons resonate at 1.41 and 1.05 ppm, respectively, significantly upfield of their resonance positions in either chenodeoxycholic acid or deoxycholic acid. Interestingly, these chemical shifts are similar to those for ursodeoxycholic acid which lacks the 12$\alpha$ hydroxyl group and has the 7 hydroxyl substituent on the $\beta$ face of the molecule.

Only two $^{13}C$ resonances show opposite trends in this work compared to those previously reported (4). $C_{20}$, a methine carbon, is assigned at 35.1 ppm, upfield of $C_1$, a methylene carbon, at 35.3 ppm, compared to 35.1 and 35.0 ppm, respectively, as reported by Iida et al. (4).

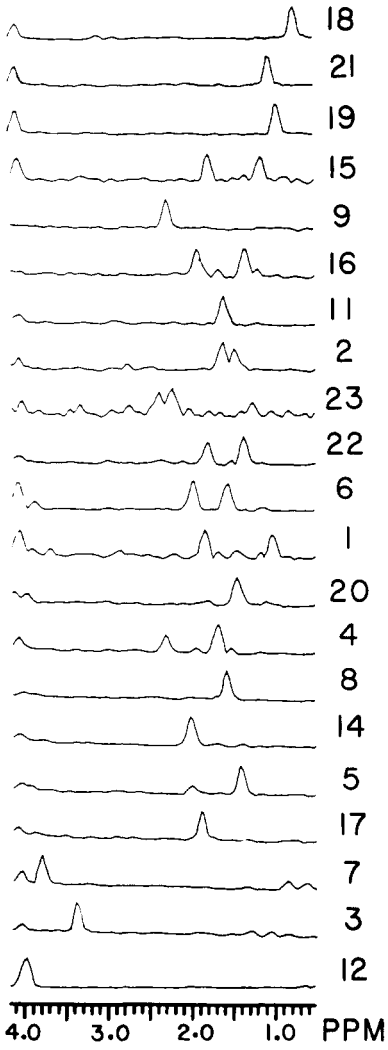


**Fig. 6.** Slices of the contour plot of cholic acid (see Fig. 5) through each $^{13}C$ resonance peak. This enables accurate assignment(s) of the $^{1}H$ resonances. The carbon atoms to which the protons are attached are given at the right of each spectrum. Except for the slice containing the $H_{12\beta}$ proton resonance, the peak that appears at 4 ppm in many of the slices is an artifact due to pulse imperfections.

These new assignments have also been confirmed by the APT experiment.

### 5β-Cholanoic acid

The assignments for the unsubstituted parent compound, 5β-cholanoic acid, are made as easily as for the hydroxylated bile acids. The contour plot for 5β-cholanoic acid (**Fig. 7**) illustrates the clear resolution of the $^{1}H$ resonances despite the close proximity of many of the $^{13}C$ resonances. In the most complex region of the contour plot, the resonances of five methylene carbons are located between 26.6 and 28.2 ppm ($C_3$, $C_4$, $C_6$, $C_7$, and $C_{16}$). In spite of the complexity in both the $^{1}H$ and $^{13}C$ domains, the $^{1}H$ resonances of the two protons attached to each of these methylene carbons are clearly observed. The assignment of these $^{1}H$ resonances by standard one-dimensional techniques would be very difficult. Indeed, spectra with an even greater degree of complexity can be assigned with the hetcor 2D NMR experiment.

The only notable changes seen for the $^{13}C$ and $^{1}H$ chemical shifts between lithocholic acid and 5β-cholanoic acid are for the resonances in the A-ring. A 2.33-ppm upfield shift occurs for the $H_{3\beta}$ geminal proton. The $^{1}H$ resonance of the $H_{4\beta}$ and $H_{2\beta}$ protons also shift upfield by 0.22 and 0.26 ppm, respectively, whereas the hydroxyl effect for the $^{1}H$ resonances of $H_{1\beta}$ and $H_{5\beta}$ is only 0.06 and 0.08 ppm, respectively.

The consistency of chemical shift changes in the $^{1}H$ spectrum led to reassignment of the $C_4$ and $C_6$ $^{13}C$ resonances. The previously reported values for these carbon atoms (4) were 27.4 ppm for $C_4$ and 27.1 ppm for $C_6$. Comparing the assigned $^{1}H$ resonances of the $H_6$ protons in lithocholic acid to the $^{1}H$ resonances in this compound suggests that the assignments of the $C_4$ and $C_6$ $^{13}C$ resonances are 27.3 and 27.6 ppm, respectively (Table 1). The APT experiment cannot distinguish between these two methylene carbons. However, this result is consistent with the 9 ppm upfield shift for $C_2$ and $C_4$, which are both in the β-position to the removed 3α hydroxyl group in going from lithocholic acid to 5β-cholanoic acid (Table 1). Since $C_6$ is in the δ-position to this substituent, no major change would be expected for this resonance (4, 14, 15).

## DISCUSSION

The present study has shown that a heteronuclear correlated 2D NMR experiment can be effectively applied to the assignment of proton resonances of all the common bile acids, and by analogy, of steroids and sterols. For the first time this has allowed the systematic study of the effect of substituents on $^{1}H$ resonances in the cyclopentanoperhydrophenanthrene ring system in bile acids. Previously, substituent effects were restricted to those on the $C_{18}$ and $C_{19}$ methyl $^{1}H$ resonances (17). The largest substituent effect from the hydroxyl groups occurs at the geminal proton, as would be expected. A 2.33-ppm downfield change in $^{1}H$ chemical shift is observed for $H_{3\beta}$ when the 3α hydroxyl group is added to 5β-cholanoic acid to form lithocholic acid. The addition of the 7α or 7β hydroxyl groups to lithocholic acid to form dihydroxy-substituted bile acids causes downfield changes in the chemical shifts of their geminal protons of 2.41 and 2.40, respectively (**Table 2**). This is consistent with the 2.3-ppm downfield change in chemical shift of the axial and equatorial geminal protons to the hydroxyl group in 4-*t*-butylcyclohexanol (18), which is generally regarded as a sigma-inductive effect due to the electronegative hydroxyl group (17). The addition of a 12α hydroxy group to lithocholic acid to form deoxycholic acid causes a 1.97-ppm downfield

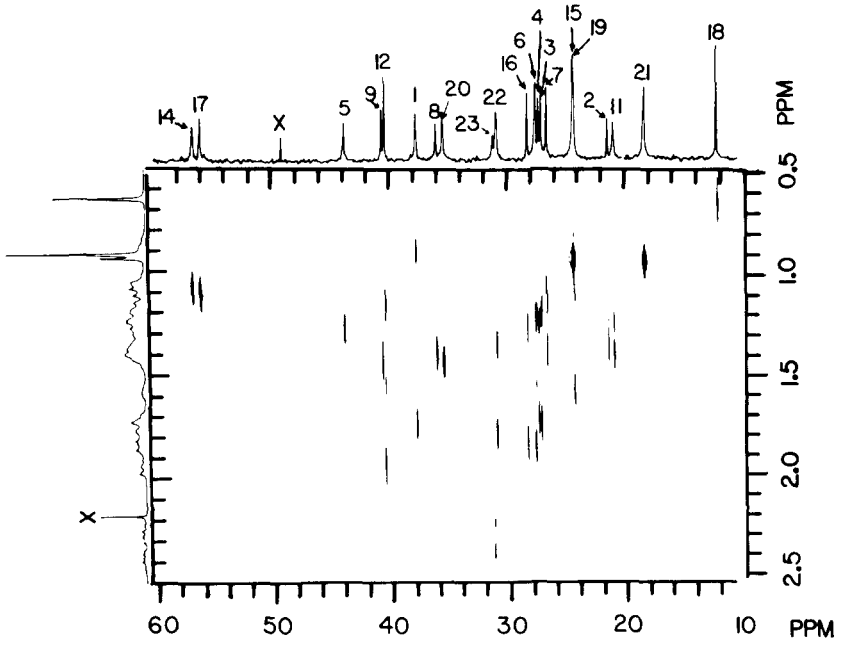


**Fig. 7.** Heteronuclear-correlated 2D NMR contour plot of 5β-cholanoic acid (see Fig. 5). Note the resolution of the peaks in the contour plot of the five closely spaced methylene carbons between 26.6 and 28.2 ppm. This enabled accurate assignments of the associated $^1$H resonances.

chemical shift for the geminal $H_{12\beta}$ proton. The smaller downfield effect for the 12α hydroxyl group than for the 3α, 7α, and 7β hydroxyl groups is consistent with the 2.02-ppm downfield chemical shift observed for the equatorial geminal proton to the hydroxy group in 2-methylcyclohexanol (19) and is due to shielding of this proton by the $C_{18}$ methyl group. In contrast, the 2.35-ppm downfield change in $^1$H chemical shift of the axial proton geminal to a 12β hydroxyl group (20) indicates that shielding from the $C_{18}$ methyl group does not occur for this proton.

TABLE 2. The substituent effects of the hydroxyl groups on individual $^1$H resonances of hydroxycholanoic acids

| | 3α-Effect | | 7α-Effect | | 7β-Effect | | 12α-Effect | | Combined Effects[a] Calculated | | Combined Effects[a] Observed | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbon # | α | β | α | β | α | β | α | β | α | β | α | β |
| 1 | +0.01, | +0.06 | +0.08, | +0.05 | +0.06, | +0.09 | +0.02, | +0.04 | +0.11, | +0.15 | +0.07, | +0.11 |
| 2 | −0.05, | +0.26 | +0.07, | −0.01 | −0.01, | +0.02 | −0.15, | +0.01 | +0.17, | +0.24 | +0.11, | +0.25 |
| 3 | — | +2.33 | — | −0.14 | — | −0.04 | — | +0.03 | — | +2.22 | — | +2.19 |
| 4 | −0.01, | +0.22 | +0.54, | +0.21 | +0.10, | +0.10 | +0.08, | +0.03 | +0.61, | +0.46 | +0.57, | +0.43 |
| 5 | — | +0.08 | — | +0.01 | — | +0.12 | — | +0.04 | — | +0.13 | — | +0.09 |
| 6 | −0.03, | +0.03 | +0.29, | +0.15 | +0.37, | −0.23 | +0.03, | +0.06 | +0.35, | +0.18 | +0.33, | +0.09 |
| 7 | +0.01, | +0.02 | — | +2.41 | +2.40 | — | +0.03, | +0.10 | — | +2.46 | — | +2.43 |
| 8 | — | −0.01 | — | +0.12 | — | +0.07 | — | +0.08 | — | +0.19 | — | +0.16 |
| 9 | +0.02 | — | +0.46 | — | +0.07 | — | +0.48 | — | +0.96 | — | +0.86 | — |
| 11 | −0.01, | −0.01 | +0.10, | +0.12 | +0.09, | +0.11 | +0.15, | +0.30 | +0.24, | +0.41 | +0.19, | +0.33 |
| 12 | +0.02, | +0.04 | +0.07, | +0.04 | +0.05, | +0.07 | — | +2.02 | | +2.10 | — | +2.05 |
| 14 | −0.02 | — | +0.43 | — | +0.04 | — | +0.57 | — | +0.98 | — | +0.93 | — |
| 15 | −0.02, | +0.03 | +0.05, | +0.18 | +0.42, | +0.34 | +0.05, | +0.06 | +0.12, | +0.21 | +0.10, | +0.17 |
| 16 | −0.03, | −0.02 | +0.05, | +0.05 | +0.01, | +0.03 | +0.02, | +0.02 | +0.04, | +0.05 | +0.02, | +0.03 |
| 17 | 0.00 | — | +0.08 | — | +0.15 | — | +0.73 | — | +0.81 | — | +0.76 | — |
| 18 | −0.01 | | +0.05 | | +0.07 | | +0.07 | | +0.11 | | +0.07 | |
| 19 | 0.00 | | +0.02 | | +0.03 | | +0.02 | | +0.04 | | +0.01 | |
| 20 | −0.02 | | +0.04 | | +0.03 | | +0.01 | | +0.03 | | 0.00 | |
| 21 | −0.03 | | +0.04 | | +0.04 | | +0.09 | | +0.10 | | +0.07 | |
| 22 | −0.04, | −0.05 | +0.04, | +0.02 | +0.05, | +0.03 | +0.03, | +0.06 | +0.03, | +0.03 | 0.00, | +0.01 |
| 23 | −0.06, | −0.12 | −0.01, | +0.12 | +0.03, | +0.09 | +0.06, | +0.11 | −0.01, | +0.11 | −0.01, | −0.03 |

[a]Combined effects are the chemical shift changes caused by the introduction of three hydroxyl groups (3α, 7α, and 12α) to 5β-cholanoic acid. The calculated combined effects are the summation of the individually observed 3α-, 7α-, and 12α-effects, whereas the observed combined effects are the chemical shift differences between cholic acid and 5β-cholanoic acid.

Substituent effects, other than those due to the proton geminal to the hydroxyl group, have been modeled by Zurcher (17) by a linear electric field (LEF) effect. The dipole moment of the C–OH bond induces an electric field along the C–H dipole. This interaction has an inverse cube distance dependence and an angular term which is consistent with a dipole-dipole interaction. Calculations using this model have been confined to only the methyl groups of the related monohydroxy cholestanes (17). These substituent effects can now be observed for all the $^1$H resonances (Table 2), and calculations are now in progress to determine the appropriateness of the models.

Notwithstanding a detailed calculation of $^1$H chemical shifts, the effects of hydroxyl substitution can be evaluated empirically. By subtracting the $^1$H chemical shifts of the protons of 5$\beta$-cholanoic acid from those of cholic acid (Table 1), the total effects of adding the 3$\alpha$, 7$\alpha$, and 12$\alpha$ hydroxyl groups are found (see Observed Combined Effect in Table 2). The primary $^1$H chemical shift changes, besides those for the geminal protons to the hydroxyl groups, are observed for $H_4$ on the A ring (0.57 ($\alpha$) and 0.43 ($\beta$) ppm), $H_{9\alpha}$ on the B ring (0.86 ppm), $H_{14\alpha}$ on the C ring (0.93 ppm), and $H_{17\alpha}$ on the D ring (0.76 ppm). In order to determine the contribution of the 3$\alpha$ hydroxyl group to these $^1$H chemical shift changes, the $^1$H chemical shifts of protons for 5$\beta$-cholanoic acid are subtracted from the corresponding values for lithocholic acid (see 3$\alpha$-effect in Table 2). The main 3$\alpha$-effects are the 0.22-ppm and 0.26-ppm downfield changes in $^1$H chemical shifts of the $H_{4\beta}$ and $H_{2\beta}$ protons. All other $^1$H chemical shift changes are small, indicating that the large downfield changes in chemical shift of the $H_{9\alpha}$, $H_{14\alpha}$, and $H_{17\alpha}$ protons in cholic acid are not due to this hydroxyl group.

The 7$\alpha$-effect can be determined by comparing $^1$H resonances for chenodeoxycholic acid and lithocholic acid (Table 2). Large downfield changes in chemical shift of 0.54 ($\alpha$) and 0.19 ($\beta$) ppm occur for the two $H_4$ protons. Thus, most of the chemical shift changes for the $H_4$ protons in cholic acid arise from the effects of the 3$\alpha$ and 7$\alpha$ hydroxyl groups. Furthermore, the $^1$H chemical shifts of the $H_{9\alpha}$ and $H_{14\alpha}$ protons move downfield by nearly equal amounts of 0.46 and 0.43 ppm, respectively, due to the 7$\alpha$-effect. These protons are each related to the 7$\alpha$ hydroxyl group by a 1,3 diaxial orientation (Fig. 1), which suggests that the through space mechanism of the LEF theory is operative. No significant change is seen for the $^1$H chemical shift of the $H_{17\alpha}$ proton due to its larger distance from the 7$\alpha$ hydroxyl group.

Even though cholic acid does not contain a 7$\beta$ hydroxyl group, a 7$\beta$-effect can be found by comparing $^1$H resonances for ursodeoxycholic acid and lithocholic acid (Table 2). One finds that, in contrast to the 7$\alpha$-effect, the $H_4$, $H_{9\alpha}$, and $H_{14\alpha}$ proton resonances undergo only small chemical shifts. Since the $H_4$, $H_{9\alpha}$, and $H_{14\alpha}$ protons are more distant from the 7$\beta$ hydroxyl group than the 7$\alpha$ hydroxyl group, it is expected that a hydroxyl group in the $\beta$ position would not affect the chemical shifts of these protons as much as a hydroxyl group in the $\alpha$ position. The 7$\beta$-effect, therefore, supports a through space mechanism for the chemical shift changes, since a through bond mechanism should have resulted in similar changes from either substitution position. Further evidence for a through space mechanism can be seen by considering the $^1$H chemical shift changes for the $H_{15}$ protons which are much larger (0.34 and 0.42 ppm) for the 7$\beta$-effect than the 7$\alpha$-effect (0.18 and 0.05 ppm).

The 12$\alpha$ effect is illustrated by comparison of the $^1$H resonances of deoxycholic acid and lithocholic acid. The $^1$H resonances of the $H_4$ protons do not change significantly (0.08 ($\alpha$) and 0.03 ($\beta$) ppm), because of their distance from the 12$\alpha$ hydroxyl group. The $^1$H resonances of the $H_{9\alpha}$ and $H_{14\alpha}$ protons show large downfield changes (0.48 and 0.57 ppm, respectively), which are similar to the changes caused by the 7$\alpha$-effect (0.46 and 0.43 ppm, respectively), dramatizing their 1,3 diaxial orientation to both of these substituents. Thus, the chemical shift changes of these protons in cholic acid arise from nearly equal contributions from the 7$\alpha$ and 12$\alpha$ hydroxyl groups. The $^1$H resonance of the $H_{17\alpha}$ proton shows a large 0.73-ppm downfield change since it is also 1,3 diaxially related to the 12$\alpha$ hydroxyl group. Hence, the downfield change in $^1$H chemical shift of $H_{17\alpha}$ in cholic acid is due almost entirely to the 12$\alpha$ hydroxyl group.

A comparison can be made between the calculated and observed $^1$H chemical shift values of every proton of cholic acid. Summation of the 3$\alpha$, 7$\alpha$, and 12$\alpha$ changes in $^1$H chemical shift generated from the mono- and dihydroxy 5$\beta$-cholanoic acids only (see Calculated Combined Effect in Table 2) can be compared to the $^1$H chemical shift changes between the trihydroxy 5$\beta$-cholanoic acid, cholic acid, and unsubstituted 5$\beta$-cholanoic acid (see Observed Combined Effect in Table 2). The calculated values are mostly within 0.05 ppm of the observed change, which is in excellent agreement given the known error of $\pm$0.03 ppm for any $^1$H resonance measured in the hetcor 2D NMR experiment. The small deviations are generally downfield and may be explained as solvent effects, since it was not possible to obtain data for all of these compounds in a single solvent. Overall, the predictions are good and it is expected that the hydroxyl substituent effects can be used to predict the $^1$H chemical shifts of 5$\beta$-cholanoic acids having any combination of these hydroxyl substituents.

## CONCLUSIONS

The evolution of higher field spectrometers and the many new pulse techniques such as 2D NMR spectroscopy now make characterization of proton resonances easier.

This work shows how the heteronuclear correlated 2D NMR experiment can be used to provide this information for a series of bile acids by taking advantage of the well-resolved $^{13}C$ resonances to make the $^{1}H$ assignments, even with the unsubstituted bile acid, 5$\beta$-cholanoic acid. The compiled set of substituent effects for the $^{1}H$ chemical shifts (Table 2) can be used to predict the $^{1}H$ chemical shift assignments for other bile acids not covered in this study. This is a first step in characterizing the dynamics and micellar properties of this biologically important class of compounds.

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