Nuclear magnetic resonance identification of the taurine conjugate of 3α,6β,7β-trihydroxy-5β,22-cholen-24-oic acid (tauro-Δ22-β-muricholate) in the serum of female rats treated with α-naphthylisothiocyanate

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Abstract Recently developed ^{1}H nuclear magnetic resonance methods, including 2-dimensional, ^{1}H-detected-{^{13}C} shift correlation spectroscopy and 1-dimensional HOHAHA spectroscopy at 500 MHz have been used to identify the major bile acid in the serum of rats treated with α-naphthylisothiocyanate as the taurine conjugate of 3α,6β,7β-trihydroxy-5β,22-cholen-24-oic acid (tauro-Δ^{22}-β-muricholate), a derivative of β-muricholate having an unsaturated bond in the acyclic side chain. Complete stereospecific assignments of the ^{1}H and protonated ^{13}C spectra of the title compound and β-muricholate are reported. The assignments were based entirely on ^{1}H-^{1}H and ^{1}H-^{13}C scalar connectivities and were made using approximately 0.5–2.0 mg of material. It is suggested that these new methods will be of general value for identifying the structure and assigning the spectra of other scarce steroid-like molecules. **—Davis, D. G., and M. B. Thompson.** Nuclear magnetic resonance identification of the taurine conjugate of 3α,6β,7β-trihydroxy-5β,22-cholen-24-oic acid (tauro-Δ^{22}-β-muricholate) in the serum of female rats treated with α-naphthylisothiocyanate. *J. Lipid Res.* 1993. **34:** 651–661.

Supplementary key words proton/carbon chemical shifts • 1D-HOHAHA • 2D-HSQC • indirect detection • β-muricholate • isotropic mixing • stereospecific proton assignments • scalar connectivity

In a previous study in our laboratory, the effects of induced hepatobiliary disruptions on the concentrations of bile acids in the serum of rats was examined using an HPLC/enzymatic method to quantitate the concentrations of individual bile acids (1). Concentrations of an unidentified bile acid, present at low levels in control animals, increased substantially (80-fold) in female rats treated with α-naphthylisothiocyanate (ANIT), a chemical that produces bile duct necrosis and cholestasis (2–6). Subsequent investigations showed that, in addition to confirming these initial findings, the serum concentration of the unknown also increased in male and female rats in which selective and total bile duct obstruction was surgically produced. This unknown was also secreted by cultured hepatocytes and by slices of rat liver and the level of its production was further increased by the addition of β-muricholate (β-MC, **Fig. 1A**) to the media. Other investigators have also reported increased metabolites of β-MC in rats after treatment with compounds that produce cholestasis (7–10). Because of these findings we considered β-MC to be the probable precursor of the unknown bile acid.

The goal of the present study was to identify this unknown bile acid and assign its ^{1}H and ^{13}C NMR spectra using recently developed NMR methods. The first method was two-dimensional ^{1}H-detected-{^{13}C} single quantum chemical shift correlation spectroscopy (HSQC) (11) which is substantially more sensitive than heteronuclear correlation methods involving ^{13}C-detected NMR. The second method was one-dimensional homonuclear Hartmann-Hahn cross polarization spectroscopy (1D-HOHAHA/TOCSY) (12–14), which provides separate subspectra (even in the presence of severe multiplet overlap) of those protons that make up mutually coupled spin networks. As demonstrated below, the controlled development of the subspectra yield topological maps of proton-proton J-connectivity.

These newer methods were developed specifically for the kind of problem presented here; namely, the analysis of scarce compounds with complex spectra. Moreover, they provide methods for assignment that are based on

Abbreviations: ANIT, α-naphthylisothiocyanate; β-MC, β-muricholate; Δ^{22}-β-MC, 3α,6β,7β-trihydroxy-5β, 22-cholen-24-oic acid; FID, free induction decay; HSQC, heteronuclear single quantum shift correlation; HPLC, high performance liquid chromatography; HOHAHA, homonuclear Hartmann Hahn cross-polarization; rf, radio frequency; T, taurine (tauro-); TMS, tetramethylsilane; TOCSY, total correlation spectroscopy; NMR, nuclear magnetic resonance.

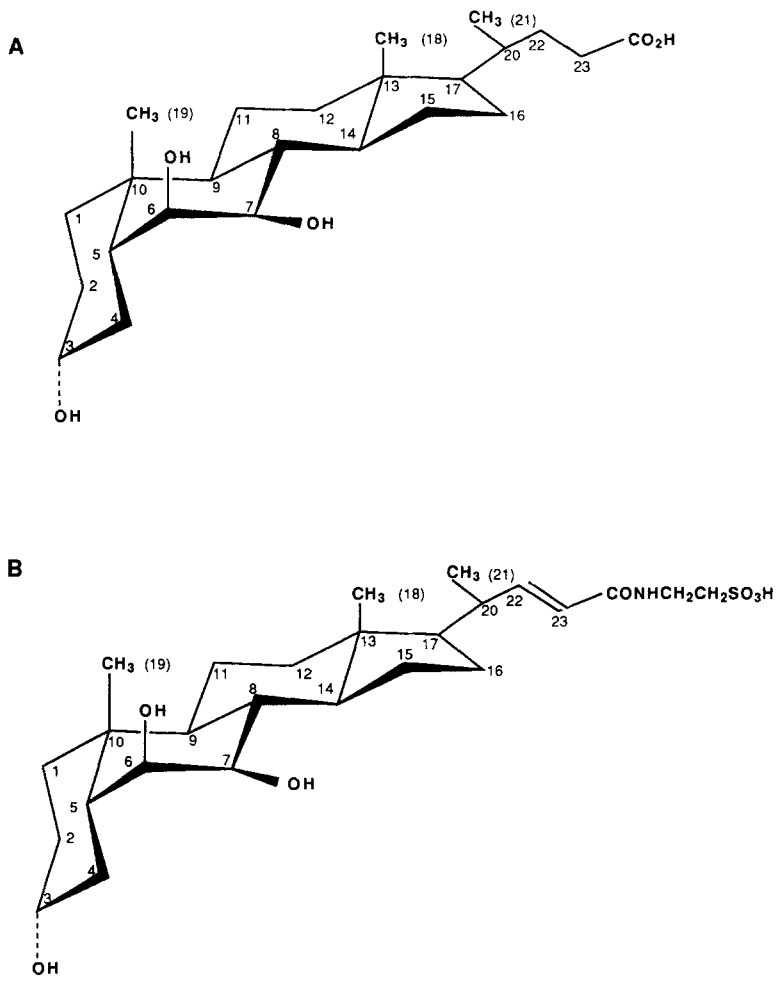

Fig. 1. Molecular structures of (A): β-muricholic acid, and (B): tauro-Δ^{22}-β-muricholate.

connectivities determined by hetero- and homonuclear scalar couplings. As originally shown by others working on bile acids (15, 16) and steroids (17), this strategy is more reliable than empirical methods based on similarities of chemical shifts in structurally related compounds (18, 19). The latter method, although widely used, may lead to errors in the assignment of functionally similar groups because of subtle effects introduced by neighboring group substituants, conformation, and solvent conditions.

This report describes the application of these NMR methods to identify an unidentified bile acid in the serum of female rats treated with ANIT as: tauro-3α,6β,7β-trihydroxy-5β,22-cholen-24-oic acid (tauro-Δ^{22}-β-muricholate; T-Δ^{22}-β-MC, Fig. 1B). Additionally, complete stereo-specific assignments of the ^{1}H and protonated ^{13}C NMR spectra of Δ^{22}-T-β-MC and its precursor, β-MC are reported. A companion paper, describes a complete analysis and comparison of the bile acid profiles in treated and control rats as well as in vitro studies using liver slices from control and ANIT-treated rats (20).

MATERIALS AND METHODS

Animals and treatments

Seven female Fischer 344 rats (Charles River Laboratories, Raleigh, NC), weighing between 181 and 201 g, were administered 20 mg/kg ANIT (Sigma Chemical Co, St. Louis, MO) in corn oil (5 mg/ml) two times a day for 3 days by gavage. Six h after the last treatment on the third day, all animals were anesthetized with CO_2 and blood samples (4 to 5 ml) were collected from the right cardiac ventricle. For all samples, serum was separated and stored at $-70°C$ for extraction of bile acids at a later time.

Sample collection, preparation, and cleanup of unknown

Extraction of serum bile acids using C18 cartridges (Sep-Pak, Waters Associates, Milford, MA) and analysis of individual bile acids using reversed-phase HPLC and post column enzymatic reaction with fluorescence detec-

tion have been described previously (1). In the current study, UV (Model 481, Waters Associates, Milford, MA) and fluorescence (Model FL-750, McPherson Instrument, Acton, MA) detectors were used simultaneously in the system during pilot runs. This procedure allowed initial fluorescence detection of the peak (365 nm excitation, zero order emission with a 420 nm cut-off filter) and recognition of the corresponding peak with the UV detector (210 nm). Subsequently, collections of the unknown bile acid were performed using the HPLC system and UV detection to isolate the peak. This procedure permitted the intact bile acid to be collected in the mobile phase (acetonitrile and ammonium phosphate buffer) and avoided the addition of the enzyme buffer (Tris/βNAD$^+$/dithiothreitol), which is required for fluorescence detection, to the collections.

The appropriate fractions, ($\sim$15 ml $\times$ 60) were pooled, evaporated under nitrogen to 175 ml, and the volumes were adjusted to 500 ml with deionized water. Aliquots of the solution were passed through C18 cartridges. The latter were washed exhaustively with deionized water and the unknown bile acid was eluted with acetonitrile. The samples were pooled, evaporated to dryness, and prepared for NMR analysis.

NMR analysis

For the NMR experiments, samples estimated to be 1.9 and 0.5 mg, respectively, of β-MC (Fig. 1A) (Steroids, Inc. Wilton, NH), and the unknown (Fig. 1B) were dissolved in $\sim$0.4 ml of 99.96% methanol-d_4 (Cambridge Isotopes Laboratories, Woburn, MA), transferred to 5-mm OD NMR tubes, and capped. For some ^{1}H NMR spectra, a 0.25-mg portion of the unknown sample was similarly prepared in 99.96% dimethyl sulfoxide-d_6 (also from CIL). After completion of the NMR studies, the actual concentrations of the samples were confirmed quantitatively by HPLC as described above and in the companion paper (20).

The NMR experiments were performed on a GE 500 MHz spectrometer (GN 500 series) equipped with a 1280 processor, a 293 pulse programmer, a reverse detection probe, and a broad-banded heteronuclear decoupling accessory. For all experiments, the temperature of the probe was kept at 30°C($\pm$ 0.2°) via the temperature control unit of the spectrometer and data were acquired without sample spinning.

The pulse sequence for the 2D HSQC-^{1}H-{^{13}C} experiment (11) is shown in **Fig. 2A.** The phase cycling, data

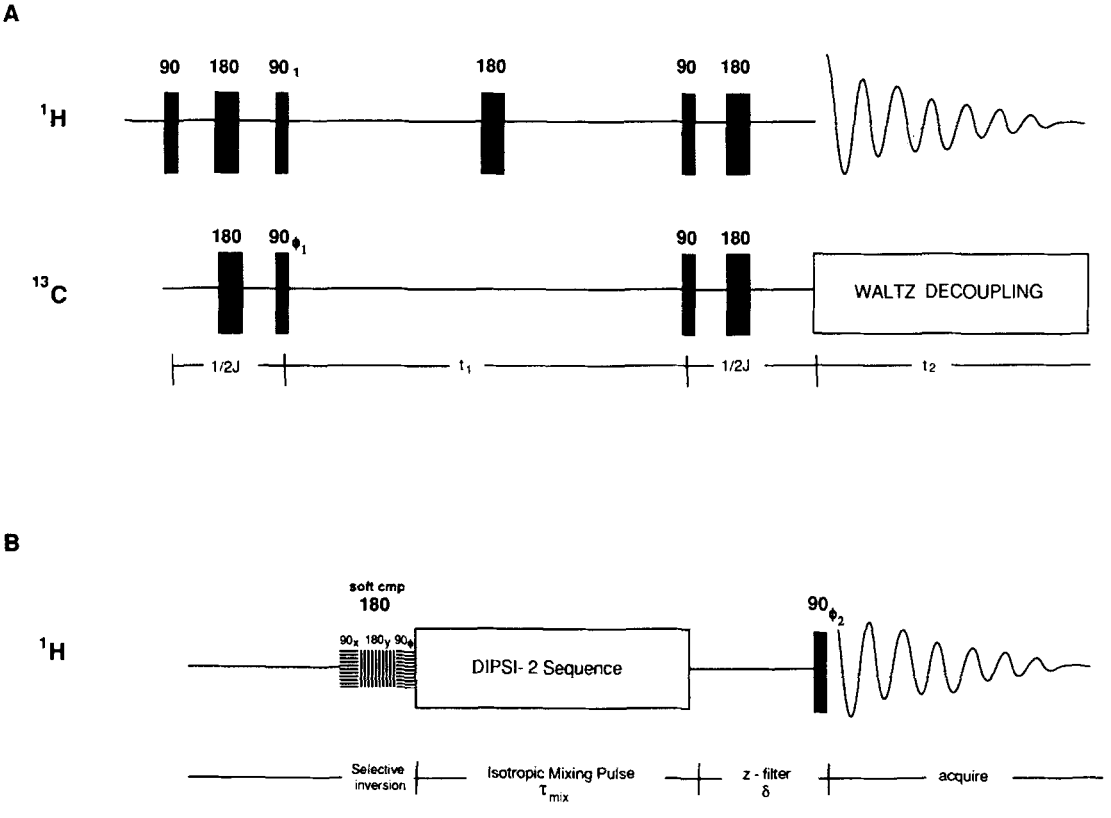

Fig. 2. NMR pulse sequences for: (A) 2D ^{1}H-{^{13}C} single quantum shift correlation (HSQC) experiment; (B) selective excitation of ^{1}H subspectra via isotropic mixing (1D-HOHAHA). Details concerning phase cycling and rf power levels are described in Methods section.

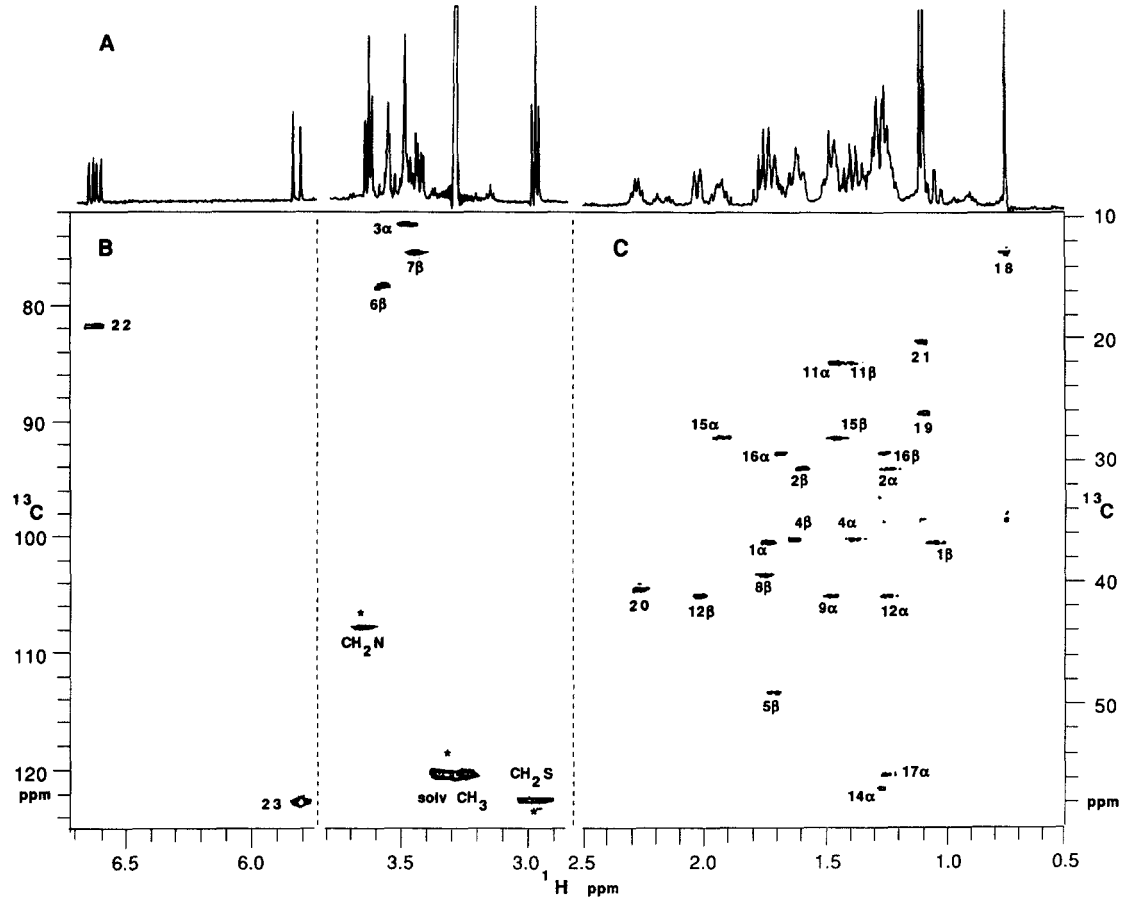

Fig. 3. ^1H and ^1H-{^{13}C} shift correlation spectra of tauro-Δ^{22}-β-muricholate: (A) 500 MHz ^1H NMR spectrum in CH_3OH-d_4; (B) 2D absorption mode contour plot of the olefinic and CHOH regions of the ^1H and ^{13}C spectra. The frequency of the ^{13}C decoupler was centered at 98 ppm. Cross peaks labeled with (*) are folded in the ^{13}C frequency dimension from the top edge of the plot; (C) 2D contour plot of the "methylene envelope" region with the ^{13}C decoupler frequency set at 35 ppm. Except for the different decoupler frequencies and ^{13}C spectral widths, the experimental conditions for acquisition of the data plotted in panels B and C are identical and are described in the Methods section.

routing, and processing were set for a pure absorption-mode presentation (21). The phases of the ^1H pulses were fixed, but in the ^{13}C channel, ϕ was cycled with each acquisition (in multiples of $\pi/2$) by: 0,1,2,3. The phase of the receiver, operated in the quadrature detection mode, was cycled by: 0,0,2,2, with even and odd acquisitions stored in alternate blocks of memory. Typically, the 90°(^1H) pulse was 22 μs, and in the decoupler channel, a 90°(^{13}C) pulse was 45 μs during the preparation and mixing periods. To minimize sample heating and interference with the observe channel during acquisition, the power in the decoupler channel was attenuated by 6 dB, to give a 90°(^{13}C) pulse of 90 μs in the WALTZ16 decoupling sequence (22). The delays, 1/2J were set to 3.2 ms, corresponding to a value of J_{CH} of ~156 Hz. The (t_1,t_2) data sets generally consisted of 2 × 128 × 512 real points (2 × 96 acquisitions/t_1 increment) with acquisition times, AT_1 and AT_2 of 20.2 ms and 256 ms (corresponding to spectral widths, SW_1 and SW_2 of 6329 Hz and 2000 Hz), respectively. The total run time was ≈16 h per 2D data set. Prior to Fourier transformation to the (F_C, F_H) frequency domain, the t_1 data (^{13}C) were zero-filled once, and the t_2 data files (^1H) were multiplied by a 4Hz Gaussian filter. No apodization was applied to the t_1 files.

The pulse sequence for the selective ^1H 1D-HOHAHA experiment (12–14) is shown in Fig. 2B, and is adapted from a study by Rucker and Shaka (14). Here, a low power composite 180° pulse from the ^1H decoupler (40–60 ms duration) is used to selectively invert an isolated proton multiplet (23); then a high power, composite pulse train or isotropic mixing pulse (14) was applied to the system. Under these conditions the selectively inverted magnetization (i.e., "excitation") is transferred to its directly coupled partners which, in turn, relay the excitation to their coupled partners (24). The propagation of the excitation through the spin-coupled network is then observed by Fourier transforming the free induction decay (FID) generated by the final nonselective 90° pulse. By taking the difference between spectra created with and without selective inversion (i.e., by alternating the phase

ϕ of the second 90° pulse in the low power composite decoupler pulse by 0° and 180° (+x,−x) and alternately adding and subtracting the FID from memory) only the subspectra of the perturbed protons are observed. The delay, δ, between the mixing pulse and the observe pulse is an optional z-filter that can be used to minimize distortions in the subspectra that arise from antiphase components which are unavoidably generated by the isotropic mixing process (13). In the subspectra shown below, z-filtering was implemented by coadding difference spectra collected with 11 values of δ, ranging from 0 to 7.0 ms in increments of 700 μs.

Because isotropic mixing sequences involve the application of moderately high power pulses (90°(^{1}H) $\approx$48 μs) for sustained periods of time (10–150 ms), the proton high power (Class C) rf amplifier and attenuators in the GN console were bypassed. Instead, the low level proton rf from the console was directed externally through an attenuator, a broad-banded 25 W linear amplifier (ENI Model 525LA) and 500 MHz band pass filter before being connected to the probe interface module.

RESULTS

Because the unknown bile acid was thought to be derived from and thus structurally related to β-MC, NMR studies were conducted in parallel on both compounds. The conventional 1-dimensional ^{1}H NMR spectrum of the unknown in methanol-d_4 (**Fig. 3A**), had three methyl group resonances at 0.75, 1.10, and 1.11 ppm; the latter was split into a 7 Hz doublet via coupling to a single proton. These three lines are common to all C_{24} bile acids and can be assigned to the C_{18}, C_{19}, and C_{21} methyl groups, respectively. Additionally, there were three partially resolved, single proton resonances in the CHOH region of the spectrum at 3.58, 3.48, and 3.45 ppm, indicating that the unknown was a triol derivative. The lowest

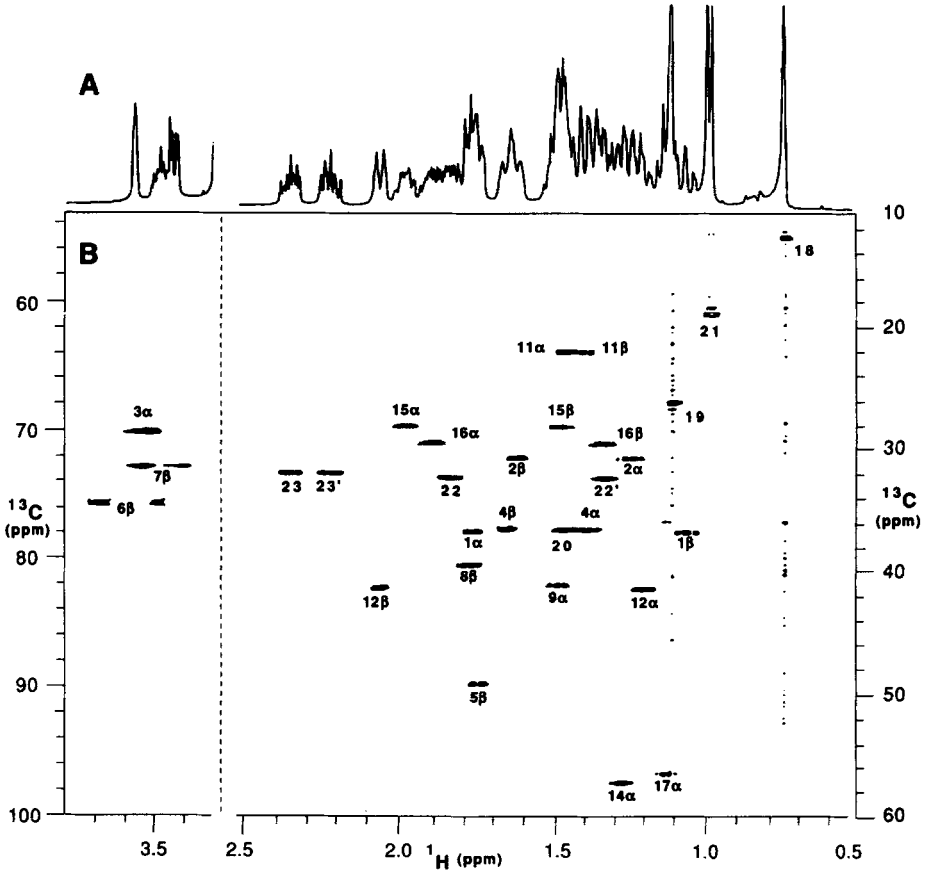

Fig. 4. ^{1}H and ^{1}H-{^{13}C} shift correlation spectra of β muricholate: (A) 500 MHz ^{1}H NMR spectrum in CH_3OH-d_4; (B) 2D absorption mode contour plot of the CHOH and "methylene envelope" regions of the ^{1}H and ^{13}C spectra. The frequency of the ^{13}C decoupler was centered at 35 ppm. Although the cross peaks for the CHOH and methylenes were present in the same data set, those for the former were, in fact, folded in the ^{13}C frequency dimension from the bottom of the plot. The left hand axis was therefore relabeled to reflect their true chemical shifts. The cross peaks for C_6H and C_7H appear as doublets in the ^{1}H dimension due to incomplete ^{13}C decoupling. The experimental conditions for B are the same as those used for Fig. 3C.

TABLE 1. ^{13}C and ^{1}H resonance assignments for Δ^{22}-Tβ-MC and 5β-MC[a,b,c]

	Tauro-Δ^{22}-β-muricholate Chemical Shifts (ppm)			β Muricholate Chemical Shifts (ppm)		
		^{1}H			^{1}H	
Carbon	^{13}C	α	β	^{13}C	α	β
1	36.71	1.75	1.06	36.75	1.76	1.07
2	30.60	1.25	1.61	30.68	1.25	1.62
3	71.46		3.50	70.40		3.49
4	36.35	1.39	1.64	36.49	1.40	1.65
5	48.94		1.74	49.19		1.74
6	76.68	3.58		76.05	3.58	
7	73.66	3.45		73.24	3.45	
8	39.30		1.77	39.38		1.76
9	41.05	1.49		40.77	1.49	
11	21.84	1.49	1.42	21.98	1.46	1.39
12	41.05	1.25	2.04	41.31	1.21	2.05
14	56.82	1.29		57.28	1.28	
15	28.08	1.94	1.47	29.50	1.98	1.49
16	29.29	1.70	1.27	30.68	1.89	1.35
17	55.79	1.27		56.50	1.13	
18	12.75	0.76		12.61	0.75	
19	26.01	1.11		26.05	1.11	
20	40.50	2.29		36.49	1.47	
21	20.04	1.12		18.82	0.99	
22	81.0	6.65		32.28	1.34, 1.83	
23	122.58	5.82		31.93	2.22, 2.35	

[a]In ppm from Me_4Si (TMS).
[b]^{13}C resonances for quarternary carbons 10, 13, and 24 are not observed.
[c]Stereo assignments of methylene Hs were made according to methods described in the text.

field line was weakly split into a "triplet" (J ~3 Hz), the middle line into a broad complex multiplet, and the high field line into a doublet of doublets with splittings of 3 and 10 Hz. As demonstrated below, these lines can be assigned to CHs with OH substituents at carbons 6, 3, and 7, respectively. These same spectral properties are essentially replicated in the ^{1}H spectrum of β-MC (**Fig. 4A**).

Unique features in the spectrum of the unknown included a pair of single proton multiplets in the olefinic, CH=CH region of the spectrum at 6.65 and 5.82 ppm. These lines were coupled to each other with a coupling constant, J = 16 Hz, which was indicative of a vicinal, *trans* interaction. Additionally, excluding the CH_3 lines, only 20 protons appeared in the so-called 'methylene envelope' region situated between 2.4 to 1.0 ppm. For β-MC and bile acids in general, 24 protons are commonly found in this region of the spectrum. The ^{1}H spectrum of the unknown also contained a pair of coupled multiplets of two protons each at 3.66 and 2.99 ppm that can be assigned to the NCH_2 and CH_2SO_3- protons of taurine. In DMSO-d_6, the spectrum of the unknown shows a low field, single proton triplet at 7.81 ppm (J = 6 Hz) which is coupled to the CH_2 line at 3.66 ppm. This proton, which is lost via solvent exchange in methanol-d_4, can be assigned to NH of the amide bond that links the taurine moiety to bile acid at C_{24}.

The similarities and differences between the unknown and β-MC were evident in the 2-dimensional ^{1}H-{^{13}C} correlation spectra of the 'methylene envelope' region for the two compounds. These spectra are presented as contour plots in Fig. 3B and Fig. 4B, respectively, and the carbon and proton chemical shifts are listed in **Table 1.** Except for the pronounced difference in the positions of the cross peaks that have been assigned (vide infra) to C_{20}, and the absence (for the unknown) of cross peaks similarly assigned to C_{22} and C_{23} (for β-MC), the coincidence of cross peaks for the compounds was sufficiently close to suggest that these peaks report or identify structurally identical elements of the steroid ring moieties. The structural correspondence in the ring portions of the compounds are also reflected in the cross peak positions for the three CHOH groups. Indeed these spectra provide a clear indication of the structural modification that converts β-MC into the unknown, namely dehydrogenation at $C_{22,23}$ to give a *trans* $C_{22,23}$ double bond. Thus it was concluded that the unknown bile salt is the taurine conjugate of 3α,6β,7β-trihydroxy-5β,22-cholen-24-oic acid (tauro-Δ^{22}-β-muricholate; T-Δ^{22}-β-MC, Fig. 1B).

To confirm this and provide experimentally independent assignments of the ^{1}H and ^{13}C spectra, the method of selective ^{1}H subspectral excitation was used to determine the J connectivites of the protons. For example, the subspectra generated by the selective excitation of the C_{21} methyl resonance of β-MC is shown in **Fig. 5.** For a short mixing time of 12 ms, magnetization from the methyl group was transferred solely to its directly coupled neighbor H_{20}, at 1.46 ppm. At a mixing time of 36 ms the magnetization has been relayed on to $H_{17\alpha}$ at 1.10 ppm, H_{22}, at 1.34 ppm, and to a lesser extent, to H_{22} at 1.83 ppm. Increasing the mixing time to 48 ms, produced additional transfers to H_{22} and $H_{22'}$, and to $H_{16\alpha,\beta}$ and $H_{15\alpha,\beta}$. The β resonances of H_{16} and H_{15} are superimposed on those of $H_{22'}$ and H_{20}, respectively (Fig. 3B). With a doubling of the mixing time to 96 ms, the aforementioned lines continue to receive magnetization transferred and relayed from the point of excitation, H_{21}, growing in intensity, but two new lines, $H_{14\alpha}$ (1.26 ppm) and $H_{8\beta}$ (1.75 ppm) now appear as well. By 144 ms, the excitation has been relayed to $H_{6\beta}$ at 3.55 ppm (insert in Fig. 4E).

Subspectra with different components and excitation pathways were generated by selective excitation introduced at other isolated multiplets such as $H_{12\beta}$ (2.05 ppm), $H_{6\beta}$ (3.58 ppm), $H_{3\alpha}$ (3.49 ppm), and $H_{1\beta}$ (2.17 ppm) (**Fig. 6**). Taken collectively, all the multiplets in the ^{1}H spectra of β-MC were eventually excited, and accounted for in these subspectra.

Similar, although less extensive studies were carried out on T-Δ^{22}-β-MC (**Fig. 7**) to confirm that the subspectral excitation patterns were comparable to those for β-MC. An exception, however, was the subspectra for the protons in the acyclic side chain of T-Δ^{22}-β-MC (Fig. 7A, B).

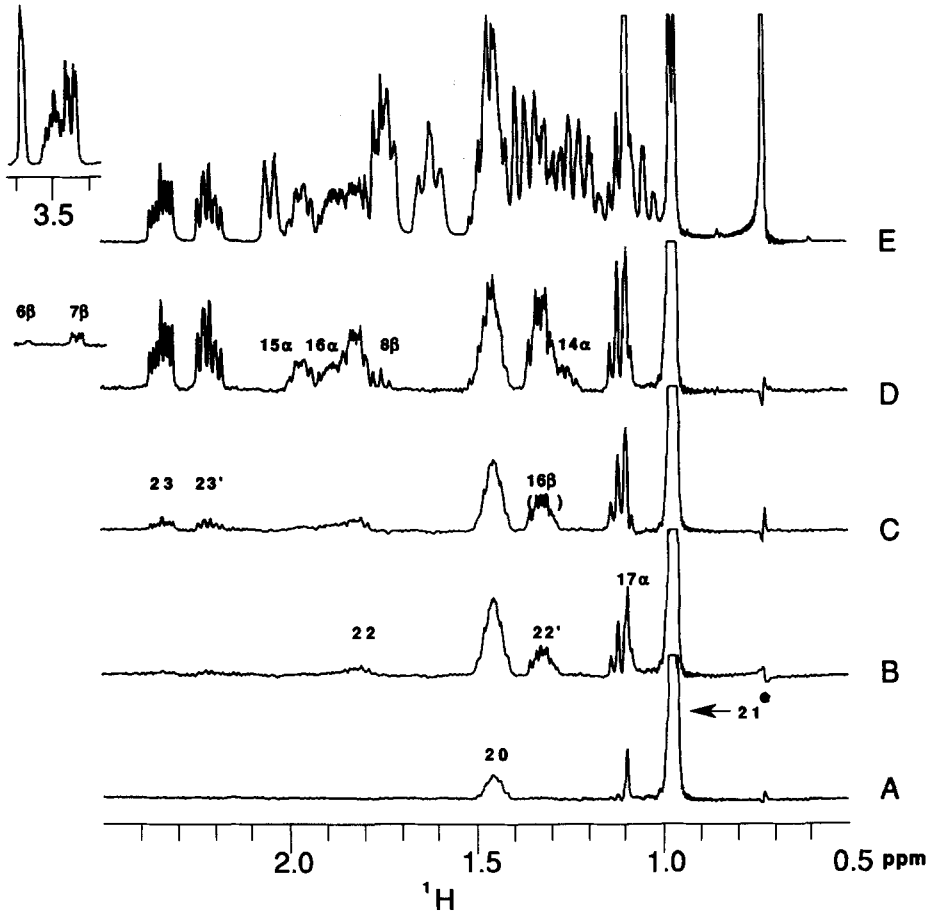

Fig. 5. Z-filtered ^{1}H 1D-HOHAHA subspectra generated by selective excitation of the C_{21} methyl protons of β-muricholate as a functon of the isotropic mixing time: (A) 12 ms; (B) 36 ms; (C) 48 ms; (D) 144 ms; (E) conventional ^{1}H spectrum. The multiplets are assigned in the order they appear with increasing mixing times. Multiplets for H_{15} and $H_{16\beta}$ overlap with those of H_{20} and H_{22}, respectively. The symbol (*) indicates the point of selective excitation.

Whether the selective excitation is introduced via the C_{21} methyl protons at 0.99 ppm or via H_{20} at 2.28 ppm, the distribution of magnetization was confined for short mixing times ($\leq$48 ms), to these two multiplets, to $H_{17\alpha}$ (1.26 ppm) and to the two multiplets in the olefinic region of the spectra (not shown). The latter were assigned accordingly to H_{22} (6.65 ppm) and H_{23} (5.82 ppm). With considerably longer mixing times (144 ms), the excitation was eventually transferred to the protons of the D ring, revealing the resonance positions of $H_{16\alpha,\beta}$, $H_{15\alpha,\beta}$, $H_{14\alpha}$, and $H_{8\beta}$.

Stereospecific assignments of the protons to α or β orientations were based on an analysis of the multiplet splitting patterns and the pattern of magnetization transfer. Both criteria are related to the particular combination of geminal and vicinal couplings that make up the multiplet (17).

DISCUSSION

Despite the advent of high field NMR instrumentation and the development of numerous 2-D homonuclear NMR techniques, the analysis of the ^{1}H spectra of steroids is still a challenging problem because of the severe congestion in the "methylene envelope" region of the ^{1}H spectra. Instead, ^{13}C NMR is the more favored choice for structural analysis, given the relative simplicity of the spectra and the large body of accumulated empirical correlations between ^{13}C chemical shifts and steroid structure (19). Likewise, the potential for obtaining proton chemical shifts and identifying geminal pairs of protons from 2D heteronuclear ^{13}C-{^{1}H} correlation spectra of steroids also makes ^{13}C NMR attractive (16). Unfortunately, direct detection of ^{13}C at natural abundance is relatively insensitive compared to ^{1}H NMR, and gener-

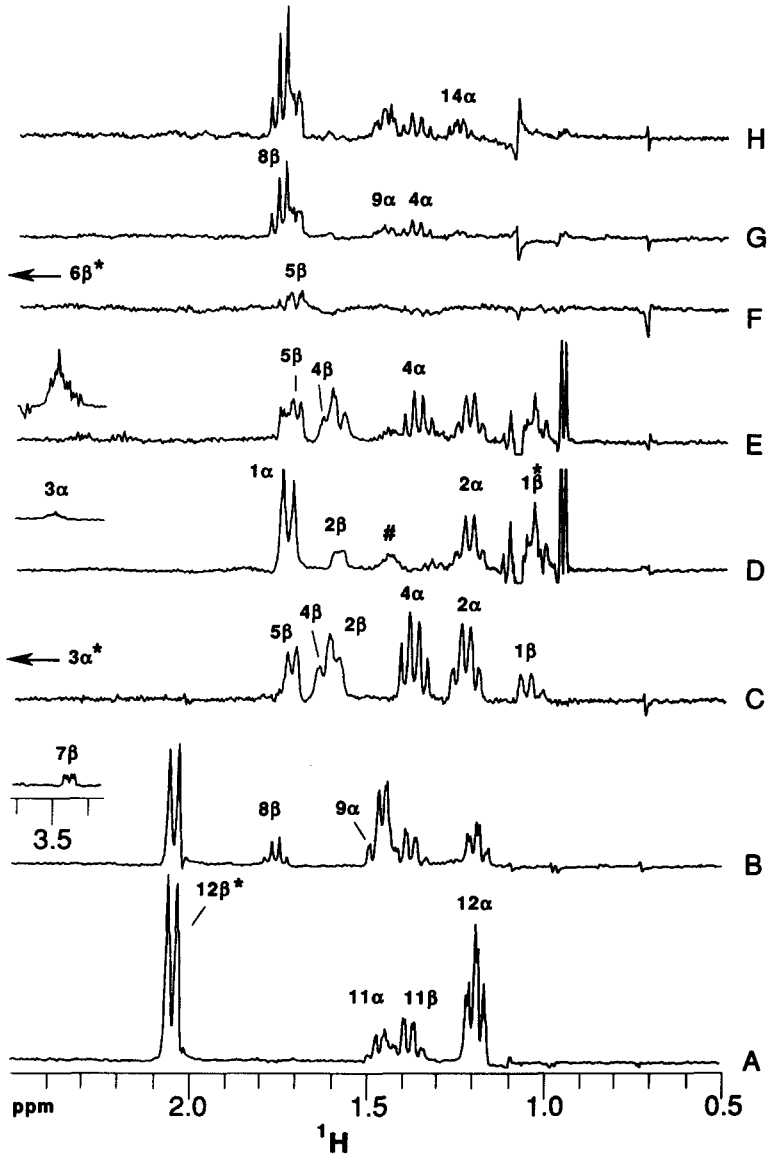

Fig. 6. Z-filtered ^{1}H 1D-HOHAHA subspectra of β-muricholate generated by selective excitation of various isolated multiplets and mixing times. The (*) indicates the points of selective excitation: (A) and (B), excitation of $H_{12\beta}$ with mixing times of 24 and 72 ms, respectively; (C) $H_{3\alpha}$, 24 ms; (D) and (E), $H_{1\beta}$, 18 and 144 ms, respectively. The # symbol indicates transfer to H_{20} due to partial excitation of H_{21}; (F), (G), and (H), $H_{6\beta}$ with mixing times of 24, 48, and 72 ms, respectively.

ally requires several milligrams of material. And, even though geminal protons may be identified, the problem of their stereospecific assignment remains (17, 18). In this study we have demonstrated the application of two new NMR techniques that overcome the limitations of these more established methods. The use of these new methods requires less material for analysis and simplifies the experimental strategies used to assign the spectra.

The strategy involved here is two staged. From the 2D HSQC ^{1}H-{^{13}C} spectra, the chemical shifts of the protonated carbons and their attached protons are determined. Additionally, nonequivalent geminal protons are readily identified, since their cross peaks are separated by their chemical shifts in the ^{1}H dimension. At this stage one may tentatively assign the ^{13}C spectra based on empirical correlations established by comparison of the ^{13}C shifts for structurally similar or related compounds. In fact, the ^{13}C spectra of β-MC was previously assigned in this manner (25) and our independent assignments, (Table 1) based on the methods described here, agree with this previous report, except for the interchange of the C_{14} and C_{17} assignments. We also confirm their tentative assignments for carbons 8 and 9.

The next strategic step is to determine how the CH and

CH_2 groups are pairwise connected via the 3-bond couplings, $^3J_{HH}$ between protons on adjacent carbon atoms. For this, selective ^1H subspectral excitation is ideal, particularly in cases where overlapping multiplets involve protons that are not directly coupled.

In spirit, if not in substance, subspectral excitation is reminiscent of one of the earliest methods used to determine connectivities between proton multiplets, namely double resonance difference spectroscopy (15, 26). It differs, however, in significant ways. First, the excitation that labels connected multiplets is applied before detection and involves a net transfer of magnetization from the initially excited multiplet to its coupled partners. Thus, after nonexcited lines are eliminated in the difference spectra, only the perturbed multiplets with essentially in-phase, absorption mode line shapes remain. Another important property is that when excitation is transferred from the initially excited multiplet to its directly coupled partners, it is also relayed farther along to adjacent protons, and eventually excites the entire spin network. The amount of excitation that is transferred depends in part on the duration of the isotropic mixing sequence, while the extent of transfer depends on the number of proton multiplets that make up the spin network.

As illustrated by the sequence of subspectra for β-MC in Fig. 5, excitation is introduced selectively into the C_{21} methyl resonance. One then follows the propagation of the excitation as a function of the mixing time, which here

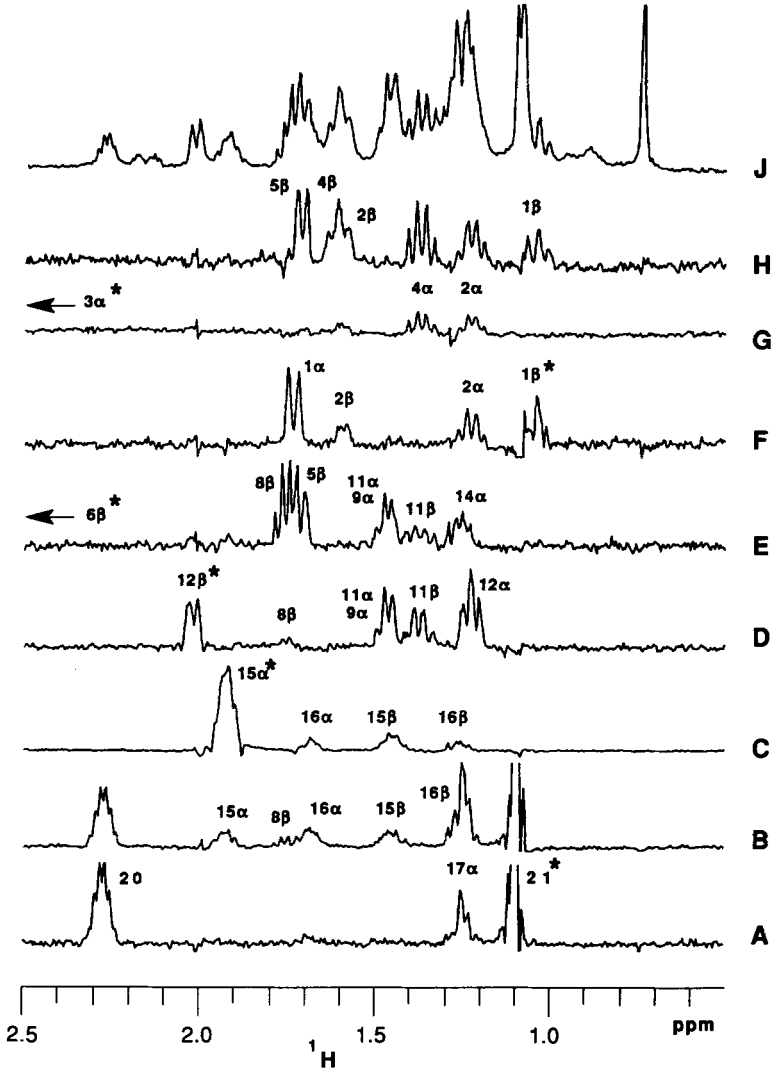

Fig. 7. Z-filtered ^1H 1D-HOHAHA subspectra of tauro-Δ^{22}-β-muricholate generated by selective excitation of various isolated multiplets and mixing times. The labeling conventions are the same as those in Fig. 5 and 6: (A) and (B) excitation of H_{21} with mixing times of 48 and 144 ms, respectively; (C) $H_{15\alpha}$, 6 ms; (D) $H_{12\beta}$, 48 ms; (E) $H_{6\beta}$, 96 ms; (F), $H_{i\beta}$, 24 ms; (G) and (H), $H_{3\alpha}$ with mixing times of 12 and 30 ms, respectively; (J) conventional ^1H spectrum.

extended to the $H_{6\beta}$ proton, some nine bonds removed from the starting point. By introducing excitation at other isolated multiplets, the experiment is repeated for a series of different mixing times until one reaches a multiplet that is common to another independently generated subspectra. Generally this procedure is sufficient to disentangle overlapping lines and determine how they are pairwise connected. Ambiguities, such as those created for example in β-MC by the overlap of $H_{16\beta}$ with H_{22} and $H_{15\beta}$ with H_{20}, may be resolved by reference to the 2D ^1H-$\{^{13}C\}$ shift correlation spectra.

Stereospecific assignments of the ^1H spectra are based on the different magnitudes of the geminal and various vicinal couplings between the protons of interest, where it generally follows that $J_{gem} \approx J_{a,a} \approx 12$–14 Hz, $J_{a,e} \approx 4$ Hz, $J_{e,e} \approx 2$ Hz. Here the subscripts, *a* and *e* refer to axial and equatorial orientations of vicinal pairs of protons. The operative values of J determine the multiplet splitting pattern (17), as well as the pattern of magnetization transfer in the 1D HOHAHA spectra examined at different mixing times.

For example, the multiplet splitting pattern for an axial proton of a geminal pair, such as $H_{12\alpha}$, will be roughly a "triplet of small doublets" due to *1)*: the dominate ~12 Hz couplings, one to its geminal partner, 12β and another to its axial vicinal partner, 11β (two spins of approximately equal coupling imposing the triplet structure) and *2)*: a weaker, ~4 Hz coupling to its equatorial vicinal partner, 11α, (one spin imposing the doublet on each component of the triplet). As another example, $H_{8\beta}$, which is coupled equally by ~12 Hz to three 1,2 diaxial neighbors, 7α,9α, and 14α, has a quartet structure. Note that to observe multiplet structure clearly, the multiplet should be isolated, free of overlap with other lines. This condition is seldom observed in conventional 1D ^1H NMR spectra even at the highest field strengths. By contrast, the selectively excited subspectra, as well as slices, taken through the appropriate cross peaks parallel to the ^1H dimension of the ^1H-$\{^{13}C\}$ 2D-spectrum, frequently provide a clear view of isolated multiplets and their structure.

The second approach to stereospecific assignments, i.e., the pattern of magnetization transfer, is based on the approximation that the rate of transfer between coupled protons is directly proportional to the magnitude of the coupling constant, J (12). This approach is best applied qualitatively in situations where the multiplet structure cannot be clearly resolved. For example, if excitation is introduced into the axial proton of a geminal pair such as 1β, which is also coupled to its vicinal axial neighbor, 2α, the transfer of magnetization to 2α, as well as to its geminal partner, 1α, occurs at about the same rate, since $J_{gem} \approx J_{a,a} \approx 12$Hz. However, very little magnetization will be transferred initially to the vicinal equatorial proton, 2β, since $J_{a,e} \approx 2$Hz (see Figs. 6F and 7F). On the other hand, if excitation is applied to an equatorial geminal proton, such as 12β, which is also coupled to an equatorial (11α) and an axial neighbor (11β), the excitation is first transferred to its geminal partner, 12α, since, here $J_{gem} \geq J_{a,e}$, $J_{e,e}$. In turn, 12α relays the excitation to its axial neighbor, 11β at about the same rate as 12$\beta \rightarrow$12α (see Figs. 6A and 7D).

In summary, we have demonstrated, through the use of recently developed ^1H and ^1H-$\{^{13}C\}$ NMR techniques, that the major bile acid found in the serum of female rats treated with ANIT is the taurine conjugate of 3α,6β,7β-trihydroxy-5β,22-cholen-24-oic acid (tauro-Δ^{22}-β-muricholate; T-Δ^{22}-β-MC, Fig. 1B), a derivative of β-muricholate with an unsaturated bond in the side chain between C_{22} and C_{23}. Additionally, we have described how these NMR methods were used to yield, for T-Δ^{22}-β-MC and its precursor, β-MC, a complete, de novo, stereospecific assignment of their ^1H and protonated ^{13}C NMR spectra. As these methods provide assignments based principally on scalar, ^1H–^1H, and ^1H–^{13}C connectivities rather than empirical correlates, they should also prove to be of general value for structure determination and spectral assignments of other unknown steroids and related compounds.

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