SYNTHESES WITH STABLE ISOTOPES: SYNTHESIS OF DEUTERIUM AND ^{13}C LABELED BILE ACIDS.*

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SUMMARY

A series of 5β-cholanic acids labeled with deuterium in the A ring were prepared by exchange labeling of the corresponding ketone by column chromatography on deuterated alumina. Factors affecting yield and labeling efficiency are discussed. 5β-Cholanic acids labeled with ^{13}C in the carboxyl position were prepared by treatment of the corresponding 23-chloro-24-norcholane with sodium cyanide-^{13}C followed by alkaline hydrolysis of the nitrile. The intermediates in the synthesis were characterized by high resolution NMR spectroscopy. Mass spectra are also reported for the ^{13}C labeled products.

Introduction

In the course of clinical studies on bile salt metabolism we required bile acids labeled with stable isotopes of carbon and hydrogen. The primary advantage to the use of deuterium and ^{13}C in clinical studies is the absence of a radiation hazard, which allows studies in pediatric and obstetric patients. Such clinical work, however, depends on the development of sensitive analytical procedures for detecting and measuring stable isotope labeled drugs.

The usefulness of stable isotope labeled bile acids in the clinical diagnosis of cholelithiasis has recently been demonstrated by Hofmann *et al.*(1).

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Similarly, a study of bile salt metabolism in newborn infants using deuterium labeled bile acids has been reported by Lester and co-workers [2,3]. The development of a gas chromatography-mass spectroscopy-accelerating voltage alternator (GC-MS-AVA) system with the required sensitivity has been described by Klein and co-workers [3,4]. Herein we wish to report details for the synthesis of deuterium and ^{13}C labeled bile acids used in the above studies.

Bile acids labeled specifically with tritium[5] and ^{14}C [6-8] have previously been described in the literature. Deuterium labeling of ketones and aldehydes is usually carried out in the solution phase using either an acid or base catalyst [9]. Less frequently, the exchange reaction is done on a chromatography column deactivated with heavy water [10]. Anthony and Brooks have also shown that keto steroids can be directly labeled for mass spectroscopy on a deuterated gas chromatography column [11]. However, for preparative scale work, the practical limitations of column geometry and temperature sensitivity of keto steroids appears to preclude the use of the GC labeling technique. In this work, 3-keto bile acids were labeled using the column chromatography technique. The method of preparation involved the following steps (Fig. 1): oxidation of the 3α-hydroxyl to the 3-keto bile acid methyl ester; exchange labeling of the ketone on a column of basic alumina containing 3% D_2O; reduction of the ketone with sodium borodeuteride; and separation of the α and β epimers by column chromatography. The final product is the 2α,2β,3β,4α,4β-^{2}H labeled bile acid methyl ester. The d_5 labeled compounds offer the advantage of high sensitivity in the mass spectrometric analysis used in the clinical procedures [4,5]. The use of ^{13}C labeled bile acids in clinical studies offers the advantage that the label is much less likely to exchange under physiological conditions than the corresponding deuterium labeled bile acids.

Previously, bile acids labeled with ^{14}C had been prepared by degradation of the side chain using the classical Hunsdiecker reation. The halogen was replaced with sodium cyanide-^{14}C followed by alkaline hydrolysis of the nitrile.

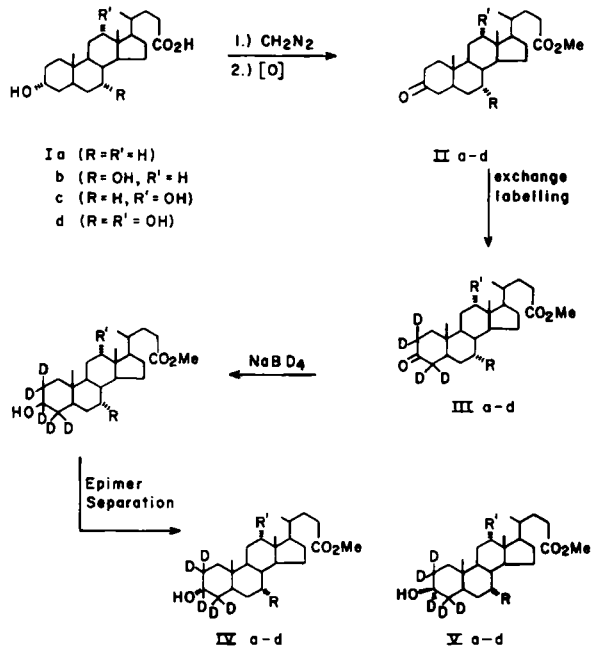

Figure 1. Synthesis of deuterium labeled bile acids.

In this work, bile acids labeled with ^{13}C in the carboxyl position (C-24) were prepared by using the following sequence (Fig.2): acetylation of the hydroxyl groups; degradation of the side chain carboxyl using the lead tetraacetate-lithium chloride modification of the Hunsdiecker reaction described by Kochi [15]; preparation of the ^{13}C nitrile; and alkaline hydrolysis of the nitrile and protecting groups. This procedure overcomes certain technical difficulties in preparing dry silver salts used in the Hunsdiecker reaction. [6-8]

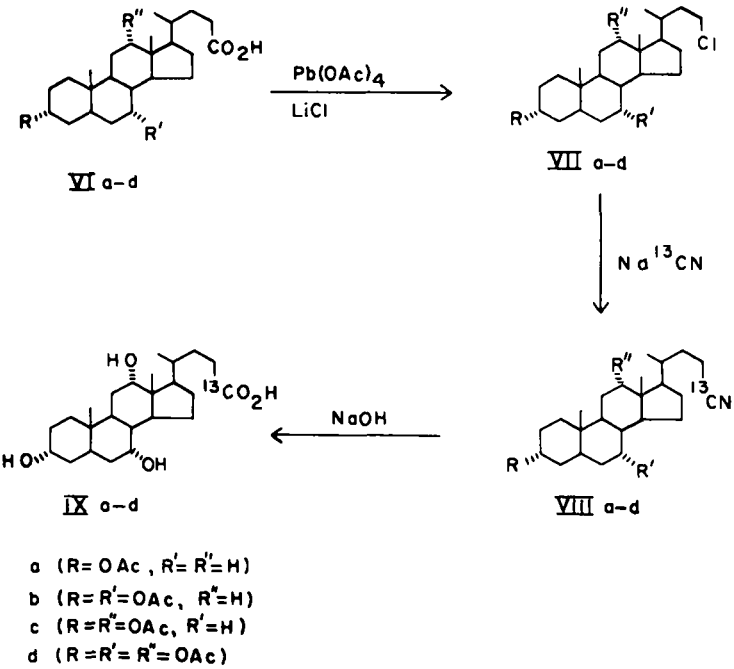

Figure 2. Synthesis of 24-^{13}C labeled bile acids.

Experimental

Elemental analyses were performed by Clark Microanalytical Laboratories, Urbana, Illinois. NMR spectra were run on a Varian HR-220, 220 MHz spectrometer. Mass spectra were run on a Perkin-Elmer Model 270 gas chromatograph-mass spectrometer. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Solvents were ACS analytical reagent grade and were used without further purification. Lithocholic acid was obtained from Nutritional Biochemicals Corp. and was 98% pure. Cholic acid and deoxycholic acid were obtained from Aldrich Chemical Co. and were 98% pure. Chenodeoxycholic

acid, a gift of Dr. Alan Hofmann, Mayo Clinic, was from Wedell Pharmaceuticals Ltd.(London) and was purified as the methyl ester by column chromatography.

Bile Acid Derivatives. Methyl esters were prepared on a small scale by treatment of the acid with diazomethane in ether. Large scale preparations can be conveniently done using acetone dimethylketal in methanolic HCl [16]. Acetylations were carried out in cold acetic anhydride:acetic acid (2:3) containing a drop of perchloric acid. The 3-keto bile acid methyl esters were prepared according to published procedures and are reported in a previous article [5]. Methyl 3-keto-7α-hydroxy-5β-cholanoate (IIb) was kindly supplied by Dr. Hofmann.

Deuterium Labeled Bile Acids. Basic alumina, 750 g (Camag, Brockmann activity grade I), was placed in a 2 liter boiling flask and heated to 350° over four hours at 0.5 torr and kept at that temperature for an additional two hours. The alumina was allowed to cool gradually under vacuum. Deuterium oxide, 22.5 ml (99.8% D), was added in small portions with vigorous shaking. The alumina was allowed to stand overnight to permit the deuterium to equilibrate on the alumina surface. The dehydration-exchange-equilibration process was repeated five more times. Fewer than this number of exchanges gave very poor deuterium labeling which could not be improved by repeated labeling[10]. Careful heating of the alumina is essential in preparing highly active alumina.

In a representative procedure for preparative scale work, methyl 3-keto-7α-hydroxy-5β-cholanoate (IIb), 6.1334 g (15 mmoles) was placed on a column of 3% D_2O on basic alumina (320 g) and eluted with 0.5 l anhydrous benzene. On four consecutive days 0.5 l portions of benzene:ethyl acetate (95:5) were applied to the column in order to elute the ketone onto a fresh deuterated surface. The labeled compound (IIIb) was finally removed from the column with 1.5 l of benzene:ethyl acetate (50:50). The product was analyzed by mass spectroscopy and was found to contain 89.20% ^2H in the 2α,2β,4α and 4β positions and contained 55.10% of the d_4 labeled compound. The labeled ketone (IIIb,

6.0 g) was reduced with 6.109 g sodium borodeuteride in isopropanol at 0°. After two hours the reduction was quenched with 2N HCl. The α and β epimers were separated by column chromatography on alumina. The β epimer was eluted with 25% ethyl acetate:benzene and the α epimer was obtained with 35-40% ethyl acetate:benzene. The yield was 2.5 g (41%) methyl chenodeoxycholate 2α,2β,3β,4α,4β-d_5. The free acid was obtained by saponification in methanolic sodium hydroxide. After crystallization from hot ethyl acetate:hexane (1:2) the yield was 1.637 g (28%, m.p. 119° (reported [17], 119°). The final product contained 85.6% deuterium and contained 54.2% of the d_5 labeled compound (IVb) and was 99% chemically pure.

Preparation of ^{13}C Labeled Bile Acids. The synthesis is described for the preparation of chenodeoxycholic acid-24-^{13}C (IXb). Chenodeoxycholic acid (Ib). 20.0 g (51 mmoles), was acetylated in 75 ml of the cold acetic acid:acetic anhydride mixture containing 5 drops of perchloric acid. The diacetate (VIb) was dissolved in 500 ml anhydrous benzene and refluxed under nitrogen. Lead tetraacetate, 45.0 g (100 mmoles), was added to the solution and reflux was continued an additional 0.5 hr. The solution was thoroughly degassed to prevent inhibition of the reaction by oxygen. Lithium chloride, 4.2 g (100 mmoles), was added slowly, in one gram portions, to minimize the vigorous evolution of carbon dioxide. Reflux was continued until evolution of gas ceased (about 2 hrs). The reaction was quenched with 2N HCl in order to destroy excess Pb^{+4} salts. The organic layer was washed free of unreacted acids with 5% sodium hydroxide, dried over magnesium sulfate and evaporated. The yield was 14.2 g of a pale yellow oil which crystallized on standing. The crude chloride was chromatographed on 300 g neutral alumina and eluted with benzene and benzene:ether (95:5). The benzene:ether fractions contained the bulk of the chloride which was finally crystallized from methanol. The final yield was 7.5 g of colorless needles, m.p. 177-78°. NMR spectrum (220 MHz, $CDCl_3$):0.67 ppm (s, 3H), 0.94 (s, 6H), 2.03 (s, 3H), 2.06 (s, 3H), 3.59

(m, 2H, $C\underline{H}_2Cl$), 4.50 (m, 1H, 3 AcO-C$\underline{H}$), 4.90 (m, 1H, 7 AcO-C$\underline{H}$). Anal. Calc. for $C_{27}H_{43}ClO_4$: C, 69.43; H, 9.28; Cl, 7.59. Found: C, 68.56; H, 8.96; Cl, 7.08.

3α,7α-diacetoxy-5β-cholanonitrile (VIIIb) was prepared by treatment of 0.60 g chloride (VIIb) with 1.50 g sodium cyanide in 10 ml dimethyl sulfoxide. The solution was heated at 100° for 3 hrs. The crude nitrile was poured into water and extracted into ether. The product was purified by column chromatography on alumina and eluted with benzene:ether (80:20). The nitrile crystallizes readily from hot ether:hexane in the form of needles, m.p. 155.5-56.0° (loss of solvent at 85-90°). NMR spectrum (220 MHz, $CDCl_3$): 0.67 ppm (s, 3H) 0.93 (s, 3H), 0.97 (d, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 2.35 (m, 2H, $-CH_2C\underline{H}_2CN$), 4.56 (m, 1H, 3 AcO-C$\underline{H}$), 4.85 (m, 1H, 7 AcO-C$\underline{H}$). Anal. Calc. for $C_{28}H_{43}NO_4$: C, 73.49; H, 9.46; N, 3.06. Found C, 73.40; H, 9.29; N, 3.15.

Chenodeoxycholic acid-24-^{13}C (IXb) was prepared using sodium cyanide-^{13}C (91.9 atom % ^{13}C, Merck, Sharp & Dohme). Thus, 3α, 7α,-diacetoxy-23-chloro-24-*nor*cholane (VIIb), 2.8214 g (6.05 mmoles), was combined with 0.2601 g (5.20 mmoles) $Na^{13}CN$ in 30 ml anhydrous DMSO. The reaction was heated at 100° for 3.5 hr. The product was isolated as described above. The yield was 2.7573 g of a white solid which was a mixture of starting chloride and nitrile (by TLC on silica gel, solvent;hexane:n-butanol:acetic acid, 95:5:0.3). The nitrile was hydrolyzed by refluxing for two days in 50 ml of 90% aqueous ethanol containing 5.5 g sodium hydroxide. The dark alcoholic solution was poured into water and washed free of neutral organic material eith ether. The aqueous solution was acidified and the acid was isolated in ether, dried over magnesium sulfate, filtered through a charcoal:celite bed and evaporated. The pale yellow oil was crystallized once from ethyl acetate:hexane to give 1.480 g (3.77 mmoles, 72.5% yield based on $Na^{13}CN$) chenodeoxycholic acid-24-^{13}C. The product was crystallized once more from hot ethyl acetate:hexane. The final yield was 1.2129 g (3.08 mmoles, 59.2%),

m.p. 119-120° (corr., reported [17] m.p. 119°). The product showed only a single spot on TLC (benzene:dioxane:acetic acid, 75:20:2). The product was analyzed by mass spectroscopy and found to contain 90.72 atom % excess ^{13}C in the 24 position.

Cholic acid-24-^{13}C, deoxycholic acid-24-^{13}C and lithocolic acid-24-^{13}C were also prepared in the same manner in about 50-60% yield. Thin layer chromatographic behavior is listed in Table I. The physical and analytical data for the chlorides (VIIa-d) and nitriles (VIIIa-d) are summarized in Table II, characteristic NMR peaks are listed in Table III. Compounds VIIId and VIId could not be induced to crystallize, hence, no analytical or physical data were obtained for these compounds.

Table I. Thin layer chromatogram R_f values for chlorides (VIIa-d) and nitriles (VIIIa-d).

Compound	Solvent A[1]	Solvent B[2]
VIIa	0.59	0.92
VIIb	0.42	0.74
VIIc	0.45	0.77
VIId	0.31	0.62
VIIIa	0.43	0.81
VIIIb	0.28	0.57
VIIIc	0.28	0.58
VIIId	0.12	0.37

(1) Solvent system A: hexane:n-butanol:HOAc (93:7:0.5).

(2) Solvent system B: benzene:acetone (95:5).

Table II. Analytical data for bile acid derivatives.

Element	Analysis		
Compound	VIIa	VIIb	VIIc
C (Calcd.)	73.41%	69.43%	69.43%
(Found)	72.49	68.56	69.37
H (Calcd.)	10.10	9.28	9.28
(Found)	9.75	8.96	9.28
Cl (Calcd.)	8.67	7.59	7.59
(Found)	8.24	7.08	6.97
m.p. (Corr.)	154-55°	177-78°	128-31°
Compound	VIIIa	VIIIb	VIIIc
C (Calcd.)	78.15	73.49	73.49
(Found)	77.84	73.40	73.01
H (Calcd.)	10.33	9.46	9.46
(Found)	10.28	9.29	9.24
N (Calcd.)	3.51	3.06	3.06
(Found)	3.48	3.15	3.30
m.p. (Corr.)	169-70°	155.5-56.0°	181-83°

Results and Discussion.

The labeling efficiency of methyl 3-keto-5β-cholanoates (IIa-d) and the acetylated derivatives is summarized in Table IV. For the studies on deuterium incorporation, 10 mg of the ketone was placed on a 4 x 100 mm column of deuterated alumina in benzene to give a loading ratio of 1 mg ketone to 150 mg alumina. The ketones were eluted directly with benzene (for IIa), with benzene:ethyl acetate (95:5 for IIb and IIc), and benzene:ethyl acetate

Table III. Characteristic NMR spectra of bile acid derivatives VII and VIII[a] (19)

Protons	Chemical Shift (ppm)			
	VIIa	VIIb	VIIc	VIId
C-19	0.92 (s)	0.94 (s)	0.91 (s)	0.92 (s)
C-18	0.66 (s)	0.67 (s)	0.74 (s)	0.76 (s)
C-21	0.96 (d)	0.97 (d)	0.84 (d)	0.85 (d)
C-3	4.60 (m)	4.50 (m)	4.58 (m)	4.46 (m)
C-7	---	4.80 (m)	---	4.79 (m)
C-12	---	---	4.97 (m)	4.98 (m)
C-23	3.57 (m)	3.59 (m)	3.57 (m)	3.53 (m)
C-3 (ACO)	2.03 (s)	2.03 (s)	2.03 (s)	2.04 (s)
C-7 (AcO)	---	2.06 (s)	---	2.08 (s)
C-12 (AcO)	---	---	2.10 (s)	2.13 (s)
	VIIIa	VIIIb	VIIIc	VIIId
C-19	0.92 (s)	0.93 (s)	0.95 (s)	0.92 (s)
C-18	0.66 (s)	0.67 (s)	0.79 (s)	0.76 (s)
C-21	0.96 (d)	0.97 (d)	0.89 (d)	0.86 (d)
C-3	4.59 (m)	4.56 (m)	4.59 (m)	4.45 (m)
C-7	---	4.85 (m)	---	4.80 (m)
C-12	---	---	4.98 (m)	4.98 (m)
C-23	2.34 (m)	2.35 (m)	2.37 (m)	2.33 (m)
C-3 (AcO)	2.03 (s)	2.03 (s)	2.07 (s)	2.04 (s)
C-7 (AcO)	---	2.06 (s)	---	2.08 (s)
C-12 (AcO)	---	---	2.15 (s)	2.15 (s)

(a) 220 MHz spectra were run in $CDCl_3$, chemical shifts are expressed in parts per million (ppm) relative to TMS.

(50:50 for IId). Comparison of the acetylated bile acid derivatives with the free alcohols reveals that the alcohols are better labeled than the acetates. Apparently the hydroxyl groups play an important role in the exchange reaction, possibly by binding the ketone

Table IV. Labeling efficiency of methyl 3-keto-5β-cholanoates (IIa-b) and the corresponding acetate derivatives (Xb-d, R=R'=OAc).

Compound	%D[a]	d_0[b]	d_1	d_2	d_3	d_4	d_5
IIa	69.0	6.3	13.2	21.6	28.3	24.2	6.3
IIb	68.2	2.9	9.9	28.1	36.7	18.2	4.2
IIc	81.2	3.0	5.6	14.0	29.7	38.6	9.1
IId	45.8	24.6	28.8	23.8	12.5	6.9	3.4
Xb	47.5	6.6	33.5	36.4	17.8	4.4	1.3
Xc	60.0	7.6	17.4	29.7	29.0	13.3	3.0
Xd	36.2	39.5	31.8	18.2	6.6	2.3	1.6

a) %D is the total amount of deuterium in the 2 and 4 positions.

b) d_n is the percent of the molecules having n deuterium atoms.

more tightly to the chromatography adsorbent (although this cannot be the only effect, since IId, which should be the most strongly adsorbed, does not label as well as IIb or IIc). Steric effects most likely play an important part in the deuteration of 3-keto steriods. Malhotra and Ringold observed that androst-4-ene-3,17-dione exchanged the 2β protons about ten times faster than the 2α protons [12]. In the case of methyl 3-keto-cholate (IId), addition of the 12α hydroxyl may sufficiently hinder exchange of the 2α and 4α protons.

Table V. Multiple pass labeling of methyl 3-keto-cholanoates.

Pass No.	% deuterium	d_0	d_1	d_2	d_3	d_4	d_5
methyl 3-keto-7α,12α-dihydroxy-5β-cholanoate (IId).							
1	73.1	1.9	8.0	24.9	34.3	24.7	6.2
2	70.0	4.6	10.4	22.0	32.4	24.3	6.4
3	78.5	2.2	4.9	17.9	35.0	31.3	8.5
4	83.2	1.8	3.0	13.1	34.5	38.1	9.5
5	84.2	1.6	3.0	12.0	35.1	38.4	9.8
Methyl 3-keto-7α-hydroxy-5β-cholanoate (IIb).							
1	62.2	5.3	16.7	29.7	29.7	14.8	3.8
2	79.5	1.5	5.2	17.5	35.3	32.7	7.8
3	85.5	1.5	3.0	10.6	32.8	42.3	9.8

Multiple-pass labeling of the keto bile acids generally does not improve the deuterium content greatly (Table V). Five exchanges of methyl 3-keto-7α, 12α-dihydroxy-5β-cholanoate (IId) on a deuterated column only raised the deuterium content from 73% to 84%. A similar run using methyl 3-keto-7α-hydroxy-5β-cholanoate (IIb) showed that the deuterium content was only raised from 62% to 86% after three passes. These data indicate that three (of four) enolic protons exchange readily, but that the fourth position exchanges only with difficulty, possibly for steric reasons.

In examining the tritium labeling of keto steroids by exchange labeling on tritiated alumina, Klein and co-workers [4,13,14] observed that the labeling activity of a compound depended on the intrinsic keto-enol equilibrium, substituent effects, activity of the alumina, and the length of time the ketone remained on the column. In the large-scale preparation of deuterated keto bile acids, we also observed that labeling time is an important factor in obtaining highly labeled

products. For our system, an equilibration period of five days gave the best compromise between ketone recovery and labeling efficiency. For long reaction times, as much as 25% of the product was lost on the column due to base-catalyzed hydrolysis of the methyl ester. The optimum loading ratio for preparative scale work was about 1 gram of ketone to 50 grams of 3% deuterated alumina. At higher loading ratios, considerable product was lost through ester hydrolysis. At lower ratios, inefficient labeling resulted.

In preparative scale work, a 6.0 gram portion of methyl 3-keto-cholate (IId) was deuterated. This compound does not incorporate deuterium as efficiently as 3-keto-chenodeoxycholate (IIb) or 3-keto-deoxycholate (IIc). Table VI shows the results of labeling 6.0 g samples of two keto bile acids on 300 g of 3% deuterated alumina. The same batch of alumina was used in both cases.

Table VI. Labeling pattern for the large scale preparation of deuterated bile acids IIb and IId (a five day on column equilibration period was used).

Compound	%D	d_0	d_1	d_2	d_3	d_4
IIb	87.7	1.6	1.7	5.1	28.7	62.7
IId	81.5	2.8	3.6	10.7	33.5	49.4

The mass spectra of bile acids has been reviewed in detail by Sjövall, Eneroth, and Ryhage (ref. 18 and references cited therein). In this work, the acetylated bile acid methyl esters were used for the gas chromatography-mass spectroscopy (GC-MS) studies. Although this derivative does not give a parent peak, it does have two major fragments above M/e 350 which would be useful dor diagnostic work (Figure 3). The first fragment at M/e 368 (at M/e 370 and 372 for dihydroxy and monohydroxy bile acids respectively) in the spectrum of tri-acetoxy cholate (VId, methyl ester) is caused by loss of the acetate

protecting groups. The second fragment at M/e 353 (M/e 355 and 357 for di- and mono-hydroxy bile acids, respectively) is caused by loss of a methyl group from the 368 fragment. In addition, minor fragments at M/e 74 and 87 due to side chain fragmentation could also be used in the isotope ratio measurements since the label is retained in that fragment. The small boxes in Figure 3 indicate major fragments in the spectrum of the ^{13}C labeled bile acids above M/e 200 which are different from the unlabeled bile acids.

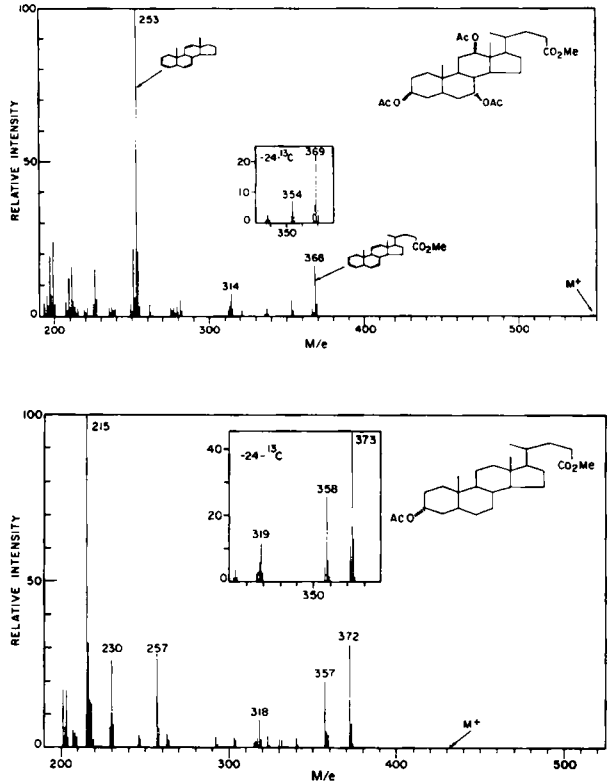

Figure 3. Mass spectra of bile acids: cholic acid (top) lithocholic acid (bottom).

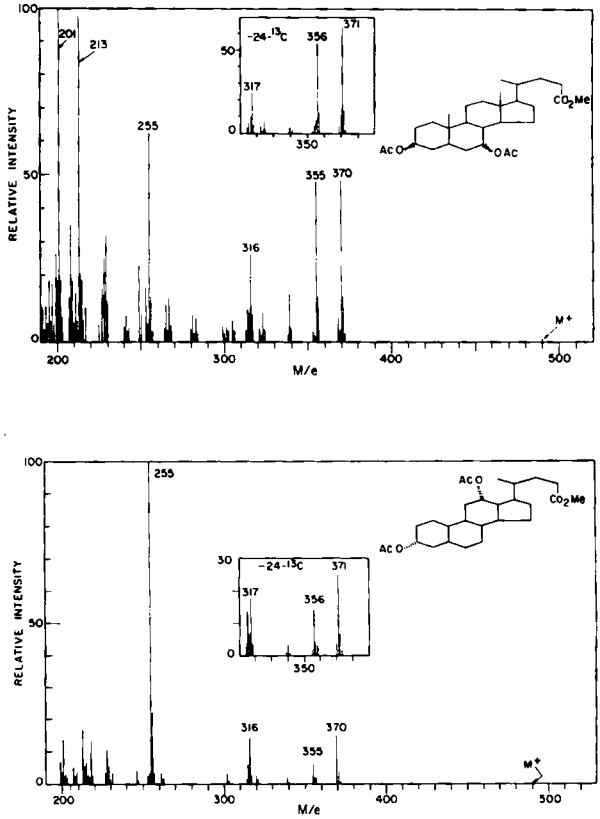

Figure 3. (cont.) Chenodeoxycholic acid (top) and deoxycholic acid (bottom).

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