NEW BILE ALCOHOLS - SYNTHESIS OF 5β-CHOLESTANE-3α,7α,25-TRIOL AND 5β-CHOLESTANE-3α,7α,25-24(^{14}C)-TRIOL[1]

Bertram I. Cohen[2], G.S. Tint[3], Taiju Kuramoto[2] and Erwin H. Mosbach[2,4]

Department of Lipid Research, Public Health Research Institute of the City of New York Inc., 455 First Avenue, New York, N.Y. 10016, and Department of Medicine, College of Medicine and Dentistry of New Jersey, Newark, N.J. 17103

Received: 12/30/74

ABSTRACT

5β-Cholestane-3α,7α,25-triol and 5β-cholestane-3α,7α,25-24(^{14}C)-triol were synthesized from 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid). Chenodeoxycholic acid was converted to the diformoxy derivative (II) using formic acid. Reaction of II with thionyl chloride yielded the acid chloride which was treated with diazomethane (CH_2N_2 or $^{14}CH_2N_2$) to produce 3α,7α-diformoxy-24-oxo-25-diazo-25-homocholane (III, A or B). 25-Homochenodeoxycholic acid (IV, A or B) was formed from III by means of the Wolff rearrangement of the Arndt-Eistert synthesis. The methyl ester of V (A or B) was treated with methyl magnesium iodide in ether to provide the desired triol, VI (A and B). The triol was identified by mass spectrometry and elemental analysis and was characterized by thin-layer and gas-liquid chromatography. The 3α,7α,25-triol is of possible significance as an intermediate in the pathway of bile acid formation from cholesterol.

INTRODUCTION

C_{27} bile alcohols have been postulated as intermediates in the formation of the primary bile acids, cholic acid and chenodeoxycholic acid (5-11). The pathway for the degradation of the sterol side chain was thought to involve 26-hydroxylation as an initial step. Recent studies have

indicated that 25-hydroxylation of the side chain may play an important role in bile acid synthesis (11).

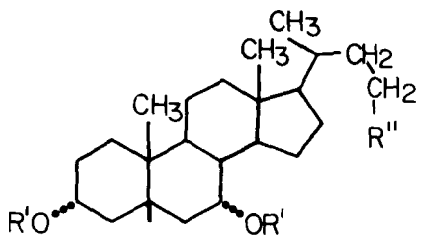
Further investigation of the importance of 25-hydroxylation as a pathway in bile acid synthesis required the synthesis of 5β-cholestane-3α,7α,25-triol and 5β-cholestane-3α,7α,25-24(^{14}C)-triol. The description of the synthesis and characterization of these compounds (Charts 1 and 2) form the basis of this report.

EXPERIMENTAL

All solvents were reagent grade or spectrophotometric grade and were used without further purification. Infrared spectra were taken on a Perkin-Elmer 421 grating spectrometer. Melting points were obtained on a Thermolyne melting point apparatus and are uncorrected. Thin-layer chromatography was carried out on 250 μ plates precoated with silica gel G (Analtech Inc.). Column chromatography was carried out on silica gel, 40-140 mesh (J.T. Baker, Inc.). The solvent systems used for thin-layer chromatography were: System A - isooctane-ethyl acetate-acetic acid, 30/6/1 (V/V/V); System B - ethyl acetate-chloroform-acetic acid, 45/45/10 (V/V/V); System C - acetone-benzene, 40/60 (V/V). All R_f values are reported relative to chenodeoxycholic acid (RR_f=1) in each solvent system. Gas-liquid chromatography was carried out as previously described (11,12). Gas-liquid chromatography-mass spectrometry was performed on a Varian MAT 111 (Varian Associates, Inc.) using conditions previously described (11). Chenodeoxycholic acid was a gift from I.P.D. Corp. (New Rochelle, N.Y.). N-Methyl-N-nitroso-p-toluenesulfonamide (Diazald) was obtained from Aldrich Chemical Company. N-Methyl-N-nitroso-p-toluenesulfonamide(methyl-^{14}C) was purchased from New England Nuclear, Inc., and had a specific activity of 2-10 mCi/mmole.

3α,7α-Diformoxy-5β-cholan-24-oic acid (II)

Formylation of chenodeoxycholic acid (I) was carried out by dissolving 10 g of I in 20 ml of formic acid and heating the solution at 55° for 4 hrs. The solution was allowed to stand overnight and was then evaporated to dryness. The product was dissolved in benzene and the solution was evaporated to remove any residual formic acid. Crystallization from ethanol-H_2O gave 9.8 g (84%) of white crystalline needles with the following properties: m.p.



	R'	R'	R''		R''
			A		B
I	H	H	^{24}COOH	or	^{24}COOH
II	CHO	CHO	^{24}COOH	or	^{24}COOH
III	CHO	CHO	^{24}CO-CHN$_2$	or	^{24}CO-[^{14}^{25}C]HN$_2$
IV	H	H	CH$_2$-^{25}COOH	or	[^{14}C]H$_2$ ^{25}COOH
V	H	H	CH$_2$-^{25}COOCH$_3$	or	[^{14}C]H$_2$ ^{25}COOCH$_3$
VI	H	H	CH$_2$-^{25}COH(CH$_3$)$_2$	or	[^{14}C]H$_2$ ^{25}COH(CH$_3$)$_2$

CHART I

SYNTHESIS OF 3α, 7α, 25 - TRIOL

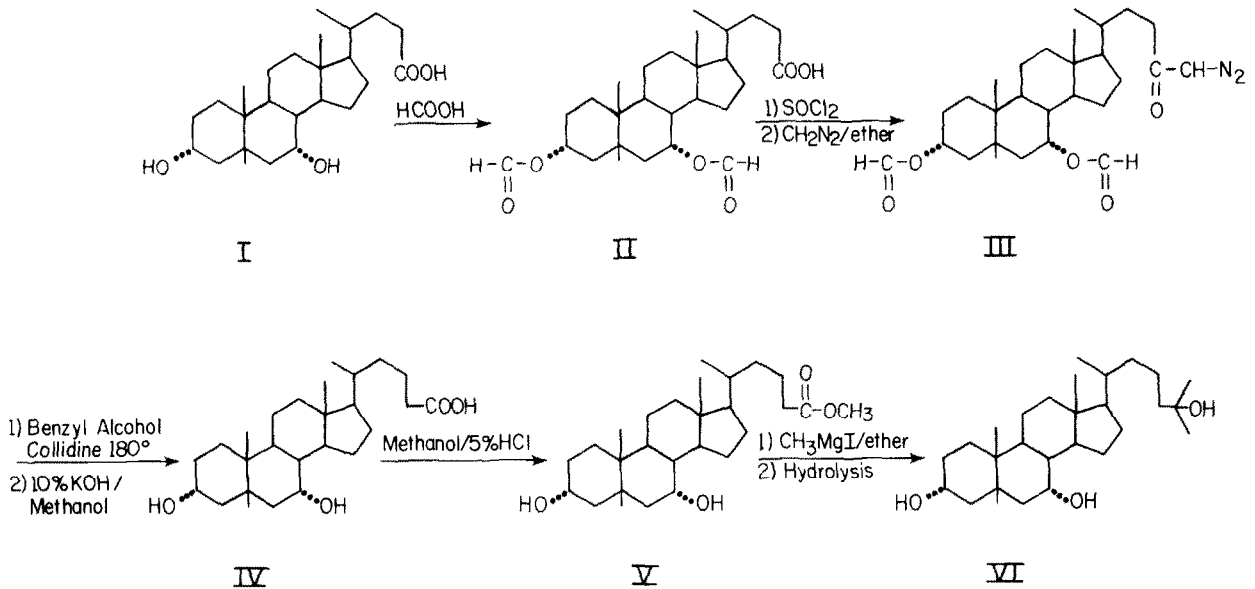


CHART 2

123-125°; single spot on tlc, relative R_f 3.4 (Solvent system A); νmax 2950, 2949, 2860, 1705, 1690 (shoulder), 1177, 1140, and 695 cm^{-1}.

3α,7α-Diformoxy-24-oxo-25-diazo-25-homocholane (III, A and B)

To 5 g of II (dried at 60° in vacuo for 24 hrs) was added 12.5 ml of thionyl chloride. The reaction was allowed to proceed at room temperature for 2 hrs. The thionyl chloride was removed at room temperature in vacuo and the acid chloride was used without further purification. Diazomethane in ether was freshly prepared using either Diazald or methyl-(^{14}C) Diazald (500 μCi). The acid chloride was dissolved in dry benzene (50 ml) and added dropwise to the diazomethane solution at 0°. The mixture was allowed to come to room temperature and was kept at room temperature for 18 hrs. Evaporation of the solvent gave a pale yellow oil. The material was crystallized from ethanol-water and gave 3.75 g (72%) of light yellow needles with the following properties: m.p. 78-80°; single spot on tlc, relative R_f 1.3 (System B); νmax 2921, 2082 (diazo), 1705, and 1170 cm^{-1}.

25-Homochenodeoxycholic acid (IV, A and B)

A flask equipped with a condenser and a thermometer was preheated to 200° in an oil bath. A solution containing 1.0 g of IIIA (or IIIB), 2 ml of benzyl alcohol and 2 ml of freshly-distilled collidine was added to the heated flask and the temperature was maintained at 180-200° for 15 min. Evolution of gas (nitrogen) was observed during the first 5 min of the reaction. The reaction mixture was cooled to room temperature, 25 ml of water was added and the solution was extracted with four 50 ml portions of ethyl ether. The ethereal extracts were combined and washed successively with water (50 ml), 0.1 N HCl (50 ml), water (50 ml), 5% sodium bicarbonate (50 ml), then with water until the pH was between 6-7. The ether solution was dried over sodium sulfate and then evaporated to dryness. The resulting gum was dissolved in 10% methanolic KOH and refluxed for 1.5 hrs. The hydrolysate was cooled to 0°; 10 ml of water followed by 20 ml of 2.5% potassium carbonate were added. The solution was acidified with HCl (pH 1-2) and extracted with ether. Evaporation of the solvents gave 0.9 g of crude homochenodeoxycholic acid. Recrystallization from acetone-water gave 0.80 g (63%) of white crystalline flakes with the following properties: m.p. 210-212°; single spot on tlc, relative R_f 1.0 (System A and System B); νmax 2935, 2865, 1692, 1365, 1265, 1068, and 965 cm^{-1}. The mass spectrum of the free acid obtained by direct inlet insertion showed peaks at m/e 406, M^+; 388, M-(18); 370, M-(2x18); 355, M-(2x18+15); 301, M-(18+87); 255, M-[115(side chain)+2x18]; and also peaks at 228, 201, 187, 173, 159, 147, 133, 121, 107, and 95 (Fig. 1).

Anal. Calcd. for $C_{25}H_{42}O_4 \cdot \frac{1}{2}H_2O$: C, 72.28% H, 10.36%
Found: C, 72.50% H, 10.56%

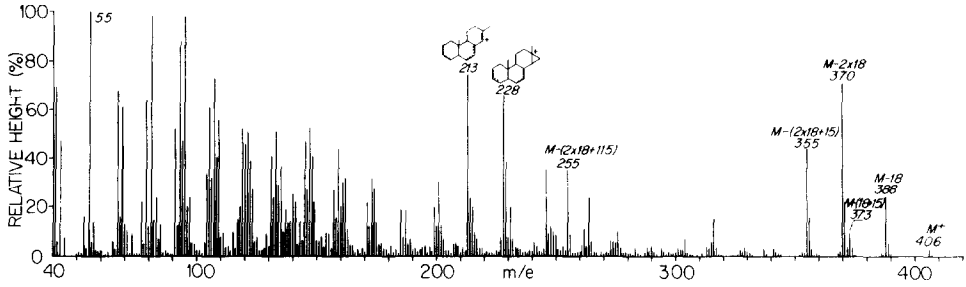


Fig. 1. Mass spectrum of homochenodeoxycholic acid obtained by direct inlet at 240°; ion source, 80 eV.

25-Homochenodeoxycholic acid methyl ester (V, A and B)

200 Mg of IV was dissolved in benzene-methanol (86:14, V/V) and the solution was evaporated to dryness. The dry acid was esterified by adding 30 ml of methanolic-HCl (5%) and allowing the reaction mixture to remain at room temperature for 18 hrs. The material was evaporated to dryness and residual traces of HCl were removed by adding 30 ml of benzene-methanol and reevaporating. Crystallization from methanol-water gave 180 mg (87%) of a white crystalline material with the following properties: m.p. 94-95°; single spot on tlc with relative R_f 3.5 (System C); retention time on gas-liquid chromatography (relative to methyl chenodeoxycholate) - 1) 3% SE-30, 1.31; 2) 3% QF-1, 1.26; νmax 3400, 2920, 2860, 1732, 1160, 1070, and 972. The mass spectrum of the trimethylsilyl ether of methyl homochenodeoxycholate exhibited peaks at m/e 564, M^+; 549, M-(15); 474, M-(90); 459, M-(90+15); 384, M-(2x90); 369, M-(2x90+15); 345, M-(90+129 side chain); 255, M-(2x90+129); with other peaks at 243, 229, 213, 147, and 129 (Fig. 2).

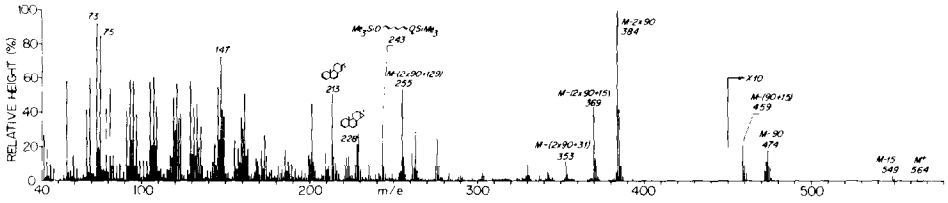


Fig. 2. Mass spectrum of trimethylsilylether derivative of methyl homochenodeoxycholate by glc-ms coupling, 3% QF-1, 260°.

5β-Cholestane-3α,7α,25-triol (VI-A) and 5β-cholestane-3α, 7α,25-24(^{14}C)-triol (VI-B)

The procedure used was similar to that of Lettré with modifications (13). A solution of 600 mg of V was dissolved in 12 ml of dry benzene and added dropwise to a flask containing 7 ml of 2 M methyl magnesium iodide (Alfa Inorganics, Inc.) dissolved in 5 ml of dry ethyl ether. The reaction mixture was kept at 0° for 15 min and then allowed to warm up to room temperature. After 1 hr, the ether was evaporated under N_2 and the reaction was refluxed at 55° for one hr. The solution was kept at 25° for 18 hrs. After cooling to 0° in ice, 10 ml of a saturated solution of ammonium chloride, 10 g of ice and 9 ml of 20% H_2SO_4 were added to decompose the Grignard product. The solution was quickly extracted with four 50 ml portions of ether. The ethereal extract was washed successively with 20 ml of 2N HCl followed by distilled water until the washings were neutral. The ether layer was dried over sodium sulfate and then evaporated to dryness. The residue was dissolved in 25 ml of methanol. After the addition of 10 ml of 10% KOH, the product was hydrolyzed for 1.5 hrs to decompose any unreacted methyl ester that was present. The hydrolysate was cooled to 0°, diluted with 20 ml of water and extracted with four 50 ml portions of ether. Chromatography on silica gel (Baker, Inc.) was used to purify the final product. Fifty grams of silica gel were used for every 50 mg of product. The pure triol was eluted using ethyl acetate:benzene, 90/10 (V/V) and emerged in fractions 40-80 (5 ml per tube). Crystallization from ethyl acetate:hexane gave 382 mg (64%) of white needles with the following properties: m.p. 181-183°; single spot on tlc, relative R_f 2.2 (System C); gas-liquid chromatographic analysis - retention time relative to 5α-cholestane - as the trimethylsilyl ethers: 1) 3% SE-30, 4.33; 2) 3% QF-1, 3.13; υ_{max} 3480, 2925, 2860, 1465, 1378, 1160, 1150, 1070, and 980 cm^{-1}. The mass spectrum of

the trimethylsilyl ether of the triol (Fig. 3) exhibited important fragments at m/e 621, M-15; 546, M-90; 531, M-(90+15); 456, M-(2x90); 441, M-(2x90+15); 366, M-(3x90); 351, M-(3x90+15); 255, M-(2x90+side chain); 253, M-(2x90+side chain+2H); 131, (base peak characteristic of a C-25 OH group).

Anal. Calcd. for $C_{27}H_{48}O_3$: C, 77.09% H, 11.50%
Found: C, 76.77% H, 11.65%

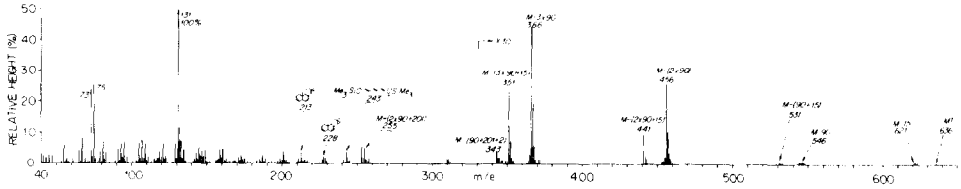


Fig. 3. Mass spectrum of the trimethylsilylether derivative of 5β-cholestane-3α,7α,25-triol, glc-ms coupling, 3% QF-1, 240°.

RESULTS AND DISCUSSION

Degradation of the cholesterol side chain to form bile acids may involve 25-hydroxylated intermediates in light of the discovery of 5β-cholestane-3α,7α,12α,25-tetrol in the feces and bile of patients with cerebrotendinous xanthomatosis (CTX) (11). It was suggested that 25-hydroxylation of the cholesterol side might be an alternate pathway in the formation of cholic acid (11).

In order to study the possibility that 25-hydroxylated intermediates participate in the biosynthesis of chenodeoxycholic acid, 5β-cholestane-3α,7α,25-triol was synthesized from chenodeoxycholic acid as illustrated in Charts 1 and 2. Protection of the free hydroxyl groups of chenodeoxycholic acid was accomplished by preparation of the formate esters.

The procedure was similar to that reported for the formation of the diformoxy ester derivatives of hyodeoxycholic acid (13). Infrared absorption at 1705 and 1177 cm^{-1} was characteristic of the formate ester (14). The diformoxy derivative of chenodeoxycholic acid was easily converted to the corresponding acid chloride using the method of Hoshita (15). This compound was not isolated but was freed of any residual thionyl chloride by repeated evaporation with benzene. The acid chloride was added to an alcohol-free ethereal solution of diazomethane to form the desired diazoketone (III). The diazoketone had strong infrared absorption at 2082 cm^{-1} which is characteristic of the $CH_2N{=}N$ functional group (16). N-Methyl-N-nitroso-p-toluenesulfonamide (Diazald) was used to generate the diazomethane because of the ease of handling, and because introduction of the (^{14}C) radioisotope could be easily accomplished using Diazald [methyl-(^{14}C)]. The mass spectrum of the diazoketone obtained by direct insertion showed a major fragment at m/e 84 (Chart 3). This is attributed to a McLafferty-type rearrangement forming the radical cation (b). The diazoketone formed from the labeled Diazald (Chart 2, IIIB) had a specific activity (S.A.) of 32500 dpm/mg.

MS FRAGMENTATION OF DIAZOKETONE

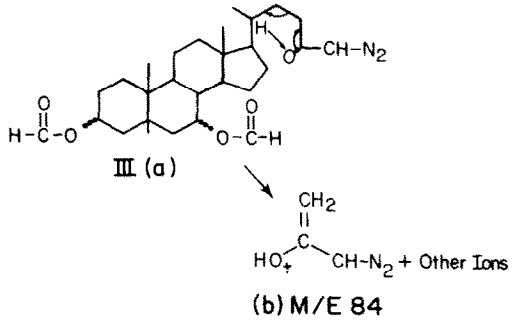


CHART 3

Homochenodeoxycholic acid (IV, A and B) was formed from the diazoketone (III, A and B) by the Wolff rearrangement of the Arndt-Eistert synthesis. This method provided a convenient way to increase the carbon chain of a carboxylic acid by one carbon atom. In the case of the (^{14}C) diazoketone (IIIB), it provided a method to introduce a (^{14}C) label into the C-24 position of the sterol side chain. We used a modification of the original Arndt-Eistert reaction using benzyl alcohol and collidine with subsequent heating of the reaction mixture to 180-200° (17). This method gave better yields than other procedures (18,19). We found the modified Arndt-Eistert reaction more convenient to carry out and obtained average yields of homochenodeoxy-

cholic acid of 63%. We also obtained smooth conversion of the (^{14}C) diazoketone to the corresponding 24-(^{14}C) homochenodeoxycholic acid (IVB).

The mass spectrum of the free acid (IV, Fig. 1) showed a molecular ion at m/e 406 and fragments corresponding to the loss of one hydroxyl group (m/e 388), two hydroxyl groups (m/e 370) and a parent peak at m/e 255 which was characteristic of a dihydroxy bile acid (20). The infrared spectrum was similar to that of chenodeoxycholic acid with peaks at 2935, 2865, 1692 and 1068 cm^{-1}.

The methyl ester of homochenodeoxycholic acid (V, A and B) was formed by dissolving the free acid in a solution of methanol/5% HCl under anhydrous conditions as described for methylation of the fecal bile acids (12). The mass spectrum of the trimethylsilylether derivative of methyl homochenodeoxycholate (Fig. 2) was identical to that of trimethylsilylether derivative of methyl chenodeoxycholate with respect to peak intensities and fragmentation patterns. The only difference was that the peaks in the spectrum of the homochenodeoxycholate were shifted 14 mass units upfield because of the additional CH_2 moiety present in the sterol side chain. Major fragments at m/e 474 and 384 (base peak) corresponded to the loss of one and two trimethylsilyl groups from the steroid nucleus. Peaks were also observed for the molecular ion (m/e 564), loss of one trimethylsilyl group + the sterol side chain (m/e 345) and loss of both trimethylsilyl groups + the side chain (m/e 255). The

retention time of methyl homochenodeoxycholate on both SE-30 and QF-1 was longer than that of methyl chenodeoxycholate. The infrared spectrum showed peaks at 1732 and 1160 cm^{-1} which were characteristic of a methyl ester (14). On the whole, the spectral properties of both methyl homochenodeoxycholate and the free acid were similar to those of chenodeoxycholic acid and its methyl ester.

Treatment of methyl homochenodeoxycholate with an excess of methyl magnesium iodide in ether followed by hydrolysis of the Grignard reagent which gave the 3α,7α,25-triol (VI, A and B) in good yield. Further purification of the triol was carried out using silica gel column chromatography. Spectral analysis permitted positive identification of the triol. The infrared spectrum showed characteristic methyl absorption at 1378 and 1465 cm^{-1} and absorption due to secondary and tertiary alcohol groups at 3480, 1169, 1150 and 1070 cm^{-1} (21). No absorption peak characteristic of the carbonyl group was present. Assignment of the structure as that of a trihydroxy compound with a C-25 hydroxyl group was established by gas-liquid chromatography-mass spectrometry. The trimethylsilylether derivative of the triol showed the following fragmentation pattern (Fig. 3): a peak at m/e 621 due to M-15, a series of fragments at 546, 456, and 366 represents the loss of one, two, and three trimethylsilylether groups, and the base peak at m/e 131 which was characteristic of a C-25 hydroxyl group in the sterol side chain of a C-27 sterol (11). The 24-(^{14}C)methyl

homochenodeoxycholate was converted to triol as efficiently as the nonlabeled analog and gave a triol (VIB) with a specific activity of 37000 dpm/mg. The fate of the (^{14}C) triol in vivo and in vitro as a possible intermediate in bile acid synthesis will be reported.

ACKNOWLEDGMENTS

This research was supported in part by grants HL 10894 and AM 05222 from the U.S. Public Health Service, GB 31919X from the National Science Foundation, and a grant from the I.P.D. Corporation.

REFERENCES

1. The following IUPAC names apply to the substances discussed in the manuscript:
chenodeoxycholic acid = 3α,7α-dihydroxy-5β-cholan-24-oic acid
homochenodeoxycholic acid = 3α,7α-dihydroxy-5β-homocholan-25-oic acid
2. Present address: Department of Lipid Research, Public Health Research Institute of the City of New York, Inc., 455 First Avenue, New York, N.Y. 10016.
3. Present address: Department of Medicine, College of Medicine and Dentistry of New Jersey, Newark, N.J. 07103.
4. Author to whom correspondence should be addressed.
5. Staple, E. and Rabinowitz, J.L., BIOCHIM. BIOPHYS. ACTA 59, 735 (1962).
6. Carey, J.B., Jr., J. CLIN. INVEST. 43, 1443 (1964).
7. Cronholm, T. and Johansson, G., EUR. J. BIOCHEM. 16, 373 (1970).
8. Hanson, R.F., J. CLIN. INVEST. 50, 2051 (1971).
9. Anderson, K.E., Kok, E. and Javitt, N.B., J. CLIN. INVEST. 51, 112 (1972).
10. Danielsson, H., THE BILE ACIDS: CHEMISTRY, PHYSIOLOGY, METABOLISM, P.P. Nair and D. Kritchevsky, Editors, Plenum Press, New York, 1973, p. 1.
11. Setoguchi, T., Salen, G., Tint, G.S. and Mosbach, E.H., J. CLIN. INVEST. 53, 1393 (1974).

12. Cohen, B.I., Raicht, R.F. and Mosbach, E.H., ANAL. BIOCHEM. in press (1975).

13. Lettré, V.H., Egle, A., Von Jena, J. and Mathes, K., LIEBIGS ANN. CHEM. 708, 224 (1967).

14. Bellamy, L.J., THE INFRA-RED SPECTRA OF COMPLEX MOLECULES, John Wiley and Sons, New York, 1958, p. 189.

15. Hoshita, N. and Okuda, K., J. BIOCHEM. (Japan) 62, 655 (1967).

16. Bellamy, L.J., op. cit. p. 273.

17. Wilds, A.L. and Meader, A.L., Jr., J. ORG. CHEM. 13, 763 (1948).

18. March, J., ADVANCED ORGANIC CHEMISTRY: REACTION, MECHANISMS, AND STRUCTURE, McGraw Hill, New York, 1968, p. 809.

19. Pearlman, W.H., J. AM. CHEM. SOC. 69, 1475 (1947).

20. Elliot, W.H., BIOCHEMICAL APPLICATIONS OF MASS SPECTROMETRY, G.R. Waller, Editor, John Wiley and Sons, New York, 1971, p. 291.

21. Bellamy, L.J., THE INFRA-RED SPECTRA OF COMPLEX MOLECULES, John Wiley and Sons, New York, 1958, p. 109.