PREPARATION OF RADIOLABELLED 3α-HYDROXY-7-KETO-5β-CHOLANIC ACID AND ITS GLYCINE AND TAURINE CONJUGATES

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SUMMARY

3α-Hydroxy-7-keto-5β-cholanic acid has been prepared from cholic acid by a route which allowed introduction of ^{3}H into positions 11 and 12 of the steroid C ring. Labelling with ^{14}C was done by halodecarboxylation and resynthesis of the carboxyl function with ^{14}C cyanide. The conjugates of both ^{3}H- and ^{14}C-labelled compounds with glycine and taurine were prepared. ^{14}C-labelled compounds were prepared by degradation of the carboxyl-containing side chain to the norchloride, which was used to prepare the homologous ^{14}C-containing nitrile. Hydrolysis afforded the acid in good yield. ^{3}H was introduced into the C ring of the steroid nucleus by dehydration of 3α,12α-dihydroxy-7-keto-5β-cholanic acid to the Δ^{11}-alkene which was catalytically tritiated. Physical and spectral data are presented to characterize the new compounds and TLC methods for purification are reported.

KEY WORDS: bile acid, radiosynthesis, 3α-hydroxy-7-keto-5β-cholanic acid, Tritium, ^{14}C labelling.

INTRODUCTION

3α,7α-dihydroxy-5β-cholanic acid (chenodeoxycholic acid, 1) is one of two primary bile acids in man (1). An epimer of chenodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanic acid (ursodeoxycholic acid, 2), has also been shown to occur in man (2) and is apparently metabolically derived from chenodeoxycholic

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acid (3,4,5). Both 1 and 2 are of considerable therapeutic interest. Chenodeoxycholic acid has been shown (both in vitro and in vivo) to increase the solubility of cholesterol in bile. This effect can be exploited to dissolve cholesterol gallstones (6), and currently, 1 is under study in an extensive national therapeutic trial. Ursodeoxycholic acid is reported to have similar effects at lower dose levels (7,8,9). It has also been reported that ursodeoxycholic acid may have a decreased incidence of side effects when compared to 1 (7,8,9).

If there is interconversion in vivo between 1 and 2, a probable intermediate is 3α-hydroxy-7-keto-5β-cholanic acid (cavicholic acid, 3). This compound, which could be derived in vivo by oxidation of either 1 or 2, has been reported to be a trace constituent of bile in man (10) and is a primary bile acid of the guinea pig (Cavia cobaya) (11).

As part of our program to study the metabolic pathways between 1 and 2, radiolabelled 3 was needed. Since 3 may reach the liver in free as well as conjugated form (like other bile acids), it was desirable to prepare both the free acid and the glycine and taurine conjugates. In order to study conjugated and nonconjugated 3 simultaneously in the same subject as the experimental design required, both ^{14}C and ^{3}H labelled 3 were needed.

RESULTS

Carboxyl ^{14}C-labelled 3 was synthesized from nonradioactive 3, which was prepared by N-bromosuccinimide oxidation according to the method of Fieser (13). The route is shown in Figure 1. The remaining hydroxyl group was protected by formylation (14) and that intermediate reacted with lithium chloride and lead tetra-acetate to prepare the 24-nor-23-chloro derivative. The carboxyl group was reintroduced using radioactive cyanide and subsequent base hydrolysis (15). Conjugates were prepared according to published methods using the purified radioactive bile acid (16).

The synthetic routes used to prepare tritiated 3 are shown in Figure 2. The availability of a commercial sample of 3α, 7α-dihydroxy-Δ^{11}-5β-cholenic acid (12)

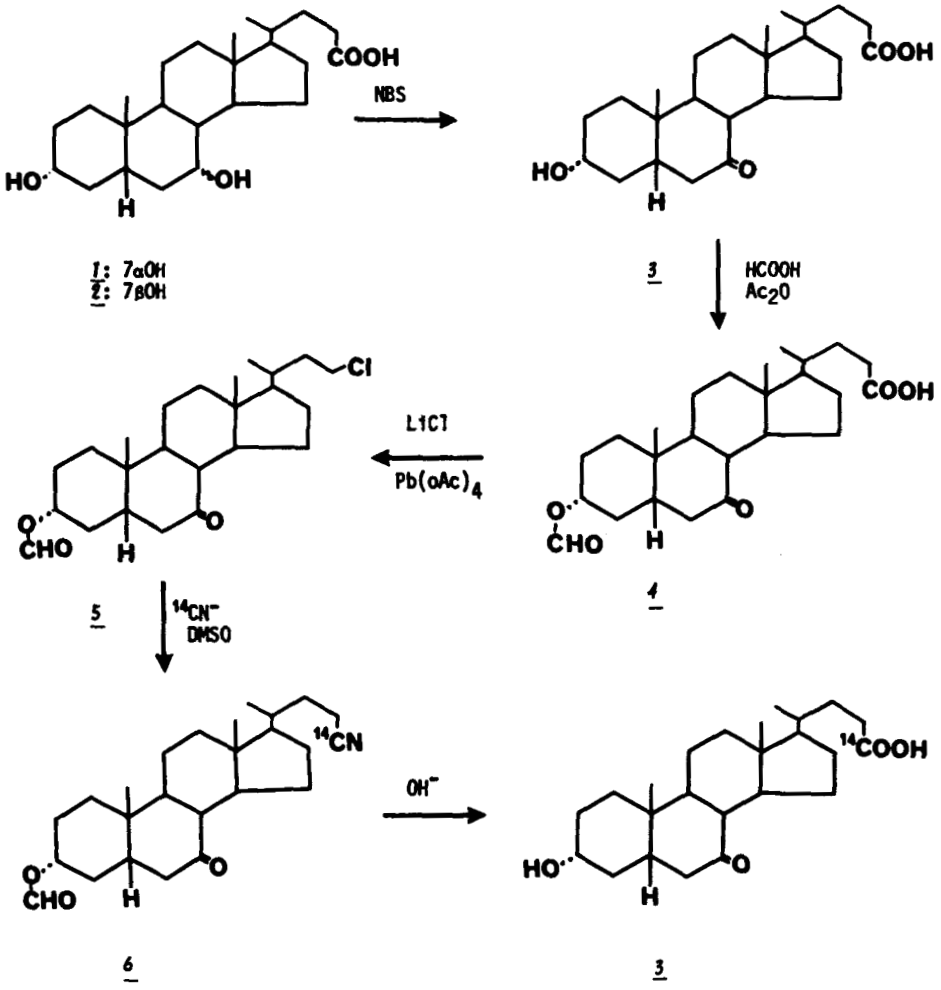

Figure 1: Preparation of ^{14}C-labeled cavicholic acid

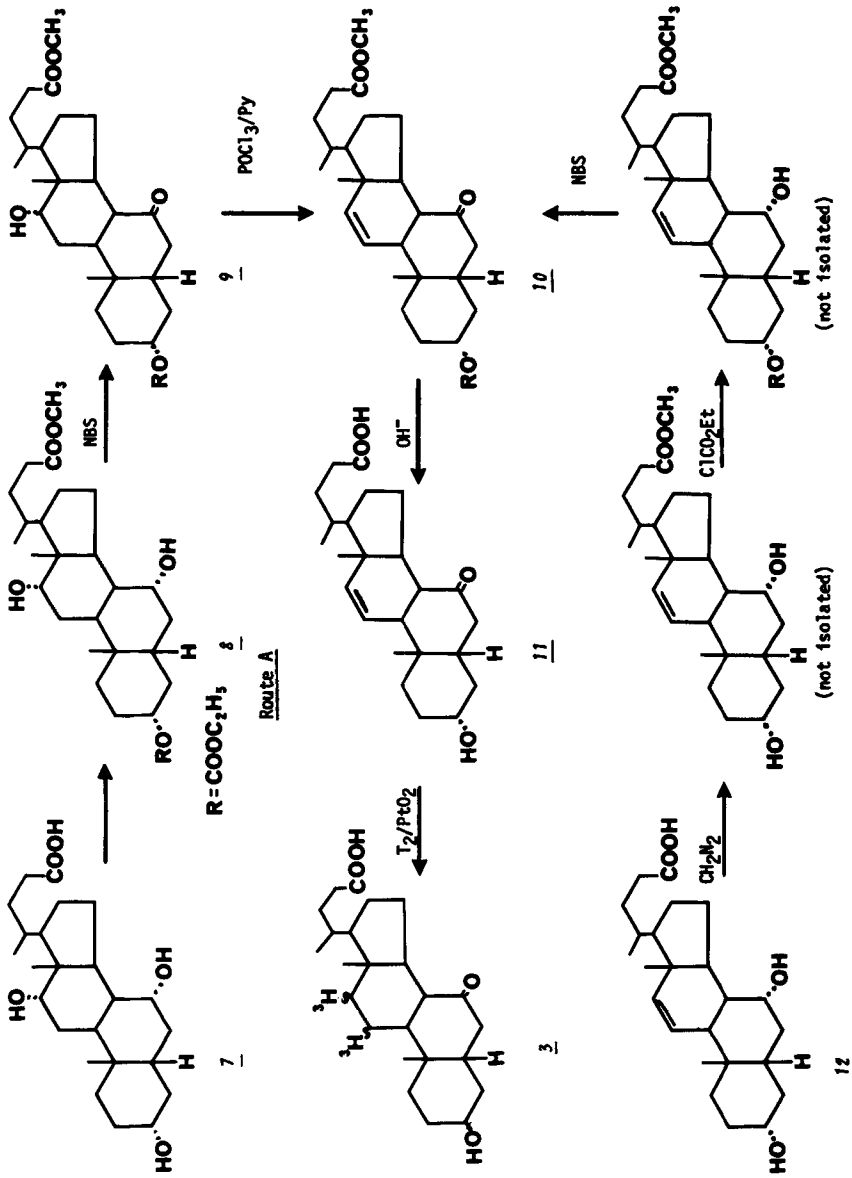

Figure 2: Introduction of unsaturation into cavicholic acid

Table 1. Physical Constants of New Compounds

Compound # Name	Formula	mp (Lit)	Recryst Solvent	Analysis	Other Data
3 3α-hydroxy-7-keto-5β-cholanic acid	$C_{24}H_{38}O_4$	201-3 (203-4)	EthOH/H_2O	C, H	
4 3α-formyloxy-7-keto-5β-cholanic acid	$C_{25}H_{38}O_5$	179-81	EtOH/H_2O	C, H	
5 24-nor-3α-formyloxy-7-keto-23-chloro-5β-cholane	$C_{24}H_{37}ClO_3$	181-3	iPrOH	C, H, Cl	
6 3α-formyloxy-7-keto-5β-cholanonitrile	$C_{25}H_{37}NO_3$	175-8	MeOH	C, H	CN 2260 cm^{-1}
10 methyl-3α-carboethoxy-7-keto-Δ^{11}-5β-cholenate	$C_{28}H_{42}O_6$	132-3	MeOH	C, H	CH_3 3.65δ =CH 6.15δ, 5.31δ
11 3α-hydroxy-7-keto-Δ^{11}-5β-cholenic acid	$C_{24}H_{36}O_4$	219-21	MeOH/H_2O	C, H	=CH 6.15δ 5.31δ
13 3α-hydroxy-7-keto-Δ^{11}-5β-cholenyl-taurine	$C_{26}H_{41}NO_6S$	168-70	EtOH/EtOAc	C, H, S	
14 3α-hydroxy-7-keto-5β-cholanyl-taurine	$C_{26}H_{43}NO_6S$	214-6	EtOH/EtOAc	C, H	+ 1½ H_2O*
15 3α-hydroxy-7-keto-Δ^{11}-5β-cholenyl-glycine	$C_{26}H_{39}NO_5$	204-4	H_2O	C, H	+ 1½ H_2O*
16 3α-hydroxy-7-keto-5β-cholanyl-glycine	$C_{26}H_{41}NO_5$	179-81	H_2O	C, H	+ 1½ H_2O*

* Water of crystallization (from elemental analysis)

allowed the synthesis of the target compound for reductive tritiation, 11, by two routes which converged on a common intermediate. Preparation of this intermediate was successful by both routes shown and overall yields were acceptable in both cases. The final intermediate was successfully tritiated by reduction with tritium gas and Adam's catalyst in dioxane. Compound 12 was also reacted with taurine and glycine to prepare the unsaturated taurine and the glycine conjugates respectively which were also tritiated under the same conditions.

The products were checked for purity by thin layer chromatography (tlc) in several solvent systems (17). Both ^{14}C-labelled and ^{3}H bile acids were purified by preparative thin layer chromatography. We observed that one of the contaminants of the tritiated material was the corresponding dihydroxy compound. Hydrolysis of the products of tritiation of unsaturated glycine or taurine conjugates revealed that the carboxyl moiety had also been reduced to some extent during tritiation. Accordingly, the glycine and taurine conjugates containing tritium were resynthesized using purified material which was not contaminated by 1 or 2.

Physical constants, spectral data, and the results of combustion analysis for the new compounds reported are presented in the Experimental Section. (See table 1).

DISCUSSION

Preparation of the ^{14}C-labelled bile acid followed known methods for the preparation of other isotopically labelled bile acids. Although the intermediates are previously unreported, no unusual complications were encountered. No effort was made to obtain high specific activity since the compounds were needed for tracer purposes only.

Bile acids have been tritiated by enol exchange (18), metal hydride (19) reduction, and by catalytic reduction of an unsaturated center introduced into the bile acid (20). The third method, which requires the sacrifice of a third hydroxyl group to prepare an unsaturated center (usually in the C ring of the steroid), has been the favored approach. It is felt that this location lessens

the chance of metabolic degradation and loss of the radiolabel because this labelling site is remote from more metabolically active parts of the bile acid (21). The method used here to introduce unsaturation in the C ring is that first used by Nakada in the synthesis of chenodeoxycholic acid from cholic acid (22, 23). A similar method has been applied to the preparation of lithocholic acid from deoxycholic acid (22,24).

The availability of a commercial sample of 3α,7α-dihydroxy-Δ^{11}-5β-cholenic acid allowed the synthesis of the target compound for tritiation by routes, both of which were successful. The reduction of the unsaturated center in the C ring was carried out according to known methods which were not modified for the present synthesis. In retrospect it seems probable that some reduction of the ketone function could have been anticipated and alternative reduction methods should be investigated for future syntheses.

It should be emphasized that the purity of these compounds cannot be adequately determined with a single tlc system. It was necessary to chromatograph the free acid and its methyl ester in different systems to be certain that the compound was free of 7-hydroxyl contaminants. In addition, the glycine or taurine conjugates must be hydrolyzed and the bile acid component chromatographed alone in addition to chromatography of the conjugate. Without such steps, the probability of detecting contaminants was low.

EXPERIMENTAL

General: IR spectra were obtained using KBr mulls on a PE-337 grating instrument and were consistent with the assigned structures. NMR spectra were run at the Hormel Institute on a Varian CFT-20 spectrometer operating in the pulsed Fourier transform mode at 79.54 MHz (^{1}H); TMS was used as internal standard. Generally, $CDCl_3$ was used as solvent. NMR spectra were consistent with the assigned structures. Melting points were done with a Mel-temp apparatus and are corrected. Elemental analyses were performed by Atlantic Microlabs (Atlanta, GA) and where indicated by element were within $\pm$ 0.4% of the calculated values for the formula shown.

Synthesis with ^{14}C.

3α-Hydroxy-7-keto-5β-cholanic acid (3). This compound was prepared by the oxidation of 1 with buffered potassium chromate or with N-bromosuccinimide, as described by Fieser (13). mp 201-3° (Lit. 203-4°).

3α-Formyloxy-7-keto-5β-cholanic acid (4). 500 mg of 3 (0.0013 mole) was dissolved in 4 mL of 88% formic acid. One drop of 70% perchloric acid was added and the solution stirred and heated to 55° for 21 hours. The solution was cooled to room temperature and 1 mL of acetic anhydride was added. The reaction was again heated to 55° and held at that temperature for 10 minutes. The cooling, addition, and heating was repeated; some gas evolution was noted after the second addition of acetic anhydride. The solution was again cooled, poured into 200 mL of cold water, and the solid collected and recrystallized from ethanol and water by dissolving in 10 mL of ethanol and diluting to the cloud point with water (about 15 mL). Yield 450 mg (84%), mp 179-181°. Anal: ($C_{25}H_{38}O_5$) C, H

24-Nor-23-chloro-3α-formyloxy-7-keto-5β-cholane (5). 4 (13.5 g, 0.032 mole) was dissolved in 300 mL of dry benzene in a 3-necked reaction flask equipped with a mechanical stirrer, reflux condenser, and provision for dry nitrogen atmosphere. Thirty mL of benzene was removed by distillation into a Dean-Stark trap under a nitrogen atmosphere to assure dryness. Lead tetracetate (14.5 g, 0.038 mole) which had been dried over P_2O_5 *in vacuo* was added carefully to avoid introduction of atmospheric water vapor or oxygen, followed by 1.3 g (0.032 mole) of dried lithium chloride. The reaction was stirred and refluxed overnight. After cooling the reaction was poured into 2N hydrochloric acid (500 mL). Caution: some chlorine may be evolved. After washing with two 200 mL portions of 2N hydrochloric acid, 5% sodium carbonate, and brine, the benzene solution was dried ($MgSO_4$) and the solvent was removed on the rotary evaporator. A yellow solid was obtained which was recrystallized from methanol and then from

isopropanol to provide 5 g (38%) of faintly yellow needles, mp 181-3°.
Anal: ($C_{25}H_{38}O_5$) C, H,Cl

3α-Formyloxy-7-keto-5β-cholanonitrile (6). The reaction was performed in a screw-cap test tube with a Teflon liner. 5 (408 mg, 0.001 mole) was dissolved in 10 mL of dry DMSO and 490 mg (0.01 mole) of sodium cyanide was added. The reaction was heated at 125° for 2 hours, cooled, diluted with 25 mL of water and extracted with three 20 mL portions of ether. After drying ($MgSO_4$) and treatment with Norit, the ether solution was evaporated, and the solid obtained was recrystallized from methanol. Yield: 200 mg (50%) col. nd., mp 175-8°.
Anal: ($C_{25}H_{37}NO_3$) C, H IR: CN, 2260 cm^{-1}.

3α-Hydroxy-7-keto-5β-cholanic acid from 6. The nitrile 6 (100 mg, 0.25 m mol) was dissolved in 0.5 mL of DMSO and 1 mL of 2N NaOH was added. The reaction was heated to 90° for 1 hour, cooled, and the product extracted and isolated as described below. After recrystallization, the product was shown to be identical to 3 by IR, mixed mp, and tlc.

24-^{14}C-3α-hydroxy-7-keto-5β-cholanic acid (3). The route described above was followed. 5 (43 mg, 0.105 mmol) was dissolved in 0.5 mL of DMSO containing 5.8 mg (0.12 mmol, 1 mCi) of ^{14}C-NaCN. The solution was stirred and heated to 130° for 2 hours, cooled, and 1 mL of 2N NaOH was added. The temperature was raised to 90° for 1 hour to hydrolyze the nitrile and formate. The reaction was cooled, acidified with 6N HCl, and the product was extracted with 7 x 5 mL of ethyl acetate. The product was chromatographed on two 20 x 20 cm silica gel H plates using ethyl acetate/iso-octane/acetic acid (25:5:0.2) solvent. The product (r_f = .4) was visualized with iodine and the silica gel-product band was scraped from the plate. Soxhlet extraction of the silica with methanol for 24 hours was used to isolate the labelled acid. The radiochemical yield was 525 μCi. The compound was shown to be homogenous by radiochromatography as the free acid in the system above and as the methyl ester in benzene/acetone (70:30).

Synthesis with ^{3}H.

Methyl 3-carbethoxy-7α,12α-dihydroxy-5β-cholanate (8) was prepared from cholic acid (7) as described by Fieser (13). mp 178-9° (Lit. 176-7°).

Methyl 3α-carbethoxy-7-keto-12α-hydroxy-5β-cholanate (9) was also prepared by previously reported methods (25). mp 184-5° (Lit. 181-2°) from NBS oxidation, or 154-5° (Lit. 154-5°) from chromate oxidation.

Methyl 3α-carbethoxy-7-keto-Δ^{11}-5β-cholanate (10)

Route A: From 9. 9(40 g, 0.081 mole) was dissolved in 250 mL of dry pyridine in a 500 mL Erlenmeyer flask. The reaction was cooled in ice and 100 mL phosphorus oxychloride was added carefully. The reaction was stoppered and sealed with Parafilm to exclude atmospheric moisture and allowed to stand at room temperature for 2 weeks. At the end of that time, the reaction was poured into a large excess of ice with vigorous stirring. The precipitated solid was filtered. Thin-layer chromatography (silica gel H, benzene/ether 9:1) showed three major components at (R_f = 0.9, 0.5, and 0.3). The crude product was chromatographed on a column containing 560 g of silica gel (60-200 mesh) using benzene/ether (9:1) eluent. The second peak eluting from the column was shown to be the desired product by IR and NMR spectrometry and by comparison with the product obtained from the alternate route (B). Yield: 15.4 g (40%) of colorless needles which recrystallized from methanol or ethanol; mp 132-3° after three recrystallizations. Anal: ($C_{28}H_{42}O_6$) C, H. NMR: CH_3 3.65δ, HC=CH 6.15δ, 5.31δ

Route B: From 3α,7α-dihydroxy-Δ^{11}-5β-cholenic acid (12). 12 (2 g, 0.005 mole, from Weddel Pharmaceuticals mp 203-4°, Lit 204-6°) was treated with an excess of diazomethane in methanol/ether solution. Excess diazomethane was destroyed with acetic acid, and the oil remaining after solvent removal was dissolved in 10 mL of dry pyridine, cooled in ice, and 5 mL of ethyl chloroformate was added dropwise. Five minutes after completion of the addition, the reaction was warmed briefly in hot water to effect solution and then allowed to stand at room temperature overnight.

The crude cathylate (2.2 g, 95%) was collected by filtration. Attempts to recrystallize it were unsuccessful and the material was accordingly oxidized without additional purification by dissolving in 25 mL of acetic acid containing 5 g of sodium acetate trihydrate and adding dropwise a solution of 1.5 g of potassium chromate (0.008 mole) in 5 mL of water. The resulting solution was stirred overnight at room temperature, then diluted with 300 mL of water. The precipitate was collected and dried. The material was recrystallized from 40 mL of methanol to give 1.9 g (96%) of hard prisms, mp 128-31°. This increased to 132-3° after several further recrystallizations. The mixed mp with the product from Route A was not depressed. Anal: ($C_{28}H_{42}O_6$) C, H

3α-Hydroxy-7-keto-Δ^{11}-5β-cholanic acid (11). 10 (4.75 g, 0.01 mole) was dissolved in 25 mL of methanol in a 250 mL flask. Twelve mL of 0.5 N sodium hydroxide and 100 mL of water was added, and the turbid suspension refluxed 4 hours to give a clear solution. The reaction was cooled, solvent removed to reduce the volume by one-half, and the reaction was acidified with 2 N hydrochloric acid. The solid was collected, washed with water, and recrystallized from methanol/water by dissolving in methanol and adding water to the cloud point. The yield was 3.8 g (98%) of crystalline material melting from 85-100°. TLC on silica gel H (benzene/acetone/acetic acid, 85:15:1) showed two components well separated which were present in a ratio of 1 to 3 (later determined by separation and weighing). The faster moving component was found to be the ester of the desired product, and the slower component the completely hydrolyzed material. The material was purified by preparative tlc in the same solvent to give a pure sample of the free acid, mp 219-22°. Anal: ($C_{24}H_{36}O_4$)· C, H

Reductive tritiation of Δ^{11} bile acids: General Procedure. Tritiations were carried out by New England Nuclear Corporation. Fifty to 100 mg of precursor was dissolved in 10 mL of dry dioxane and an equal weight of platinum oxide was added. $^3H_2$ (5-25 Ci) was introduced and the reaction stirred at atmospheric pressure for 1 hour. An excess of hydrogen was then added and the reduction completed at 2 atm for 4 hours. Labile ^3H was removed *in vacuo* using ethanol. After removing the catalyst by filtration, the ethanol was removed and the product redissolved in ethanol for storage and shipment.

The free acid was purified by chromatography as described for the ^{14}C derivative. Samples of conjugates were fully hydrolyzed prior to chromatography. Typically, 20-30% of the total ^{3}H measured in crude preparations was found to be due to 3, 7-dihydroxycholanic acids. Because of this co-reduction of the carbonyl group, the tritiated conjugates were hydrolyzed, the bile acid purified by preparative tlc, and the conjugates resynthesized as described below.

Preparation of Glycine and Taurine Conjugates

3α-Hydroxy-7-keto-Δ^{11}-5β-cholenyl taurine (13). 11 (789 mg, 0.002 mole) was dissolved in 5 mL of dry dimethylformamide (DMF) and N-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (EEDQ) (0.8 g, 0.003 mole) was added. Taurine (350 mg, 0.002 mole) and triethylamine (0.5 mL, 0.0036 mole) were added and the reaction warmed to 90° with stirring overnight. The reaction was cooled, and poured into cold ethyl ether. The precipitated product was isolated by decanting the solvent and dissolving in dichloromethane. The addition of HCl-saturated ether precipitated the free acid which was collected after cooling at 5° overnight. The solid was recrystallized from ethanol/ethyl acetate to give 800 mg (80%) colorless needles, mp 168-70°. Anal: ($C_{26}H_{41}NO_6S$) C, H

3α-Hydroxy-7-keto-5β-cholanyl taurine (14) was prepared from 1.95 g (0.005 mmole) of 3 in a similar manner to the preparation of 13. Yield 1.8 g (72%) of needles, mp 214-6°. Anal: ($C_{26}H_{43}NO_6S$) C, H One batch of material, apparently pure, melted at 201-4° and had a different crystal habit.

3α-Hydroxy-7-keto-Δ^{11}-5β-cholenyl glycine (15). 11 (190 mg, 0.5 mmol) was added to a stirred solution of 100 mg (0.7 mmole) of glycine ethyl ester hydrochloride, EEDQ (200 mg, 0.75 mmol) and 0.3 mL (2.2 mmol) of triethylamine in 7 mL of DMF. The reaction was refluxed overnight, cooled, and poured into 50 mL of water. The suspension was extracted twice with 25 mL of ethyl acetate and the combined ethyl acetate extracts were washed with 50 mL each of 0.5 N sodium hydroxide, 0.5 N hydrochloric acid and water. The solution was dried ($MgSO_4$), and ethyl acetate was removed on the rotary evaporator. The remaining oil was dissolved in 10 mL of ethanol and 10 mL of 10% potassium carbonate solution was added while boiling gently on the steam bath. After refluxing for 15 minutes, the solution was evaporated to half its volume, diluted with one volume of water, and acidified.

The precipitate was collected, washed with water, and recrystallized from water to give 140 mg (63%) of almost colorless needles, mp 202-4°.

Anal: ($C_{26}H_{39}NO_5 + \frac{1}{2} H_2O$) C, H

3α-Hydroxy-7-keto-5β-cholanyl glycine (16) was prepared from 1.95 g of 3 with the same procedure as 15. Yield 1.5 g (68%), mp 179-81°. Anal: ($C_{26}H_{41}NO_5 + \frac{1}{2} H_2O$) C, H

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REFERENCES

1. Nair P. and Kritchevsky D. - The Bile Acids, Volume 2, Plenum, New York (1973) pp. 55-8.
2. Sjovall J. - Acta Chem. Scand., 13:711 (1959)
3. Samuelsson B. - Acta Chem. Scand., 13:976 (1959)
4. Salen G., Tint G. S., Eliav B., Deering N., Mosbach E. H. -Clin. Invest., 53:612 (1974)
5. Fromm H., Erbler H. C., Eschler A., and Schmidt F. W. -Klin. Wschr., 54:1125 (1976)
6. Thistle J. L., Hofmann A. F., and Stephens D. A., -JAMA, (In Press).
7. Makine I., Shinozaki K., Yoshino K., and Nakagawa S., -Jap. J. Gastr., 72:690 (1975)

8. Ashizawa S., Ishii N., Isihara F., Ito Y., Veno Y., Osawa H., Osuga T. - Igakv. No. Ayumi., (Progress in Medicine), 101:922 (1977)
9. Nakagawa S., Makino I., Ishizaki T., Dohi I. - Lancet 367 (1977)
10. Eneroth P., Gordon B., Ryhage R., Sjovall J. - J. Lip. Res., 7:511, (1966)
11. Imai A. - Physiol. Chem. 248:65 (1937)
12. Nair P., Kritchevsky D. - The Bile Acids, Volume 2, Plenum, New York (1973), pp 63-4.
13. Fieser L. F., Rajagopalan S. - J. Am. Chem. Soc., 7 71:35-39 (1949)
14. Tserng K. Y., Klein P. - Steroids 29:635 (1977)
15. Hachey D. L., Szczepanik P., Berngruber O., Klein P. - J. Lab. Compounds 9:703 (1973)
16. Tserng K. Y., Hackey D. L. Klein P. D. - J. Lip. Res. 18:404 (1977)
17. Hofmann A. F. - In New Biochemical Separations, L. J. Morris and A. T. James, eds. Van Nostrand, Princeton (1964), p. 362.
18. Hofmann A. F., Szczepanik P., Klein P. D. - J. Lip. Res. 9:707 (1968)
19. Stahl E., Arnesjo B. - Scand. J. Gastroenterol., 7:559 (1972)
20. Cowen A. E., Hofmann A. F., Hachey D., Thomas P., Belobaba D., Klein P., Tokes L. - J. Lip. Res. 17:231 (1976)
21. Panveliwalla D., Pertsemlidis D., Ahrens E., Jr. - J. Lip. Res. 15:530 (1974)
22. Nakada F., Yamasaki K. - Steroids 1:131 (1963)
23. McKenzie B. F., McQuckin W. F., Kendall E. C. - J. Biol. Chem. 162:555 (1946)
24. Nakada F. - Steroids, 2:45 (1964)
25. Fieser L. F., Rajagopalan S. - J. Am. Chem. Soc. 72:5530 (1950)