SYNTHESIS OF DEUTERIUM-LABELLED LITHOCHOLIC ACID

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SUMMARY

A convenient synthesis is reported of lithocholic acid labelled in high isotopic purity with two atoms of deuterium at C-11. Incorporation of deuterium is achieved by base-catalyzed equilibration of a 12-oxo bile acid in heavy water. The product is converted *in situ* into its tosylhydrazone derivative which is reduced with sodium borohydride to afford the labelled 12-deoxy bile acid.

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INTRODUCTION

The majority of investigations of bile acid pool size and synthesis rate in man have been carried out by isotope dilution procedures using tritium or ^{14}C-labelled tracers (see 1). Recently, Hofmann, Klein and co-workers have demonstrated that bile acids labelled specifically with stable isotopes also may be employed as tracers in turnover studies (2,3), thereby extending the scope of the technique to investigations in obstetric and paediatric patients (4). Both ^{13}C- and deuterium-labelled bile acids have been prepared (5) and mass spectrometric techniques have been developed for the measurement of stable isotope ratios in molecules of this type (6-9).

The synthetic requirements for the preparation of tracer bile acids labelled with stable isotopes are two-fold: (i) that the label(s) is introduced in high isotopic purity, and (ii) that the heavy atom(s) occupies a non-exchangeable position, remote from sites of metabolic attack. Although these requirements can best be met by labelling with ^{13}C, the relatively high cost of this isotope has hitherto restricted its use, particularly for the synthesis of multiply-labelled compounds. Deuterium, on the other hand, is readily available at low cost and remains the isotope of choice for most applications. Bile acids have been synthesized with deuterium at C-2 and C-4 (5), but partial loss of label from both positions occurs *in vivo*, thus limiting the clinical usefulness of these tracers (10). Ring C, however, has proved to be a more suitable area for labelling and di-deutero analogues of chenodeoxycholic and lithocholic acids, labelled at the 12,12- and 11,12-positions, have been prepared by catalytic deuteration procedures (3,11). Previous studies in our laboratory (12) have shown that incorporation of two atoms of deuterium at the 11 position may be carried out conveniently by base-catalysed exchange of a 12-keto steroid in heavy water (13) to give a product of very high isotopic purity. The object of the present investigation was to determine whether this reaction could also be employed to prepare [11,11-$^2H_2$]bile acids, and lithocholic acid was chosen as a model compound for this study.

EXPERIMENTAL

GENERAL

Melting points were determined on a Gallenkamp hot-stage apparatus and are corrected.

Infrared spectra were recorded either on solutions in carbon tetrachloride or on discs of potassium bromide using a Perkin-Elmer 254 Infrared Spectrophotometer.

Thin-layer chromatography (TLC) was carried out on glass plates (5 x 20 cm) pre-coated with 0.25 mm layers of silica gel 60 F_{254} (Merck AG, Darmstadt, Germany). The mobile phase was toluene/dioxan/glacial acetic acid (75:20:2 by vol.) and spots were visualized either by viewing under UV light (λ = 254 nm) or by spraying the plate with a vanillin-sulphuric acid solution and subsequent heating at 110° for 5 min.

Gas-liquid chromatography (GLC) was performed with a Pye Series 104 instrument, equipped with 2 m x 3 mm i.d. glass columns packed with 1% SE-30 on Gas Chrom Q, 100-120 mesh. Flame ionization detectors were used with nitrogen (30 ml/min) as carrier gas, and the column oven temperature was 250°. Bile acids were methylated with diazomethane prior to analysis and free hydroxyl groups were converted to trimethylsilyl (TMS) ether derivatives. Retention times (t_R) were measured relative to that of 5α-cholestane.

Mass spectrometry. Spectra obtained by the direct insertion technique were obtained using an LKB 2091 instrument. The temperature of the ion source was 225°, the energy of the bombarding electrons was 70 eV and the accelerating voltage was 3.5 kV.

Gas chromatography-mass spectrometry (GC-MS) was carried out on a modified LKB 9000 instrument (14), equipped with a 3 m x 3 mm i.d. glass column packed with 1.5% SE-30 on Gas Chrom Q, 100-120 mesh, with helium (25 ml/min) as carrier gas. The column was maintained at 230°, while the molecular separator and ion source temperatures were 255° and 290°, respectively. The accelerating voltage was 3.5 kV, the ionizing energy was 22.5 eV and the trap current was 60 µA. Multiple spectra were recorded on magnetic tape and processed off-line using an IBM 1800 computer (9).

Reagents. Deoxycholic acid (puriss) was purchased from Kebo AB (Stockholm, Sweden).

Deuterium oxide (99.8%) was obtained from Norsk Hydro (Oslo, Norway) and monodeuteromethanol (CH_3O^2H, $\geq$ 99%) from Merck AG (Darmstadt, Germany). All other chemicals were used as purchased and were reagent grade where available.

SYNTHESIS

Methyl 3α-acetoxy-12-oxo-5β-cholanate (IV)

Deoxycholic acid (I) was methylated overnight at room temperature in 0.1 N methanolic HCl to give (II), which was selectively acetylated and oxidized by published procedures (15). The crude product was recrystallized from ethanol to give IV as colourless needels, m.p. 150-152° (lit.(15) 151-153°). IR (CCl_4): 1737 (s), 1706 (s), 1243 (s), 1173 (w) and 1030 cm^{-1} (m). TLC: one spot with R_f = 0.60. GLC: single peak with t_R = 3.91. MS: (GC inlet) m/e 446 (M^+), 386, 371, 354, 231 (base peak) and 229.

Methyl 3α-hydroxy-12-oxo-5β-[11,11,23,23-$^2H_4$]cholanate tosylhydrazone (V)

To a stirred mixture of deuterium oxide (15 ml) in monodeuteromethanol (600 ml) was added sodium metal (4.17 g; 181 mmole) in small pieces. After the reaction was complete, methyl 3α-acetoxy-12-oxo-5β-cholanate (IV; 15.0 g; 33.6 mmole) was added and the mixture was heated under reflux for 48 h. The resulting solution was cooled to ice-bath temperature and treated, dropwise, with acetyl chloride (15 ml). The reaction mixture was allowed to warm up to room temperature, tosyl hydrazine (20.2 g; 100 mmole) was added and the mixture heated under reflux for 24 h. On cooling, the tosylhydrazone (V) crystallized from solution as needles m.p. 258-261° (yield = 19.6 g). IR (KBr): 3440 (w), 2558 (s), 1736 (s), 1445 (w), 1332 (m) and 1171 cm^{-1} (w). TLC: one tailing spot with R_f = 0.24. MS (direct inlet): m/e 574-576 (M^+), 559-561, 543-545, 419-421, 276, 275, 257 and 91 (Fig. 2).

Methyl 3α-hydroxy-5β-[11,11,23,23-$^2H_4$]cholanate ($^2H_4$-methyl lithocholate; VI)

The tosylhydrazone V (3.14 g; 5.34 mmole) was dissolved in methanol (300 ml) and sodium borohydride (6.0 g; 160 mmole) was added in portions with ice-bath cooling. The resulting mixture was heated under reflux for 4 h, during which time a further two portions of sodium borohydride (6 g each) were added. The reaction product was cooled, acidified with conc. HCl and the solvent was evaporated in vacuo.

The residue was diluted with water (750 ml) and extracted with ether (3 x 600 ml). The combined ether extracts were washed with 2 N NaOH (2 x 50 ml) and water (5 x 100 ml), dried ($MgSO_4$) and evaporated to give 1.96 g of an amorphous solid. Analysis by TLC and GC-MS indicated that the major component (~70%) of the reaction product was the labelled methyl lithocholate (VI), a pure sample of which was obtained by crystallization from methanol. This gave fine needles, m.p. 122.5-125° (lit (16) 125-127.5°). IR (CCl_4): 3620 (w), 2202 (w), 2120 (w), 1741 (s) and 1171 cm^{-1} (w). TLC: one spot with R_f = 0.45. GLC (TMS ether): single peak at t_R = 1.99. MS (GC inlet): m/e 464-466 (M^+), 449-451, 374-376, 359-361, 259, 232 and 217 (base peak).

3α-Hydroxy-5β-[11,11,23,23-$^2H_4$]cholanic acid ($^2H_4$-lithocholic acid; VII)

A sample of the labelled methyl ester VI was saponified under strong alkaline conditions by treatment with a 15% solution of NaOH in 50% aqueous ethanol at 110° for 10 h. The identity of the product as the $^2H_4$-lithocholic acid VII was confirmed by analysis by IR, TLC and (following derivatization) by GLC and GC-MS. Deuterium content (as determined by GC-MS analysis of the methyl ester-TMS ether derivative) was found to be as follows: (atoms% excess) - m/e 372 (M-90) 0.7% $^2H_0$, 4.3% $^2H_1$, 65.0% $^2H_2$, 22.5% $^2H_3$ and 7.5% $^2H_4$; m/e 257 (M-90-side-chain) 4.6% $^2H_0$, 3.7% $^2H_1$ and 91.7% $^2H_2$.

RESULTS AND DISCUSSION

The reaction sequence leading to the labelled lithocholic acid is summarized in Fig. 1. Deoxycholic acid (I) was transformed by published procedures into the 12-oxo methyl ester IV, which was subjected to base-catalyzed equilibration with deuterium oxide. The resulting deuterium-labelled bile salt was not isolated, but the reaction mixture was acidified and treated with tosylhydrazine; under these conditions simultaneous re-methylation at C-24 and derivatization at the 12 position took place to yield the crystalline tosylhydrazone V. The mass spectrum of this compound (Fig. 2) indicated that the above sequence of reactions had resulted in incorporation of deuterium not only into the steroid nucleus but also into the side-chain (probably at C-23), as evidenced by the shift in mass of fragments re-

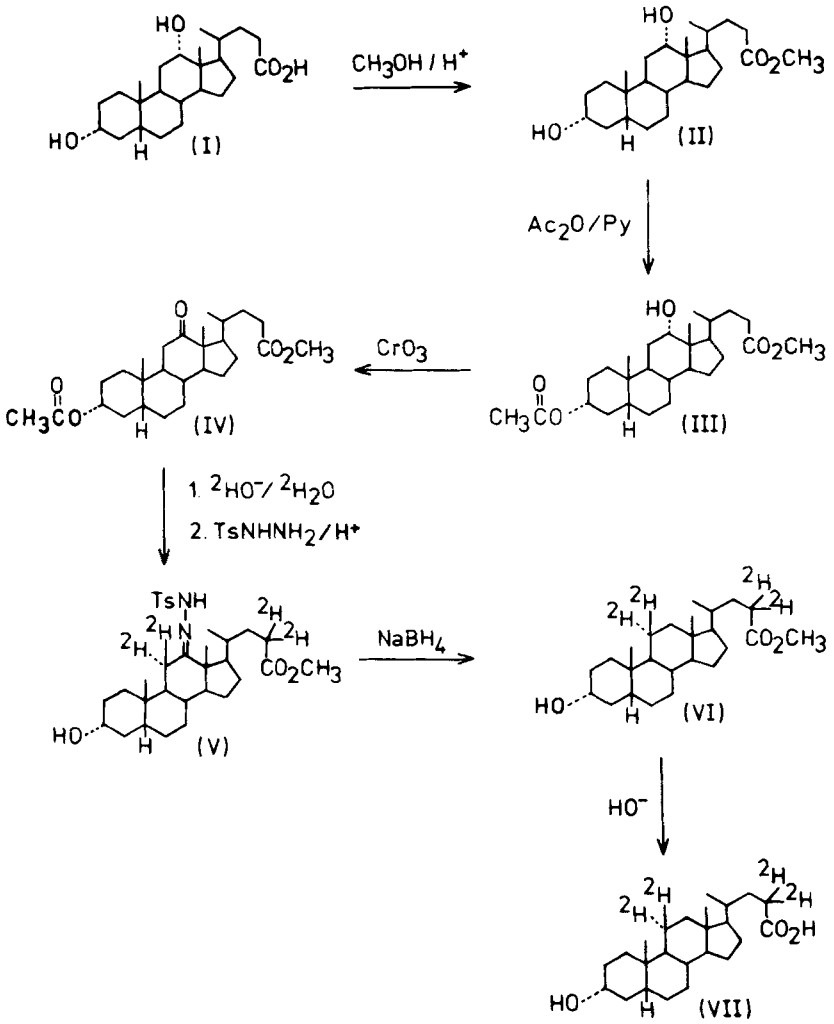

Figure 1. Reaction scheme for the synthesis of deuterium-labelled lithocholic acid.

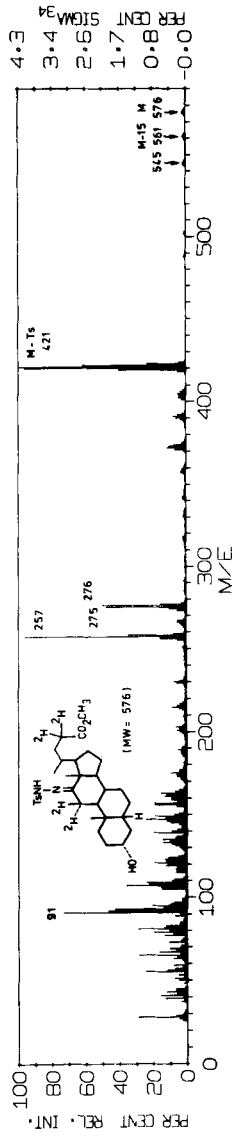

Figure 2. Mass spectrum (direct inlet) of the tosylhydrazone derivative of methyl 3α-hydroxy-12-oxo-5β-[11,11,23,23-$^{2}H_{4}$]cholanate (V).

taining carbons 20-24. Reduction of V with a large excess of sodium borohydride afforded the labelled methyl lithocholate (VI), which was saponified to give the corresponding lithocholic acid (VII). The isotopic purity of this product at C-11 was greater than 90% $^2H_2$, although side-chain labelling was still evident from its mass spectrum (Fig. 3A).

Deuterium-labelled lithocholic acid prepared by the above route should prove valuable for the following applications: (i) as an internal standard for the quantitative determination of lithocholic acid in biological fluids by stable isotope dilution and selected ion monitoring GC-MS, (ii) as a starting material for the synthesis of 5β-reduced pregnane derivatives labelled with deuterium at C-11 and (iii) as a tracer for studies of lithocholic acid metabolism in humans.

The limiting factor for its use in (i) is the proportion of unlabelled molecules in the deuterated lithocholic acid; the figure in this case is less than 1% which is acceptable for most applications. In addition, the deuterium atoms must be stable towards back-exchange under the conditions of sample extraction and analysis - no loss of label from C-11 would be expected to occur in a 12-deoxy bile acid. It appears that forcing conditions are necessary to remove deuterium substituents at C-23; despite the severity of the reaction employed to saponify the methyl ester VI, less than 50% of the deuterium at this position was removed by exchange. Similar conditions of alkaline hydrolysis, however, have been found to be more effective when used on conjugated bile acids (17).

[11,12-$^2H_2$]Chenodeoxycholic and lithocholic acids have been synthesized (11) and the biological stability of the labelled atoms has been verified in studies with human subjects (2). The metabolism of 3β-hydroxy-5α-pregnan-20-one sulphate in the rat has been investigated using the corresponding [3α,11,11-$^2H_3$]derivative as a tracer, when no loss of label from C-11 was observed (18). Based on these findings, it would appear that, at least in the case of 11- and 12-deoxy steroids, ring C is the area of choice for the introduction into the steroid nucleus of chemically and metabolically stable hydrogen isotopes. The synthesis reported in this communication may be extended readily to the preparation of [11,11-$^2H_2$]chenodeoxy-

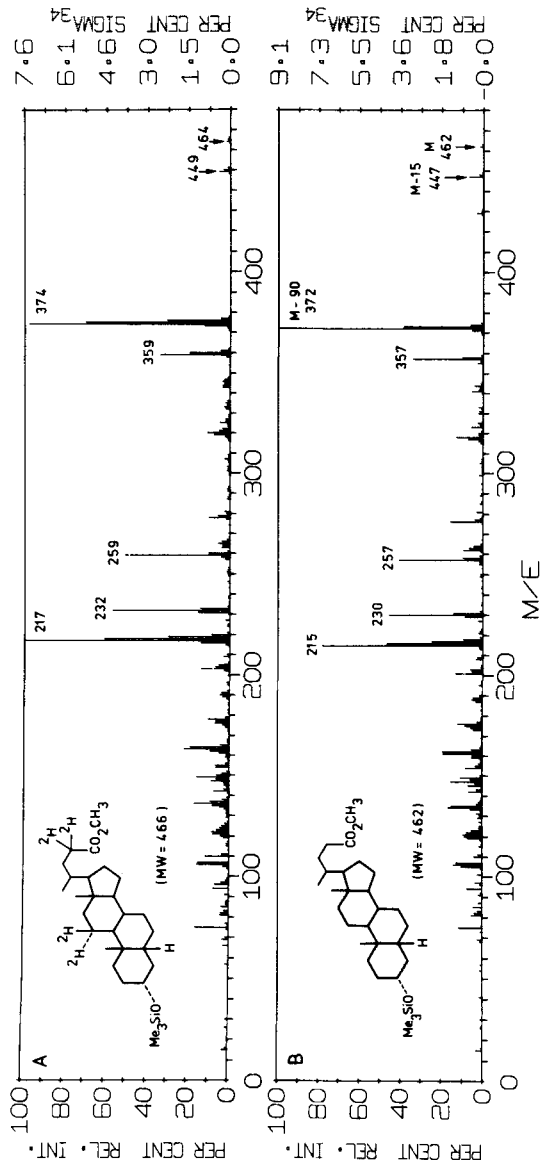

Figure 3B. Mass spectrum of unlabelled methyl lithocholate TMS ether, obtained under the same conditions as used for Fig. 3A.

Figure 3A. Mass spectrum (GC inlet) of the methyl ester-TMS ether derivative of $^{2}H_{4}$-lithocholic acid (VII).

cholic acid or to 11,11,12,12-tetradeutero analogues by reduction of the 12-tosylhydrazone with sodium borodeuteride (19). This approach should provide a useful alternative to existing methods for the preparation of bile acids labelled specifically and in high isotopic purity with deuterium.

Deuterium-labelled lithocholic acid may be degraded by classical procedures to afford deuterated analogues of 5β-reduced pregnane derivatives and of hormones such as progesterone and testosterone (20). We are currently evaluating this approach to the synthesis of [11,11-$^2H_2$]progesterone and some of its metabolites for use in studies on the turnover of these compounds in pregnant women (21).

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REFERENCES

1. Nair, P.P. and Kritchevsky, D. (eds.) - The Bile Acids: Chemistry, Physiology and Metabolism. Vol. II. Plenum Press, New York, 1970.
2. Balistreri, W.F., Cowen, A.E., Hofmann, A.F., Szczepanik, P.A. and Klein, P.D. - Pediat. Res. 9: 757 (1975)
3. Hofmann, A.F., Klein, P.D., Thistle, J.L., Hachey, D.L., Hoffman, N.E., La Russo, N.F., Thomas, P.J. and Szczepanik, P.A. - Proceedings of the First International Conference on Stable Isotopes in Chemistry, Biology, and Medicine (Klein, P.D. and Peterson, S.V., eds.) - Nat. Tech. Inf. Service, US Dept. of Commerce, document code CONF-730525, 1973, p. 369.
4. Watkins, J.B., Ingall, D., Szczepanik, P., Klein, P.D. and Lester, R. - New Engl. J. Med. 288: 431 (1973)
5. Hachey, D.L., Szczepanik, P.A., Berngruber, O.W. and Klein, P.D. - J. Labelled Compounds 9: 703 (1973)
6. Klein, P.D., Haumann, J.R. and Eisler, W.J. - Clin. Chem. 17: 735 (1971)

7. Klein, P.D., Haumann, J.R. and Eisler, W.J. - Anal. Chem. 44: 490 (1972)
8. Klein, P.D., Haumann, J.R. and Hachey, D.L. - Clin. Chem. 21: 1253 (1975)
9. Axelson, M., Cronholm, T., Curstedt, T., Reimendal, R. and Sjövall, J. - Chromatographia 7: 502 (1974)
10. Hepner, G.W., Sturman, J.A., Hofmann, A.F. and Thomas, P.J. - J. Clin. Invest. 52: 433 (1973)
11. Cowen, A.E., Hofmann, A.F., Hachey, D.L., Thomas, P.J., Belobaba, D.T.E., Klein, P.D. and Tökés, L. - J. Lipid Res. 17: 231 (1976)
12. Baillie, T.A., Sjövall, J. and Herz, J.E. - Steroids 26: 438 (1975)
13. Tökés, L., Jones, G. and Djerassi, C. - J. Am. Chem. Soc. 90: 5465 (1968)
14. Reimendal, R. and Sjövall, J. - Anal. Chem. 44: 21 (1972)
15. Gallagher, T.F. and Long W.P. - J. Biol. Chem. 162: 521 (1946)
16. Chang, F.C., Blickenstaff, R.T., Feldstein, A., Gray, J.R., McCaleb, G.S. and Sprunt, D.H. - J. Am. Chem. Soc. 79: 2164 (1957)
17. Cronholm, T., Burlingame, A.L. and Sjövall, J. - Eur. J. Biochem. 49: 497 (1974)
18. Baillie, T.A., Eriksson, H., Herz, J.E. and Sjövall, J. - Eur. J. Biochem. 55: 157 (1975)
19. Tökés, L. and Throop, L.J. - In: "Organic Reactions in Steroid Chemistry, Vol. 1" (Fried, J. and Edwards, J.A., eds.) Van Nostrand Reinhold, New York, 1972, p. 145.
20. Koechlin, B.A., Kritchevsky, T.H. and Gallagher, T.F. - J. Biol. Chem. 184: 393 (1950)
21. Baillie, T.A., Sjövall, K., Herz, J.E. and Sjövall, J. - In: "Advances in Mass Spectrometry in Biochemistry and Medicine, Vol. 2" (Frigerio, A. ed.) Spectrum Publications, New York, 1976, p. 161.