An improved synthesis of 24-^{13}C-labeled bile acids using formyl esters and a modified lead tetraacetate procedure

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Summary An improved synthesis of 24-^{13}C-labeled bile acids has been achieved using formyl derivatives of bile acids and a modified lead tetraacetate procedure. The formylated bile acids were degraded by lead tetraacetate and lithium chloride to formylated 23-chloronorcholanes in 72–83% yield. Formylated 23-chloronorcholanes were converted to nitriles in dimethylformamide, which were then hydrolyzed to obtain C-24 labeled bile acids in yield of 80–90% of labeled sodium cyanide used. This method results in a higher yield and a purer product with less manipulation than previously reported procedures for synthesis of labeled bile acids.

Supplementary key words [24-^{13}C]cholic acid · [24-^{13}C]chenodeoxycholic acid · [24-^{13}C]deoxycholic acid · [24-^{13}C]lithocholic acid · [24-^{13}C]ursodeoxycholic acid

Bile acids labeled specifically at carbon-24 are important compounds for the tracer study of bile acid metabolism. They have an advantage over the use of tritium- or deuterium-labeled bile acids for metabolic study in that the label is metabolically and chemically stable. In the past several years, the use of compounds labeled with stable isotopes as metabolic tracers has gained importance in clinical investigation (1–3). The primary advantage of stable isotopes is the lack of radiation hazard, so that they can be used in infants, pregnant women, and the general population.

Recently, the application of deuterium-labeled bile acids as tracers in the study of bile acid kinetics has been reported (4, 5). However, the metabolic instability of labeled hydrogen introduced into enolic positions and the chemical inaccessibility of some of the deutero-bile acids limit their applicability to metabolic studies. On the contrary, C-24 labeled bile acids do not have these disadvantages. The label at C-24 is metabolically stable and bile acids so labeled are generally accessible by chemical synthesis. In preliminary studies, the sensitivity of 24-^{13}C-labeled bile acids in isotopic dilution assays was found to be comparable to the currently available deutero-bile acids by using a GC–CIMS–AVA system. The only previous disadvantage of C-24-labeled bile acids has been the higher cost of ^{13}C-labeled compounds when compared to deutero compounds. The development of an efficient synthesis to maximize the use of starting material and to lower the cost of synthesis by simplifying the procedure would reduce the cost of ^{13}C-labeled bile acids for clinical application. This account describes such a procedure.

Materials and methods

Formylated bile acids were obtained in quantitative yield by heating the bile acids in 90% formic acid containing perchloric acid and then adding acetic anhydride.[1] A typical example is given: A stirred solution or a suspension of bile acid (5 g) in 20 ml of 90% formic acid containing 4 drops of 70% perchloric acid was heated at 55°C for 1.5 hr. The solution was removed from the bath and allowed to cool to about 40°C. Acetic anhydride was then added dropwise at a rate that maintained the temperature between 55°–60°C until a large quantity of bubbles appeared (ca. 16 ml of acetic anhydride required). The solution was then cooled to room temperature and poured into 200 ml of stirred water. The precipitate was collected, washed with water, and dried to yield formylated bile acids. The products were chromatographically pure and could be recrystallized by dissolving them in boiling ethanol (50 ml) and diluting slowly with 50 ml of boiling water.

All chemicals used were of commercial reagent grade. Sodium [^{13}C]cyanide (90 atom % C-13) was purchased from Merck, Sharp & Dohme, Canada Ltd., Montreal, Canada.

Melting points were determined on a Fischer–Johns melting point apparatus and are uncorrected. The purity of the products was checked by thin-layer chromatography and gas–liquid chromatography. Their identities were established by comparing their retention time with authentic samples under various conditions, and also by cochromatography.

Thin-layer chromatography was carried out on precoated silica gel plates (Kontes Q1) using the following solvent systems, designated by letters: A, acetone–benzene 2:98; B, benzene–dioxane–acetic acid 75:20:2.0; C, benzene–dioxane–acetic acid 20:10:2.0 (13). The plates were visualized by spraying with 10% H_2SO_4 in ethanol and heating at 120°C.

Gas–liquid chromatography was carried out by using methyl ester acetates of labeled bile acids. A

Abbreviations: DMF, dimethylformamide; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GC–CI–MS–AVA, gas chromatograph–chemical ionization mass spectrometric–accelerating voltage alternator system; DMSO, dimethyl sulfoxide.

[1] A fully detailed synthesis of pure formylated bile acids in quantitative yield will appear elsewhere.

Varian Aerograph series 1400 gas chromatograph (Varian Assoc., Palo Alto, CA) with a glass column (1 mm × 180 cm) packed with 1% Poly S-179 on gas chrom Q (100/120 mesh) was used (6). The column temperature was 260°C; the injection port was 275°C and the detector was 280°C. The nitrogen flow rate was 20 ml/min.

The methyl ester acetates for GLC were prepared by methylating bile acid (2 mg/ml, 0.5 ml) with 0.5 ml of dimethoxypropane (7) containing 1 drop of conc. HCl at 25°C for 1 hr. After evaporating to dryness with nitrogen, the residue was acetylated with 1 ml of acetylating mixture (chloroform–acetic acid–acetic anhydride–70% perchloric acid 78:10:14:0.1) at 25°C. After 15 min, 4 ml of water was added and the contents of the tube were mixed by shaking. The chloroform layer was withdrawn by a syringe, evaporated to dryness with nitrogen, and dissolved in 1 ml of acetone. The amount injected was 1 μl.

Formylated 23-chloronorcholanes. A solution of bile acid formate (0.035 mol) in 300 ml of benzene was azeotropically dried by refluxing for 0.5 hr with a Dean Stark apparatus (20 ml). After cooling to room temperature, lead tetraacetate (30 g, 0.07 mol) and LiCl (3 g, 0.07 mol) was added to this solution. The stirred suspension was deoxygenated by bubbling N_2 gas through it and the bath was then heated to 83°C. After 2 hr, several portions of LiCl (1.5 g, 0.035 mol, each) were added at 1-hr intervals until the benzene solution showed a negative $Pb(OAc)_4$ reaction (no brown precipitate is formed when one drop of benzene solution is shaken with water). The end point can be easily detected by the change of the clear solution with a sticky residue on the flask wall to a turbid suspension. This change usually required three or four additions of LiCl. The turbid suspension was stirred and allowed to cool to room temperature. It was filtered and washed with benzene. The combined filtrate was washed with ice-cold 2% NaOH (150 ml × 2) and water (200 ml × 4), dried over anhydrous $MgSO_4$, and evaporated in vacuo to a solid or oily residue. The residue was dissolved in 30 ml of absolute ethanol at room temperature. A solution was formed first, which was followed by a heavy crystallization within a few minutes. The crystalline solid was then collected, washed with ice-cold ethanol, and air dried. The following chlorides were obtained in yields of 62–68%:

23-chloro-3α,7α,12α-triformyloxynorcholane, mp 165–167°C; TLC (solvent A), R_f 0.36; elemental analysis, $C_{26}H_{39}O_6Cl$:Calc. C, 64.65, H, 8.14; Found. C, 64.44, H, 8.07.

23-chloro-3α,7α-diformyloxynorcholane, mp 158–159°C; TLC (solvent A), R_f 0.49; elemental analysis, $C_{25}H_{39}O_4Cl$:Calcd. C, 68.40, H, 8.95; Found. C, 68.30, H, 8.94.

23-chloro-3α,12α-diformyloxynorcholane, mp 123–125°C; TLC (solvent A), R_f 0.53; elemental analysis, $C_{25}H_{39}O_4Cl$:Calcd. C, 68.40, H, 8.95; Found. C, 68.26, H, 8.65.

23-chloro-3α,7β-diformyloxynorcholane, mp 142–144°C; TLC (solvent A), R_f 0.50; elemental analysis, $C_{25}H_{39}O_4Cl$:Calcd. C, 68.40, H, 8.95; Found. C, 68.25, H, 8.96.

23-chloro-3α-formyloxynorcholane, mp 131–133°C; TLC (solvent A), R_f 0.78; elemental analysis, $C_{24}H_{39}O_2Cl$:Calcd. C, 72.97, H, 9.95; Found. C, 72.85, H, 10.03.

The mother liquor was concentrated in vacuo at room temperature and then chromatographed on a silica gel column (2 × 30 cm). After eluting with 5% acetone in benzene, an additional batch (10–15%) of formylated 23-chloronorcholane could be obtained. The total yields were 72–83%.

The formylated 23-chloronorcholanes can be recrystallized from ethanol. However, if trace amounts of either acid or base were trapped in the crude product, the recrystallized product would often be contaminated with trace amounts of partially deformylated compounds and recovery would be low. Pure formylated 23-chloronorcholanes are stable in boiling ethanol for at least 5 min.

24-^{13}C-Labeled bile acids

A stirred suspension of 23-chloronorcholane formate (0.009 mol) and $Na^{13}CN$ (0.43 g, 0.0085 mol) in 30 ml DMF was heated at 100°C for 3 hr. After cooling to room temperature, 150 ml of water was added and the suspension was stirred for 1 hr at room temperature. The precipitate (23-cyanonorcholane formate + unreacted 23-chloronorcholane formate) was collected, washed with water, and then dissolved in 40 ml of hot ethanol. To the solution was added an aqueous alkali solution (5 g of NaOH in 40 ml of water), and the mixture was then refluxed in a 100°C bath with stirring for 48 hr. The cooled solution was poured into 100 ml of water and washed with ether (50 ml × 2). The aqueous alkali solution was heated on a steam bath to remove the dissolved ether and filtered. The filtrate was acidified with dilute HCl and the white precipitate was collected, washed with water, and recrystallized from various solvents (as described below) in yields of 80–90%.

1. [24-^{13}C]Cholic acid was recrystallized from ethanol and dried at 150°C, in vacuo, overnight; mp 198–200°C, lit (8) mp 195.2–195.8°C; TLC (solvent B), R_f 0.04, (Solvent C), R_f 0.39; GLC, R_t 28.9 min.

2. [24-^{13}C]Chenodeoxycholic acid was recrystal-

lized from ethyl acetate and hexane; mp 140–142°C, sintered at 115°C, lit. mp 140–141.5°C (8), 119°C (9); TLC (Solvent B), R_f 0.30, (Solvent C), R_f 0.68; GLC, R_t 20.7 min.

3. [24-^{13}C]Deoxycholic acid was crystallized from acetone; mp 176–177°C, lit. (8) mp 176–177°C; TLC (solvent B), R_f 0.28, (Solvent C), R_f 0.68; GLC, R_t 15.0 min.

4. [24-^{13}C]Ursodeoxycholic acid was crystallized from aqueous ethanol as plates and dried at 80°C, in vacuo, overnight; mp 204–205°C, lit (8) mp 202°C; TLC (solvent B), R_f 0.36, (solvent C), R_f 0.71; GLC, R_t 29.5 min.

5. [24-^{13}C]Lithocholic acid was crystallized from aqueous ethanol; mp 191–192°C, lit (8) mp 185–186°C; TLC (solvent B), R_f 0.58, (solvent C), R_f 0.80; GLC, R_t 10.1 min.

Discussion

Conventionally, C-24-labeled bile acids have been synthesized by the procedure of Bergström, Rottenberg, and Voltz (10). They were obtained by bromine degradation of the silver salt of the acetylated acid to the corresponding 23-bromonorcholane. The bromide is then reconverted into the acid via a nitrile synthesis with labeled potassium cyanide. Besides the disadvantage (to be discussed) of using the acetyl group to protect the bile acids used as starting material, this procedure also presents certain difficulties. First, Hachey et al. (11) have pointed out that it is difficult to prepare dry silver salts for use in the classical Hunsdiecker reaction. Second, the yields of the important precursors, the 23-bromonorcholanes, were not reported except for 23-bromo-3α-acetoxynorcholane, which was 57% (crude) as calculated from the reported data. The corresponding 23-bromonorcholanes of di- and trihydroxycholanic acids were generally found to be more difficult to prepare, and to give lower yields.

The improved procedure (11) developed in this laboratory, of using lead tetraacetate and lithium chloride degradation of acetylated bile acids to the acetylated chloronorcholanes, overcame the difficulty of drying the silver salts but did not increase the yield of the corresponding 23-chloronorcholanes. In both methods, the isolation of pure acetylated halonorcholanes has been found to be relatively difficult and unpredictable with the single exception of 3α-acetoxy halonorcholane. In several instances, the purification could only be accomplished by resorting to repeated time-consuming column chromatography. Furthermore, the hydrolysis of acetylated 23-cyanonorcholanes from the corresponding bromides or chlorides into the labeled bile acids usually resulted in a mixture of several compounds. This mixture had to be converted to the methyl ester form and purified by column chromatography. Close examination of the reaction mixture by TLC revealed that most of these difficulties resulted from the use of the acetyl group to protect the bile acid used as starting material.

Generally, acetylated bile acids are synthesized by treatment with either acetic anhydride–pyridine (10) or acetic anhydride–acetic acid–perchloric acid (11). TLC examination of the reaction mixture and products revealed that neither of the methods produced pure acetylated bile acids. Instead, the product was a mixture of several compounds. In addition to the anticipated fully acetylated bile acids, there were substantial amounts of mixed anhydrides, dehydrated products, incompletely acetylated products, and numerous other unidentified side products. In fact, the so-called "bile acid acetate" was never obtained in pure, crystalline form[2] (8). It was generally obtained as an oil (8) or amorphous powder[3] (10) and was used as such. The use of this mixture as starting material further complicated the product obtained by the degradation using either bromine on the silver salt or lead tetraacetate–lithium chloride on the free acid.

In contrast, formylated bile acids are all well-defined crystalline compounds (8) and can be prepared easily in high yield. The infrequent use of formyl protecting groups in organic synthesis reflects concern about the stability of the formate group under the reaction conditions. However, formylated bile acids were found to be stable under the conditions used for lead tetraacetate–lithium chloride degradation. The reaction of bile acid formates and lead tetraacetate–lithium chloride in boiling benzene under an oxygen-free atmosphere readily produced formylated 23-chloronorcholanes. These could easily be isolated in pure form in 62–68% yield by simply triturating the residue with absolute ethanol. In previous procedures (11) two equivalents each of lead tetraacetate and lithium chloride were used for the conversion of acetylated bile acids to the 23-chloronorcholanes. This proportion not only resulted in incomplete conversion (typically below 60%), but also required a cumbersome isolation procedure, due to the unreacted lead tetraacetate. It was found that by using an excess of lithium chloride

[2] Although acetates of lithocholic acid and chenodeoxycholic acid have been obtained in crystalline form in moderate yield, TLC revealed the presence of impurity (possibly a mixed anhydride) in these crystalline products.

[3] The experience in this laboratory has been that it is impossible to obtain the so-called "crystalline acetylated bile acids" from dihydroxy- and trihydroxy cholanic acids using the procedure described.

(4- or 5-fold excess), added in several portions over a one to two hour interval, the conversion was substantially increased (to about 90% conversion as estimated from TLC) and the isolation was simplified because the reaction had used up all of the excess lead tetraacetate. The only detectable by-products resulting from this degradation were the unreacted starting material, which could be removed from alkali washing, and traces of partially deformylated product, which could be removed by ethanol. A slight excess of formylated 23-chloronorcholane was then reacted with sodium [^{13}C]cyanide in hot DMF to obtain the corresponding nitrile and small amounts of starting material. Upon subsequent hydrolysis of the mixture, pure labeled bile acids were obtained in 80–90% yield by crystallization. The use of DMF as solvent for the preparation of nitrile not only retains the advantage of using DMSO (11) over aqueous ethanol (10) in shortening the reaction time and decreasing the amount of 23-halonorcholane used, but also eliminates the possible side reaction of producing an aldehyde compound as a result of the action of DMSO and base upon the alkyl halide (12). The conversion of formylated 23-chloronorcholanes into labeled bile acids also resulted in a much cleaner product than the corresponding acetylated 23-halogenated norcholanes. The reason is not fully understood at present, and may reflect the rate at which the protecting group can be removed.

In summary, the use of formylated bile acids and modified lead tetraacetate procedure produces a higher yield of pure formylated 23-chloronorcholanes. Their conversion into labeled bile acids is also favored in comparison to acetylated counterparts in both yield and purity. Virtually all the time-consuming and yield-limiting chromatographic purification steps of the old methods (10, 11) have been eliminated. This procedure should be the method of choice for synthesizing C-24 labeled bile acids on either a small or a large scale.

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