# An improved procedure for the synthesis of glycine and taurine conjugates of bile acids

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**Summary** Glycine and taurine conjugates of 5β-cholanic acids have been synthesized using improved procedures based on the peptide coupling reagent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. The conjugates are obtained in chromatographically pure form in yields higher than 90%. The use of this procedure in the large scale preparation of cholyl[1,2-$^{13}C_2$]glycine is described.

**Supplementary key words** 5β-cholanic acids · glycine conjugates · taurine conjugates · N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline · cholyl[1,2-$^{13}C_2$]glycine

Our interest in the use of stable isotopes in clinical studies of bile acid metabolism, in gas–liquid chromatographic–mass spectrometric determination of plasma bile acids by inverse isotope dilution, and in the detection of bacterial overgrowth in the gastrointestinal tract by deconjugation of labeled cholylglycine (1) required the synthesis of a number of conjugated bile acids. In particular, the large quantity of cholylglycine required, and the relatively high cost of $^{13}C$-labeled glycine used in its synthesis, indicated that an efficient and convenient method was needed. Moreover, the internal standards used in inverse isotope dilution assays needed to be of the highest possible purity to achieve maximum sensitivity in the method, and this meant that a synthetic procedure yielding pure products was also required.

Conventionally, conjugated bile acids have been synthesized by the procedure of Norman (2). These conjugates are prepared by reacting the bile acid mixed anhydride with the sodium salt of taurine or glycine. It was our experience that Norman's procedure had a number of disadvantages. First, the reaction is invariably incomplete, regardless of the amount of glycine or taurine used, because the water used as a cosolvent hydrolyzed a portion of the mixed anhydride to the free bile acid. This free acid formed during the reaction is relatively difficult to remove in its entirety without a heavy loss in yield. Second, the procedure involves the low-temperature removal of large quantities of water, which is a time-consuming step. Third, as noted by Hofmann (3), all glycine conjugates prepared by this method contain a polar impurity (2–8%) in the crude preparation which is not easily removed by further purification. Although the modifications introduced by Hofmann gave a better product, the procedures were still lengthy and time-consuming. Also, a satisfactorily pure glycine conjugate of chenodeoxycholic acid could never be obtained without resorting to adsorption chromatography.

In 1973, Lack et al. (4) described the use of EEDQ as a coupling agent for the synthesis of conjugated bile acids. Unfortunately, similar difficulties were found in their procedure. These authors used ethanol and water as the solvent for the synthesis of taurine conjugates, and both of these components are relatively reactive with the mixed anhydride intermediate. According to the authors (4), the conjugation reaction was only 75–80% complete; and, despite their claim that the unreacted bile acid could be easily removed by extraction, it was our experience that a clean-cut separation could not always be achieved. When an aqueous sodium taurate solution was added to the reaction mixture, it precipitated out almost quantitatively as crystalline free taurine, while the free bile acid was converted to the sodium salt. At the end of the reaction, upon the addition of water, both the unreacted free taurine and the sodium salts of the bile acid remained in the aqueous solution and could not be readily removed by solvent extraction. Thus there is a strong possibility that such products contain both taurine and free bile acid as contaminants. Similar difficulties were encountered with the synthesis of glycine conjugates by this method.

Since none of the available methods were suitable for our purposes, a new procedure has been developed in our laboratory, based on the EEDQ method, but with substantial modifications to overcome the difficulties described. This method yields conjugate products that are chromatographically pure at the end of the reaction, even before purification by crystallization. The method is convenient and gives reproducibly high yields; the yields of crude products are quantitative and the final recrystallized products were obtained in overall yields of greater than 90%.

## Materials and methods

Deoxycholic acid (Aldrich Chemical Company, Inc. Milwaukee, WI, 99% pure by TLC and GLC), chenodeoxycholic acid (Canada Packers Ltd., Toronto, Canada, 99% pure by GLC), lithocholic acid (ICN Pharmaceuticals, Inc. Cleveland, OH, 98%

Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DMF, dimethylformamide; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

pure by GLC), ethyl glycinate hydrochloride (Pierce, Rockford, IL), and EEDQ (99 + %, Aldrich) were used without further purification. All organic solvents were of commercial reagent grade. Cholic acid (Eastman Kodak Co. Rochester, NY) was recrystallized from ethanol to remove the contaminant dihydroxycholanic acid and then dried at 150°C in vacuo overnight. Ethyl [1,2-$^{13}$C]glycinate hydrochloride was prepared by bubbling dry HCl gas through a stirred ethanolic solution of [1,2-$^{13}$C]glycine (90 atom percent $^{13}$C, synthesized by Donald G. Ott of Los Alamos Scientific Laboratory, Los Alamos, NM) in 98% yield. DMF was dried over molecular sieves and used directly.

Melting points were determined on a Fisher–Johns melting point apparatus and are uncorrected. The purity of the products was checked by thin-layer chromatography on precoated silica gel plates (Kontes, Vineland, NJ) using the following solvent systems: (*A*) benzene–*p*-dioxane–acetic acid 75:20:2 (6); (*B*) ethyl acetate–*n*-butanol–acetic acid–water 40:30:15:15 (8); and (*C*) propionic acid-isoamyl acetate-n-propanol-water 15:20:10:5 (7). The spots were visualized by spraying the plates with 10% sulfuric acid in ethanol and heating them at 120°C. The progress of the reaction was monitored by TLC using appropriate proportions of benzene and acetone as developing solvent.

*Synthesis of taurine conjugated bile acids: general procedure.* Taurine (690 mg, 5.5 mmol) and triethylamine (0.9 ml) were added to a stirred solution of bile acid (5 mmol) and EEDQ (1.73 g, 7 mmol) in 10 ml of DMF, and the resulting suspension was heated at 90°C until a clear solution was formed (ca. 15–20 min). The solution was stirred at 90°C for 15 min longer and then cooled to room temperature with stirring (0.5 hr). The solution was poured slowly into 100 ml of stirred, chilled, anhydrous diethyl ether in an ice bath. A heavy precipitate or resinous product was formed. Individual preparations were worked up as described below.

*a. Taurolithocholic acid and sodium taurolithocholate.* The suspension was stirred at 0° for 0.5 hr and then filtered and washed several times with anhydrous ether. The collected solid was dissolved in methylene chloride (30 ml) and filtered to remove the unreacted, insoluble taurine. The clear filtrate was poured slowly into 100 ml of stirred, chilled anhydrous ether in an ice bath. After 0.5 hr at 0°C, the crystalline precipitate was collected, washed with ether, and airdried to obtain the triethylammonium salt of taurolithocholic acid (3.0 g) mp 178–180°C.

For the preparation of taurolithocholic acid, the triethylammonium salt obtained above (3.0 g) was dissolved in $CH_2Cl_2$ (75 ml). Dry HCl gas was bubbled through the stirred chilled solution at 5°C. The precipitate was collected, washed with $CH_2Cl_2$, and air dried. The solid (2.4 g, 99%) was recrystallized from ethanol–ethyl acetate as needles, mp 222–223°C (lit. (2) mp 212–213°C).

For the preparation of sodium taurolithocholate, the triethylammonium salt (585 mg) was dissolved in 5 ml of 0.2 N methanolic NaOH. The solution was diluted with 20 ml of anhydrous ether. After a period at 0°C, the solid was collected, washed with methanol–ether 1:3, ether, and then was air dried. It weighed 457 mg (90%), mp 199–201°C.

*b. Sodium taurocholate, sodium taurochenodeoxycholate, and sodium taurodeoxycholate.* The resinous substance was kept at 5°C overnight and the supernatant was decanted. The resinous substance was washed several times with ether, dissolved in $CH_2Cl_2$ (30 ml) and filtered. The filtrate was worked up as before. The triethylammonium salt was very hygroscopic and was immediately dissolved in 25 ml of 0.2 N methanolic NaOH. After diluting the solution with 50 ml of anhydrous ether, the suspension was kept at 5°C for several hours and then filtered. The collected solid was washed thoroughly with ether. The yields were all above 90%. Thin-layer chromatography showed a single spot for each compound. The sodium salts of the taurine conjugates can be recrystallized from absolute ethanol [sodium taurodeoxycholate, mp 172–173°C, lit (2) mp 171–175°C] or from ethanol–ethyl acetate [sodium taurocholate, mp 182–184°C, lit (2) mp 225–235°C, (5) 130–145°C, and 225–235°C, and sodium taurochenodeoxycholate, mp 182–184°C, lit mp 182–184°C (3), 193–196°C (4), 165–175°C (2)].

The acid form of taurine conjugates can be obtained in the same way as taurolithocholic acid; however, the free acid forms of these three conjugates are all extremely hygroscopic. No attempt was made to characterize them.

*Synthesis of glycine conjugates of bile acids; general procedure.* A suspension of ethyl glycinate hydrochloride (0.98 g, 7 mmole) in 70 ml of ethyl acetate containing 1 ml of triethylamine was stirred at 25°C for 0.5 hr. Bile acid (5 mmol) and EEDQ (1.73 g, 7 mmol) were then added to the solution. After stirring at 25°C for 10 min, the suspension was refluxed on a steam bath overnight. The resulting suspension was cooled to room temperature and worked up individually as described below.

*a. Glycolithocholic acid and glycocholic acid.* The crystalline suspension was filtered and washed with water (50 ml) and ethyl acetate (10 ml). The ethyl acetate layer was separated, washed successively with 0.5

N NaOH (50 ml), water (50 ml), 0.5 N HCl (50 ml, 2×), water (50 ml, 2×), and evaporated to dryness. The residue was combined with the solid collected before and dissolved in 10 ml of boiling ethanol on the steam bath, while 10 ml of 10% $K_2CO_3$ solution was added slowly to the ethanolic solution to maintain a clear solution. The solution was heated on the steam bath for 15 min, and then evaporated in vacuo to half its original volume. After diluting the solution with 20 ml of water, it was acidified with 0.5 N HCl. The precipitate was collected and washed with water to obtain glycine conjugate products that were pure as judged by TLC.

Glycocholic acid was recrystallized by dissolving the product in 30 ml of ethanol and diluting it with 90 ml of water. It crystallized as small needles with 94% yield (mp 140–142°, lit (2) mp 132–134°C).

Glycolithocholic acid was recrystallized from ethanol (40 ml) and water (20 ml) as plates with 92% yield (mp 184–186°C, lit (2) mp 180–181°C).

*b. Glycochenodeoxycholic acid and glycodeoxycholic acid.* The suspension obtained after refluxing the reaction mixture overnight was worked up directly without filtering. The ethyl ester was isolated and hydrolyzed to the free acid as described previously.

Glycochenodeoxycholic acid was recrystallized from aqueous ethanol and kept at 25°C for several days to obtain a crystalline solid in 90% yield, mp 114–117°C.

Glycodeoxycholic acid was recrystallized from ethanol (10 ml) and water (20 ml) as needles with 95% yield (mp 191–192°C, lit (2) 187–188°C).

*Synthesis of [1,2-$^{13}C_2$]glycine-labeled glycocholic acid (choly[1,2-$^{13}C_2$]glycine).* A suspension of ethyl [1,2-$^{13}C_2$]glycinate hydrochloride (4.26 g, 30 mmol) in 300 ml of ethyl acetate was treated with 6 ml of triethylamine and stirred at 25° for 10 min. Cholic acid (11.832 g, 29 mmol) and EEDQ (10.38 g, 42 mmol) were then added to the solution and, after mixing, the suspension was refluxed on the steam bath for 48 hr. The cooled suspension was then worked up as described under the procedures for glycocholic acid above. The product was crystallized from ethanol (30 ml) and ethyl acetate (150 ml) as needles in 90% yield (based on ethyl glycinate hydrochloride) mp 168–170°C. Thin-layer chromatography showed a single spot without contamination by cholic acid or other by-products.

## Results and discussion

*Taurine conjugates.* Taurine conjugates were synthesized by reacting taurine (1.1 eq), bile acid (1 eq), and EEDQ (1.4 eq) in DMF in the presence of triethylamine at 90°C for 30–40 min. The triethylammonium salt of the conjugates was isolated by diluting the DMF solution with ether. Conversion to the sodium salt was achieved by dissolving the product in a stoichiometric amount of alcoholic NaOH and diluting with ether or ethyl acetate. The free acids were obtained by acidifying the methylene chloride solution of the triethylammonium salts with dry hydrogen chloride gas.

The use of DMF as the solvent instead of aqueous ethanol not only eliminates the problem of hydrolysis of the mixed anhydride and the resulting contamination by free bile acid but also reduces the reaction time. The reaction was essentially complete after heating for 20–30 min, as judged by TLC monitoring of the reaction mixture and by the formation of a clear solution. Prolonged heating darkened the color of the reaction mixture, presumably from the oxidation of quinoline products. The ratio of starting materials was determined to be optimal for the synthesis of unlabeled taurine conjugates and for taurine conjugates with an isotopic label on the bile acid portion on the basis of convenience and yield. The unreacted taurine is insoluble and can be readily removed by filtering the methylene chloride solution, in which the triethylammonium salt of the taurine conjugate is freely soluble. Decreasing the amount of EEDQ to 1.2 equivalents resulted in a slight decrease in yield. For the synthesis of conjugates with an isotopic label on the taurine, one equivalent of taurine, instead of 1.1 equivalent, can be used. This decreases the yield (in terms of bile acid used) slightly. In this case, the trace amount of unreacted bile acid can be removed by subsequent treatment of the DMF with ether. Although taurolithocholic acid can be prepared by acidifying the aqueous solution of the salt (2), this procedure usually resulted in a silky precipitate that was very difficult to filter. Using HCl in methylene chloride gives a product that is much easier to filter. The by-product, triethylammonium hydrochloride, is very soluble in methylene chloride and remains in the filtrate after filtration of the product.

*Glycine conjugates.* The procedure used for the synthesis of taurine conjugates failed in the synthesis of glycine conjugates when either glycine or ethyl glycinate hydrochloride was used. Therefore, the procedure of Lack et al. (4), using ethyl acetate as solvent, was modified in the ratio of reactants and the substitution of triethylamine for sodium carbonate. Glycine conjugates were obtained by refluxing ethyl glycinate hydrochloride (1.4 eq), EEDQ (1.4 eq) bile acid (1 eq), and triethylamine in ethyl acetate solution overnight. After washing successively with aqueous alkali and acid solutions, the ethyl esters of glycine conjugates were hydrolyzed in ethanol and

aqueous 10% potassium carbonate for 15 min to obtain the glycine conjugates.

The use of triethylamine simplifies the procedure and eliminates the loss of labeled ethyl glycinate in preparing the free base from the hydrochloride salt, using sodium carbonate (4). The loss was found to be significant in our experience. The reaction was complete after refluxing for 5–10 hr, but was usually allowed to proceed overnight. The ratio of reactants is critical for the synthesis in terms of yield, purity of product, and convenience. When the amount of EEDQ is reduced, the reaction is incomplete, as noted by Lack et al. (4). Decreasing the amount of ethyl glycinate hydrochloride required an increase in the reaction time for completion. However, when bile acid was present in amounts in excess of the ethyl glycinate hydrochloride, two difficulties were noted. First, the reaction time had to be increased to insure complete utilization of the ethyl glycinate hydrochloride; second, and more troublesome, the excess bile acid and EEDQ formed a relatively stable mixed anhydride (or its precursor) (9) that could not be removed by alkali or acid washing. Upon subsequent hydrolysis of the ethyl ester product, it was also hydrolyzed to the free bile acid and contaminated the product. Thus, when one wishes to synthesize glycine conjugates with isotopically labeled glycine (as in the case of the choly[1,2-$^{13}C_2$]glycine, one cannot use the bile acid in excess to maximize the reaction of labeled glycine. Instead, a slight excess of ethyl glycinate hydrochloride is used, and the reaction time is extended to 48 hr. In this manner, it was possible to obtain chromatographically pure cholyl[1,2-$^{13}C_2$]glycine in 90% yield, based upon labeled ethyl glycinate. In order to hydrolyze the ethyl ester to the free acid, aqueous potassium carbonate and ethanol at 100°C for 15 min was used instead of the widely used alcoholic NaOH for 24 hr at room temperature (3, 4). The free acid forms of glycine conjugates were obtained conveniently in this way without evidence of hydrolysis of the amide linkage.

Finally, in addition to the advantages described above, this procedure for the synthesis of taurine conjugates can also be adapted easily to the one step, high yield synthesis of taurine conjugate sulfate esters as will be reported subsequently.

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