Synthesis of sulfate esters of lithocholic acid, glycolithocholic acid, and taurolithocholic acid with sulfur trioxide–triethylamine

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Abstract The facile synthesis of lithocholic acid sulfates by a procedure that produced the desired products in over 90% yield is described. Lithocholic acid sulfate and glycolithocholic acid sulfate were synthesized by reacting lithocholic acid or glycolithocholic acid with sulfur trioxide–triethylamine complex in dimethylformamide for 0.5–1 hr. Taurolithocholic acid sulfate was obtained by conjugating lithocholic acid sulfate with taurine in dimethylformamide at 90°C for 0.5 hr. The one-pot synthesis of taurolithocholic acid sulfate starting from lithocholic acid is also described. This procedure, which generated lithocholic acid sulfate in situ, produced taurolithocholic acid sulfate in 98% yield, compared to an overall yield of less than 10% obtained by previously published procedures.

Supplementary key words bile acid sulfates · demethylformamide

Since the first observation that sulfation is an important metabolic pathway for lithocholic acid excretion appeared in 1967 (1), there has been increasing evidence that sulfation also plays an important role in the metabolism of other bile acids (2–6). Although a number of these publications deal with the assay of sulfated bile acids in clinical pathological states of liver, many of the basic properties of bile acid sulfates remained unstudied. These studies require the ready availability of chemically pure bile acid sulfates in either labeled or unlabeled form. Here we report the facile synthesis of sulfate esters of lithocholic acid, glycolithocholic acid, and taurolithocholic acid by using a sulfur trioxide–triethylamine complex **(Fig. 1).**

EXPERIMENTAL PROCEDURE

Materials and Methods

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Lithocholic acid was purchased from ICN Pharmaceuticals, Inc., Cleveland, OH. Glycolithocholic acid and taurolithocholic acid were synthesized as described (7). DMF was commercial reagent grade and was dried over Linde molecular sieves (1/16″ pellets, Linde Co.). All other reagents were of commercial reagent grade.

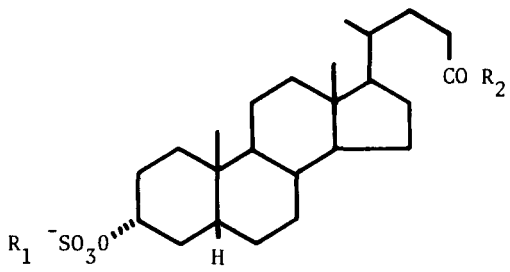
Thin-layer chromatography was carried out on precoated silica gel plate (Kontes Q1, Kontes, Vineland, NJ). They were developed by solvent system EBAW, ethyl acetate–*n*-butanol–acetic acid–water 40:30:15:15 (v/v); BAW, *n*-butanol–acetic acid–water 10:1:1 (v/v) (8); and CMAW, chloroform–methanol–acetic acid–water 65:24:15:9 (v/v) (13). They were detected by spraying the plates with 10% sulfuric acid in ethanol and heating at 120°C. Infrared (IR) spectra were determined on a Perkin-Elmer 337 infrared spectrometer.

Elemental analyses were performed by Microtech Lab., Skokie, IL. Each sample was dried to constant weight at 100°C before analysis.

Preparation of sulfur trioxide–triethylamine complex

Chlorosulfonic acid (46 g, 0.4 mol) was added dropwise over 2 hr into a stirred solution of triethylamine (80.8 g, 0.8 mol) in methylene chloride (150 ml) in an ice bath. The resulting solution was washed with ice-cold water (20 ml × 4) and dried over anhydrous $MgSO_4$. The filtered solution was evaporated to about 100 ml with a stream of dry nitrogen. It was then heated to boiling and diluted with ethyl ether (100 ml). A heavy crystallization occurred. After standing at room temperature and then at 5°C for several hr, the crystals were collected, washed with ether, and dried. The product was colorless crystals with mp 90–92°C, lit. (9) mp 92–93°C. The yield was 83%.

Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DMF, demethylformamide; TLC, thin-layer chromatography; 3-sulfolithocholic acid, 3α-sulfooxy-5β-cholan-24-oic acid; 3-sulfoglycolithocholic acid, 3α-sulfooxy-5β-cholan-24-oylglycine; 3-sulfotaurolithocholic acid, 3α-sulfooxy-5β-cholan-24-oyl-taurine.



I, $R_1 = (C_2H_5)_3N^+H$, $R_2 = OH$

II, $R_1 = Na^+$, $R_2 = O^- Na^+$

III, $R_1 = Na^+$, $R_2 = NHCH_2CO_2^-Na^+$

IV, $R_1 = Na^+$, $R_2 = NHCH_2CH_2SO_3^-Na^+$

Fig. 1. Lithocholic acid sulfates.

3-Sulfolithocholic acid, disodium salt, *II*

Method A. To a solution of lithocholic acid (1.88 g, 5 mmol) in 10 ml of DMF was added sulfur trioxide–triethylamine complex (997 mg, 5.5 mmol) and the solution was mixed well. After standing at room temperature for 0.5 hr, 2 drops of water were added to the solution which was then stirred at 40°C for 1 hr. It was then poured into a stirred anhydrous diethyl ether solution (125 ml). After standing at 5°C for several hours, the crystalline solid was collected, washed with ether, and air dried to obtain triethylammonium salt, *I*, (2.8 g), which was contaminated with some triethylammonium hydrogen sulfate from the excess sulfating agent. The solid was dissolved in 120 ml of 0.1 N methanolic NaOH (the pH of final solution was 9) and filtered to remove insoluble sodium sulfate. The filtrate was diluted with 100 ml of ether, and the resulting precipitate was collected and washed with ether. The procedure was repeated with methanol (100 ml) and ether (100 ml) to yield the disodium salt, *II*, (2.25 g, 90% yield), mp 233–235°C; IR (Nujol) 3450 (broad), 1550 (c = 0), 1200, 1060, 965 cm^{-1}; elemental analysis, $C_{24}H_{38}O_6SNa_2$, calcd. C, 57.58, H, 7.65, S, 6.41, found C, 57.47, H, 7.81, S, 6.20.

Method B. The reaction was carried out as above, but the reaction mixture, after 0.5 hr at 25°C, was added to 10 ml of 1 M aqueous *p*-toluidine hydrochloride solution. After mixing well, the suspension was diluted with 40 ml of water. The precipitate was collected and washed with water until a negative silver nitrate test of the filtrate was obtained. The dried solid was then suspended in 30 ml of 0.4 N methanolic NaOH. After heating to boiling on a steam bath, the methanolic solution was diluted with 75 ml of ether. The precipitated disodium salt was then treated and isolated as described before to obtain 2.46 g (98% yield) of pure *II*.

3-Sulfoglycolithocholic acid, disodium salt, *III*

Method A. To a stirred suspension of glycolithocholic acid (433 mg, 1 mmol) in 2 ml of DMF was added sulfur trioxide–triethylamine (200 mg, 1.1 mmol). The suspension was stirred at 25°C for 0.5 hr, and then additional sulfur trioxide–triethylamine (200 mg, 1.1 mmol) was added. After further stirring at 25°C for 0.5 hr, 5 drops of water were added and the suspension was heated at 40°C. After 1 hr, the suspension was poured slowly into 40 ml of stirred ether. The reaction flask was washed with small amounts of methylene chloride several times and the washings were added to the ether solution. The suspension was stirred at 25°C for 10 min; it was then kept at 25°C for 2 hr, and filtered. The solid was washed thoroughly with ether and air dried. It was then dissolved in 24 ml of 0.2 N methanolic NaOH (final pH > 9) and filtered to remove insoluble sodium sulfate. The filtrate was diluted with 30 ml of ether and the precipitate was collected, washed with ether, and air dried. It was then treated with methanol (15 ml) and ether (30 ml) to yield chromatographically pure disodium salt, III, (536 mg, 93%); mp 248–250°C (darkened at 230°C); IR (Nujol) 3350 (broad), 1600 (c = 0), 1215, 1053, 1050, 965 cm^{-1}; elemental analysis, $C_{26}H_{41}O_7SNNa_2 \cdot H_2O$, calcd. C, 54.25, H, 7.53, S, 5.57, found C, 54.09, H, 7.58, S, 5.61.

Method B. The reaction was carried out as above, but the reaction mixture was poured into 40 ml of water. The milky suspension was then added to 6 ml of 1 M *p*-toluidine hydrochloride. the precipitated *p*-toluidinium salt of 3-sulfoglycolithocholic acid was collected, washed with water, and dried. It was converted to disodium salt, III, as described in method *B* of compound *II*. The yield was 91%.

3-Sulfotaurolithocholic acid, disodium salt, *IV*

Method A. To a solution of crude 3-sulfolithocholic acid triethylammonium salt, *I*, (prepared from 1 mmol of lithocholic acid as described) and EEDQ (346 mg, 1.4 mmol) in 2 ml of DMF was added taurine (138 mg, 1.1 mmol) and triethylamine (0.2 ml). The stirred suspension was heated at 90°C for 0.5 hr. It was then cooled to room temperature and poured slowly into 40 ml of stirred, chilled ether. The reaction flask was washed several times with small amounts of methylene chloride and the washings were added to the ether solution. After stirring at 5°C for 0.5 hr, the crystalline precipitate was collected, washed thoroughly with ether, and then dissolved in methylene chloride (15 ml) and filtered to remove

unreacted taurine. The combined filtrate and washing (10 ml) was added slowly to 40 ml of stirred, chilled ether in an ice bath. After stirring for 0.5 hr, the solid was collected, and washed thoroughly with ether. The triethylammonium salt (mp 140–141°C, after drying in vacuo) thus obtained was hygroscopic and was immediately converted to the sodium salt as follows. The solid was dissolved in 12 ml of 0.2 N methanolic NaOH and filtered. The filtrate was diluted with ether (25 ml). After storage at 0° for 1 hr, the precipitate was collected, washed with ether–methanol 5:3 and ether, and then was air dried. The disodium salt weighed 608 mg (94% yield): mp 212–214°C; IR (Nujol) 3425 (broad), 1650 (c = 0), 1540, 1200, 1050, 965 cm^{-1}; elemental analysis, $C_{26}H_{43}O_8S_2NNa_2$, calcd. C, 51.39, H, 713, S, 10.55, found C, 51.32, H, 7.34, S, 10.71.

Method B. To a stirred solution of lithocholic acid (752 mg, 2 mmol) in 4 ml of DMF was added sulfur trioxide–triethylamine (400 mg, 2.2 mmol). After stirring at 25°C for 0.5 hr, EEDQ (692 mg, 2.8 mmol), taurine (276 mg, 2.2 mmol), and triethylamine (0.4 ml) were added; the solution was then heated at 90°C with stirring for 0.5 hr. The reaction mixture was cooled to 25°C, diluted with 4 ml of methylene chloride, and filtered to remove unreacted taurine. Four drops of water were added to the filtrate and the mixture was then heated at 70°C for 0.5 hr. After cooling, the solution was poured into 100 ml of ether. Thereafter, it was worked up in the same way as in Method *A*, except that the quantity of reagents was doubled. The disodium salt thus obtained weighed 1.269 g (98%) and was identical with the compound obtained in Method *A*.

Method C. A suspension of 3-sulfolithocholic acid, disodium salt, *II* (125 mg, 0.25 mol), EEDQ (86.5 mg), and taurine (34.5 mg) in 2 ml of DMF was stirred at 90°C (bath temp) for 0.5 hr; additional EEDQ (86.5 mg) was then added. After 0.5 hr, another portion of EEDQ (86.5 mg) was added. The stirring was continued for 0.5 hr. The reaction mixture was then cooled to 25°C, diluted with 3 ml of methanol and filtered. The combined filtrate and methanol wash (1 ml × 3) was diluted with 20 ml of ether. The precipitated disodium salt, *IV*, was isolated and purified as described above. The yield was 83%.

RESULTS AND DISCUSSION

The sulfate esters of lithocholic acid and its conjugates have been synthesized previously by Palmer and Bolt (10) by using chlorosulfonic acid in pyridine. However, the procedure was tedious and time-consuming. Moreover, 3-sulfotaurolithocholic acid was obtained in low yield and was contaminated with the starting compound, taurolithocholic acid.

Recently, the use of sulfur trioxide–tertiary amine complex has gained wide popularity in preparing the sulfate esters of alcohols (11). It is preferred to chlorosulfonic acid in its ease of handling and measurement. In addition, Dusza, Joseph, and Berstein (9) have demonstrated a simple procedure using sulfur trioxide–triethylamine complex to synthesize the sulfate esters of neutral steroids and have pointed out the extraordinary stability of the triethylammonium salts of sulfate esters. In order to optimize the procedure for bile acid sulfate synthesis, the following experiments were performed.

1. *Effect of sulfating agent.* The three common sulfur trioxide–tertiary amine complexes, sulfur trioxide–pyridine, sulfur trioxide–trimethylamine, and sulfur trioxide–triethylamine, were tried as sulfating agents. It was found that the reactivity in pyridine or DMF was in the order sulfur trioxide–triethylamine > sulfur trioxide–pyridine > sulfur trioxide–trimethylamine. Furthermore, among the three, only sulfur trioxide–triethylamine gave a crystalline, nonhygroscopic, easily isolable sulfate salt. Thus, sulfur trioxide–triethylamine was chosen as the sulfating agent.

2. *Effect of solvent.* Chloroform and pyridine are solvents commonly used for sulfation when employing the sulfur trioxide–tertiary amine complex. The reaction in chloroform is very slow. Lithocholic acid sulfate can be prepared by overnight reaction in chloroform or by refluxing for several hours. However, the limited solubility of glycolithocholic acid and taurolithocholic acid in chloroform preclude its use as a solvent. Pyridine was used successfully by Dusza, Joseph, and Berstein (9) in synthesizing sulfate esters of neutral steroids. However, when the same solvent was used for the synthesis of sulfate esters of lithocholic acid, two spots were detected after thin-layer chromatography of the reaction mixture. Apparently, they resulted from the formation of the pyridinium salt as well as the triethylammonium salt. Consequently, the product was a syrup and could only be isolated with some effort. DMF was found to be the best solvent for sulfation when using sulfur trioxide–triethylamine. The sulfur trioxide complex, lithocholic acid, and lithocholic acid conjugates are all highly soluble in DMF. The reaction mixture is a homogeneous solution and the reaction rate is the fastest among all the solvents studied.

The triethylammonium salt of 3-sulfolithocholic acid can be synthesized easily by simply mixing lithocholic acid with a slight excess of sulfur trioxide–

triethylamine in DMF. After 30 min at 25°C, the excess sulfating agent was decomposed by adding water and the solution was diluted with ether. However, under the same reaction conditions, glycolithocholic acid failed to give a quantitative conversion. The unreacted glycolithocholic acid is somewhat more difficult to remove due to its limited solubility in ether. To simplify the preparation procedure, larger quantities of sulfur trioxide–triethylamine and a longer reaction time had to be used in order to give a quantitative conversion. The triethylammonium salt is then converted to disodium salt by dissolving in methanolic NaOH solution. Triethylammonium hydrogen sulfate, the by-product from the excess sulfating agent, is converted to sodium sulfate, which is insoluble in methanol and is removed by filtration. The disodium salts of bile acids are obtained by diluting this solution with ether. Another equally convenient way of obtaining bile acid sulfates free from contaminating inorganic salts is the conversion of triethylammonium salt of bile acid sulfates to *p*-toluidinium salt by the addition of *p*-toluidine hydrochloride solution. The *p*-toluidinium salts of lithocholic acids, which are insoluble in water, are isolated and separated from water soluble inorganic salts. They are converted to sodium salts by methanolic NaOH solution. When taurolithocholic acid, in the acid form or as the triethylammonium salt, was reacted with sulfur trioxide–triethylamine, it was not quantitatively converted to the sulfate form even in the presence of a 2–3-fold excess of reagent over an extended period of time. The same phenomena were also observed by Palmer and Bolt (10). They obtained only 50% conversion, even when using a several fold excess of chlorosulfonic acid in pyridine for a period of 7 days. The reactivity toward sulfur trioxide–triethylamine complex is in the sequence lithocholic acid > glycolithocholic >> taurolithocholic acid. The possible reason for the differences in reactivity may be the shielding of the 3α-hydroxy group by the side chain of the lithocholic acid conjugates. The conjugation with glycine or taurine results in an extension in the length of the side chain, which could then fold back and shield the 3α-hydroxyl group. The longer side chain and the stronger acidic sulfonate group of taurolithocholic acid, coupled with the fact that it is much less reactive than glycolithocholic acid, tend to support this assumption.

Since the sulfate ester of taurolithocholic acid could not be readily obtained by the sulfation of taurolithocholic acid, an alternate route was sought. It was found that the sulfate ester of taurolithocholic acid could be obtained in high yield if the synthetic sequence was reversed, e.g., lithocholic acid first converted to sulfate ester and this sulfate ester then conjugated with taurine. Either lithocholic acid sulfate or lithocholic acid can be used as the starting material for this synthesis. In the latter case, the sulfate ester was synthesized in situ and was then converted to the taurine conjugate in the usual way. The one-pot synthesis of the diconjugate produced the pure product in over 90% yield with minimal manipulation. The *p*-toluidinium salt of 3-sulfotaurolithocholic acid is a gelatinous substance in aqueous medium, so this sulfate cannot be purified by its *p*-toluidinium salt. The disodium salt of 3-sulfotaurolithocholic acid can also be prepared directly by coupling disodium 3-sulfolithocholic acid, *II*, with taurine in the presence of EEDQ. Although this method is not the method of choice in preparing disodium salt, *IV*, it is the best method to prepare monosulfate of taurine conjugates of other bile acids, as will be reported subsequently.

All the sulfate salts prepared were chromatographically pure in the solvent systems studied. Their R_f values (thin-layer chromatography) and those of their unsulfated counterparts, are listed in **Table 1**. Their infrared spectra are identical to those of diammonium salts reported by Palmer and Bolt (10) except the region above 3000 cm^{-1} which is attributed to the difference in salt form. They all show the characteristic sulfate absorption around 1200, 1060, and 965 cm^{-1} (12). The triethylammonium salt of lithocholic acid sulfate and the disodium salts of all three sulfates are all relatively non-hygroscopic and very stable. They can be kept at room temperature over a period of months without any sign of decomposition.

Much work has been done on the quantitation and the clinical significance of bile acid sulfates (2–6), but very little work has been devoted to the basic study of the fundamental properties of bile acid sulfates. These include such properties as solvolysis, hydrolysis of the amide linkage, and their adsorption onto macroreticular non-ionic adsorbents such as

TABLE 1. Thin-layer chromatographic mobilities (R_f) of sulfated and unsulfated lithocholates

Bile Salt	Solvent System		
	EBAW[a]	BAW[b]	CMAW[c]
3-Sulfolithocholic acid	0.77	0.60	0.82
3-Sulfoglycolithocholic acid	0.56	0.34	0.60
3-Sulfotaurolithocholic acid	0.37	0.13	0.43
Lithocholic acid	0.98	0.95	0.98
Glycolithocholic acid	0.84	0.71	0.93
Taurolithocholic acid	0.60	0.41	0.74

[a] Ethyl acetate–*n*-butanol–acetic acid–water 40:30:15:15.
[b] *n*-Butanol–acetic acid–water 10:1:1.
[c] Chloroform–methanol–acetic acid–water 65:24:15:9.

Amberlite XAD-2. Although some of these characteristics have been mentioned briefly in various investigations, there has been no systematic and fully detailed investigation of these areas. For example, it is important to ascertain the influence of the sulfate group on the enzymatic hydrolysis by using cholylglycine hydrolase (5,6), to determine the influence of a conjugate group on the solvolysis of sulfates, and to measure the recovery of adsorption onto XAD-2 of bile acid sulfates other than glycochenodeoxycholic acid sulfate (2). Without extensive investigation into the basic properties of bile acids, as recently pointed out by Haslewood and Haslewood (12), the interpretation of the clinical data could be misleading in the absence of this information. With the ready availability of bile acid sulfates and their labeled counterparts by this procedure, the validation and re-evaluation of existing analytical procedures can be undertaken. Solvolytic experiments on these lithocholic acid sulfates and the synthesis of other bile acid sulfates are now under way.

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