BILE ACID SULFATES. III. SYNTHESIS OF 7- AND 12-MONOSULFATES OF BILE ACIDS AND THEIR CONJUGATES USING A SULFUR TRIOXIDE-TRIETHYLAMINE COMPLEX

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ABSTRACT

The 7- and 12-monosulfates of chenodeoxycholic acid, deoxycholic acid, and cholic acid were prepared by sulfation of the protected bile acids with sulfur trioxide-triethylamine in pyridine overnight and were isolated by precipitation as the p-toluidinium salt after removing the protecting group(s). The taurine conjugates were obtained by conjugating the bile acid sulfates with taurine in hot dimethylformamide (DMF) in the presence of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). A new procedure of preparing glycine conjugated bile acid sulfates by direct conjugation of the bile acid sulfate triethylammonium salt with ethyl glycinate in boiling chloroform in the presence of EEDQ is also described. The advantage of these procedures over other procedures are their simplicity and their higher yields (typically above 90%) The thin layer chromatographic mobilities of these sulfates are presented. The influence of side chain and hydroxyl group configurations on the properties of bile acid sulfates is briefly discussed.

INTRODUCTION

In order to validate analytical methodology for bile acid sulfates (1) and to examine further 7- sulfates of chenodeoxycholic acid and cholic acid conjugates reported to occur in mice (2) and rats (3), a simple method of preparing the 7- as well as 12-monosulfates of common

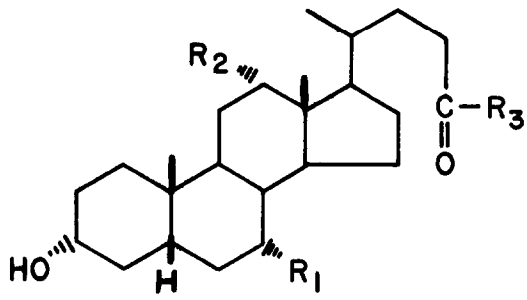
bile acids is needed. In a recent communication, Parmentier and Eyssen (4) described the synthesis of most of the 7- and 12-monosulfates using the lengthy chlorosulfonic acid-pyridine reaction, following by purification on XAD-2, and Sephadex LH-20. This procedure resulted in relatively low yields. In this paper we report the simple, high-yield synthesis of all 7- and 12-monosulfates of chenodeoxycholic acid, deoxycholic acid, and cholic acid by the sulfur trioxide-triethylamine procedure described by us previously (5,6). The method is also used to prepare the glycine and taurine conjugates of 7- and 12-monosulfates of cholic acid, which cannot be made by the older procedure (7).

EXPERIMENTAL PROCEDURE

Materials and Methods - Melting points were determined on a Fisher Jones melting point apparatus and are uncorrected. The sources of deoxycholic acid, chenodeoxycholic acid, cholic acid, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), taurine, ethyl glycinate hydrochloride, p-toluidine hydrochloride, dimethylformamide (DMF), sulfur trioxide-triethylamine, and other reagents were the same as used previously (6). Methyl 3-carboethoxydeoxycholate and methyl 3-carboethoxy-chenodeoxycholate were prepared according to Fieser and Rajagopalan (8). They were purified by column chromatography on silica gel with 1.25% acetone in benzene as eluent. Methyl cholate 3,7-diacetate was synthesized by the method described in reference (8). Ethyl glycinate was prepared by neutralization of ethyl glycinate hydrochloride with barium hydroxide in chloroform (9). It was kept at 5°C for no longer than 1 week, because prolonged storage results in the appearance of a precipitate.

Thin layer chromatography was carried out on precoated silica gel G plates (250 μM, Applied Science Lab., College Station, Pa.). They were developed by use of the following systems: 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) (7); and CMAW, chloroform-methanol-acetic acid-water, 65:24:15:9 (v/v) (10). Compounds were detected after spraying the plate with 10% sulfuric acid in ethanol and heating at 120°C.

Dowex column chromatography was carried out on a column (1 x 13 cm) of Dowex AG 50W-X8 (H^+ form, prewashed with methanol) that was treated with 10% triethylamine in methanol (20 ml) at a flow rate of 1 ml/min to convert it to the triethylammonium form. After washing with 50 ml of 1% triethylamine in methanol, the column is ready for use to convert sodium salts of bile acid sulfates to their triethylammonium salts. The used column can be regenerated by washing successively with



I	$R_1 = OSO_2O^-Na^+$,	$R_2 = H$,	$R_3 = O^-Na^+$
II	$R_1 = OSO_2O^-Na^+$,	$R_2 = H$,	$R_3 = NHCH_2CO_2^-Na^+$
III	$R_1 = OSO_2O^-Na^+$,	$R_2 = H$,	$R_3 = NHCH_2CH_2SO_3^-Na^+$
IV	$R_1 = H$,	$R_2 = OSO_2O^-Na^+$,	$R_3 = O^-Na^+$
V	$R_1 = H$,	$R_2 = OSO_2O^-Na^+$,	$R_3 = NHCH_2CO_2^-Na^+$
VI	$R_1 = H$,	$R_2 = OSO_2O^-Na^+$,	$R_3 = NHCH_2CH_2SO_3^-Na^+$
VII	$R_1 = OSO_2O^-Na^+$,	$R_2 = OH$,	$R_3 = O^-Na^+$
VIII	$R_1 = OSO_2O^-Na^+$,	$R_2 = OH$,	$R_3 = NHCH_2CO_2^-Na^+$
IX	$R_1 = OSO_2O^-Na^+$,	$R_2 = OH$,	$R_3 = NHCH_2CH_2SO_3^-Na^+$
X	$R_1 = OH$,	$R_2 = OSO_2O^-Na^+$,	$R_3 = O^-Na^+$
XI	$R_1 = OH$,	$R_2 = OSO_2O^-Na^+$,	$R_3 = NHCH_2CO_2^-Na^+$
XII	$R_1 = OH$,	$R_2 = OSO_2O^-Na^+$,	$R_3 = NHCH_2CH_2SO_3^-Na^+$

Figure 1. 7-Mono- and 12-mono sulfates of bile acids and their glycine and taurine conjugates.

conc. HCl-methanol, 5:95 (20 ml), methanol (50 ml), and finally 10% triethylamine.

Infrared (ir) spectra were determined on a Perkin-Elmer 457 infrared spectrophotometer. Elemental analyses were performed by Microtech Lab., Skokie, IL. Each sample was dried to constant weight at 100°C before analysis.

Methyl 7-oxo-3α,12α-diacetoxy-5β-cholan-24-oate: This compound was synthesized by a modification of the published method (11). A solution of methyl cholate (8.44 g, 20 mmole) in 20 ml of acetone (dissolved by heating on a steam bath) was diluted with 10 ml of water, cooled to 25°C, and sodium bicarbonate (2.1 g, 25 mmol) was added. N-Bromosuccinimide (recrystallized from water, 4.5 g, 25 mmol) was then added in small portions to keep the temperature of the reaction mixture at about 25°C. After the last addition, the mixture was kept at 25°C for 8 hr with occasional swirling, and was then diluted with 50 ml of water and extracted with 50 ml of ether. The ether extract was washed with 0.5 N NaOH (50 ml x 2) then with water until neutral and finally was dried over anhydrous $MgSO_4$. The dried solution was evaporated at 25°C, *in vacuo*, to a syrupy residue. If the temperature exceeded 25°C at this step, the residue became discolored. The residue was then evaporated under high vacuum to remove the last trace of solvent. The solid was then acetylated in 48 ml of the acetylation mixture (acetic anhydride-acetic acid-70% perchloric acid, 10:14:0.1 (v/v) at 0°C for 15 min. The resulting solution was diluted with 50 ml of water and extracted with benzene (50 ml). The benzene extract was washed with water, saturated $NaHCO_3$, water again, and finally dried over anhydrous $MgSO_4$. The dried solution was evaporated to dryness. The resulting residue was chromatographed on a silica gel column, and developed with benzene-acetone (95:5). The fractions containing the desired product were pooled and evaporated to dryness to yield a crystalline solid. The results from GLC and TLC analysis showed that this product contained about 5% of methyl 12-oxo-3α,7α-diacetoxy-5β-cholan-24-oate. It was crystallized from petroleum ether (bp 60-110°C) to yield pure methyl 7-oxo-3α,12α-diacetoxy-5β-cholan-24-oate as prisms. The final product weighed 6.66 g, (65% yield) mp 124-125°C, lit mp 118.0-118.4°C (8).

Methyl 7α-hydroxy-3α,12α-diacetoxy-5β-cholan-24-oate: A stirred solution of methyl 7-oxo-3α,12α-diacetoxy-5β-cholan-24-oate (4.55 g) in 20 ml of methanol at 0°C was treated with 300 mg of sodium borohydride (Fisher). The solution was then stirred at 0°C for an hour, and poured into 100 ml of 0.5 N HCl. The oily suspension was extracted with ethyl acetate (50 ml). The organic layer was washed with 0.5 N NaOH, water, and then dried over anhydrous Na_2SO_4. The dried solution was evaporated to dryness and the resulting residue was chromatographed on a silica gel column. The column was eluted with benzene-acetone (95:5) and the pure fractions were pooled and evaporated to a syrupy residue which was then dried under high vacuum to a crystalline solid. The product weighed 3.52 g, (77% yield), mp 146-147°C.

Ethyl glycochenodeoxycholate: This compound was synthesized using the same procedure reported for glycochenodeoxycholic acid (12). However, instead of hydrolyzing the reaction mixture to the free acid, the residue was dissolved in benzene-acetone (70:30) and chromatographed on

a silica gel column. The column was eluted with benzene-acetone (70:30) to obtain a 68% yield of pure ethyl glycochenodeoxycholate as a solid when dried under high vacuum, mp, 78-80°C; ir (Nujol) 3320 (OH), 1740, 1650, 1560 (C=O), 1200 (-O-) cm^{-1}.

Anal. calcd. for $C_{28}H_{47}NO_5 \cdot C_3H_6O$: C, 69.50, H, 9.97, N, 2.61; found: C, 69.38, H, 9.63, N, 2.67.

Ethyl glycodeoxycholate: The same procedure as described for ethyl glycochenodeoxycholate yielded ethyl glycodeoxycholate (74% yield) from deoxycholic acid as a powder when dried under high vacuum. The characteristics were: mp 80-82°C; ir (Nujol) 3320 (OH), 1735, 1650 1560 (C=O), 1200 (-O-) cm^{-1}; anal. calcd. for $C_{28}H_{47}NO_5$: C, 70.40, H, 9.92, N, 2.93; found: C, 70.32, H, 10.08, N, 2.77.

Ethyl 3-carboethoxyglycochenodeoxycholate: A solution of ethyl glycochenodeoxycholate (4.77 g, 10 mmol) in 25 ml of pyridine and 80 ml of ethyl acetate at 0°C was treated gradually with 3.27 g of ethyl chloroformate (30 mmol) in 40 ml of ethyl acetate. After 0.5 hr, the reaction mixture was poured into 100 ml of water. The ethyl acetate layer was washed with 0.5 N HCl, water, and dried over anhydrous $MgSO_4$. The solution was evaporated *in vacuo* to dryness and the resulting residue was chromatographed on silica gel. The column was eluted with benzene-acetone (90:10) to yield ethyl 3-carboethoxyglycochenodeoxycholate (4.62 g, 85%) as a crystalline solid, mp 58-60°C, ir (Nujol) 3320 (OH), 1735, 1650, 1560 (C=O), 1260, 1200 (-O-) cm^{-1}; anal. calcd. for $C_{31}H_{51}NO_7$: C, 67.73, H, 9.35, N, 2.55; found: C, 67.66, H, 9.60, N, 2.42.

Ethyl 3-carboethoxyglycodeoxycholate: Starting from ethyl glycodeoxycholate (4.77 g), the same procedure as described for ethyl 3-carboethoxyglycochenodeoxycholate yielded ethyl 3-carboethoxyglycodeoxycholate (5.36 g, 97%) as a crystalline powder, mp 53-55°C; ir (Nujol) 3320 (OH), 1725, 1650, 1525 (C=O), 1260, 1200 (-O-) cm^{-1}; anal. calcd. for $C_{31}H_{51}NO_7$: C, 67.73, H, 9.35, N, 2.55; found: C, 67.84, H, 9.51, N, 2.27.

7-Sulfochenodeoxycholic acid, disodium salt, I; A solution of methyl 3-carboethoxychenodeoxycholate (1.434 g, 3 mmol) and sulfur trioxide-triethylamine (1.2 g) in 6 ml of pyridine was kept at 25°C overnight. The light yellow solution was then poured into 100 ml of petroleum ether (bp 30-60°C). After standing at 25°C for several hours, the resulting oily suspension changed to a crystalline solid. The solid was collected, washed with petroleum ether, and dried with a stream of nitrogen. It was then suspended in 30 ml of 1 N NaOH and heated on a steam bath for 10 min. After cooling, the alkaline hydrolysate was neutralized to pH 5-6 with conc. HCl, transferred to a centrifuge tube, and 8 ml of 1 M p-toluidine HCl was added. After mixing well, the resulting reddish oily suspension was centrifuged. The upper aqueous layer was decanted and the lower syrupy layer was washed with water 1 ml x 2). After drying in a vacuum, the syrupy residue was dissolved in 30 ml of 0.2 N methanolic NaOH and then diluted with 200 ml of ether. After several minutes at 25°C the precipitate was collected and washed thoroughly with ether. It was purified by redissolving in 15 ml of methanol, filtering, and diluting with 60 ml of ether. After washing with ether and drying *in vacuo*, the product appeared as a white powder which weighed 1.5 g (96% yield), mp 196-197°C, lit mp. 224°C (3), 190-194°C (4).

Anal. calcd. for $C_{24}H_{38}O_7SNa_2$: C, 55.80, H, 7.41, S, 6.21: found: C, 55.52, H, 7.59, S, 6.25.

12-Sulfodeoxycholic acid, disodium salt, IV: Methyl 3-carboethoxydeoxycholate (1.434 g, 3 mmol) was sulfated and worked up as described for I. The p-toluidinium salt of IV was precipitated first as an oily suspension which changed to a crystalline solid after scratching the glass wall of the flask with a stirring rod. The crystalline p-toluidinium salt was then converted to the disodium salt and purified as described for I. The product was a white powder and weighed 1.5 g (97% yield), mp 213-215°C, lit. mp 205-206°C (4).

Anal. calcd. for $C_{24}H_{38}O_7SNa_2$: C, 55.80, H, 7.41, S, 6.21: found: C, 55.68, H, 7.77, S, 6.08.

7-Sulfocholic acid, disodium salt, VII: Methyl 7α-hydroxy-3α, 12α-diacetoxy-5β-cholan-24-oate (1.518 g, 3 mmol) was sulfated as described for I. The precipitated triethylammonium salt remained as an oil despite efforts to induce its solidification. The oily suspension was kept at 25°C until the supernatant became clear. The supernatant was decanted and the syrupy residue was triturated with petroleum ether several times. The residue was then dried in a stream of nitrogen and hydrolyzed in 30 ml of 1 N NaOH on a steam bath for 2 hr. The alkaline hydrolysate was then worked up as described for I. The product weighed 1.293 g (78% yield), mp 208-209°C, lit mp 192-195°C (13): its ir and TLC characterisitics were identical to those of an authentic sample (13).

12-Sulfocholic acid, disodium salt, X: Methyl 12α-hydroxy-3α, 7α-diacetoxy-5β-cholan-24-oate, (1.518 g, 3 mmol) was sulfated and worked up as described for VII to yield 1.46 g (91% yield) of X as a white powder, mp 202-204°C, lit mp 194-195°C (15): the ir and TLC properties were identical with those of an authentic sample (13).

7-Sulfoglycochenodeoxycholic acid, disodium salt, II: Ethyl 3-carboethoxyglycochenodeoxycholate (550 mg, 1 mmol) was sulfated as described for I. The syrupy triethylammonium salt precipitate was treated as described for VII and hydrolyzed by heating on a steam bath for 10 min. The alkaline hydrolysate was acidified and precipitated by adding 6 ml of 1 M p-toluidine HCl. The syrupy p-toluidinium salt was worked up as described for I, except that less water was used for washing. The white powdered product weighed 517 mg (96% yield), mp 191-192°C; lit mp 179-180°C (4).

Anal. calcd. for $C_{26}H_{41}O_8NSNa_2 \cdot H_2O$: C, 52.78, H, 7.33, S, 5.42; found: C, 52.81, H, 7.42, S, 5.65.

12-Sulfoglycodeoxycholic acid, disodium salt, V; Ethyl 3-carboethoxyglycodeoxycholate (550 mg, 1 mmol) was sulfated and worked up as described for II, except that the oily triethylammonium salt of the protected sulfate crystallized as prisms after standing. The prisms were then treated as described for IV. The product weighed 525 mg (91% yield), mp 210-212°C, lit mp 210° (4).

Anal. calcd. for $C_{26}H_{41}O_8NSNa_2 \cdot H_2O$: C, 52.78, H, 7.33; found: C, 52.65, H, 7.34.

12-Sulfoglycocholic acid, disodium salt, XI: A solution of 12-sulfocholic acid, disodium salt, X, (267 mg, 0.5 mmol) in 2 ml of methanol containing 5% triethylamine was converted to the triethylammonium salt form by passing through a column (1. x 13 cm) of Dowex AG 50W-X8

in the triethylammonium form and eluting with the same solvent at a flow rate of 1 ml/min. The first 8 ml of eluate were collected and evaporated to dryness *in vacuo* at 55°C. The residue was dissolved in 15 ml of chloroform. The chloroform solution was then treated with triethylamine (0.2 ml), ethyl glycinate (206 mg, 2 mmol), and EEDQ (173 mg, 0.7 mmol) and was refluxed on a steam bath for 24 hr. An additional portion of EEDQ (173 mg) was then added and the refluxing was continued for another 24 hr. The solution was evaporated to approximately 5 ml, and diluted with 30 ml of ether. The resulting precipitate was collected and washed with ether several times. It was then dissolved in 10 ml of methylene chloride and filtered. The filtrate was diluted with 30 ml of ether and the precipitate was collected, washed with ether, and air dried. The dried powder was then dissolved in a mixture of 10 ml of methanol, 4 ml of 0.4 N methanolic NaOH, and 0.1 ml of water. The solution was then refluxed on a steam bath for 20 min and evaporated almost to dryness *in vacuo*. The residue was dissolved in 5 ml of methanol and the solution was diluted with 40 ml of ether. The precipitate was collected, washed with ether, and redissolved in 10 ml of methanol. The methanolic solution was filtered and the filtrate was diluted with 30 ml of ether. The precipitate was collected, washed with methanol:ether (1:3), ether, and air dried. The white powdered product weighed 270 mg (91% yield); mp 208-209°C.

Anal. calcd. for $C_{26}H_{41}O_9NSNa_2 \cdot H_2O$: C, 51.39, H, 7.13, S. 5.28; found: C, 51.00, H, 7.22, S, 4.98.

7-Sulfoglycocholic acid, disodium salt, VIII: 7-Sulfocholic acid, disodium salt, VII, (267 mg, 0.5 mmol) was converted to the triethylammonium salt, conjugated with ethyl glycinate, and worked up as described for XI to obtain VIII as a white powder. The product weighed 290 mg (98% yield), mp 189-190°C.

Anal. calcd. for $C_{26}H_{41}O_9NSNa_2 \cdot H_2O$: C, 51.39, H, 7.13, S, 5.28; found: C, 51.09, H, 7.19, S, 5.15.

7-Sulfotaurochenodeoxycholic acid, disodium salt, III: A stirred suspension of 7-sulfochenodeoxycholic acid, disodium salt, I, (516 mg, 1 mmol), EEDQ (346 mg, 1.4 mmol), and taurine (138 mg, 1.1 mmol) in 2 ml of DMF was heated in 90°C constant temperature bath for 0.5 hr, and then at 25°C for 0.5 hr. The reaction mixture was diluted with 4 ml of methanol, filtered, and the filter was washed with three 1 ml portions of methanol. The combined filtrate was diluted with 30 ml of ether. The resulting precipitate was collected, washed with ether and dried. It was then redissolved in 10 ml of methanol, and the solution was diluted with 20 ml of ether. The precipitate was collected, washed with ether-methanol (2:1), ether, and dried *in vacuo*. The product weighed 561 mg (90% yield), mp 160°C (sintered), melted at 195°C, lit mp 174°C(4).

Anal. calcd. for $C_{26}H_{43}O_9NS_2Na_2 \cdot 2\ 1/2\ H_2O$: C, 46.70, H, 7.23, S, 9.59, found: C, 46.77, H, 7.25, N, 9.89.

12-Sulfotaurodeoxycholic acid, disodium salt, VI: 12-Sulfodeoxycholic acid, disodium salt, IV, (516 mg, 1 mmol) was conjugated with taurine and worked up as described for III to afford 12-sulfotaurodeoxycholic acid, disodium salt, VI (538 mg, 86% yield), mp 189-191°C; lit mp 185°C (4).

Anal. calcd. for $C_{26}H_{43}O_9NS_2Na_2 \cdot H_2O$: C, 48.66, H, 7.07, S, 9.99; found: C, 48.11, H, 7.05, S, 10.17.

12-Sulfotaurocholic acid, disodium salt, XII: 12-Sulfocholic acid, disodium salt, X, (533 mg, 1 mmol) was conjugated with taurine and worked up as described for III to give 12-sulfotaurocholic acid, disodium salt, XII (596 mg, 93% yield), mp 180-181°C.

Anal. calcd. for $C_{26}H_{43}O_{10}NS_2Na_2 \cdot 3/2\ H_2$: C, 46.83, H, 6.95, S, 9.62; found: C, 46.87, H, 6.91, S, 9.89.

7-Sulfotaurocholic acid, disodium salt, IX: A stirred suspension of 7-sulfocholic acid, disodium salt, VII (533 mg, 1 mmol), taurine (138 mg, 1.1 mmol), and EEDQ (346 mg, 1.4 mmol) in 4 ml of DMF was heated at 90°C for 0.5 hr. Another portion of EEDQ (346 mg, 1.4 mmol) was then added and the suspension was heated for an additional 0.5 hr. After stirring at 25°C for another 0.5 hr, the reaction mixture was worked up as described for III to give 7-sulfotaurocholic acid, disodium salt, IX (456 mg, 71% yield), mp 178-180°C.

Anal. calcd. for $C_{26}H_{43}NS_2Na_2 \cdot H_2O$: C, 47.48, H, 6.90, S, 9.75; found: C, 47.49, H, 7.07, S, 9.37.

RESULTS AND DISCUSSION

The superiority of sulfur trioxide-triethylamine over other sulfating agents has been demonstrated by us in previous studies (5,6). The use of p-toluidine hydrochloride as a precipitating agent to isolate the bile acid sulfate from aqueous solution also simplified the procedure and raised the product yields (6). With some modifications, the present paper now applies the same procedures to the synthesis of the 7-mono- and 12-mono- sulfates. As starting materials we used methyl or ethyl esters of bile acids or glycine conjugated bile acids with the 3α-hydroxyl and other appropriate hydroxyl groups blocked by a protecting group. Since the triethylammonium salts, obtained from the sulfation of bile acid esters whose 3α-hydroxyl groups are protected, are all relatively soluble in ethyl ether, the less polar petroleum ether has to be used to precipitate these salts quantitatively from the reaction medium. This requirement precludes the use of DMF, which is the best solvent for sulfation employing sulfur trioxide-triethylamine (5),

since DMF has limited miscibility with petroleum ether. For this reason, pyridine was substituted for DMF in the syntheses described here. With this solvent substitution, complete sulfation required more than 6 hours instead of the usual 0.5-1 hour when DMF was used as solvent, and the reaction mixture was usually left overnight.

For the synthesis of the 7-sulfate of chenodeoxycholic acid and the 12-sulfate of deoxycholic acid, the methyl esters of the individual bile acids with their 3α-hydroxyl groups protected with carboethoxyl group were used as starting materials. The acid form used by Eyssen, Parmentier, and Mertens (2) can not be synthesized readily since a mixture of acid and mixed anhydride is formed. For the synthesis of 7-sulfocholic acid and 12-sulfocholic acid, we used the same starting materials, i.e. methyl 7α-hydroxy-3α,12α-diacetoxy-5β-cholan-24-oate and methyl 12α-hydroxy-3α,7α-diacetoxy-5β-cholan-24-oate, used by Parmentier and Eyssen (4). However, the synthesis of methyl 7α-hydroxy-3α,12α-diacetoxy-5β-cholan-24-oate was achieved by sodium borohydride reduction of the corresponding ketone, methyl 7-oxo-3α,12α-diacetoxy-5β-cholan-24-oate. This reduction procedure yielded the 7α-isomer as the only product and no 7β-isomer could be detected in the reaction mixture by TLC or GLC, either before or after acetylation. The reaction of these protected bile acid esters with sulfur trioxide-triethylamine in pyridine overnight resulted in quantitative sulfation. The resulting triethylammonium salts of these bile acids were readily isolated by diluting the pyridine solution with petroleum ether. They were isolated either as oils or as crystalline solids. These triethylammonium salts were then hydrolyzed and the bile acid sulfates in the alkaline hydrolysate were

isolated by acidifying and precipitating them as p-toluidinium salts. The p-toluidinium salts were usually crystalline and were washed free of inorganic contaminants and excess p-toluidinium chloride before being converted to their disodium salts with methanolic NaOH.

Ethyl 3-carboethoxyglycochenodeoxycholate and ethyl 3-carboethoxyglycodeoxycholate were synthesized by reacting ethyl esters of glycine conjugates of the individual bile acids with ethyl chloroformate in pyridine in a similar manner to that described for the ethyl carbonation of the methyl esters of the unconjugated bile acids (8). These were used as starting materials for the synthesis of 7-sulfoglycochenodeoxycholic acid and 12-sulfoglycodeoxycholic acid, respectively. The procedures for these syntheses were the same as described for their unconjugated counterparts. However, the procedure used to synthesize 7-sulfocholic acid and 12-sulfocholic acid presented several difficulties when applied to the glycine conjugates. First, the corresponding protected glycine conjugated bile acids were not easy to obtain. When the procedure used for the synthesis of methyl cholate 3,7-diacetate was applied to the corresponding ethyl glycocholate, a mixture of several partially acetylated bile acids and the fully acetylated bile acid was produced. Second, the N-bromosuccinimide oxidation procedure, which will specifically oxidize the 7α-hydroxyl group of cholic acid, did not give clear-cut results with ethyl glycocholate. These two examples emphasize the influence of the side chain substitution on the reactivity of nuclear hydroxyl groups in bile acids toward different reagents. The same influence was also observed in our synthesis of glycolithocholic sulfate with sulfur trioxide-triethylamine (5). However, even if the protected

glycine conjugated bile acids could be synthesized by another elaborate synthetic sequence, there remains a further difficulty. The 7- and 12-acetyl groups used to protect the conjugated bile acids are resistant to hydrolysis. These groups take more than 2 hours (6) for complete hydrolysis to be achieved under the usual conditions. During this extended period of alkaline hydrolysis, a considerable amount of the glycine conjugate is also deconjugated. Separation of the deconjugated bile acid sulfate from the desired glycine conjugated sulfate becomes a very tedious procedure.

In order to avoid these difficulties, the glycocholic acid 7- and 12-monosulfates were synthesized by the alternative sequence of conjugation of their sulfated counterparts with ethyl glycinate in chloroform in the presence of EEDQ. Disodium salts of bile acid sulfates are practically insoluble in the organic solvents used for the synthesis of glycine conjugates by the EEDQ procedure (12). Nevertheless, because these disodium salts have an appreciable solubility in DMF, we made initial attempts to conjugate these bile acid sulfate disodium salts with ethyl glycinate in DMF. These attempts failed to produce the desired glycine conjugated cholic acid sulfates. However, the triethylammonium salts of sulfates are known to be relatively soluble in organic solvents (14). Therefore the disodium salts of sulfates were converted to their triethylammonium salts by ion exchange on Dowex AG50W-X8 columns in the triethylammonium form. The triethylammonium salts of sulfates are relatively unstable in methanol solution, and some triethylamine has to be added to the methanol eluent to stabilize these salts. The triethylammonium salts of these sulfates have very limited solubility in ethyl acetate, a solvent used in the

synthesis of glycine conjugated bile acids by using the EEDQ procedure (12), but they are very soluble in chloroform. The conjugation reaction took a considerably longer time in boiling chloroform than in ethyl acetate, due in part to the lower boiling point of chloroform. At the end of the reaction, the addition of ether removed all the excess reagents and precipitated the glycine conjugated sulfates as triethylammonium salts. This preparation sequence is also applicable to the synthesis of other glycine conjugated bile acid sulfates. It is especially useful for the preparation of isotopically labeled species, in which a common labeled bile acid is all that is needed to prepare unconjugated bile acid sulfate, glycine conjugated sulfate, and taurine conjugated bile acid sulfate.

The taurine conjugated bile acid sulfates were obtained by direct conjugation of bile acid sulfate disodium salts with taurine in DMF in the presence of EEDQ. Only one addition of EEDQ was required for the reaction to proceed to completion for most of the 7- and 12-monosulfates compared to the two additions required for the corresponding 3-monosulfates (5). The difference in reactivity probably can be accounted for by their different solubilities in DMF. The 7- and 12-monosulfate disodium salts of bile acids are relatively soluble in hot DMF, while all 3-monosulfate disodium salts have limited solubility in the same solvent. The only exception is 7-sulfocholic acid disodium salt. This compound has the same limited solubility in hot DMF as 3-monosulfates do, so that two additions of EEDQ are required to obtain its taurine conjugate in high yield.

Table 1 summarizes the precipitability and solubility of the p-toluidinium salts of 7- and 12-monosulfates of unconjugated and conjugated bile acids.

Table 1. Precipitability and solubility of p-toluidinium salts of bile acid 7- and 12-monosulfates.

	Unconjugated	Glycine Conjugates	Taurine Conjugates
7-Sulfochenodeoxycholic	++++	++	-
12-Sulfodeoxycholic	++++	++	-
7-Sulfocholic	+++	-	-
12-Sulfocholic	+++	-	-

Degree of precipitability is represented by the following symbols: ++++, precipitable by the addition of 2 ml of 1 M p-toluidinium chloride to a solution of 1 mmole of bile acid sulfate in 10 ml of water at pH ~ 6, and the precipitates are practically insoluble in water; +++, precipitable by the addition of 2 ml of the reagent, but the precipitated salts are sparingly soluble in water; ++, precipitable by the addition of 6 ml of the reagent, and the precipitated salts are relatively soluble in water; +, precipitable by the addition of 6 ml of the reagent, and the precipitated salt is freely soluble in water (not represented in this table); -, not precipitable by the reagent at all concentrations.

From the data in this table and those in the previous report (5) it can be concluded that monosulfates of unconjugated bile acids are all readily precipitated by p-toluidine hydrochloride and that the resulting p-toluidinium salts are relatively insoluble in water. However, as the

number of hydroxyl groups on the steroid nucleus increases, or if the molecule is conjugated with glycine, most of the resulting sulfates still can be precipitated quantitatively by using higher concentrations of p-toluidine chloride, but the resulting p-toluidinium salts of the sulfates are much more soluble in water. The more polar taurine conjugated bile acid sulfates usually do not precipitate as p-toluidinium salts. The influence of the conjugating groups and the number of hydroxyl groups on the precipitability of bile acid sulfates as p-toluidinium salt is so profound that one would expect other properties of these sulfates to be influenced by these changes. Thus, even the structurally similar 3-sulfotaurochenodeoxycholic acid and 3-sulfotaurodeoxycholic acid have different precipitability by p-toluidine chloride. The former is precipitable by this reagent, while the latter is not precipitated at all. These discrepancies emphasize the wide intra-group variation in physical properties in bile acid sulfates and suggest that the validation of any analytical procedure using a limited number of sulfate standards can not be regarded as a general procedure for other unvalidated sulfates.

The thin layer chromatographic mobilities of 7- and 12-monosulfates in three common solvents for bile acid sulfates are listed in Table 2. In all three solvent systems, the mobilities of bile acid sulfates are in the sequence of 3-sulfate > 7-sulfate > 12-sulfate for unconjugated and glycine conjugated bile acids. But the mobilities for the taurine conjugated bile acids are generally in the reverse sequence, e.g. 12-sulfate > 7-sulfate > 3-sulfate. Reliance on TLC mobilities to assign the sulfate position of possible isomeric bile acid sulfates (3)

Table 2. Thin layer chromatographic mobilities of 7- and 12-monosulfates of bile acids.[a]

Bile salt	Solvent System		
	EBAW	BAW[b]	CMAW[c]
7-Sulfocholic acid	0.94	0.91	0.91
12-Sulfocholic acid	0.90	0.90	0.86
7-Sulfoglycocholic acid	0.66	0.47	0.80
12-Sulfoglycocholic acid	0.64	0.51	0.80
7-Sulfotaurocholic acid	0.25	0.21	0.43
12-Sulfotaurocholic acid	0.29	0.25	0.46
7-Sulfochenodeoxycholic acid	0.99	0.95	1.08
7-Sulfoglycochenodeoxycholic acid	0.76	0.61	0.86
7-Sulfotaurochenodeoxycholic acid	0.30	0.38	0.46
12-Sulfodeoxycholic acid	1.07	1.05	1.13
12-Sulfoglycodeoxycholic acid	0.71	0.56	1.00
12-Sulfotaurodeoxycholic acid	0.36	0.33	0.61
3-Sulfocholic acid	1.00	1.00	1.00
3-Sulfoglycocholic acid	0.71	0.65	0.80
3-Sulfotaurocholic acid[d]	0.22	0.23	0.33

[a] *The chromatographic conditions and solvent systems are given in Experimental Procedures. The TLC mobilities are presented as relative mobilities to 3-sulfocholic acid which is designated as 1.00 in all three solvent systems. The absolute Rf values for 3-sulfocholic acid are as follows: EBAW (0.55), BAW (0.61), and CMAW (0.45).*

[b] *Tailing of all glycine conjugated sulfates occurred.*

[c] *This solvent system must be freshly prepared. Storage results in phase separation.*

[d] *For chromatographic mobilities of other 3-sulfates of free, glycine or taurine conjugated bile acids, see (6).*

is thus not without hazard in the present solvent systems.

The infrared spectra of these sulfates are not distinguishable from their corresponding 3-monosulfates. The familiar absorption bands due to the sulfate group are prominent (6).

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