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Facile Synthetic Routes to Prepare α-Muricholic Acid, Hyocholic Acid, and Their Taurine Conjugates

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α-Muricholic acid was synthesized through 9 steps from chenodeoxycholic acid with 26% overall yield. Hyocholic acid was synthesized through 8 steps from the same starting material with 63% overall yield. Taurine conjugates of α-muricholic acid and hyocholic acid were also prepared *via* their pentafluorophenyl ester.

Key Words : Bile acids, α-Muricholic acid, Hyocholic acid, Taurine conjugates, Metabolites

Introduction

Bile acids are important endogenous molecules associated with myriad biological functions, including absorption and excretion of cholesterols. The intrahepatic accumulation of hydrophobic bile acid induces cell injury[1] and can result in various diseases such as hepatitis and jaundice.

Hydroxylation of hydrophobic bile acids by cytochrome P450 (CYP) enzymes is believed to be a defensive mechanism against bile acid toxicity.[2] Both α-muricholic acid and hyocholic acid are potential metabolites in this detoxification mechanism. However, since most CYPs produce multiple hydroxylated products, it is difficult to characterize these metabolites without knowledge of their chemical composition and constitution. Synthetic bile acids are needed to further understand the dynamics of bile acid biology. Knowledge of such conversions can contribute to the development of drugs for cholestasis, which modulate bile acid accumulation.

α-Muricholic acid and hyocholic acid are important bile acids, both are monohydroxylated forms of chenodeoxycholic acid distinguished only by the stereochemistry of the C-6 hydroxyl group (Fig. 1).

Therefore, efficient stereospecific synthetic routes to these isobaric bile acids are required to facilitate metabolomics studies. We report here a facile synthetic route to α-muricholic acid and hyocholic acid that afford these materials in good quantity and high purity. In addition, we also describe a new method for high yield taurine conjugation of α-muricholic and hyocholic acids. These bile acid salts are key metabolic intermediates in bile acid secretion and represent a distinct pool of soluble bile acids in circulation. These preparations have already found utility in bile acid homeostasis studies in rodents.[3]

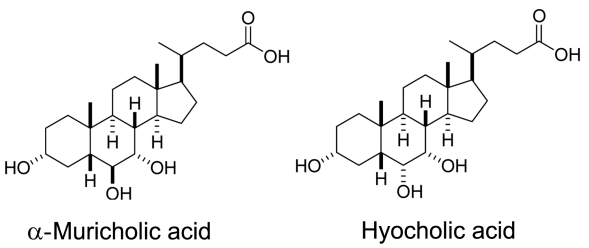


Figure 1. Structures of α-muricholic acid and hyocholic acid.

Experimental

General. ^{1}H NMR (nuclear magnetic resonance) spectra were obtained with a Varian Gemini 300 spectrometer. When using $CDCl_3$ and CD_3OD as a solvent, the chemical shifts are expressed relative to $CHCl_3$ (7.26 ppm) and HOD (4.87 ppm).

High-resolution mass spectra were recorded on a Micromass/Waters LCT Premier Electrospray TOF (time of flight) mass spectrometer coupled to a Waters HPLC system.

4,4'-Thiobis-(6-*tert*-butyl-3-methylphenol) was purchased from Wako Pure Chemical (Japan). Other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical TLC (thin-layer chromatography) was performed on precoated silica gel (0.25 mm layer thickness; Fluka 60763, Germany).

Methyl 3α-Hydroxy-7-oxo-5β-cholanoate (2). Chenodeoxycholic acid (**1**, 2.01 g, 4.86 mmol) solution in water (40 mL) was added sodium bicarbonate (1.49 g, 17.7 mmol) and *N*-bromosuccinimide (1.30 g, 7.23 mmol) at room temperature. The reaction mixture was stirred for 16 h at room temperature and then warmed to 50 °C for 2 h. The mixture was cooled to 0 °C and acidified with 1 M aqueous hydrochloride solution to pH 1 to 2. Solid was filtered, washed with water, and dried under vacuum. Methanol (100 mL) and sulfuric acid (0.3 mL) was added to the solid. The mixture was refluxed for 16 h and then cooled to room temperature. Methanol was removed *in vacuo* and the resulting residue was dissolved in water and extracted with

ethyl acetate. The combined organic layers were dried with magnesium sulfate, filtered and evaporated. Flash column chromatography using *n*-hexane/ethyl acetate (10:1 to 1:1, v/v) afforded compound **2** in 74% yield. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.66 (s, 3H), 0.93 (d, 3H, *J* = 6.3 Hz), 2.86 (m, 1H), 3.62 (m, 1H), 3.67 (s, 3H).

Methyl 3α-Acetoxy-6α-bromo-7-oxo-5β-cholanoate (3). To a solution of compound **2** (1.45 g, 3.58 mmol) in methylene chloride (30 mL) was added triethylamine (1.45 g, 14.3 mmol), acetic anhydride (1.46 g, 13.4 mmol), and *N,N*-dimethylaminopyridine (10 mg). The reaction mixture was stirred for 20 h at room temperature and then evaporated. The oily residue was purified by flash column chromatography eluting with *n*-hexane/ethyl acetate (10:1 to 2:1, v/v) afforded 3α-acetoxy-7-oxo-5β-cholanoic acid methyl ester. It was added acetic acid (30 mL), slowly dropped bromine (593 mg, 3.71 mmol), and then stirred for 20 h at room temperature. The reaction mixture was poured into ice-water (150 mL) and extracted with ethyl acetate. Organic layer was dried with magnesium sulfate, filtered, and evaporated. The residue was lyophilized to give the desired compound as a pale yellow solid. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.67 (s, 3H), 0.93 (d, 3H, *J* = 6.3 Hz), 1.29 (s, 3H), 2.02 (s, 3H), 3.67 (s, 3H), 4.70 (m, 1H), 5.19 (d, 1H, *J* = 4.5 Hz).

Methyl 3α-Acetoxy-5β-chol-6-enoate (4). To a solution of compound **3** (1.01 g, 1.92 mmol) in methanol (6 mL) and methylene chloride (3 mL) was very slowly added sodium borohydride and then stirred for 20 minutes at room temperature. It was added water and extracted with ethyl acetate. The organic layer was dried with magnesium sulfate, filtered, and concentrated *in vacuo* to give 3α-acetoxy-6α-bromo-7α-hydroxy-5β-cholanoic acid methyl ester which was added acetic acid (20 mL) and refluxing. Zinc powder (1.2 g) was added to the refluxing solution and additional 30 minutes continued reflux and then cooled to room temperature. Zinc powder was removed by filtration through celite pad and washed with methylene chloride.

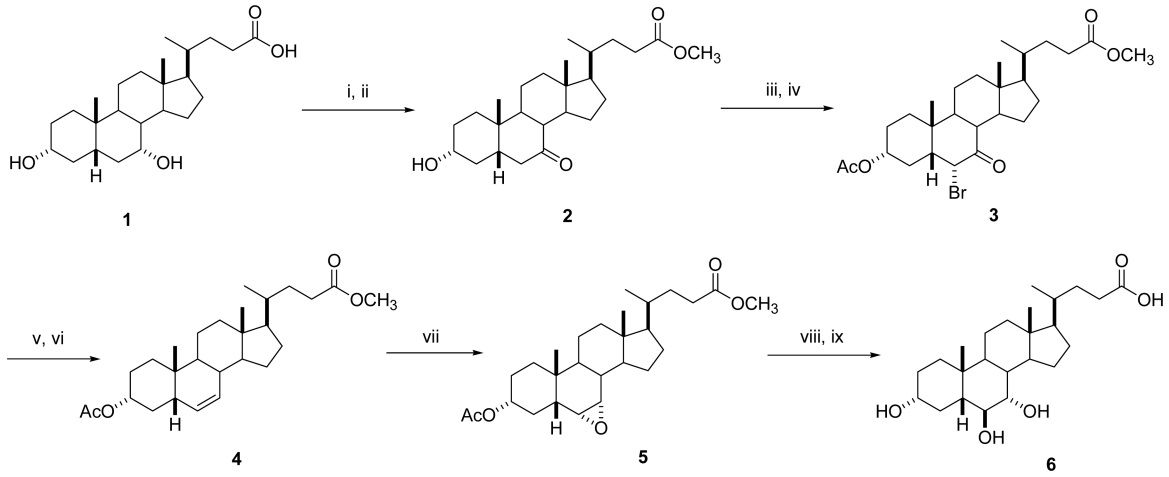
The organic layer was washed with water, 5% aqueous sodium bicarbonate solution, and brine. It was dried with magnesium sulfate, filtered, and evaporated. The residue was purified by flash column chromatography eluting with *n*-hexane/ethyl acetate (20:1 to 4:1, v/v) afforded the desired compound. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.70 (s, 3H), 0.87 (s, 3H), 0.94 (d, 3H, *J* = 6.3Hz), 2.03 (s, 3H), 3.68 (s, 3H), 4.68 (m, 1H), 5.47 (m, 2H).

Methyl 3α-Acetoxy-6α,7α-epoxy-5β-cholanoate (5). To a solution of compound **4** (445 mg, 1.03 mmol) in 1,2-dichloroethane (20 mL) was added 3-chloroperbenzoic acid (70%, 0.73 g, 2.96 mmol) and 4,4'-thiobis-(6-*tert*-butyl-3-methylphenol) (9 mg) at room temperature. The reaction mixture was refluxed for 2 h and then cooled to room temperature. It was extracted with ethyl acetate. Organic layer washed with 5% aqueous sodium thiosulfate (60 mL), 5% aqueous sodium bicarbonate (60 mL) and water and then dried with magnesium sulfate, filtered, and evaporated. The residue was purified by flash column chromatography eluting with *n*-hexane/ethyl acetate (10:1 to 4:1, v/v) afforded the desired compound. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.70 (s, 3H), 0.84 (s, 3H), 0.93 (d, 3H, *J* = 6.3 Hz), 3.10 (m, 2H), 3.68 (s, 3H), 4.70 (m, 1H).

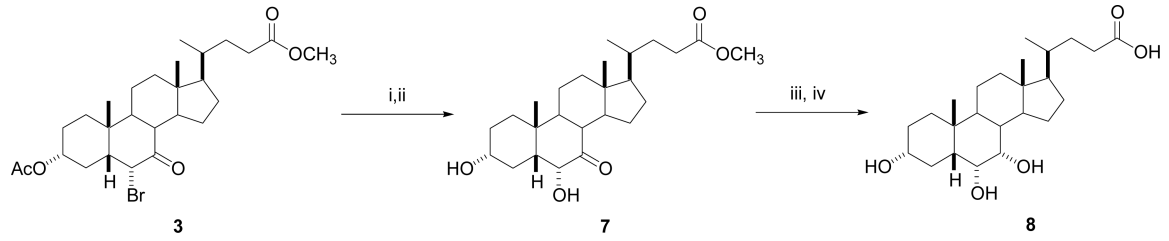
3α,6β,7α-Trihydroxy-5β-cholanoic acid: α-Muricholic acid (6). To a solution of compound **5** (310 mg, 0.694 mmol) in *N,N*-dimethylformamide (10 mL) was added boron trifluoride diethyl etherate (0.6 mL, 4.78 mmol) and then stirred for 16 h at room temperature. The reaction mixture was added water and extracted with methylene chloride which was washed with water. Organic layer was dried with magnesium sulfate, filtered, and concentrated under vacuum. The residue was added 10% methanolic potassium hydroxide (25 mL) and refluxed for 2 h and then cooled to room temperature. Methanol was removed by evaporation. It was added water and acidified with 10% aqueous sulfuric acid to pH 1-2. The mixture was extracted with ethyl acetate. Organic layer was washed with water, dried with magnesium sulfate, filtered, and concentrated under vacuum. The residue was lyophilized to give the desired compound as a white solid. ^{1}H-NMR (CD_3OD, 300 MHz) δ 0.75 (s, 3H), 0.99 (d, 3H, *J* = 6.6 Hz), 1.10 (s, 3H), 3.37 (m, 1H), 3.66 (m, 2H). High-resolution mass (ES^-) calculated for $C_{24}H_{39}O_5$: 407.2798. Found: 407.2797.

Methyl 3α,6α-Dihydroxy-7-oxo-5β-cholanoate (7). 8% Methanolic potassium hydroxide (15 mL) was slowly added dropwise to a solution of **3** (817 mg, 1.55 mmol) in methanol (12 mL) over 10 minutes and then stirred for 16 h at room temperature. Methanol was removed and water was added, and the mixture was acidified with 10% aqueous sulfuric acid to pH 1-2. The resulting solution was extracted with methylene chloride. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated under vacuum. To the residue was added methanol (30 mL), sulfuric acid (0.3 mL, catalytic amount), and refluxed for 3 h and then cooled to room temperature. Methanol was removed by evaporation, water was added, and extracted with ethyl acetate. The organic layer was dried with magnesium sulfate, filtered, and evaporated. The residue was purified by flash column chromatography eluting with methylene chloride/methanol (30:1 to 10:1, v/v) afforded the desired compound. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.67 (s, 3H), 0.93 (d, 3H, *J* = 6.3 Hz), 1.24 (s, 3H), 3.41 (d, 1H, *J* = 5.1 Hz), 3.60 (m, 1H), 3.68 (s, 3H), 4.53 (m, OH).

3α,6α,7α-Trihydroxy-5β-cholanoic acid: Hyocholic acid (8). To a solution of compound **7** (285 mg, 0.677mmol) in methanol (10 mL) and methylene chloride (4 mL) was slowly added sodium borohydride (31 mg, 0.819 mmol) and stirred for 20 minutes at room temperature. The reaction mixture was added water and extracted with ethyl acetate. Organic layer was washed with brine, dried with magnesium sulfate, filtered, and concentrated under vacuum. The residue was added 5% methanolic potassium carbonate (20 mL) and refluxed for 3 h and then cooled to room temperature. Methanol was removed and then added water, acidified with 10% aqueous sulfuric acid to pH 1-2. The mixture was extracted with ethyl acetate. Organic layer was washed with water, dried with magnesium sulfate, filtered, and concen-



Scheme 1. Synthesis of α-muricholic acid; i) $NaHCO_3$, NBS, H_2O; ii) H_2SO_4, MeOH, 74% two steps; iii) Et_3N, Ac_2O, DMAP, DCM; iv) Br_2, AcOH, 97% two steps; v) $NaBH_4$, MeOH, DCM; vi) zinc powder, AcOH, 54% two steps; vii) *m*-CPBA, 4,4'-thiobis-(6-*tert*-butyl-3-methylphenol), 1,2-dichloroethane, 68%; viii) $BF_3{\cdot}O(Et)_2$, DMF, ix) 10% KOH in MeOH, 99% two steps;.



Scheme 2. Synthesis of hyocholic acid; i) KOH, MeOH; ii) H_2SO_4, MeOH, 91% two steps; iii) $NaBH_4$, MeOH, DCM; iv) 10% KOH in MeOH, 96% two steps.

trated under vacuum. The residue was lyophilized to give the desired compound as a white solid. ^{1}H-NMR (CD_3OD, 300 MHz) δ 0.72 (s, 3H), 0.95 (s, 3H), 0.99 (d, 3H, J = 6.3 Hz), 3.37 (m, 1H), 3.80 (m, 2H). High-resolution mass (ES^-) calculated for $C_{24}H_{39}O_5$: 407.2798. Found: 407.2803.

Tauro 3α,6β,7α-Trihydroxy-5β-cholanoate (9). Compound **6** (115 mg, 0.281 mmol) was added methylene chloride (20 mL), pentafluorophenol (65 mg, 0.353 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (65 mg, 0.339 mmol), *N,N*-dimethylformamide (0.1 mL), and stirred for 20 h at room temperature. Methylene chloride was removed by evaporation. The residue was purified by flash column chromatography eluting with methylene chloride/methanol (30:1 to 10:1, v/v) afforded the pentafluorophenyl ester. The product fraction was concentrated under vacuum. It was solved in methylene chloride (5 mL), and added taurine (32 mg, 0.255 mmol), 1,8-diazabicyclo[5,4,0]undec-7-ene (37 mg, 0.243 mmol), and then stirred for 20 h at room temperature. Solvent was evaporated and the residue was purified by flash column chromatography eluting with methylene chloride/methanol (10:1 to 3:1, v/v). The product fraction was concentrated under vacuum and lyophilized to obtain the desired compound as a white solid. ^{1}H-NMR (CD_3OD, 300 MHz) δ 0.74 (s, 3H), 1.00 (d, 3H, J = 6.3 Hz), 1.09 (s, 3H), 2.98 (t, 2H, J = 6.9 Hz), 3.38 (m, 3H), 3.61 (m, 2H). High-resolution mass (ES^-) calculated for $C_{26}H_{44}NO_7S$: 514.2838. Found: 514.2828.

Tauro 3α,6α,7α-Trihydroxy-5β-cholanoate (10). Compound **7** (115 mg, 0.281 mmol) was added methylene chloride (20 mL), pentafluorophenol (63 mg, 0.342 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (67 mg, 0.350 mmol), *N,N*-dimethylformamide (0.1 mL), and stirred for 20 h at room temperature. Methylene chloride was removed by evaporation. The residue was purified by flash column chromatography eluting with methylene chloride/methanol (30:1 to 10:1, v/v) afforded the pentafluorophenyl ester. The product fraction was concentrated under vacuum. It was solved in methylene chloride (7 mL), and added taurine (30 mg, 0.240 mmol), 1,8-diazabicyclo[5,4,0]undec-7-ene (40 mg, 0.263 mmol), and then stirred for 20 h at room temperature. Solvent was evaporated and the residue was purified by flash column chromatography eluting with methylene chloride/methanol (10:1 to 3:1, v/v). The product fraction was concentrated under vacuum and lyophilized to obtain the desired compound as a white solid. ^{1}H-NMR (CD_3OD, 300 MHz) δ 0.72 (s, 3H), 0.95 (s, 3H), 0.99 (d, 3H, J = 6.3 Hz), 2.98 (t, 2H, J = 6.9 Hz), 3.37 (m, 3H), 3.80 (m, 2H). High-resolution mass (ES^-) calculated for $C_{26}H_{44}NO_7S$: 514.2838. Found: 514.2817.

Result and Discussion

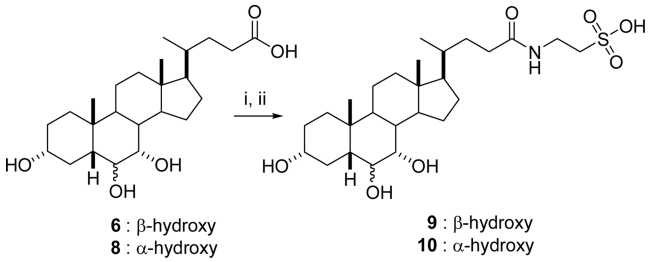
Synthesis of α-Muricholic Acid. Previous work demon-

strated a viable synthetic route to prepare α-muricholic acid with an overall yield of 27% from chenodeoxycholic acid.[4] In that study, the C-3 hydroxyl of chenodeoxycholic acid was protected by carbethoxylation, whereas our method employed an acetylation protecting group strategy and the use of 7-oxo-chenodeoxycholic acid methyl ester **2** resulting in a significant improvement in yield.

The detailed synthetic approach involves selective oxidation of chenodeoxycholic acid using *N*-bromosuccinimide in aqueous sodium bicarbonate solution and esterification of 3α-hydroxy-7-oxo-5β-cholanic acid with acidic methanol, affording methyl 3α-hydroxy-7-oxo-5β-cholanoate **2**. 3α-Hydroxyl derivative of compound **2** was protected by acetylation, and was treated with acetic anhydride and triethylamine in methylene chloride. Methyl 3α-acetoxy-7-oxo-5β-cholanoic acid was treated with bromine in acetic acid to prepare a 6α-brominated compound. The 7-oxo of compound **3** was reduced to 7α-alcohol through reaction with sodium borohydride in methanol and methylene chloride, to form methyl 3α-acetoxy-6α-bromo-7α-hydroxy-5β-cholanoate, which underwent elimination with treatment of zinc powder in acetic acid to provide methyl 3α-acetoxy-5β-chol-6-enoate **4** in 54% yield over two step yield from compound **3**. We anticipate that yield of this sequence could be improved by optimizing the zinc powder reaction conditions to minimize unwanted elimination products. Compound **4** was treated with 3-chloroperbenzoic acid and catalytic 4,4'-thiobis-(6-*tert*-butyl-3-methylphenol) in 1,2-dichloroethane to create 6α,7α-epoxide **5** in 68% yield. This was accompanied by a small amounts of deacetylation due to prolonged (2 h) reflux. α-Muricholic acid could be prepared in a good yield through reacting 6α,7α-epoxide **5** with boron trifluoride diethyl etherate in *N,N*-dimethylformamide[5] to give methyl 3α-acetoxy-6β,7α-dihydroxy-5-cholanoate which was hydrolyzed with 10% methanolic potassium hydroxide (Scheme 1).

Synthesis of Hyocholic Acid. Previous studies reported synthetic access to hyocholic acid in 61% yield from methyl 3α-acetoxy-7-oxo-5β-cholanoate.[4,6,7] Our results demonstrate improved synthetic utility through the use of methyl 3α-acetoxy-6α-bromo-7-oxo-5β-cholanoic acid **3** as an intermediate. The overall yield from 3α-hydroxy-7-oxo-5β-cholanoate **2** to hyocholic acid was 85% (Scheme 1 and 2). Therefore, this 4 step approach represents a major synthetic optimization hyocholic acid preparation. We also have prepared hyocholic acid with an overall yield of 63% from chenodeoxycholic acid. This provides modest improvement over a previously reported synthetic route, but nevertheless represents a good yield over 8 synthetic steps.

Methyl 3α-acetoxy-6α-bromo-7-oxo-5β-cholanoic acid **3** was hydrolyzed with potassium hydroxide, and continuous esterification in acidic methanol gave methyl 3α,6α-dihydroxy-7-oxo-5β-cholanoate **7** in 91% yield. It was treated with sodium borohydride in methanol and methylene chloride to prepare methyl 3α,6α,7α-trihydroxy-5β-cholanoate, which was hydrolyzed with potassium hydroxide in methanol to afford hyocholic acid **8** (Scheme 2). The ketone reduction



Scheme 3. Synthesis of taurine conjugates; i) pentafluorophenol, EDCI, DMF, DCM; ii) taurine, DBU, DCM, two steps (**9**, 48%; **10**, 52%).

(iii) and ester hydrolysis (iv) steps were performed in one-pot as partial hydrolysis was observed in conjunction with sodium borohydride treatment.

Synthesis of Taurine Conjugates of α-Muricholic Acid and Hyocholic Acid. Taurine conjugates of bile acids have previously been prepared using several different synthetic routes.[8-12] We hypothesized that pentafluorophenyl esters of α-muricholic acid, hyocholic acid and other bile acids would be attractive precursors for preparation of taurine and glycine bile salts based on the utility of pentafluorophenol in peptide synthesis and selectivity for addition of amines[13-15]

α-Muricholic acid and hyocholic acid were treated with pentafluorophenol, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimde hydrochloride and a catalytic amount of *N,N*-dimethylformamide in methanol to obtain its pentafluorophenyl ester intermediates, which were reacted with taurine and 1,8-diazabicyclo[5,4,0]undec-7-ene in methylene chloride to afford taurine conjugates (**9** and **10**, Scheme 3). The taurine conjugates were synthesized in approximately 50% yield from α-muricholic acid and hyocholic acid. This unoptimized yield should be improved by modifying the EDCI coupling conditions to increase the yield of the intermediate pentafluorophenyl esters.

Conclusion

We have developed modified synthetic approaches for the synthesis of bile acid metabolites. α-Muricholic acid was prepared 9 steps from chenodeoxycholic acid in comparable yield (26%) to previous reports. Importantly, our 8 step route to hyocholic acid from chenodeoxycholic acid represents a marked improvement (63%) over previous published routes.

We also present an efficient new method to prepare tauro cholic acids in high yield and purity. We demonstrate the utility of this approach for taurine conjugates of α-muricholic acid and hyocholic acid, which were prepared in 2 steps *via* pentafluorophenyl esters in 48% and 52% yield, respectively. However, we anticipate that this approach will have general utility in the preparation bile acid salts using a variety of acid and amine precursors. Taken together, these approaches will allow the synthesis of bile acid metabolites and facilitate quantitative metabolomics studies in complex biological samples.

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