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Synthesis of bile acid 24-acyl glucuronides

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*The synthesis of acyl glucuronides of common bile acids is described. By means of the Mitsunobu reaction employing diethylazodicarboxylate and triphenylphosphine, bile acids were condensed through the inherent C-24 carboxy group with benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate, which was prepared from 1-*O*-methyl-α-D-glucose. The separation and purification of the β-anomers at the anomeric position of the sugar moiety were attained by preparative thin-layer chromatography and/or high-performance liquid chromatography on a column packed with phenyl-bonded silica using H_2O-MeOH as a mobile phase. The removal of the benzyl group on the sugar moiety was achieved by catalytic hydrogenation with 10% palladium on carbon to yield the desired acyl glucuronides of bile acids. The structures of these acyl glucuronides were confirmed by proton nuclear magnetic resonance spectral properties.* (*Steroids* **63**:180–185, 1998) © *1998 by Elsevier Science Inc.*

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Introduction

Bile acids are synthesized from cholesterol in hepatocytes, followed by secretion into the small intestine, reabsorbed from the ileum-proximal colon and then returned to the liver via the portal vein. This enterohepatic circulation is responsible for the low concentration of bile acids in the peripheral blood and urine of healthy subjects. In hepatobiliary diseases, disturbances of synthesis and clearance by the liver and absorption by the intestine cause changes in the level and the metabolic profile of bile acids in biological fluids.

The conjugation with D-glucuronic acid to produce glucuronides plays an important role in the elimination of endogenous substances such as steroid hormones and bile acids as well as drugs.[1] The principal purpose of this metabolism is believed to be transformation of lipophilic biological materials into strongly dissociated compounds which are more soluble under physiological conditions. The D-glucuronic acid moiety of UDP-glucuronic acid is easily transformed not only to a hydroxy group but also to carboxy, amino, imino and sulfhydryl groups of various biological importance by hepatic glucuronyltransferases. It has been believed that common bile acids are converted into the ether-type glucuronides through the 3α-hydroxy group on the steroid nucleus.[2–6] In the recent decade, it has also been reported that unusual bile acids such as 24-nor lithocholic acid are metabolized into carboxy-linked glucuronides, so called acyl glucuronides.[7–11] In a previous communication,[12] we have disclosed the formation of common bile acid acyl glucuronides, which have been identified as their acetate-methyl ester derivatives by liquid chromatography (LC)-atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS), in an incubation mixture of liver microsomes from male rats. These observations strongly imply the occurrence of similar acyl glucuronides of common bile acids in humans. Our continuing interests in the biodynamics of bile acids in normal subjects and patients with liver disease have led to the development of a reliable method for the simultaneous determination of the bile acid 24-glucuronides. However the instability of acyl glucuronides, for example, which are almost as susceptible to transesterification and hydrolysis under mild conditions, has prevented preparation of authentic specimens for use in their direct identification and quantitation in biological materials. The present paper deals with the synthesis of the titled compounds by selective coupling of the carboxy group of bile acids with a D-glucuronic acid benzyl ester-benzyl ether derivative employing the Mitsunobu reaction.[13]

Experimental

Material

Cholic (CA), chenodeoxycholic (CDCA), deoxycholic (DCA) and lithocholic (LCA) acids were purchased from Sigma (St. Louis, Missouri, USA). Ursodeoxycholic acid (UDCA) was kindly donated by Tokyo Tanabe Co. (Tokyo, Japan). Preparative thin-layer chromatography (TLC) was performed using silica gel (Kiesel gel 60 F_{254}, 20 cm × 20 cm × 0.5 mm, Merck KGaA, Darmstadt, Germany). Melting points were taken on a micro hot-stage apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Varian Gemini-2000 and Hitachi FT-NMR R-3000 spectrometers at 300 MHz. Chemical shifts are

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given as the δ value with tetramethylsilane as the internal standard (s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet). Column chromatography was performed using silica gel (Kieselgel 60, 200–300 mesh, Merck KGaA) and Cosmosil 140C_{18}-OPN (140 μm, Nacalai Tesque, Inc., Kyoto, Japan). The apparatus used for semi-preparative high-performance liquid chromatography consisted of an injector (Rheodyne 7125, Catati, California, USA), a Hitachi model L-4200 UV-VIS detector (254 nm) (Hitachi, Ltd., Tokyo) and a chromatographic pump (110B Solvent Delivery Module, Beckman Instruments, Inc., California, USA). A Cosmosil 5-Ph column (5 μm, 150 × 4.6 mm i.d.) (Nacalai Tesque, Inc.) was used at ambient temperature. LC/MS was carried out using a Hitachi M-1000H quadrupole mass spectrometer with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) systems in the negative and positive ion detection modes, respectively. The mass spectrometer was set at a drift voltage of −60 V for an ESI mode and at +30 V for an APCI mode with a nebulizer gas temperature of 130°C.

A Cosmosil 5C_8 column (5 μm, 150 × 4.6 mm i.d., Nacalai Tesque, Inc.) was also used for the separation of bile acid 24-glucuronide acetate-methyl esters at a flow rate of 1 mL/min according to the method previously reported.[12]

1-O-*Methyl-6*-O-*trityl-α-D-glucopyranose (2)*

A solution of 1-*O*-methyl-α-D-glucose (**1,** 20 g) and triphenylmethyl chloride (34.7 g) in anhydrous pyridine (200 mL) was stirred at room temperature overnight. The resulting solution was diluted with H_2O and extracted with AcOEt. The organic layer was washed successively with 5% HCl, 5% $NaHCO_3$ and saturated NaCl, dried on Na_2SO_4, and then evaporated in vacuo. Recrystallization of the resulting product from acetone-hexane afforded **2** (42.2 g, 91.2%) as colorless needles. m.p. 97–99°C. ^{1}H NMR ($CDCl_3$) δ: 3.38–3.69 (6H, unresolved m, 2-, 3-, 4-, 5- and 6-H), 3.43 (3H, s, OCH_3), 4.77 (1H, d, J = 3.7 Hz, 1-H), 7.24–7.47 (15H, m, $C(C_6H_5)_3$). Analysis calculated for $C_{26}H_{28}O_6 \cdot 1/4H_2O$: C, 70.81; H, 6.51. Found: C, 70.68; H, 6.36.

1-O-*Methyl-2,3,4-tri*-O-*benzyl-6*-O-*trityl-α-D-glucopyranose (3)*

Benzylbromide (8.1 mL) and sodium hydride (1.65 g) were successively added to a stirred solution of **2** (5 g) and tetrabutylammonium iodide (138 mg) in anhydrous tetrahydrofuran (20 mL), and the reaction mixture was further stirred at room temperature for 24 h. After concentration of the solvent, the resulting solution was diluted with H_2O and extracted with AcOEt, which in turn was washed with 5% HCl, 5% $NaHCO_3$ and saturated NaCl, dried on Na_2SO_4, and then evaporated in vacuo. The residue was subjected to column chromatography on silica gel (100 g) using *n*-hexane-AcOEt (5:1, v/v) to give **3** (6.9 g, 85.2%) as a semicrystalline product. ^{1}H NMR ($CDCl_3$) δ: 3.23 (1H, dd, J = 3.9 and 9.0 Hz, 2-H), 3.44 (3H, s, OCH_3), 3.48 (1H, t, J = 9.1 Hz, 4-H), 3.61 (2H, m, 6-H), 3.80 (1H, m, 5-H), 3.96 (1H, t, J = 9.1 Hz, 3-H), 4.28 (1H, d, J = 10.6 Hz), 4.62–4.91 (6H, m), 7.19–7.48 (30H, m, 3 × $CH_2C_6H_5$ and $C(C_6H_5)_3$). Analysis calculated for $C_{47}H_{46}O_6$: C, 79.86; H, 6.56. Found: C, 79.75; H, 6.76.

1-O-*Methyl-2,3,4-tri*-O-*benzyl-α-D-glucopyranose (4)*

To a stirred solution of **3** (4.5 g) in EtOH (50 mL), concentrated H_2SO_4 (2.6 mL) was added dropwise under ice-cooling. After stirring for another 3 h at room temperature, the solution was neutralized with 10% NaOH and concentrated in vacuo, followed by extraction with AcOEt. The organic layer was washed with H_2O and saturated NaCl, dried on Na_2SO_4, and evaporated in vacuo. The residue was subjected to column chromatography on silica gel (100 g) using *n*-hexane-AcOEt (1:2, v/v) to give **4** (2.54 g, 85.3%) as a semicrystalline product. ^{1}H NMR ($CDCl_3$) δ: 3.35 (3H, s, OCH_3), 3.49–3.71 (5H, unresolved m, 2-, 4-, 5- and 6-H), 4.00 (1H, t, J = 9.8 Hz, 3-H), 4.56–4.96 (7H, m, 1-H and 3 × $CH_2C_6H_5$), 7.21–7.36 (15H, m, $C(C_6H_5)_3$). Analysis calculated for $C_{28}H_{32}O_6$: C, 72.39; H, 6.94. Found: C, 72.18; H, 6.92.

Benzyl 1-O-*methyl-2,3,4-tri*-O-*benzyl-α-D-glucopyranuronate (5)*

To an ice-cooled solution of **4** (1.7 g) in acetone (10 mL), anhydrous chromic acid (3.7 g) in 17% H_2SO_4 (v/v, 11 mL) was added dropwise, and the mixture was stirred for 30 min at room temperature. After addition of MeOH (10 mL) to decompose the excess reagents, the resulting solution was extracted with AcOEt, which in turn was washed with H_2O, dried on Na_2SO_4, and evaporated in vacuo. To the crude product obtained in dimethylsulfoxide (5 mL) were added $NaHCO_3$ (850 mg) and benzylbromide (3.5 mL), and the resulting mixture was stirred for 8 h at room temperature, followed by extraction with AcOEt. The organic layer was washed with H_2O and saturated NaCl, dried on Na_2SO_4, and evaporated in vacuo. The residue was subjected to column chromatography on silica gel (50 g) using *n*-hexane-AcOEt (5:1, v/v) to give **5** (2.06 g, 98.8%) as a semicrystalline product. ^{1}H NMR ($CDCl_3$) δ: 3.40 (3H, s, OCH_3), 3.59 (1H, dd, J = 3.6 and 9.6 Hz, 2-H), 3.71 (1H, dd, J = 9.0 and 9.9 Hz, 4-H), 3.98 (1H, t, J = 9.3 Hz, 3-H), 4.21 (1H, d, J = 9.9 Hz, 5-H), 4.62 (1H, d, J = 3.6 Hz, 1-H), 4.42–5.20 (8H, m, 4 × $CH_2C_6H_5$), 7.23–7.33 (20H, m, 4 × $CH_2C_6H_5$). Analysis calculated for $C_{35}H_{36}O_7$: C, 73.92; H, 6.38. Found: C, 73.64; H, 6.23.

Benzyl 1-O-*acetyl-2,3,4-tri*-O-*benzyl-D-glucopyranuronate (6)*

A mixed solution of H_2SO_4-AcOH (1:10, v/v) (2.8 mL) was added dropwise to a cooled solution of **5** (1.6 g) in acetic acid (16 mL) and acetic anhydride (2.6 mL), and the resulting solution was stirred for 5 h at room temperature. The reaction mixture was poured into ice water and extracted with AcOEt, which in turn was washed successively with 5% $NaHCO_3$, H_2O and saturated NaCl, dried on Na_2SO_4, and evaporated in vacuo. The residue obtained was subjected to column chromatography on silica gel (50 g) using *n*-hexane-AcOEt (6:1, v/v) to give **6** (1.36 g, 81.1%) as a semicrystalline product. ^{1}H NMR ($CDCl_3$) δ: 2.03 (0.6H, s, $OCOCH_3$ (β-anomer)), 2.15 (2.4H, s, $OCOCH_3$ (α-anomer)), 3.71–3.81 (2.2H, unresolved m, 2- (α- and β-anomer), 3- (β-anomer), and 4-H (α- and β-anomer), 3.92 (0.8H, t, J = 9.3 Hz, 3-H (α-anomer)), 4.10 (0.2H, d, J = 10.2 Hz, 5-H (β-anomer)), 4.30 (0.8H, d, J = 10.2 *Hz*, 5-H (α-anomer)), 4.45–5.19 (8H, m, 4 × $CH_2C_6H_5$), 5.65 (0.2H, d, J = 7.7 Hz, 1-H (β-anomer)), 6.34 (0.8H, d, J = 3.7 Hz, 1-H (α-anomer)), 7.06–7.35 (20H, m, 4 × $CH_2C_6H_5$). Analysis calculated for $C_{36}H_{36}O_8$: C, 72.46; H, 6.08. Found: C, 72.57; H, 5.97.

Benzyl 2,3,4-tri-O-*benzyl-D-glucopyranuronate (7)*

2-Aminoethanol (0.3 mL) was added to a solution of **6** (1.7 g) in AcOEt (20 mL) and dimethylsulfoxide (0.2 mL), and the mixture was stirred at room temperature for 7 h. The resulting solution was diluted with AcOEt, washed successively with 5% HCl, 5% $NaHCO_3$ and saturated NaCl, dried on Na_2SO_4, and evaporated in vacuo. The crude product was subjected to column chromatography on silica gel (50 g) using *n*-hexane-AcOEt (5:1, v/v) to give a mixture of α- and β-anomer **7** (1.1 g, 71%), which was subjected to crystallization from acetone-hexane to give **7** as colorless nee-

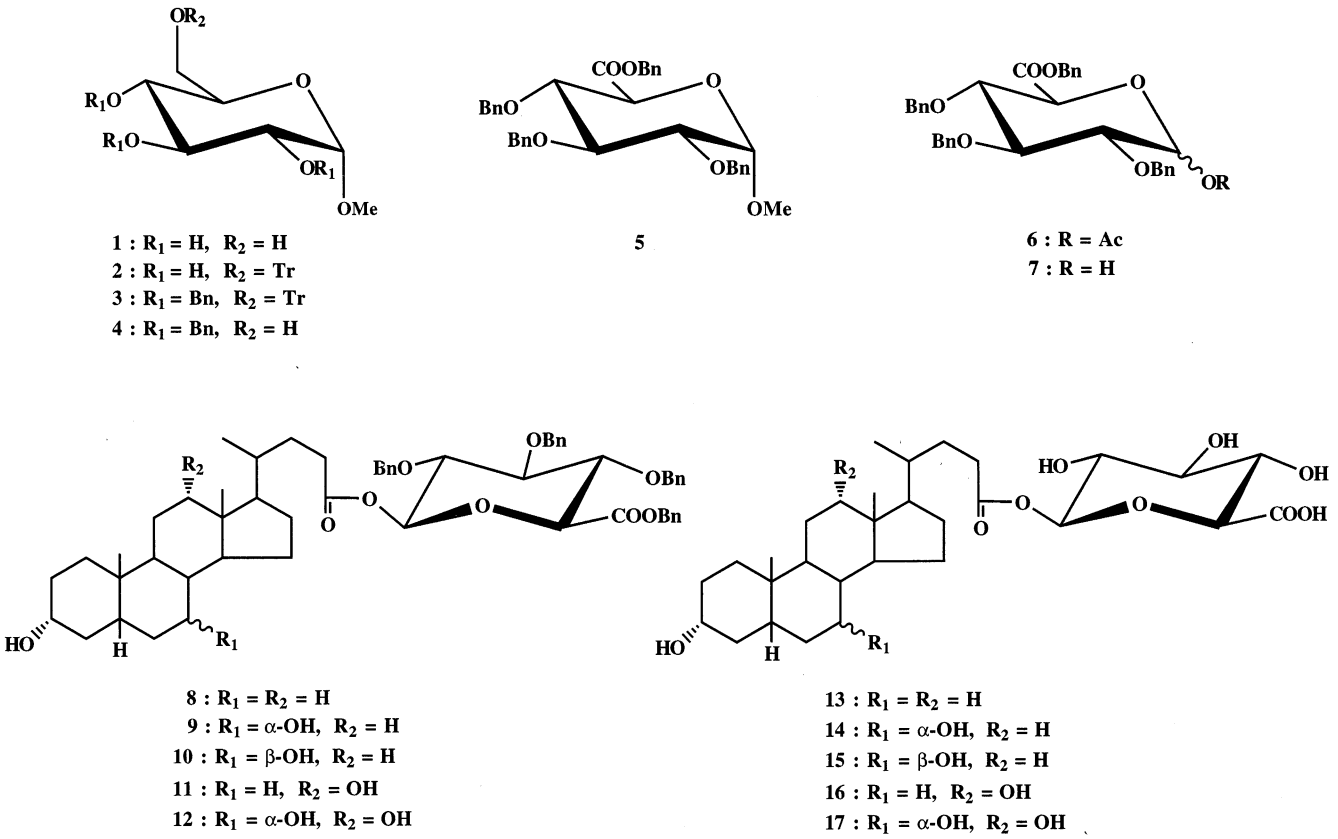

Figure 1 Structure of bile acid acyl glucuronides and its related compounds.

dles. m.p. 123–125°C. ^{1}H NMR ($CDCl_3$) δ: 3.45 (0.4H, dd, J = 6.9 and 9.3 Hz, 2-H (β-anomer)), 3.60 (0.6H, dd, J = 3.3 and 9.1 Hz, 2-H (α-anomer)), 3.66 (0.4H, t, J = 9.9 Hz, 4-H (β-anomer)), 3.76 (0.6H, t, J = 9.2 Hz, 4-H (α-anomer)), 3.86 (0.4H, t, J = 9.6 Hz, 3-H (β-anomer)), 3.94-4.03 (1.6H, unresolved m, 3- (α-anomer), 5-H (α- and β-anomer)), 4.46–5.24 (9H, m, 1-H (α- and β- anomer) and 4 × $CH_2C_6H_5$), 7.12–7.34 (20H, m, 4 × $CH_2C_6H_5$). Analysis calculated for $C_{34}H_{34}O_7$: C, 73.63; H, 6.18. Found: C, 73.89; H, 5.84.

Coupling reaction of bile acid with benzyl 2,3,4-tri-O-benzyl-D-glucopyranuronate (7)

Diethylazodicarboxylate (43 μL) was added to a stirred solution of **7** (160 mg) and bile acid (0.24 mmol) in the presence of triphenylphosphine (60 mg) in anhydrous tetrahydrofuran (2 mL) under an Ar gas stream. After stirring for another 3 h at room temperature, the solution was evaporated in vacuo and the residue obtained was diluted with AcOEt, which in turn was washed successively with 5% HCl, H_2O and saturated NaCl, dried on Na_2SO_4, and evaporated in vacuo. The crude product obtained was then purified as follows.

Benzyl 1-*O*-(24-lithocholyl)-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate (**8**): The crude product was subjected to column chromatography on silica gel (5 g) using *n*-hexane-AcOEt (7:1, v/v). The residue obtained was then submitted to preparative TLC using *n*-hexane-acetone (2:1, v/v) as a developing solvent. The spot corresponding to R_f value of 0.45 was scraped off and the desired compound was eluted with AcOEt. The residue was further purified by semipreparative HPLC with a Cosmosil 5-Ph column using H_2O-MeOH (1:10. v/v) as a mobile phase at a flow rate of 1 mL/min to give **8** (20.0 mg, 9.1%) as a semicrystalline product. ^{1}H NMR ($CDCl_3$) δ: 0.62 (3H, s, 18-H), 0.88 (3H, d, J = 6.3 Hz, 21-H), 0.92 (3H, s, 19-H), 3.63 (1H, m, 2′- and 3β-H), 3.73 (1H, t, J = 8.6 Hz, 4′-H), 3.85 (1H, t, J = 9.3 Hz, 3′-H), 4.10 (1H, d, J = 9.3 Hz, 5′-H), 4.42–5.16 (8H, m, 4 × $CH_2C_6H_5$), 5.67 (1H, d, J = 7.8 Hz, 1′-H), 7.10–7.33 (20H, m, 4 × $CH_2C_6H_5$). Analysis calculated for $C_{58}H_{72}O_9$: C, 76.28; H, 7.95. Found: C, 76.09; H, 8.30.

Benzyl 1-*O*-(24-chenodeoxycholyl)-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate (**9**): The crude product was subjected to column chromatography on silica gel (5 g) using *n*-hexane-AcOEt (2:1, v/v) and then semipreparative HPLC with a Cosmosil 5-Ph column using H_2O-MeOH (1:10, v/v) to give **9** (15.1 mg, 6.8%) as a semicrystalline product. ^{1}H NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-H), 0.89 (3H, d, J = 6.0 Hz, 21-H), 0.90 (3H, s, 19-H), 3.47 (1H, m, 3β-H), 3.63 (1H, t, J = 8.4 Hz, 2′-H), 3.73 (1H, t, J = 8.7 Hz, 4′-H), 3.85 (2H, m, 3′- and 7β-H), 4.10 (1H, d, J = 9.5 Hz, 5′-H), 4.42–5.20 (8H, m, 4 × $CH_2C_6H_5$), 5.67 (1H, d, J = 7.7 Hz, 1′-H), 7.09–7.32 (20H, m, 4 × $CH_2C_6H_5$). Analysis calculated for $C_{58}H_{72}O_{10}$: C, 74.97; H, 7.81. Found: C, 74.73; H, 8.13.

Benzyl 1-*O*-(24-ursodeoxycholyl)-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate (**10**): The crude product was subjected to column chromatography on silica gel (5 g) using *n*-hexane-acetone (5:1, v/v) and then submitted to preparative TLC using *n*-hexane-acetone (3:2, v/v) as a developing solvent. The spot corresponding to R_f value of 0.45 was scraped off and the desired compound was eluted with AcOEt to give **10** (25.2 mg, 11.3%) as a semicrystalline product. ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-H), 0.90 (3H, d, J = 6.3 Hz, 21-H), 0.94 (3H, s, 19-H), 3.56 (2H, m, 3β- and 7α-H), 3.63 (1H, t, J = 8.4 Hz, 2′-H), 3.73 (1H, t, J = 9.2 Hz, 4′-H), 3.84 (1H, t, J = 9.0 Hz, 3′-H), 4,10 (1H, d, J = 9.2 Hz,

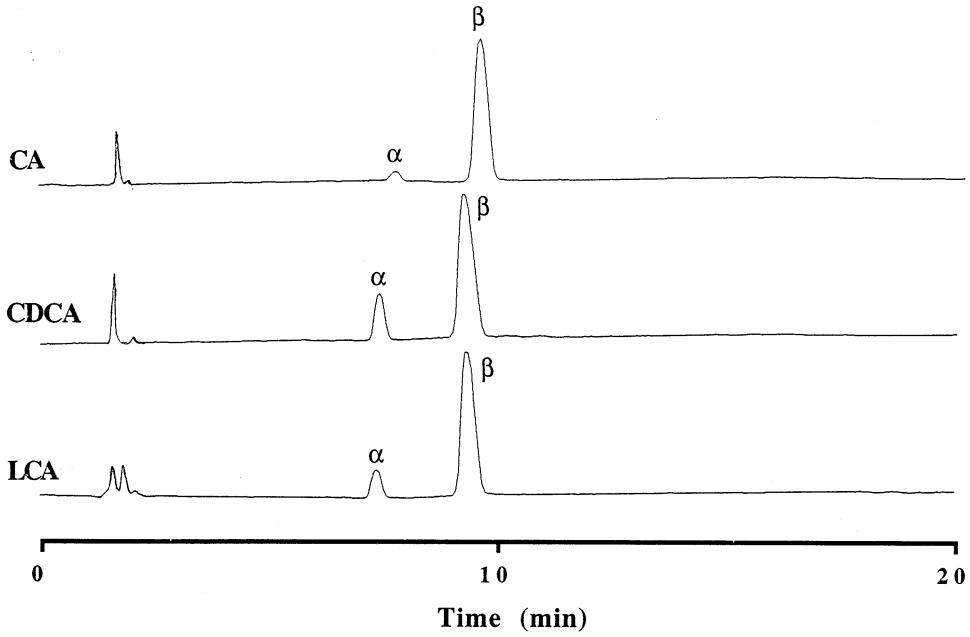

Figure 2 A typical high-performance liquid chromatogram of a synthetic product of benzyl 1-*O*-(24-chenodeoxycholyl)-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate. Conditions: column, Cosmosil 5-Ph; mobile phase, H_2O-MeOH (1:10, v/v); flow rate, 1.0 mL/min; detection, 260 nm.

5′-H), 4.42–5.17 (8H, m, 4 × CH_2C$_6$H$_5$), 5.67 (1H, d, J = 7.8 Hz, 1′-H), 7.03–7.31 (20H, m, 4 × CH$_2$C$_6$$H_5$). Analysis calculated for $C_{58}H_{72}O_{10}$: C, 74.97; H, 7.81. Found: C, 74.25; H, 7.58.

Benzyl 1-*O*-(24-deoxycholyl)-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate (**11**): The crude product was subjected to column chromatography on silica gel (5 g) using *n*-hexane-AcOEt (2:1, v/v) and then on Cosmosil 140C_{18}-OPN (8 g) using MeOH to give **11** (67.8 mg, 30.4%) as a semicrystalline product. ^{1}H NMR (CDC1$_3$) δ: 0.65 (3H, s, 18-H), 0.91 (3H, s, 19-H), 0.94 (3H, d, J = 6.3 Hz, 21-H), 3.63 (2H, m, 2′- and 3β-H), 3.73 (1H, t, J = 8.6 Hz, 4′-H), 3.84 (1H, t, J = 9.2 Hz, 3′-H), 3.96 (1, m, 1 2β-H), 4.10 (1H, d, J = 9.5 Hz, 5′-H), 4.41–5.15 (8H, m, 4 × CH_2C$_6$H$_5$), 5.67 (1H, d, J = 7.7 Hz, 1′-H), 7.03–7.31 (20H, m, 4 × CH$_2$C$_6$$H_5$). Analysis calculated for $C_{58}H_{72}O_{10}$: C, 74.97; H, 7.81. Found: C, 74.45; H, 8.18.

Benzyl 1-*O*-(24-cholyl)-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronate (**12**): The crude product was subjected to column chromatography on silica gel (5 g) using *n*-hexane-acetone (2:1, v/v) and then by semipreparative HPLC using H_2O-MeOH (1:8, v/v) to give **12** (21 mg, 9.3%) as a semicrystalline product. ^{1}H NMR (CDCl$_3$) δ: 0.66 (3H, s, 18-H), 0.89 (3H, s, 19-H), 0.95 (3H, d, J = 6.3 Hz, 21-H), 3.45 (1H, m, 3β-H), 3.63 (1H, t, J = 8.2 Hz, 2′-H), 3.73 (1H, t, J = 8.8 Hz, 4′-H), 3.84 (2H, m, 3′- and 7β-H), 3.96 (1H, m, 1 2β-H), 4,10 (1H, d, J = 9.5 Hz, 5′-H), 4.41–5.16 (8H, m, 4 × CH_2C$_6$H$_5$), 5.67 (1H, d, J = 7.7 Hz, 1′-H), 7.04–7.31 (20H, m, 4 × CH$_2$C$_6$$H_5$). Analysis calculated for $C_{58}H_{72}O_{11}$: C, 73.70; H, 7.68. Found: C, 73.32; H, 7.72.

Catalytic reduction

A mixture of 10% palladium on carbon (100 mg) and the benzyl ester-benzyl ether derivatives of bile acid 24-glucuronides (**8–12**, each 16 μmol) in AcOEt containing 1% AcOH (1 mL) was shaken under a H_2 atmospheric condition with a pressure of 5 Kbar at room temperature for 12 h. The resulting solution was centrifuged at 2600 rpm for 10 min and the supernatant was discarded. The precipitate was washed several times with 1 mL of AcOEt to remove less polar compounds and then layered onto the top of Cosmosil 140C_{18}-OPN (5 g) packed on a sintered glass filter. After washing with 30 mM AcOH, the adsorbates were eluted with 10 mM AcOH-EtOH (1:9, v/v) to give the desired compounds as colorless semicrystalline substances.

1-*O*-(24-Lithocholyl)-β-D-glucopyranuronic acid (**13**): m.p. 140–142°C. ^{1}H NMR (CD$_3$OD) δ: 0.69 (3H, s, 18-H), 0.94 (3H, s, 19-H), 0.95 (3H, d, J = 6.6 Hz, 21-H), 3.37 (1H, t, J = 10.0 Hz, 2′-H), 3.42 (2H, m, 3′- and 4′-H), 3.53 (1H, m, 3β-H), 3.67 (1H, d, J = 10.6 Hz, 5′-H), 5.49 (1H, d, J = 8.5 Hz, 1′-H). ESI-MS (*m*/*z*): 551 ([M-H]$^-$, 100%).

1-*O*-(24-Chenodeoxycholyl)-β-D-glucopyranuronic acid (**14**): m.p. 144–145°C. ^{1}H NMR (CD$_3$OD) δ: 0.69 (3H, s, 18-H), 0.92 (3H, s, 19-H), 0.96 (3H, d, J = 6.6 Hz, 21-H), 3.35–3.52 (5H, unresolved m, 2′-, 3′-, 4′-, 5′- and 3β-H), 3.79 (1H, m, 7β-H), 5.49 (1H, d, J = 7.8 Hz, 1′-H). ESI-MS (*m*/*z*): 567 ([M-H]$^-$, 100%).

1-*O*-(24-Ursodeoxycholyl)-β-D-glucopyranuronic acid (**15**): m.p. 121–123°C. ^{1}H NMR (CD$_3$OD) δ: 0.71 (3H, s, 18-H), 0.96 (3H, s, 19-H), 0.96 (3H, d, J = 6.6 Hz, 21-H), 3.34–3.57 (6H, unresolved m, 2′-, 3′-, 4′-, 5′-, 3β- and 7α-H), 5.49 (1H, d, J = 7.7 Hz, 1′-H). ESI-MS (*m*/*z*): 567 ([M-H]$^-$, 100%).

1-*O*-(24-Deoxycholyl)-β-D-glucopyranuronic acid (**16**): m.p. 164–166°C. ^{1}H NMR (CD$_3$OD) δ: 0.71 (3H, s, 18-H), 0.93 (3H, s, 19-H), 1.00 (3H, d, J = 6.2 Hz, 21-H), 3.36–3.69 (5H, unresolved m, 2′-, 3′-, 4′-, 5′- and 3β-H), 3.95 (1H, m, 1 2β-H), 5.50 (1H, d, J = 7.7 Hz, 1′-H). ESI-MS (*m*/*z*): 567 ([M-H]$^-$, 100%).

1-*O*-(24-Cholyl)-β-D-glucopyranuronic acid (**17**): m.p. 159–161°C. ^{1}H NMR (CD$_3$OD) δ: 0.71 (3H, s, 18-H), 0.91 (3H, s, 19-H), 1.01 (3H, d, J = 6.2 Hz, 21-H), 3.34–3.52 (5H, unresolved m, 2′-, 3′-, 4′-, 5′- and 3β-H), 3.80 (1H, m, 7β-H), 3.95 (1H, m, 12β-H), 5.50 (1H, d, J = 7.7 Hz, 1′-H). ESI-MS (*m*/*z*): 583 ([M-H]$^-$, 100%).

Results and discussion

The synthesis of acyl glucuronides requires D-glucuronic acid of which the hydroxy groups at C-2, 3 and 4 are

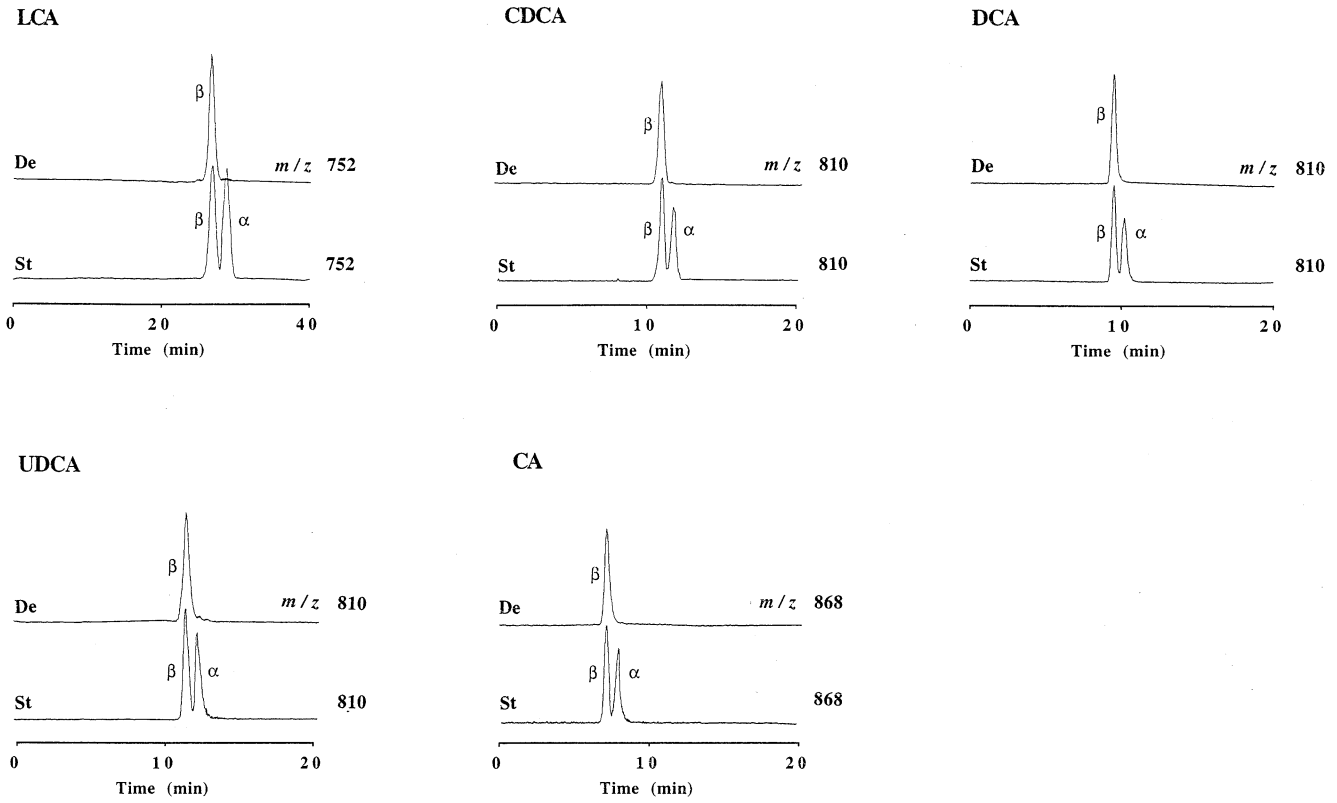

Figure 3 Selected ion recording of bile acid 24-glucuronide acetate-methyl esters monitored with corresponding adduct ion $[M + NH_4]^+$. Conditions: column, Sumichiral OA-2500 for DCA, UDCA and CA, and Cosmosil $5C_8$ for LCA and CDCA; mobile phase, 200 mM ammonium acetate (pH 7.0)-MeOH (1:4, v/v); flow rate, 1.0 mL/min. De, newly prepared sample; St, authentic specimen.

suitably protected. In the previous communication, we reported the synthesis of bile acid 24-glucuronide acetate-methyl esters by the use of the Mitsunobu reaction employing methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate.[12] This procedure is not satisfactory for the synthesis of the target compounds, because of the instability of the produced acyl glucuronides under basic conditions, under which the cleavage of the glycosyl linkage to give liberated free bile acids easily occurs. Therefore, for this purpose, D-glucuronic acid having groups easily removable under mild and non-hydrolytic conditions not only on hydroxy moieties but also on a carboxy moiety is really required. Recently, several methods have been published employing a D-glucuronic acid derivative protected with a benzyl,[14] a trichloroethyl,[15] an α-ethoxyethyl[16] or an allyloxy group.[16] Panfil et al. reported a prominent method for the synthesis of lithocholic acid 24-glucuronide using benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate which was derived from benzyl 2,3,4-tri-*O*-benzyl-1-*O*-methyl-D-glucopyranuronate.[16] The benzyl groups on the hydroxy and carboxy moieties were easily removed by catalytic hydrogenation. The latter compound, however, is not commercially available. Initial effort was therefore directed to the synthesis of benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate from methyl α-D-glucose employing the novel procedure (Figure 1). The primary hydroxy group at C-6 was protected with a trityl ether group (**2**) by treatment with triphenylmethyl chloride in pyridine. The protection of hydroxy groups at C-2, 3 and 4 to give a benzyl ether derivative (**3**) was attained by the condensation reaction of **2** with benzylbromide in the presence of sodium hydride and tetrabutyl ammonium iodide. Upon treatment with H_2SO_4, a trityl group of **3** was readily removed to produce a 6-hydroxy derivative (**4**). Subsequent oxidation of the primary hydroxy group with Jones reagent, followed by esterification with benzyl bromide in the presence of sodium bicarbonate in dimethylsulfoxide gave a benzyl ester derivative (**5**). The acetolysis of a methyl glycidic group with an equimolar amount of H_2SO_4 in a mixed solvent of acetic acid and acetic anhydride yielded a mixture of α- and β-anomers of an 1-*O*-acetyl derivative (**6**). The hydrolysis with 2-aminoethanol in AcOEt containing 1% dimethylsufoxide afforded a 3:2 mixture of α- and β-anomers of the desired benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate (**7**) in a satisfactory yield.

The synthesis of acyl glucuronides of bile acids were then undertaken. The condensation of a carboxy group of bile acids with an anomeric hydroxy group of the glucuronic acid derivatives (**7**) was achieved by the use of the Mitsunobu reaction employing triphenylphosphine and diethylazodicarboxylate in tetrahydrofuran.[17] No alternative coupling with a hydroxy group on a steroid nucleus to form a bile acid dimer was observed. In the previous paper, we have disclosed that the β-anomer of bile acid 24-glucuronides was produced only in rat liver microsomal fractions.[12] Advantageously, the condensation reaction gave exclusive formation of the β-anomer with a small amount of

the α-anomer. Purification of the desired β-anomers of the acyl glucuronides derived from lithocholic, chenodeoxycholic and cholic acids were accomplished by semi-preparative HPLC on a column packed with phenyl-bonded silica using H_2O-MeOH (1:8 or 1:10) as a mobile phase. The β-anomers of deoxycholic and ursodeoxycholic acid 24-glucuronides could be separated and purified by preparative TLC. A typical high-performance liquid chromatogram of a mixture of α- and β-anomers of benzyl 1-*O*-(24-chendeoxycholyl)-2,3,4-tri-*O*-benzyl-D-glucopyranuronate is illustrated in Figure 2. The structural confirmation of the isolated bile acid 24-glucuronide benzyl ester-benzyl ether derivatives was performed by ^{1}H NMR. The anomeric proton signal of the sugar moiety appeared at 5.67 ppm (J = 7.7–7.8 Hz), indicating the β-glucuronosyl linkage. Finally, the removal of the protecting groups was attained by hydrogenation with palladium on carbon in AcOEt containing 1% AcOH to give the acyl glucuronides. ^{1}H NMR spectra of these glucuronides also showed the anomeric proton signals at 5.49–5.50 ppm (J = 7.7–8.5 Hz). On the ESI-MS analysis, these bile acid 24-glucuronides exhibited a singly charged intact ion $[M\text{-}H]^-$ at *m/z* 551 for lithocholic acid 24-glucuronide, 567 for chenodeoxycholic, deoxycholic and ursodeoxycholic acid 24-glucuronides, and 583 for cholic acid 24-glucuronide, respectively. It is noteworthy that bile acid acyl glucuronides did not undergo acyl migration under these reaction and purification conditions described above. In the previous paper, we have reported the separatory determination of bile acid 24-glucuronide acetate-methyl esters by LC/APCI-MS.[12] Accordingly, structural confirmation was further carried out by this method with the aid of authentic specimens. After conversion of bile acid 24-glucuronides into their acetate-methyl esters in the manner previously reported, the derivatives were subjected to an LC/APCI-MS analysis with selected ion monitoring. The retention times of peaks on the chromatogram were definitely identical with those of corresponding authentic β-anomers (Figure 3). No contamination of the α-anomer in the synthesized glucuronides was observed.

The availability of these authentic samples may be helpful for studies on the metabolism and disposition of acyl glucuronides of bile acids, and details will be reported elsewhere in the near future.

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