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Bile alcohol glucuronides: regioselective *O*-glucuronidation of 5β-cholestane-3α,7α,12α,25-tetrol and 24-nor-5β-cholestane-3α,7α,12α,25-tetrol *

Bishambar Dayal [a], Gerald Salen [a], Janak Padia [a], Sarah Shefer [a], George S. Tint [a], Gino Sasso [b] and Tomas H. Williams [b]

[a] *Department of Medicine, University of Medicine and Dentistry of New Jersey – New Jersey Medical School, Newark, NJ 07103 (USA) and Veterans Administration Medical Center, East Orange, NJ 07109 (USA)*

[b] *Chemical Research Department, Hoffmann La Roche, Nutley, NJ 07110 (USA)*

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ABSTRACT

A facile and regiocontrolled procedure for the preparation of 5β-cholestane-3α,7α,12α,25-tetrol-3-*O*-β-D-glucuronide and its corresponding C-26 analogue is described. The method involves direct coupling of bile alcohols, namely, 5β-cholestane-3α,7α,12α,25-tetrol and 24-nor-5β-cholestane-3α,7α,12α,25-tetrol to methyl (tetra-*O*-acetyl-β-D-glucopyranuronate) in the presence of a Lewis acid, tin(IV) chloride, in dichloromethane. The resulting anomeric pairs of 1,2-*trans*- and 1,2-*cis*-glucuronides of tetrols were resolved by analytical and preparative thin-layer chromatography, and their identities were established by high-resolution ^{1}H NMR spectroscopy and by chemical-ionization and fast-atom-bombardment mass spectrometry. The method described has a practical advantage over the traditional two-step synthesis involving bromides as it is more efficient and uses inexpensive and less toxic materials. It is suggested that these compounds will be useful for studying permeability of the blood–brain barrier in cerebrotendinous xanthomatosis (CTX).

INTRODUCTION

Recent observations from our laboratory have indicated that bile acid synthesis is abnormal in patients with the rare inherited lipid storage disease, cerebrotendinous xanthomatosis (CTX) and that a severe neurologic disease develops in these subjects[1–4]. As a consequence of this abnormal bile acid biosynthesis, stereochemically defined bile acid precursors, namely, 5β-cholestane-3α,7α,12α,25-tetrol,

Correspondence to: Dr. B. Dayal, Gastrointestinal Research Laboratory, Veterans Administration Medical Center, East Orange, NJ 0701, USA.

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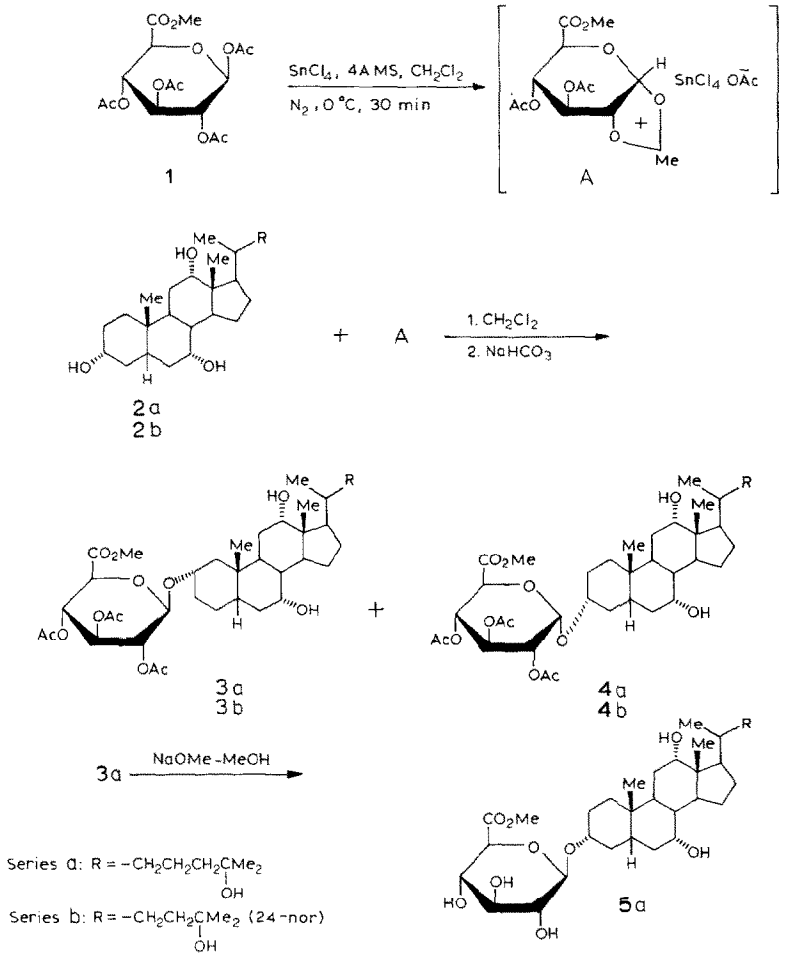
(23*R*)-5β-cholestane-3α,7α,12α,23,25-pentol and (24*R*)-5β-cholestane-3α,7α,12α,24,25-pentol[5–9], which are glucuronidated at C-3[10–12] and circulate in the plasma[13], may damage the blood–brain barrier[14] and lead to the development of neurologic disease. Furthermore, it has been suggested[15–17] that a defect in hepatic microsomal (24*S*)-hydroxylation blocks the transformation of 5β-cholestane-3α,7α,12α,25-tetrol into (24*S*)-5β-cholestane-3α,7α,12α,24,25-pentol and cholic acid in CTX patients. As a result, the 5β-cholestanetetrol accumulates in the liver where it undergoes glucuronidation[16]. A portion of the newly formed 5β-cholestane-3α,7α,12α,25-tetrol glucuronide is secreted into the bile with the remainder overflowing into the plasma where it reaches the kidney and undergoes renal hydroxylation to give the 5β-cholestane-3α,7α,12α,23,25-pentols, 5β-cholestane-hexols and -heptols. Furthermore, a mitochondrial 26-hydroxylation is also abnormal, and that process may also lead to the accumulation of the same bile alcohol glucuronides[18–20]. Therefore, in order to study these transformations of bile alcohols, we required an efficient preparation of 5β-cholestane-3α,7α,12α,25-tetrol-3-*O*-β-D-glucuronide in sufficient quantities to permit evaluation of its biological effects on the blood–brain barrier and kidney hydroxylation reactions in CTX.

Scheme 1 illustrates a facile and regioselective approach that provides access to the novel group of 5β-cholestanetetrol-3-*O*-glucuronides. In this report we have exemplified our approach by the synthesis of 5β-cholestane-3α,7α,12α,25-tetrol-3-*O*-glucuronides, as well as by the first preparation of their corresponding C-26 analogue having one carbon less in the side-chain. The method involved direct coupling of bile alcohols, namely, 5β-cholestane-3α,7α,12α,25-tetrol and 24-nor-5β-cholestane-3α,7α,12α,25-tetrol to methyl (tetra-*O*-acetyl-β-D-glucopyranuronate) in the presence of the Lewis acid, tin(IV) chloride, in dichloromethane. Although the use of tin(IV) chloride as a coupling reagent has previously been described[21–23], these studies were limited to monohydroxy compounds, only.

EXPERIMENTAL

Materials and general procedures. — Melting points were determined on a Thermolyne apparatus (Thermolyne Corp., Dubuque, IA), model MP-126000, and are uncorrected. The anomeric mixtures of C-27 and C-26 5β-cholestanetetrol glucuronides were resolved and analyzed by thin-layer chromatography using 0.25-mm E. Merck precoated Silica Gel-60 plates (F-254) with the solvent system 70:20:0.5 $CHCl_3$–$(Me)_2CO$–MeOH. The spots were made visible with phosphomolybdic acid (3.5% in 1:1 2-propanol–H_2SO_4). Preparative thin-layer chromatography was performed on 0.25 mm × 20 × 20 cm E. Merck precoated Silica Gel-60 (F-254). The bile alcohols (5β-cholestane-3α,7α,12α,25-tetrol and its corresponding C-26 analogue), as their trimethylsilyl (Me_3Si) derivatives, were analyzed on a 180 cm × 4 mm column packed with 3% OV-17 on 80/100 mesh Gas-Chrom Q (Applied Science Laboratories); column temperature 270°C, N_2 flow rate 40



Scheme 1.

mL/min (Hewlett–Packard model 7610 gas chromatograph, Palo Alto, CA). High-resolution mass spectra (HRMS) were obtained by the chemical-ionization (CI) technique (isobutane, source temperature 200°C, Varian MAT-731 spectrometer). Low-resolution mass data were obtained from electron-impact (EI) spectra (Varian MAT-5), and optical rotations were measured in $CHCl_3$ on a Perkin–Elmer (Norwalk, CT) model 141 polarimeter. Direct-probe mass spectrometric analyses of synthesized bile alcohol (C-27 and C-26-tetrol) glucuronides were performed by FAB-mass spectrometry using a ZAB-IF mass spectrometer as previously described[10]. The FAB technique has emerged as a simple and extremely versatile ionization process that is particularly well suited to biomedical applications. Although negative-ion FABMS is much more satisfactory than the positive-ion mode, this technique was not available to us at the time of this research. In the FAB mode (positive ions), the sample was dissolved in thioglycerol, and a small

aliquot (1–2 μL) was placed on the direct inlet-probe. After insertion into the mass spectrometer, the sample was bombarded with a neutral-atom Xe beam having approximately 6 kV of translational energy. ^{1}H NMR spectra were recorded on a Varian Associates XL-400 spectrometer operating in the Fourier-transform mode. All spectra were taken in $CDCl_3$ solution with Me_4Si as the internal standard. 5β-Cholestane-3α,7α,12α,25-tetrol (mp 188–190°C) and 24-nor-5β-cholestane-3α,7α,12α,25-tetrol (mp 124–126 °C) were synthesized and characterized according to Dayal et al.[6,24].

All reactions were carried out under anhydrous conditions under N_2 in flame-dried glass apparatus using dry, freshly distilled solvents. Dichloromethane was distilled under N_2 from CaH_2 and stored over activated 3A molecular sieves. The progress of the glucuronidation reaction was monitored by thin-layer chromatography (TLC) on silica gel plates (0.25 mm thickness). Activation of crushed or pellet 4A molecular sieves involved heating in a vacuum oven at 160°C and 0.05 mm pressure for at least 3 h. Upon workup, the powdered 4A sieves remained with the aqueous phase, and no filtration was generally necessary. Glucuronolactone and tin(IV) chloride in Sure/Seal™ bottles were used as obtained from Aldrich Chemical Co. Enzymic (β-glucuronidase) hydrolysis of anomeric glucuronides was performed as previously described[25].

*Preparation of methyl tetra-O-acetyl-β-*D*-glucopyranuronate* (**1**). — Compound **1** was prepared according to the procedure of Bollenback et al.[26]. Briefly, glucuronolactone (17.6 g) was dissolved in MeOH which contained 32 mg of NaOH. After stirring the mixture at room temperature for 3 h, unchanged glucuronolactone was removed by filtration, and the methanolic solution was concentrated to dryness in vacuo. The residue (methyl glucuronate) when subjected to pyridine-catalyzed acetylation, followed by storing the mixture of 0°C overnight provided, after filtration, 10.5 g of white crystals of methyl tetra-*O*-acetyl-β-D-glucopyranuronate. Recrystallization from acetone yielded 8.90 g of pure **1**; mp 177–178°C; $[\alpha]_D^{25}$ +7.5° (*c* 1.0, $CHCl_3$); (lit.[26] mp 176–178°C); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.04 (s, 3 H, OAc), 2.05 (s, 3 H, OAc), 2.06–2.1 (s, 6 H, 2OAc), 3.76 (s, 3 H, CO_2CH_3), 4.18 (d, 1 H, H-5, $J_{5,4}$ 9.3 Hz), 5.25 (t, 1 H, H-4, $J_{4,5}$ 9.3 Hz), 5.31 (t, 1 H, H-3, $J_{2,3}$ 9.3 Hz), 5.15 (dd, 1 H, H-2, $J_{2,3}$ 9.3 Hz), 5.77 (d, 1 H, H-1, $J_{1,2}$ 7.8 Hz).

*Preparation of 7α,12α,25-trihydroxy-3α-[*O*-(methyl 2,3,4-tri-*O*-acetyl-β-*D*-glucopyranosyluronate)]-5β-cholestane* (**3a**) *and 7α,12α,25-trihydroxy-3α-[*O*-methyl 2,3,4-tri-*O*-acetyl-α-*D*-glucopyranosyoluronate)]-5β-cholestane* (**4a**). – To a 3-necked, 50-mL round-bottom flask equipped with an N_2 inlet, a magnetic stirrer and two septum inlets, were added, under strictly anhydrous conditions, 18.8 mg (0.05 mmol) of methyl tetra-*O*-acetyl-β-D-glucopyranuronate (**1**) in 15 mL of CH_2Cl_2 and 0.1 mL of $SnCl_4$ (not equimolar) containing 100–150 mg of crushed 4A molecular sieves. The flask and the contents were cooled in an ice bath under N_2. After 15 min a solution of 5β-cholestane-3α,7α,12α,25-tetrol (21.8 mg, 0.05 mmol) in the same solvent (2mL) was added over a period of 12 min. Stirring was continued for 0.5–3 h at room temperature, and the reaction was monitored by

TLC. When no further reaction could be detected, the mixture was extracted with CH_2Cl_2, followed by $NaHCO_3$ and NaCl washings of the organic phase, and drying (Na_2SO_4). After solvent removal, the crude material (16 mg) was purified by chromatography through a Pasteur pipet filled with a slurry of silica gel in 97:3 $CHCl_3$–$(Me)_2CO$ to give 13 mg (60%) of product, which was further resolved by analytical and preparative TLC in 70:20:0.5 $CHCl_3$–$(Me)_2CO$–MeOH. The two glucuronide bands were scraped off and extracted to give **3a** (R_f 0.69, 5 mg) and **4a** (R_f 0.62, 4.8 mg) for a total yield of 46.6%. CIMS data for both anomers, **3a** and **4a**: *m/z* 718 [18%, (M + H − H_2O − OH)], 694 [100%, (M + H − AcO)], 632 (47%, (M − 2AcOH)], 555 [45%, (M + H − 3AcOH − H_2O)], 504 [94%, (M − 3AcOH − MeOH − H_2O)], 462 [87%, (504 − CH_2=C=O)], 461 [85%, (504 + H − CH_2=C=O)], 317 [63%, ($C_{13}H_{17}O_8$, pyronium ion)], and 257 [95%, ($C_{11}H_{13}O_7$)]. FABMS data on the penta-*O*-acetyl-acetyl derivative of both **3a** and **4a**: *m/z* 837 (M + H), 795 (M + H − CH_2=C=O), and 753 (M + H − 2CH=C=O).

Regioselective O-*glucuronidation of 24-nor-5β-cholestanetetrol: Preparation of 7α,12α,25-trihydroxy-3α-[*O-*(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)]-24-nor-5β-cholestane* (**3b**) *and 7α,12α,25-trihydroxy-3α-*[O-*(methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate)]-24-nor-5β-cholestane* (**4b**).—Both α and β anomers (**3b** and **4b** of 24 nor-5β-cholestane-3α,7α,12α,25-tetrol were obtained in essentially a 1:1 ratio according to the protocol as detailed above for the preparation of 5β-cholestane-3α,7α,12α,25-tetrol-3-*O*-glucuronides **3a** and **4a**. The two anomers were resolved by analytical and preparative TLC in the same solvent system as described, with R_f 0.46 and 0.58, respectively, for **3b** and **4b**.

Partial ^{1}H NMR data (400 MHz, $CDCl_3$): *A*. For **3b** (β anomer). δ 4.69 (d, 1 H, H-1, $J_{1,2}$ 7.5 Hz), 5.02 (dd, 1 H, H-2, $J_{2,3}$ 9.5 Hz), 5.27 (dd, 1 H, H-3, $J_{2,3} = J_{3,4} = 9.5$ Hz), 5.24 (dd, 1 H, H-4), 3.98 (d, 1 H, H-5, $J_{4,5}$ 9.5 Hz). *B*. For **4b** (α anomer). δ 5.36 (d, 1 H, H-1, $J_{1,2}$ 3 Hz), 4.84 (dd, 1 H, H-2, $J_{2,3}$ 10 Hz), 5.67 (dd, 1 H, H-3, $J_{3,4}$ 10 Hz), 5.14 (dd, 1 H, H-4, $J_{3,4} = J_{4,5} = 10$ Hz), 4.58 (d, 1 H, H-5, $J_{4,5}$ 10 Hz). FABMS data (positive-ion) for **3b**: *m/z* 739 (M + H), 721 (M + H − H_2O), 703 (M + H − H_2O), 685 (M + H − $3H_2O$); for **4b**: an *m/z* 761 (M + Na) was observed, in addition to the peaks observed for **3b**.

*Hydrolysis of 7α,12α,25-trihydroxy-5β-cholestan-3α-*O-*(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)* (**3a**) to *7α,12α,25-trihydroxy-5β-cholestan-3α-*O-*(methyl β-D-glucopyranosiduronate* (**5a**).—To a solution of (4 mg, 0.009 mmol, dried over P_2O_5 under vacuum) of 7α,12α,25-trihydroxy-5β-cholestan-3α-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate) in hot, dry MeOH (0.5 mL) were added 2 drops of NaOMe (prepared from 15 mg of Na metal in 1 mL of dry MeOH). The solution was warmed to 45°C for 10 min and then allowed to cool. The MeOH was evaporated, and the residue was dissolved in 4 mL of distilled water. Addition of a few drops of dil acetic acid resulted in a white, flocculent precipitate which was centrifuged. The supernatant liquid was decanted, and the residue was purified by passage through a Pasteur pipet filled with a slurry of silica gel in 1:3 $CHCl_3$–$(CH_3)_2CO$, which then provided 2.6 mg of compound **5**.

Enzymatic hydrolysis of 7α,12α,25-trihydroxy-5β-cholestan-3α-O-(methyl β-D-glucopyranosiduronate (**5a**).—Compound **3** (ca 500 μg) was dissolved in acetate buffer (5 mol/L, pH 4.8), and an enzyme preparation [aryl sulfatase (EC 3.1.6.1) with β-glucuronidase activity, Sigma type 1-H, from *Helix pomatia*] was added. Hydrolysis was allowed to proceed for 48 h at 37°C. The incubated solution was treated with 3 drops of dil KOH[25] and extracted with $CHCl_3$. The $CHCl_3$ layer was evaporated to dryness at 60°C under N_2, and the liberated 5β-cholestane-3α,7α,12α,25-tetrol which was then identified by direct comparison (GLC–MS) with an authentic standard as previously described[6–9]. For 5β-cholestane-3α,7α,1α,25-tetrol, $T_R = 2.60$ min ($T_R = 6.42$ min, 5α-cholestane standard). Under identical conditions the α anomer (prepared from **4a**) was resistant to the action of β-glucuronidase.

DISCUSSION

This paper describes a one-step, low temperature, rapid glulcuronidation procedure that is carried out directly on the unprotected 3-hydroxy group of bile alcohols, 5β-cholestane-3α,7α,12α,25-tetrol and 24-nor-5β-cholestane-3α,7α,12α,25-tetrol that employs a Lewis acid, tin(IV) chloride[21–23,28], as an activation agent, which leads to the formation of α,β anomeric tetrol-3-*O*-glucuronides **3a** and **4a** and **3a** and **4b** (Scheme 1).

The usual reaction of the classical Koenigs–Knorr type[29] involves the prior preparation of the α-D-glycosyl bromide[29–31] and condensation with the alcohol using silver carbonate or silver oxide. These intermediates and promoters are necessarily prepared fresh before use. Also, glycosyl bromides, which in particular allow greater reactivity at the anomeric center, have proven in many cases to either be available in poor yield, or to be inaccessible by the traditional procedure which involves the use of anhydrous HBr in CH_2Cl_2 or CH_3COOH acting upon an anomeric acetate. Furthermore, the recently discovered brominating agents such as $(Me)_3SiBr$ Or $TiBr_4$ can enhance the yield of the glycosyl bromides, but the isolation procedure is cumbersome and time consuming[30,31].

In contrast, $SnCl_4$-mediated reaction conditions are simpler to carry out requiring only the preparation of methyl tetra-*O*-acetyl-β-D-glucopyranuronate (**1**). In this procedure the preliminary activation of acetylated glucuronic acid methyl ester with $SnCl_4$ provides the activated complex (Scheme 1), which when reacted with the 3α-hydroxy group of either bile alcohol **2a** or **2b** yielded a mixture of α- and β-D-glucuronides in a ratio of ~ 1 : 1. These α,β anomeric ratios were determined by isolation of pure anomers by preparative thin-layer chromatography[32].

The structures of the tetrol-3-*O*-glucuronides **3a** and **4a** were confirmed by chemical-ionization (CI) and fast-atom-bombardment (FAB) mass spectrometry. Analysis of the fragmentation patterns of **3a** and **4a** in the chemical-ionization (CI) mode furnished no molecular ion, but showed a strong fragmentation at *m/z* 694

(100%), indicating the loss of acetic (or methyl formate) from the molecular ion. This loss of $CH_3CO_2^+$ ion or CH_3CO_2H (mass units 59 or 60) has been rationalized either from the molecular ion (m/z 752) or m/z 753 (M + H). In the case of tetrol glucuronide, loss of 59 units from the (M + 1) fragment constitutes a major peak at m/z 694 (100% intensity). Subsequent ions at m/z 632 (47%), 555 (45%), and 504 (94%) include the fragments (M − $2CH_3COOH$), [(M + 1) − $3CH_3COOH$ + H_2O] and [M − ($3CH_3COOH$ + CH_3OH + $2H_2O$ + 2H)], respectively. Furthermore, the loss of ketene [CH_2=C=O (42) or CH_3CO^+ (43)] from the fragment 504 exhibited ions at m/z 462 and 461, respectively. Ions at m/z 718 (18%) are the result of the loss of one molecule of water and hydroxyl ion (H_2O + OH) from (M + 1). Due to the presence of a tertiary hydroxyl group at C-25 in the sterol part of the molecule, a simultaneous loss of hydroxyl ion radical, accompanied by the loss of an H_2O molecule from the nucleus, gives the ion at m/z 718[27]. The sugar moiety was detected intact as the m/z 317 $(C_{13}H_{17}O_9)^+$, (pyronium ion) and was also represented by the ion m/z 257 $(C_{11}H_{13}O_7)^+$, formed via loss of CH_3COOH or methylformate) from the 317 fragment.

During the course of these studies, it was found that when the reaction was left stirring overnight transacetylation of the tetrol moiety at positions C-7 and C-12 took place. This compound having R_f 0.92 in 70:20:0.5 $CHCl_3$–$(Me)_2CO$–MeOH was also resolved by analytical and preparative TLC in the same solvent system. The fast-atom-bombardment (FAB) mass spectra in the positive-ion mode gave a major significant molecular ion species as its penta-*O*-acetyl derivative at m/z 837 $(M + H)^+$. Subsequent ions at m/z 795 [$(M + H)^+ - (CH_2CO)$], and 753 [$(M + H)^+ - (2CH_2CO)$], indicating the loss of one and two ketene units, were also observed. This method unambiguiously determined the molecular weight of the synthesized sample of tetrol glucuronide as its penta-acetyl derivative, **3a** or **4a** (Scheme 1).

In order to illustrate further that only the least hindered 3-hydroxy group of the bile sterol is involved in the direct coupling of glucuronic acid, model studies were performed on its easily accessible analogue, namely, 24-nor-5β-cholestane-3α,7α,12α,25-tetrol[24], which is a C-26-analogue of the naturally occurring C-27 bile alcohol, 5β-cholestane-3α,7α,12α,25-tetrol.

As summarized in the experimental section, the (FABMS) spectra in the positive-ion mode for α,β anomeric 24-nor-tetrol glucuronides **3b** and **4b** (Scheme 1) gave a molecular-ion peak at m/z 739 $(M + H)^+$ and other major ions at 721, 703, and 685, resulting from the subsequent loss of one, two, and three water molecules, respectively.

In the ^1H NMR spectra of 3-*O*-β-D-glucuronides of 24-nor tetrol, the ratio of intensities of sugar signals to steroidal signals indicated the presence of one glucuronic acid per bile alcohol molecule. Signals for the methoxyl and acetoxyl groups were compatible with the sugar being attached to a 3-hydroxyl group of the bile alcohols. Moreover, the C-7 and C-12 hydroxyl groups in the tetrols are extremely hindered, and glycosylation at C-7 does not take place as this position is

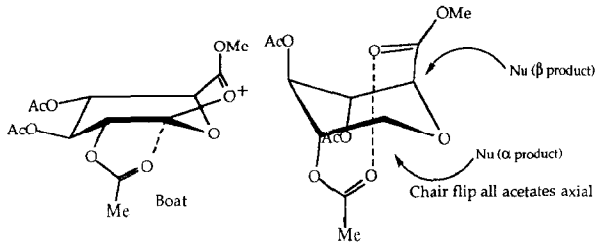


Fig. 1. The C-2 acetate in the boat and chair forms of the tetra-*O*-acetyl-D-glucoronic acid methyl ester moiety.

extremely hindered due to an unfavorable 1,3-diaxial interaction with the C-4 methylene in the A ring. Similar 1,3 diaxial interaction is also operative at C-12.

The assignment of the α- and β-anomeric configurations was confirmed by the chemical shifts of the glucuronic acid moiety, which are characteristic for a hydroxyl-linked glucuronide. The large coupling constant between H-1 and H-2 ($J_{1,2}$ 7.5 Hz) indicates a trans-diaxial configuration relative to both protons and thus established the β-glycosidic linkage. Examination of the proton NMR spectrum of the α anomer revealed doublets at δ 3.98 (H-5) and 4.69 (H-1), and a doublets of doublets at δ 5.02, 5.24, and 5.27. The above five-proton subspectrum was indicative of α-D-glucopyranosyluronate stereochemistry. Further assignment of the α-anomeric configuration was based on the sequence of coupling constants for this subspectrum. The equatorial–axial relative configuration of both protons was illustrated by the coupling constant between H-1 and H-2 ($J_{1,2}$ 3 Hz) in its ^{1}H NMR spectrum.

Insofar as the issue of the 1 : 1 α,β-stereoselectivity is concerned, we think that it is quite possible that the C-6 ester functionality is participating in the transition state in which case the α-product would result. The C-2 acetate, as shown in both boat and chair forms of the tetra-*O*-acetyl-D-glucuronic acid methyl ester moiety (Fig. 1), is also expected to participate, in which case the β-product would result. Along these lines we have reasoned that in order for the methyl ester of C-6 to participate, the conformation of the glucuronic acid moiety would be such that either a boat or chair flip would have to occur. If this is indeed the case, then changing the C-1 acetate (**1**, Scheme 1) to a glycosyl bromide would probably not be much better, as participation from the both substituents (C-6 and C-2) would still occur.

Complete hydrolysis of acetylated α and β anomers (**3a** and **4a**, Scheme 1) to yield the free glucuronide of the tetrol was readily achieved by treatment with sodium methoxide in methanol. The deacetylated β anomer, 7α,12α,25-trihydroxy-5β-cholestan-3α-*O*-(methyl-β-D-glucopyranosiduronate) (**5**) was efficiently hydrolyzed by the enzyme, β-glucuronidase, and 5β-cholestane-3α,7α,12α,25-tetrol was released[25]. On the other hand, the α anomer was resistant to hydrolysis by this enzyme, thus establishing a biochemical difference between the pair of conjugates.

CONCLUSIONS

We have described a mild method for *O*-glucuronidation of the highly reactive 3-hydroxyl group of 5β-cholestane-3α,7α,12α,25-tetrol and its corresponding C-26 analogue using $SnCl_4$. The characteristic features of the proton NMR spectra of the α and β anomers of the 24-nor tetrol glucuronide, which are presented, are helpful in the assignment of product stereochemistry and anomeric ratios of bile alcohol glucuronides. The extremely mild conditions utilized (ambient or lower temperatures, neutrality, convenient and inert solvents) should make this procedure the method of choice in complex and sensitive cases. Finally, the availability of the 3-*O*-β-D-glucuronide of 5β-cholestane-3α,7α,12α,25-tetrol and its analogue having the correct anomeric configuration will help us to study its physiological function in the disease, cerebrotendinous xanthomatosis (CTX).

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