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Chemical synthesis of 3β-sulfooxy-7β-hydroxy-24-nor-5-cholenoic acid: An internal standard for mass spectrometric analysis of the abnormal Δ^5-bile acids occurring in Niemann-Pick disease

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ABSTRACT

In Niemann-Pick disease, type C1, increased amounts of 3β,7β-dihydroxy-5-cholenoic acid are reported to be present in urinary bile acids. The compound occurs as a tri-conjugate, sulfated at C-3, *N*-acetylglucosamidated at C-7, and *N*-acylamidated with taurine or glycine at C-24. For sensitive LC–MS/MS analysis of this bile acid, a suitable internal standard is needed. We report here the synthesis of a satisfactory internal standard, 3β-sulfooxy-7β-hydroxy-24-nor-5-cholenoic acid (as the disodium salt). The key reactions involved were (1) the so-called "second order" Beckmann rearrangement (one-carbon degradation at C-24) of hyodeoxycholic acid (HDCA) 3,6-diformate with sodium nitrite in a mixture of trifluoroacetic anhydride and trifluoroacetic acid, (2) simultaneous inversion at C-3 and elimination at C-6 of the ditosylate derivatives of the resulting 3α,6α-dihydroxy-24-nor-5β-cholanoic acid with potassium acetate in aqueous *N*,*N*-dimethylformamide, and (3) regioselective sulfation at C-3 of an intermediary 3β,7β-dihydroxy-24-nor-Δ^5 derivative using sulfur trioxide–trimethylamine complex. Overall yield of the desired compound was 1.8% in 12 steps from HDCA.

1. Introduction

Alvelius et al. recently reported that the urine of a patient with Niemann-Pick disease type C1 (NP-C1) had a unique bile acid pattern as evidenced by the presence of Δ^5-bile acid constituents when analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) [1]. Increased excretion of the 3β,7β-dihydroxycholest-5-en-24-oic acid and its 7-oxo derivative (which occur in conjugated form) might be pathognomonic for this disease and its detection in urine could well aid in its diagnosis.

In a previous paper, we have prepared a series of the multi-conjugates of naturally occurring 3β,7β-dihydroxy-5-cholen-24-oic acid and 3β-hydroxy-7-oxo-5-cholen-24-oic acid as authentic reference compounds (**1a**–**1c** and **2a**–**2c**). These compounds were prepared as "multi-conjugates" in that they were esterified with sulfuric acid at C-3, with *N*-acetylglucosamine (GlcNAc) at C-7, and/or with glycine (or taurine) at C-24 (Fig. 1) [2].

For the purpose of a highly selective, sensitive, accurate analysis of such polar and hydrophilic Δ^5-bile acid multi-conjugates, the use of LC–MS/MS, coupled with the availability of a suitable internal reference standard, seems to be the best analytical method [3–7]. A satisfactory internal reference standard for the LC–MS/MS analysis is, therefore, desirable. As part of a program in our laboratory to prepare novel bile acids that have clinical utility, we undertook the chemical synthesis of the four variants of unnatural bile acid conjugates (Fig. 2). Of the four candidate compounds prepared, 3β-sulfooxy-7β-hydroxy-24-nor-5-cholenoic acid (**IV**) was found to be the most suitable for the LC–MS/MS analysis of a complicated mixture of naturally occurring **1a**–**1c** and **2a**–**2c**. We also report here the mobility of **IV** on C_{18} reversed-phase high-performance liquid chromatography (RP-HPLC) and its suitability as an internal standard.

2. Results and discussion

The four variants of candidate compounds (**I**–**IV**) were designed on the basis of the hydrophilic–hydrophobic balance in steroid molecules. The literature indicates that multiple factors influence the mobility of bile acids when separated by RP-HPLC. These include the position, number, and stereochemical configuration of functional groups [3,8], as well as their mode of conjugation, *i.e.*, with amino acid (glycine or taurine) [8–10], sulfuric acid [5,6]

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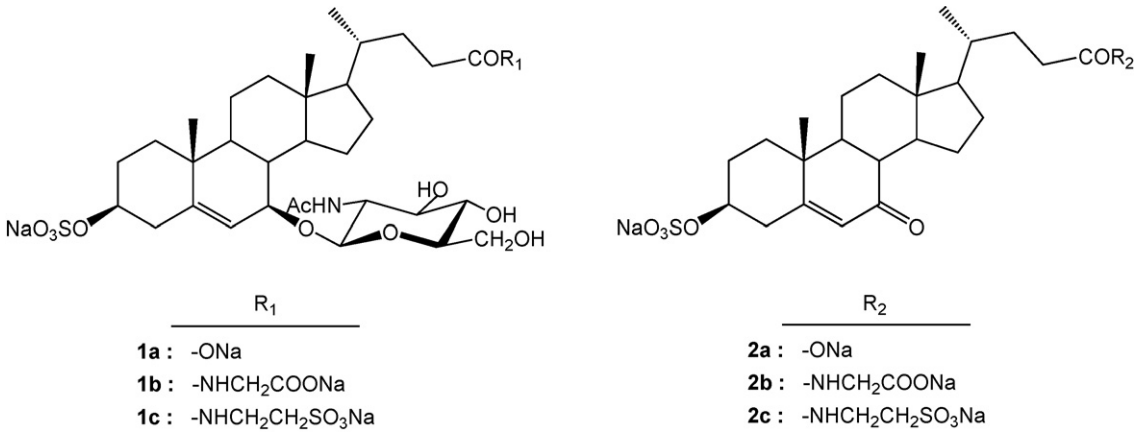


Fig. 1. Structures of the 3β,7β-dihydroxy-5-cholen-24-oic acid and 3β-hydroxy-7-oxo-5-cholen-24-oic acid multi-conjugates in a patient with NP-C1.

and/or monosaccharide (D-glucose, *N*-acetyl-D-glucosamine or D-glucuronic acid) [11–13]. The Δ^5 bile acid has an A/B-ring juncture that is pseudo-*trans*; as a result, the 3β-hydroxy group is equatorial, diminishing the hydrophilicity of the α-side of the bile acid molecule; on the other hand, the 7β-hydroxy group of ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid, UDCA) also impinges on the hydrophobic β-side of the molecule, greatly diminishing the retention time, as compared to chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid, CDCA) in which the hydroxy group at C-7 is α. Such polar substituents have a marked effect on bile acid hydrophilicity which in turn influences partitioning between the mobile and stationery phases during RP-HPLC. In addition, shape factors such as the A/B-ring juncture can influence binding to the C_{18} stationary phase. The number of carbon atoms at the C-17 side chain also influences binding to the stationery phase [3].

Based on above the findings and the structures of naturally occurring C_{24} Δ^5-bile acid multi-conjugates (**1a**–**1c** and **2a**–**2c**), we designed four variants of the unnatural types of analogous bile acid conjugates (**I–IV**), which differed from one another in the position and number of the conjugation modes, the presence or absence of a Δ^5-bond, and/or the length of the cholane side chain (C_3–C_5) (Conventional C_{24} bile acids have the isopentanoic ($C_5H_9O_2$) side chain). The chemical syntheses of **I**–**IV** have been attained and their synthetic routes were shown in Figs. 3–6; in the case of the compounds **I**–**III**, the physical and spectral data are given in Section 4, but those of the intermediary compounds have been omitted.

Our results showed that 3α-*N*-acetylglucosaminyl-7α-sulfooxy-5β-cholan-24-oic acid (**I** as sodium salt) and 3α-*N*-acetylglucosaminyl-7β-sulfooxy-5β-cholan-24-oic acid (**II** as sodium salt) were unsuitable as an internal reference standard, because both **I** and **II** had too long retention times using RP-HPLC. Variant 3β-sulfooxy-7β-hydroxy-23,24-bisnor-5-cholen-22-oic acid (**III** as sodium salt) was also unsuitable because it generated an unknown interfering ion when added to urine samples that were analyzed by LC–MS/MS (unpublished observations). On the other hand, 3β-sulfooxy-7β-hydroxy-24-nor-5-cholen-23-oic acid (**IV** as sodium salt) when added as an internal standard permitted an LC–MS/MS analysis of the targeted molecules (**1a–1c** and **2a–2c**) that was both sensitive and specific and thus useful for clinical application.

As shown in Fig. 3, the desired **IV** was prepared starting from hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-oic acid; HDCA, **3**). A key reaction involved is the so-called "second order" Beckmann rearrangement of the performate derivative (**3a**) of **3** with sodium nitrite in a mixture of trifluoroacetic anhydride and trifluoroacetic acid [14]. Subsequent alkaline hydrolysis of

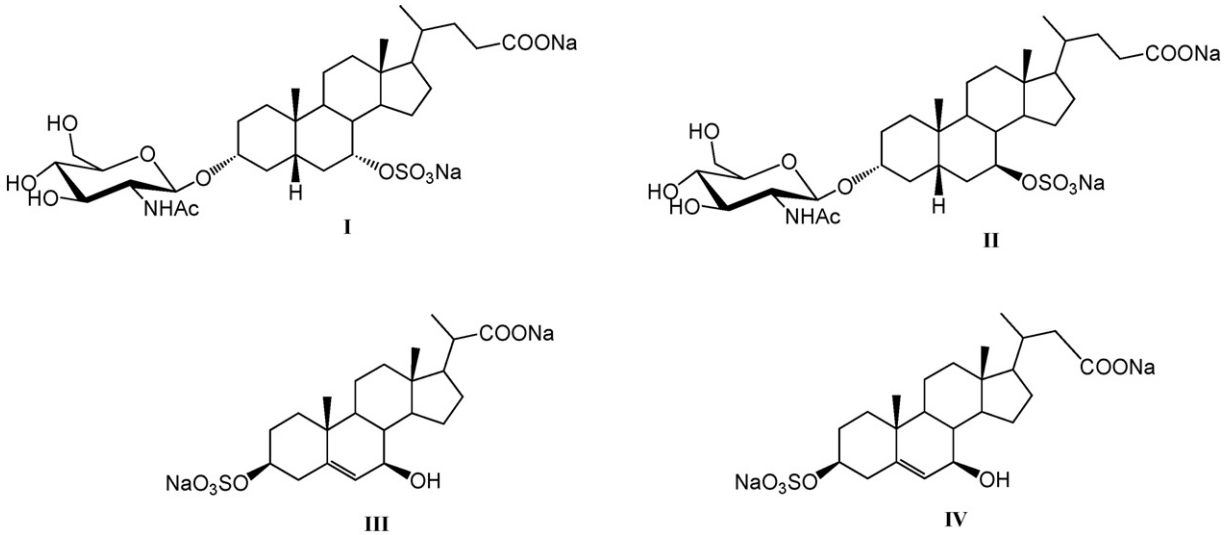


Fig. 2. Structures of the candidate compounds for an internal standard for the LC–MS/MS analysis of the multi-conjugates (**1a**–**1c** and **2a**–**2c**) reported to be present in urinary bile acids in a patient with NP-C1.

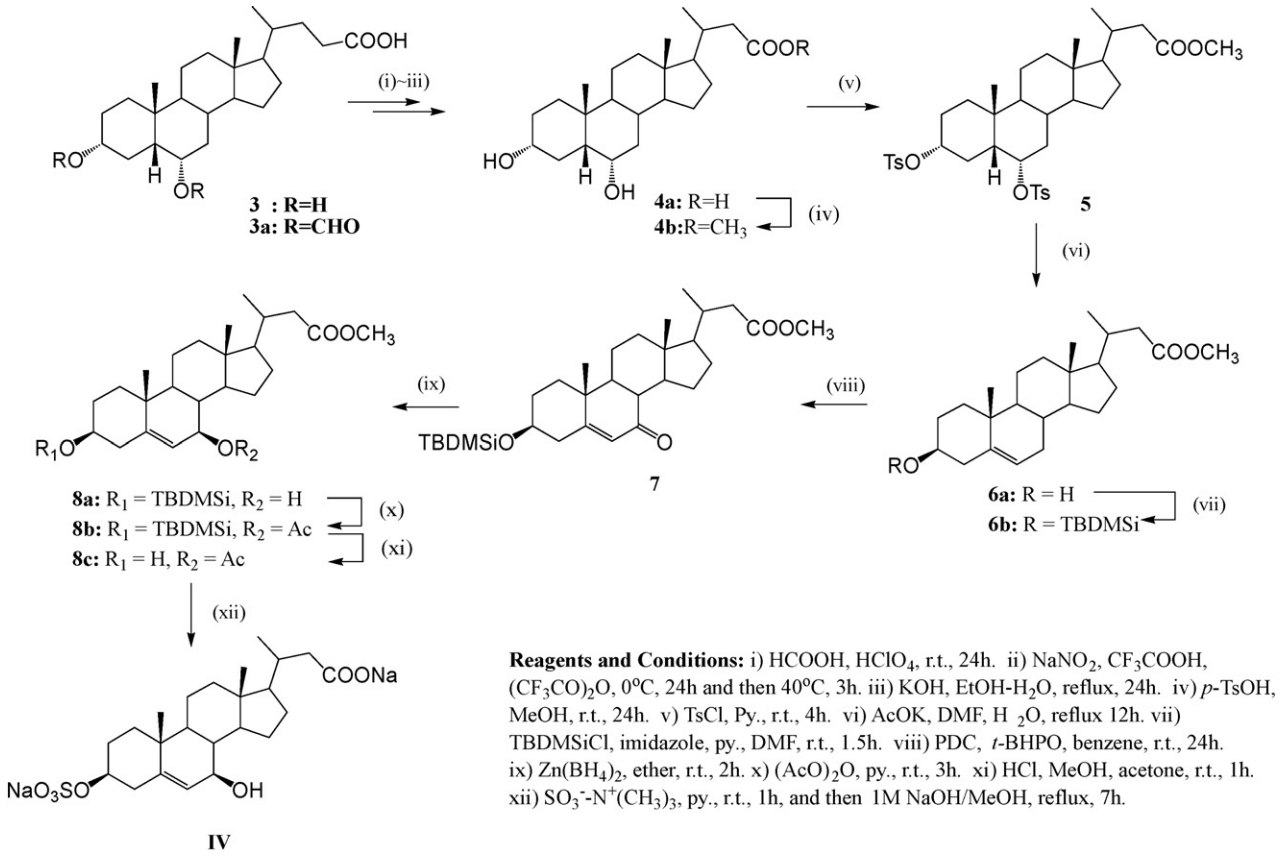


Fig. 3. Synthetic route to 3β-sulfooxy-7β-hydroxy-24-nor-5-cholen-23-oic acid disodium salts (**IV**) from HDCA (**3**).

the resulting 24-nor-23-nitrile intermediate afforded 24-nor-HDCA (**4a**) in isolated yield of 44% [14]. The procedure was found to be an easy, efficient method for one-carbon degradation of the isopentanoic (C_5) side chain in natural C_{24} bile acids.

Esterification of **4a** with methanol/*p*-toluenesulfonic acid, followed by tosylation of the resulting methyl ester **4b** with tosyl chloride in pyridine yielded readily 3α,6α-ditosyloxy-24-nor-5β-cholan-23-oate (**5**). When **5** was refluxed in aqueous *N*,*N*′-dimethylformamide (DMF) in the presence of potassium acetate, simultaneous S_N2 inversion at C-3 and E2 elimination at C-6 [2,15] took place smoothly to give methyl 3β-hydroxy-24-nor-5-cholen-23-oate (**6a**) in 38% isolated yield after chromatographic purification on silica gel.

tert-Butyldimethylsilylation (TBDMSi) of **6a** with *tert*-butyldimethylsilyl chloride in dry DMF and pyridine and subsequent allylic oxidation of the resulting 3β-TBDMSi-24-nor-Δ^5 ester **6b** with pyridinium dichromate and *tert*-butylhydroperoxide (TBHP) [16] yielded the corresponding 3β-TBDMSi-7-oxo-24-nor-Δ^5 ester **7** in 84% isolated yield after chromatographic purification on silica gel.

Reduction of the 7-ketone **7** with $Zn(BH_4)_2$ [2,17] afforded exclusively the equatorially-oriented 3β-TBDMSi-7β-hydroxy-24-

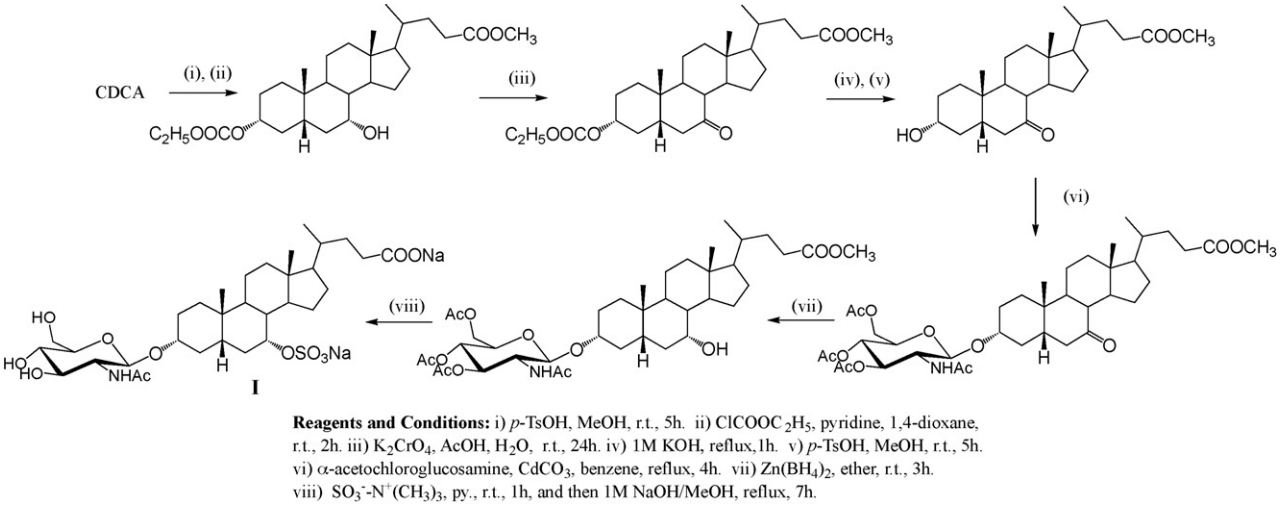
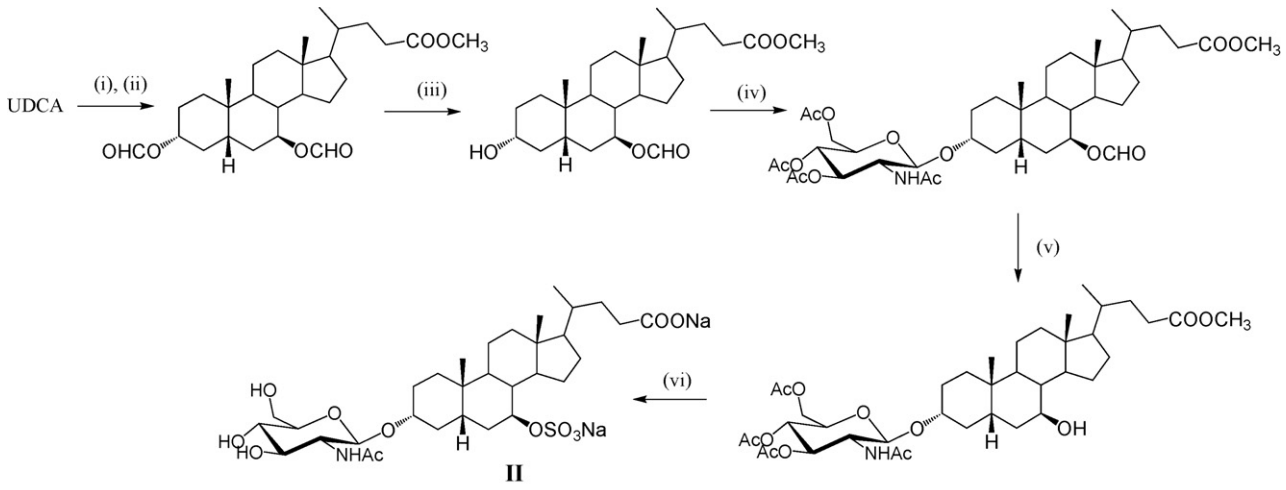


Fig. 4. Synthetic route to 3α-(2-acetamide-2-deoxy-β-D-glucopyranosyl)-7α-sulfooxy-5β-cholan-24-oic acid disodium salts (**I**) from CDCA.



Reagents and Conditions: i) *p*-TsOH, MeOH, r.t., 5h. ii) HCOOH, HClO, r.t., 1h. iii) NH_3, MeOH, r.t., 5min. iv) α-acetochloroglucosamine, $CdCO_3$, benzene, reflux, 4h. v) NH_3, MeOH, r.t., 20min. vi) SO_3^--$N^+(CH_3)_3$, py., r.t., 1h, and then 1M NaOH/MeOH, reflux, 7h.

Fig. 5. Synthetic route to 3α-(2-acetamide-2-deoxy-β-D-glucopyranosyl)-7β-sulfooxy-5β-cholan-24-oic acid disodium salts (**II**) from UDCA.

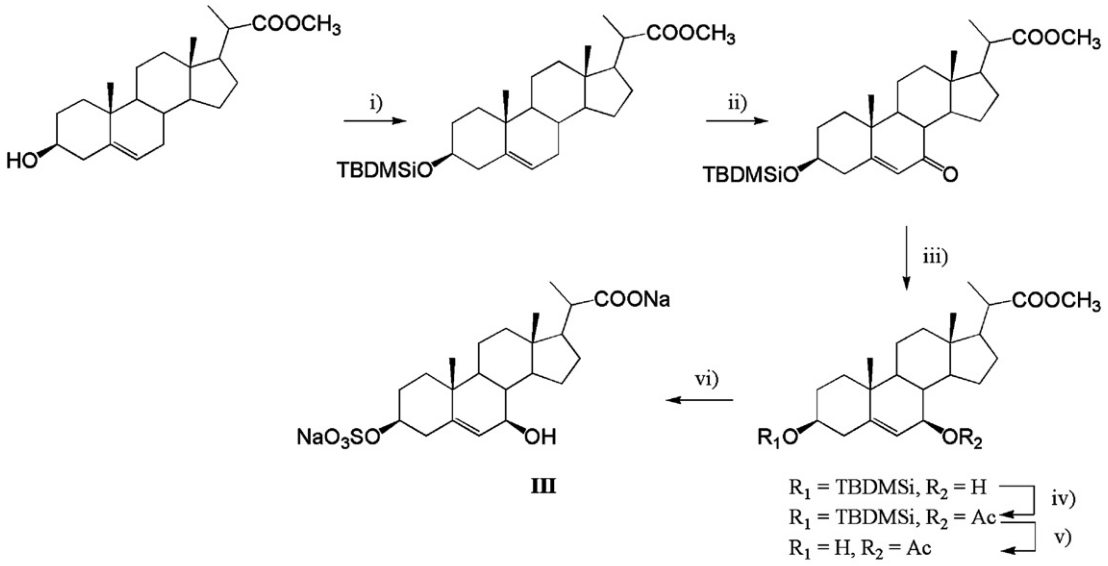
nor-Δ^5 ester **8a** (50%), which in turn was acetylated by the usual method to yield the corresponding 3β-TBDMSi-7β-acetoxy-24-nor-Δ^5 ester **8b**. Selective deprotection of the TBDMSi group at C-3 in **8b** with HCl gave the corresponding 3β-hydroxy-7β-acetoxy-24-nor-Δ^5 ester **8c**.

Regioselective sulfation at C-3 in **8c** was successfully attained with sulfur trioxide–trimethylamine complex in pyridine [18]. After the reaction, the product was purified by passing through a Sep-Pak C_{18} cartridge for solid-phase extraction, and then eluting with a mixture of methanol and water. Alkaline hydrolysis of the effluent with NaOH afforded the desired 3β-sulfooxy-7β-hydroxy-Δ^5-24-nor-5β-cholan-23-oic acid (**IV**) in 76% isolated yield. Thus, **IV** was obtained from HDCA (**3**) in 12 steps with total yield of 1.8%.

3. Characterization of the four synthetic compounds by HPLC

Fig. 7 shows a typical chromatogram of a mixture of the four synthetic compounds **I**–**IV**, together with some naturally occurring **1a** and **2a** on RP-HPLC with an ELSD. They showed much different retention times from one another, emerging from a C_{18} column in the order of **III**, **IV**, **II**, and then **I**. Of these compounds, **IV** was found to be the best choice as an internal reference standard. In addition, the sulfooxy compound **IV** was also found to be stable after addition to urine and during the LC–MS analysis.

The desired compound **IV** is now available for the highly sensitive, selective, and accurate LC–MS/MS determination of the



Reagents and Conditions: i) TBDMSiCl, imidazole, py., DMF, r.t., 1.5h. ii) PDC, *t*-BHPO, benzene, r.t., 24h. iii) $Zn(BH_4)_2$, ether, r.t., 2h. iv) Ac_2O, py., r.t., 3h. v) 10%-HCl, MeOH, acetone, r.t., 15min. vi) SO_3^--$N^+(CH_3)_3$, py., r.t., 1h, and then 1M NaOH/MeOH, reflux, 8h.

Fig. 6. Synthetic route to 3β-sulfooxy-7β-hydroxy-23,24-bisnor-5-cholen-22-oic acid (**III**) from 3β-hydroxy-23,24-bisnor-5-cholen-22-oic acid.

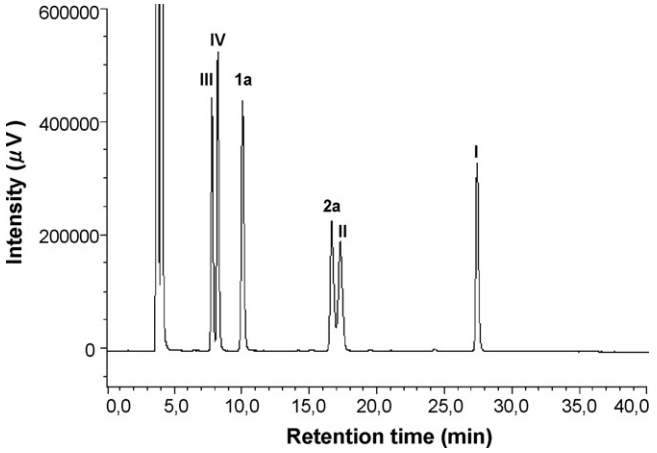


Fig. 7. A typical RP-HPLC chromatogram of a mixture of the four candidates for internal standards (compounds **I–IV**), together with two of the Δ^5-bile acid conjugates (**1a** and **2a**) identified from the urine of a patient with NP-C1.

multi-conjugates of 3β,7β-dihydroxy-5-cholen-24-oic acid and 3β-hydroxy-7-oxo-5-cholen-24-oic acid, compounds reported to be present in the urine of a patient with NP-C1 [1,2]. A further detailed study on a method for the clinical application is now progressing in our laboratory and the result will be reported elsewhere.

4. Experimental

4.1. Materials and reagents

HDCA (**3**) and 3β-hydroxy-23,24-bisnor-5-cholen-24-oic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Steraloids Inc. (Newport, RI, USA), respectively. UDCA and CDCA were kindly donated from Mitsubishi Tanabe Pharma Co. Ltd. (Osaka, Japan). All other reagents and solvents used were of analytical reagent grade.

4.2. General experimental procedure

Melting points (mp) were determined on a micro hot-stage apparatus and are uncorrected. IR spectra were obtained in KBr discs on a JASCO FT-IR 460 plus spectrometer (Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were obtained on a JEOL JNM-EX 270 FT instrument at 270 and 68.8 MHz, respectively. Low-resolution mass spectra (LR-MS) were recorded on a JEOL JMS-DX 303 mass spectrometer with an electron ionization (EI) source at 70 eV. High-resolution mass spectra (HR-MS) were measured using a JEOL JMS-700 with an EI source under the positive ion mode. For some polar compounds, HR-MS were also recorded on Shimadzu LC–MS-IT-TOF with ESI source under the negative ion mode. Normal phase TLC for nonsulfated compounds was performed on pre-coated silica gel plates (0.25 mm layer thickness; E. Merck, Darmstadt, Germany) using hexane–ethyl acetate mixtures (95:5–60:40, v/v) as the developing solvent. Reversed-phase (RP) TLC for the sulfated compounds was carried out on pre-coated RP-18F_{254S} plates using methanol–water–acetic acid mixtures (90:10:1, v/v/v) as the developing solvent. Sep-Pak Vac tC_{18} cartridges (5 g; Waters, Milford, MA, USA) were used for solid-phase extraction.

4.3. RP-HPLC analysis of the synthetic internal standards

The apparatus used was a LC-2000plus HPLC system (two PU-2085 high-pressure pumps, an MX-2080-32 solvent mixing module, and a CO-2060 column heater) equipped with a ChromNAV data-processing system (JASCO Co., Tokyo, Japan). A Capcell Pak-type MGII column [250 mm × 3.0 mm inner diameter (ID); particle size, 5 μm; Shiseido, Tokyo, Japan] was employed and kept at 37 °C. An Alltech 2000ES ELSD (Deerfield, IL) was used under the following conditions; the flow rate of purified compressed air as nebulizing gas was 1.6 L/min, the temperature of the heated drift tube was 82 °C, and the gain was 8. A mixture of two solvent systems, which is composed of methanol for the organic solvent A and 20 mM ammonium acetate (pH 5.4) for the aqueous buffer solution B, was used as the mobile phases [9]. The composition of the solvent A was gradually increased as follows: initial—12.0 min, 60% A (constant); 12.1–30.0 min, 60% A→75% A (linear gradient); 30.1 min-end, 75% A (constant). The flow rate was kept constant at 0.3 mL/min during analysis.

The synthetic internal standards **I–IV** and naturally occurring Δ^5 compounds **1a** and **2a** were dissolved in methanol at the concentration of 200 μg/mL as stock solutions, respectively. An aliquot (100 μL) of each stock solution was mixed, and then 7.5 μL of the resulting mixture was injected into the above HPLC-ELSD system.

4.4. Preparation of *I–IV*

4.4.1. 3α,6α-Dihydroxy-24-nor-5β-cholan-23-oic acid (24-Nor-hyodeoxycholicacid)(*4a*) and its methyl ester (*4b*)

Compound **4a** was prepared from HDCA **3** (25 g), according to the procedure of Schteingart and Hofmann [14]: yield, 10 g (44%); mp, 197–198 °C (EtOAc–hexane) [lit. [14] 218–219.5 °C (EtOAc)]. IR ν_{max} cm^{-1}: 1728 (C=O), 3297 (OH). ^{1}H NMR (CD_3OD) δ: 0.72 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 1.01 (3H, d, J 5.4, 21-CH_3), 3.51 (1H, brm, 3β-H), 4.01 (1H, brm, 6β-H). LR-MS (EI), m/z: 360 (M–H_2O, 100%), 345 (M–H_2O–CH_3, 41%), 342 (M–2H_2O, 97%), 327 (M–2H_2O–CH_3, 40%), 255 (M–2H_2O–SC, 16%), 246 (M–H_2O–side chain (SC)–part of ring D, 27%), 231 (M–H_2O–CH_3–SC–part of ring D, 46%), 228 (M–2H_2O–SC–part of ring D, 24%), 213 (M–2H_2O–CH_3–SC–part of ring D, 60%). HR-MS (ESI$^-$), calculated as $C_{23}H_{37}O_4$ [M−H]$^-$: 377.2692; found, m/z: 377.2707.

A mixture of the 24-nor-hyodeoxycholic acid (**4a**) (10 g) and p-toluenesulfonic acid (1.2 g) in methanol (120 mL) was left to stand at room temperature for over night. Most of the solvent was evaporated, and the residue was extracted with EtOAc. The combined extract was washed with brine, dried with Drierite, and evaporated. Recrystallization of the oily residue from EtOAc–hexane gave the desired methyl ester **4b** as colorless amorphous solids: yield 6 g (60%); mp, 82.5–84 °C. IR ν_{max} cm^{-1}: 1735 (C=O), 3309 (O—H). ^{1}H NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-CH_3), 0.86 (3H, s, 19-CH_3), 0.93 (3H, d, J 5.4, 21-CH_3), 3.57 (1H, brm, 3β-H), 3.63 (3H, s, —$COOCH_3$), 4.00 (1H, brm, 6β-H). ^{13}C NMR ($CDCl_3$) δ: 12.0 (C-18), 19.5 (C-21), 20.7 (C-11), 23.5 (C-19), 24.1 (C-15), 28.2 (C-16), 29.2 (C-4), 30.1 (C-2), 33.7 (C-20), 34.8 (C-8), 35.5 (C-1), 35.9 (C-10), 39.8 (C-9), 41.4 (C-22), 42.9 (C-13), 48.4 (C-5), 51.3 (—$COOOCH_3$), 56.0 (C-17), 56.1 (C-14), 68.0 (C-6), 71.5 (C-3), 174.0 (C-24). LR-MS (EI), m/z: 374 (M–H_2O, 100%), 359 (M–H_2O–CH_3, 39%), 356 (M–2H_2O, 81%), 341 (M–2H_2O–CH_3, 39%), 301 (26%), 255 (M–2H_2O–SC, 28%), 246 (M–H_2O–SC–part of ring D, 32%), 231 (M–H_2O–CH_3–SC–part of ring D, 45%), 228 (M–2H_2O–SC–part of ring D, 28%), 213 (M–2H_2O–CH_3–SC–part of ring D, 71%). HR-MS (EI), calculated as $C_{24}H_{38}O_3$ [M−H_2O]: 374.2821; found, m/z: 374.2804.

4.4.2. Methyl 3α,6α-ditosyloxy-24-nor-5β-cholan-23-oate (*5*)

To a solution of methyl 24-nor-hyodeoxycholate **4b** (5.2 g, 13.2 mmol) in dry pyridine (15 mL), a solution of tosyl chloride (7.5 g, 39.6 mmol) in dry pyridine (8 mL) was added, and the mixture was stirred at room temperature for 4 h. Ice chips were added gradually to the mixture, and the precipitated solid was filtered. The precipitate was redissolved in CH_2Cl_2, washed with water, dried with Drierite, and then evaporated to dryness. The yellow

oily residue was passed through a short column of silica gel (120 g) and eluted with EtOAc–hexane (6:4, v/v). Although the ditosylate **5** was pure according to the TLC and NMR analyses, it resisted crystallization attempts: yield, 6.5 g (70%). IR ν_{max} cm^{-1}: 1733 (C=O). ^{1}H NMR ($CDCl_3$) δ: 0.63 (3H, s, 18-CH_3), 0.80 (3H, s, 19-CH_3), 0.95 (3H, d, *J* 5.4, 21-CH_3), 2.47 (6H, s, 3β- and 6β-$O_3SC_6H_4CH_3$), 4.30 (1H, brm, 3β-H), 4.78 (1H, brm, 6β-H), 7.32–7.37, and 7.71–7.80 (8H, m, 3β- and 6β-$O_3SC_6H_4CH_3$). LR-MS (EI), *m*/*z*: 356 (M-2TsOH, 100%), 341 (M–2TsOH–CH_3, 23%), 255 (M–2TsOH–SC, 23%), 248 (17%), 235 (30%), 213(M–2TsOH–CH_3–SC–part of ring D, 15%). HR-MS (EI), calculated for $C_{24}H_{36}O_2$ [M−2TsOH]: 356.2715; found *m*/*z*: 356.2693.

4.4.3. Methyl 3β-hydroxy-24-nor-5-cholen-23-oate (**6a**)

A solution of the ditosylate **5** (6 g, 8.6 mmol) and CH_3COOK (840 mg) dissolved in DMF (65 mL) and water (7 mL) was refluxed for 12 h. The solution was cooled at room temperature, and the reaction product was extracted with EtOAc. The organic layer was washed with brine, dried over Mg_2SO_4, and evaporated under reduced pressure. The brown oily residue was passed through a column of silica gel (140 g) and eluted with EtOAc–hexane (2:8, v/v). Recrystallization of the oil from methanol gave the desired 3β-hydroxy-Δ^5-ester **6a** in the form of colorless needles: yield, 1.2 g (38%); mp, 123–124 °C. IR ν_{max} cm^{-1}: 1739 (C=O), 3391 (O—H). ^{1}H NMR ($CDCl_3$) δ: 0.72 (3H, s, 18-CH_3), 0.99 (3H, d, *J* 5.4, 21-CH_3), 1.01 (3H, s, 19-CH_3), 3.53 (1H, brm, 3α-H), 3.66 (3H, s, —$COOCH_3$), 5.36 (1H, brs, 6-H). LR-MS (EI), *m*/*z*: 374 (M, 100%), 359 (M–CH_3, 40%), 356 (M–H_2O, 74%), 341 (M–H_2O–CH_3, 47%), 289 (46%), 263 (M–CH_3–ring A, 70%), 255 (M–H_2O–SC, 28%), 231 (M–CH_3–SC–part of ring D, 30%), 213 (M–H_2O–CH_3–SC–part of ring D, 47%). HR-MS (EI), calculated for $C_{24}H_{38}O_3$ [M]: 374.2821; found *m*/*z*: 374.2808.

4.4.4. Methyl 3β-tert-butyldimethylsilyloxy-24-nor-5-cholen-23-oate (**6b**)

To a solution of the 3β-hydroxy-Δ^5 ester **6a** (750 mg, 2.0 mmol) in anhydrous DMF (11 mL) and pyridine (0.5 mL), imidazole (1.8 g) and *tert*-butyldimethylsilyl chloride (TBDMSiCl; 1.0 g, 6.6 mmol) were added. The mixture was stirred at room temperature for 1.5 h. The reaction product was extracted with EtOAc, and the combined extract was washed with brine, dried with Drierite, and evaporated. The residue was passed through a short column of silica gel (30 g) by elution with EtOAc–hexane (2:98, v/v) and recrystallized from methanol to give the 3β-TBDMSi derivative **6b** in the form of colorless plates: yield, 890 mg (91%); mp, 163–165 °C. IR ν_{max} cm^{-1}: 1747 (C=O). ^{1}H NMR ($CDCl_3$) δ: 0.05 (6H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 0.71 (3H, s, 18-CH_3), 0.89 (9H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 0.99 (3H, d, *J* 5.4, 21-CH_3), 1.00 (3H, s, 19-CH_3), 3.48 (1H, brm, 3β-H), 3.66 (3H, s, —$COOCH_3$), 5.31 (1H, brs, 6-H). LR-MS (EI), *m*/*z*: 431 (M–C(CH_3)$_3$, 100%). HR-MS (EI), calculated for $C_{30}H_{51}O_3Si$ [M]: 487.3607; found *m*/*z*: 487.3600.

4.4.5. Methyl 3β-tert-butyldimethylsilyloxy-7-oxo-24-nor-5-cholen-23-oate (**7**)

To a magnetically stirred suspension of the 3β-TBDMSi-Δ^5 ester **6b** (650 mg, 1.3 mmol), pyridinium dichromate (1.5 g, 4.0 mmol) and Celite (1.3 g) in benzene (12 mL) and then 70%TBHP (1.1 mL) was added gradually with ice-bath cooling. The whole mixture was stirred at room temperature for 24 h. After filtration of the reaction mixture on Celite, the mother liquor was evaporated under reduced pressure. A dark brown residue was passed through a column of silica gel (25 g) and elution with EtOAc–hexane (5:95, v/v) gave the title compound **7**, which was recrystallized from methanol in the form of colorless needles: yield, 550 mg (84%); mp, 216–217 °C. IR ν_{max} cm^{-1}: 1670 (C=O, enone), 1741 (C=O, ester). ^{1}H NMR ($CDCl_3$) δ: 0.06 (6H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 0.72 (3H, s, 18-CH_3), 0.89 (9H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 0.99 (3H, d, *J* 5.4, 21-CH_3), 1.19 (3H, s, 19-CH_3), 3.60 (1H, brm, 3β-H), 3.66 (3H, s, —$COOCH_3$), 5.66 (1H, s, 6-H). LR-MS (EI), *m*/*z*: 487 (M–CH_3, 3%), 459 (11%), 445 (M–C(CH_3)$_3$, 100%), 413 (M–C(CH_3)$_3$–CH_4O, 36%) 371 (M–TBDMSiOH, 27%). HR-MS (EI), calculated for $C_{30}H_{50}O_4Si$ [M]: 502.3478; found *m*/*z*: 502.3468.

4.4.6. Methyl 3β-tert-butyldimethylsilyloxy-7β-hydroxy-24-nor-5-cholen-23-oate (**8a**)

To a freshly prepared solution of $Zn(BH_4)_2$ in Et_2O (*ca.* 1 M, 6 mL) [16], a solution of the 3β-TBDMSi-7-oxo ester **7** (490 mg, 0.97 mmol) in benzene (6 mL) was added slowly. After standing at room temperature for 2 h, the mixture was diluted with benzene, and the combined organic layer was washed with water, dried with Drierite, and evaporated to dryness. The residue was subjected to a column of silica gel (30 g) and eluted with EtOAc–hexane (1:9, v/v). Recrystallization of the residue from aqueous methanol gave the 3β-TBDMSi-7β-hydroxy ester **8a** in the form of colorless amorphous solids: yield, 240 mg (50%); mp, 131–132 °C. IR ν_{max} cm^{-1}: 1734 (C=O), 3310 (O—H). ^{1}H NMR ($CDCl_3$) δ: 0.06 (6H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 0.73 (3H, s, 18-CH_3), 0.89 (9H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 1.00 (3H, d, *J* 8.1, 21-CH_3), 1.04 (3H, s, 19-CH_3), 3.50 (1H, brm, 3α-H), 3.83 (1H, m, 7α-H), 5.25 (1H, brs, 6-H). LR-MS (EI), *m*/*z*: 486 (M–H_2O, 100%), 429 (M–H_2O–C(CH_3)$_3$, 12%), 354 (M–H_2O–TBDMSiOH, 69%), 339 (M–H_2O–TBDMSiOH–CH_3, 75%), 313 (69%), 281 (11%), 253 (M–H_2O–TBDMSiOH–SC, 12%), 235 (14%), 211 (M–H_2O–TBDMSiOH–CH_3–part of ring D, 10%). HR-MS (EI), calculated for $C_{30}H_{52}O_4Si$ [M]: 504.3635; found *m*/*z*: 504.3620.

4.4.7. Methyl 3β-tert-butyldimethylsilyloxy-7β-acetoxy-24-nor-5-cholen-23-oate (**8b**)

The 7β-hydroxy ester **8a** was converted into the corresponding 7β-acetate **8b** by the acetic anhydride-pyridine method. After the usual work-up, **8b** was recrystallized from methanol in the form of colorless needles: yield, 180 mg (88%); mp, 146–147 °C. IR ν_{max} cm^{-1}: 1742 (C=O). ^{1}H NMR ($CDCl_3$) δ: 0.04 (6H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 0.73 (3H, s, 18-CH_3), 0.88 (9H, s, —Si(CH_3)$_2$C(CH_3)$_3$), 0.99 (3H, d, *J* 8.1, 21-CH_3), 1.06 (3H, s, 19-CH_3), 2.02 (3H, s, —$OCOCH_3$), 3.49 (1H, brm, 3α-H), 5.02 (1H, d, *J* 8.1, 7α-H), 5.18 (1H, brs, 6-H). LR-MS (EI), *m*/*z*: 486 (M–CH_3COOH, 100%), 429 (M–CH_3COOH–C(CH_3)$_3$, 7%), 355 (49%), 354 (M–CH_3COOH–TBDMSiOH, 32%), 339 (29%), 313 (25%). HR-MS (EI), calculated for $C_{30}H_{50}O_3Si$ [M−CH_3COOH]: 486.3529; found *m*/*z*: 486.3520.

4.4.8. Methyl 3β-hydroxy-7β-acetoxy-24-nor-5-cholen-23-oate (**8c**)

To a solution of the 3β-TBDMSi-7β-acetoxy ester **8b** (120 mg, 0.22 mmol) in methanol (6 mL) and acetone (2 mL), 10% HCl (40 μL) was added. After being left to stand at room temperature for 1 h, the mixture was extracted with EtOAc. The EtOAc layer was washed with brine, dried with Drierite, and evaporated to dryness. Recrystallization of the residue from methanol gave the title compound **8c** in nearly quantitative yield in the form of colorless needles: yield, 95 mg; mp, 137–138 °C. IR ν_{max} cm^{-1}: 1742 (C=O). ^{1}H NMR ($CDCl_3$) δ: 0.73 (3H, s, 18-CH_3), 0.99 (3H, d, *J* 8.1, 21-CH_3), 1.07 (3H, s, 19-CH_3), 2.03 (3H, s, —$OCOCH_3$), 3.55 (1H, brm, 3α-H), 3.66 (3H, s, —$COOCH_3$), 5.02 (1H, d, *J* 10.8, 7α-H), 5.21 (1H, brs, 6-H). LR-MS (EI), *m*/*z*: 354 (M–H_2O–CH_3COOH, 100%), 339 (M–H_2O–CH_3COOH–CH_3, 14%), 253 (M–H_2O-CH_3COOH–SC, 13%), 235 (24%). HR-MS (EI), calculated for $C_{24}H_{36}O_3$ [M−CH_3COOH]: 372.2664; found *m*/*z*: 372.2647.

4.4.9. 3β-Sulfooxy-7β-hydroxy-24-nor-5-cholen-23-oic acid disodium salts (**IV**)

To a solution of the 3β-hydroxy-7β-acetoxy ester **8c** (33 mg, 0.08 mmol) in dry pyridine (1.6 mL), sulfur trioxide–trimethylamine complex (50 mg, 0.4 mmol) was added, and the mixture was stirred

at room temperature for 1 h. The reaction mixture was poured into ice-cooled petroleum ether (10 mL), and the precipitated solid was collected by filtration. After being washed with petroleum ether, the solid product was dissolved in 5% methanolic NaOH (10 mL) and the solution was refluxed 7 h. The resulting solution was adjusted to pH 8 with 10% HCl, diluted with water (90 mL), and loaded onto a Sep-Pak® tC_{18} cartridge. The cartridge was washed with water (20 mL) and 20% methanol (20 mL). The desired 3β-sulfate (**1a**) was eluted with methanol (20 mL) and recrystallized from methanol–EtOAc in the form of colorless amorphous solids: yield, 28 mg (76%); mp, *ca.* 250 °C (decomposition). ^{1}H NMR (CD_3OD) δ: 0.76 (3H, s, 18-CH_3), 1.02 (3H, d, *J* 5.4, 21-CH_3), 1.08 (3H, s, 19-CH_3), 3.74 (1H, d, *J* 8.1, 7α-H), 4.15 (1H, brm, 3α-H). LR-MS (EI), *m*/*z*: 340 (M–H_2SO_4–H_2O, 100%), 325 (M–H_2SO_4–H_2O–CH_3, 9%), 253 (M–H_2SO_4–H_2O–SC, 9%), 221 (30%). HR-MS (ESI^-), calculated for $C_{23}H_{34}O_7S$ [M−2Na+2H−H]: 455.2103; found *m*/*z*: 455.2122.

*4.4.10. 3α-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-7α-sulfooxy-5β-cholan-24-oic acid disodium salts (**I**) (Chenodeoxycholic acid-3α-N-acetylglucosaminide-7β-sulfate)*

This compound was prepared in 8 steps (see Fig. 4) from CDCA: mp, 203–205 °C (recrystallized from methanol–EtOAc). ^{1}H NMR (CD_3OD) δ: 0.69 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 0.95 (3H, d, *J* 5.4, 21-CH_3), 1.99 (3H, s, —NHCO*C*H_3), 3.50 (1H, brm, 3β-H), 3.23–3.89 (7H, m, 2′-, 3′-, 4′-, 5′-, and 6′-H), 4.44 (1H, brs, 7β-H), 4.56 (1H, d, *J* 8.1, 1′α-H), 7.95 (3H, *J* 8.1, —N*H*COCH$_3$). LR-MS (EI), *m*/*z*: 356 (M–GlcNAc–H_2SO_4, 100%), 341 (M–GlcNAc–H_2SO_4–CH_3, 69%), 255 (M–GlcNAc–H_2SO_4–SC, 31%), 228 (M–GlcNAc–H_2SO_4–part of ring D, 14%), 213 (M–GlcNAc–H_2SO_4–CH_3–part of ring D, 28%). HR-MS (ESI^-), calculated for $C_{32}H_{51}NO_{12}S$ [M−2Na+2H−H]$^-$: 674.3209; found *m*/*z*: 674.3241.

*4.4.11. 3α-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-7β-sulfooxy-5β-cholan-24-oic acid disodium salts (**II**) (Ursodeoxycholic acid-3α-N-acetylglucosaminide-7β-sulfate)*

This compound was prepared in 7 steps (see Fig. 5) from UDCA: mp, 212–214 °C (recrystallized from methanol–EtOAc). ^{1}H NMR (CD_3OD) δ: 0.69 (3H, s, 18-CH_3), 0.96 (3H, d, *J* 5.4, 21-CH_3), 0.97 (3H, s, 19-CH_3), 1.98 (3H, s, —NHCO*C*H_3), 3.23–3.88 (7H, m, 2′-, 3′-, 4′-, 5′-, 6′-H, and 3β-H), 4.29 (1H, brm, 7α-H), 4.55 (1H, d, *J* 8.1, 1′α-H), 8.0 (1H, d, *J* 8.1 Hz —N*H*COCH$_3$). LR-MS (EI), *m*/*z*: 356 (M–GlcNAc–H_2SO_4, 100%), 341 (M–GlcNAc–H_2SO_4–CH_3, 57%), 302 (26%), 255 (M–GlcNAc–H_2SO_4–SC, 34%), 228 (M–GlcNAc–H_2SO_4–part of ring D, 14%), 213 (M–GlcNAc–H_2SO_4–CH_3–part of ring D, 27%). HR-MS (ESI^-), calculated for $C_{32}H_{51}NO_{12}S$ [M−2Na+2H−H]$^-$: 674.3209; found *m*/*z*: 674.3247.

*4.4.12. 3β-Sulfooxy-7β-hydroxy-23,24-bisnor-5-cholen-22-oic acid disodium salts (**III**)*

This compound was prepared in 8 steps (see Fig. 6) from 3β-hydroxy-23,24-bisnor-5-cholen-22-oic acid: mp, *ca.* 140 °C (decomposition) (recrystallized from methanol–EtOAc). ^{1}H NMR (CD_3OD) δ: 0.73 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 1.15 (3H, d, *J* 5.4, 21-CH_3), 3.74 (1H, d, *J* 8.1 Hz, 7α-H), 4.14 (1H, brm, 3α-H), 5.29 (1H, s, 6-H). LR-MS (EI), *m*/*z*: 326 (M–H_2O–H_2SO_4, 100%), 311 (M–H_2O–H_2SO_4–CH_3, 19%), 253 (M–H_2O–H_2SO_4–SC, 14%), 237 (10%), 207 (16%). HR-MS (ESI^-), calculated for $C_{22}H_{32}O_7S$ [M−2Na+2H−H]$^-$: 441.1946; found *m*/*z*: 441.1958.

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