に一人で課題遂行ができるようになれば、母親のよりいっそうの負担軽減につながるだろう。また、対象児が自主的に課題学習を開始することができるようになれば、問題となっている常同行動が減少することになるかもしれない。

障害児・者の社会参加と自立に向けた支援の効果を確認するには、自然な環境での行動の変化が評価されなければならない（志賀, 1990）。対象児とその家族の自然な日常生活にどのような影響があるかを検討することで初めて、この取り組みが意味のある支援であったかどうかを確認することになると思われる。たとえば、井上・井上・菅野（1995）は、地域生活技能援助教室において料理指導プログラムによる指導を行い、家庭における料理行動の自発の増加と援助の減少を確認するとともに、2年後のスキルの長期的な維持についてのフォローアップを行っている。

将来、対象児の成長とともに、家族や本人のニーズも課題の内容も変化していくであろう。本研究のような支援を長期にわたって継続的に行い、家族や本人のニーズに合った課題の選択や、新しい標的行動の選択等を通して、自立的行動への支援が対象児と家族のライフスタイルに及ぼす影響について検討していくことを、今後の課題としたい。

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―2003.9.10 受稿, 2005.2.5 受理―

STEROIDS 71 (2006) 18–29

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Chemical synthesis of the 3-sulfooxy-7-N-acetylglucosaminyl-24-amidated conjugates of 3β,7β-dihydroxy-5-cholen-24-oic acid, and related compounds: Unusual, major metabolites of bile acid in a patient with Niemann-Pick disease type C1

Takashi Iida[a,*], *Genta Kakiyama*[a], *Yohei Hibiya*[a], *Shohei Miyata*[a], *Takehiko Inoue*[b], *Kohsaku Ohno*[b], *Takaaki Goto*[c], *Nariyasu Mano*[c], *Junichi Goto*[c], *Toshio Nambara*[c], *Alan F. Hofmann*[d]

[a] *Department of Chemistry, College of Humanities and Sciences, Nihon University, Setagaya, Sakurajousui, Tokyo 156-8550, Japan*
[b] *Division of Child Neurology, Faculty of Medicine, Tottori University, Nishi-machi, Yonago 683-8504, Japan*
[c] *Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Sendai 981-8578, Japan*
[d] *Division of Gastroenterology, Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0813, USA*

ARTICLE INFO

Article history:
Received 17 June 2005
Received in revised form 8 July 2005
Accepted 28 July 2005
Available online 28 September 2005

Keywords:
Unsaturated bile acid
Unusual bile acid multi-conjugates
Electrospray ionization mass spectrometry
Tandem mass spectrometry

Abbreviations:
NP-C1, Niemann-Pick disease type C1
GlcNAc, β-D-N-acetylglucosamine
α-acetochloroglucosamine, 2-acetamido-1α-chloro-1,2-dideoxy-3,4,6-tri-O-acetyl-D-glucopyranose
m.p., melting point

ABSTRACT

The chemical synthesis of 3β,7β-dihydroxy-5-cholen-24-oic acid, triply conjugated by sulfuric acid at C-3, by N-acetylglucosamine (GlcNAc) at C-7, and by glycine or taurine at C-24, is described. These are unusual, major metabolites of bile acid found to be excreted in the urine of a patient with Niemann-Pick disease type C1. Analogous double-conjugates of 3β-hydroxy-7-oxo-5-cholen-24-oic acid were also prepared. The principal reactions involved were: (1) β-D-N-acetylglucosaminidation at C-7 of methyl 3β-*tert*-butyldimethylsilyloxy (TBDMSi)-7β-hydroxy-5-cholen-24-oate with 2-acetamido-1α-chloro-1,2-dideoxy-3,4,6-tri-O-acetyl-D-glucopyranose in the presence of $CdCO_3$ in boiling toluene; (2) sulfation at C-3 of the resulting 3β-TBDMSi-7β-GlcNAc with sulfur trioxide-trimethylamine complex in pyridine; and (3) direct amidation at C-24 of the 3β-sulfooxy-7β-GlcNAc conjugate with glycine methyl ester hydrochloride (or taurine) using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride as a coupling agent in DMF. The structures of the multi-conjugated bile acids were characterized by liquid chromatography-mass spectrometry with an electrospray ionization probe under the positive and negative ionization modes.

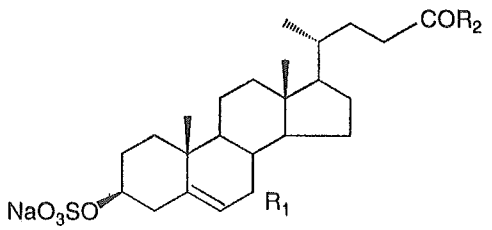
* Corresponding author. Tel.: +81 3 3329 1151; fax: +81 3 3303 9899.
E-mail address: takaiida@chs.nihon-u.ac.jp (T. Iida).

0039-128X/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2005.07.008

IR, infrared
^{1}H-NMR, proton nuclear magnetic resonance
GC/MS, gas chromatography/mass spectrometry
LC/MS, liquid chromatography/mass spectrometry
LR-MS, low-resolution mass spectra
HR-MS, high-resolution mass spectra
MS/MS, tandem mass spectrometry
EI, electron ionization
APCI, atmospheric pressure chemical ionization
ESI, electrospray ionization
PIM, positive ion mode
NIM, negative ion mode
TLC, thin layer chromatography
DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
Me_4Si, tetramethylsilane; EtOAc ethyl acetate
Et_2O, diethyl ether
DMF, *N*,*N*'-dimethylformamide

1. Introduction

Bile acids in biological material are known to exist in single- and/or double-conjugated form, rather than in unconjugated ones [1]. The carboxyl group at C-24 in bile acids is amidated with glycine or taurine and the hydroxy group at C-3 is conjugated with sulfuric acid or β-D-glycopyranose (i.e., glucuronic acid or glucose) to form the corresponding sulfate



	R_1	R_2
1a :	β-GlcNAc	-ONa
1b :	β-GlcNAc	-$NHCH_2COONa$
1c :	β-GlcNAc	-$NHCH_2CH_2SO_3Na$
12a:	= O	-ONa
12b:	= O	-$NHCH_2COONa$
12c:	= O	-$NHCH_2CH_2SO_3Na$

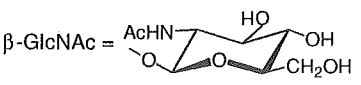
β-GlcNAc = 

Fig. 1 – Structures of 7-oxygenated 3β-hydroxy-Δ^5 bile acid multi-conjugates.

or glycosidic conjugates. The specific, selective conjugation of 7β-hydroxylated bile acids with β-D-N-acetylglucosamine (GlcNAc) has also been reported in connection with hepatobiliary diseases [2–5].

Meanwhile, Niemann-Pick disease type C1 (NP-C1) is a fatal autosomal recessive neurovisceral disorder in humans and in animals, characterized clinically by progressive neurodegeneration in the central nervous system, and by hepatosplenomegaly [6,7]. Biochemically, NP-C1 is characterized by the intracellular accumulation of unesterified cholesterol, sphingomyelin, glycosphingolipids, glycolipids, and other lipids within the endosomal/lysosomal system in various tissues, particularly in visceral organs.

Recently, Alvelius et al. [8] have reported that an NP-C1 patient with hepatosplenomegaly, mild signs of cholestasis, hepatic inflammation, and extramedullary erythropoiesis, together with chronic airway disease, excreted abnormal amounts of a new type of unusual 7-oxygenated bile acid multi-conjugates in urine. The structures of the unusual bile acids were elucidated by a combined use of electrospray ionization (ESI) mass spectrometry and gas chromatography-mass spectrometry (GC-MS). After enzymatic and solvolytic removal of the conjugating moieties, these acids were shown to have a 3β-hydroxy-Δ^5 structure and to carry either a hydroxy or oxo group at C-7. They were sulfated at C-3, and either nonamidated, or amidated with glycine or taurine at C-24. Part of the 7-hydroxy acid, presumably the 7β-hydroxylated one, was also conjugated with *N*-acetylhexosamine, probably GlcNAc, at the 7-hydroxy group. Based on previous data concerning the effect of 3β-hydroxy-Δ^5 bile acids on bile acid transport, Alvelius et al. [8] suggested that the formation of such unusual bile acid multi-conjugates was responsible for the neonatal cholestasis in the NP-C1 patient.

A more direct proof had to await chemical synthesis and the demonstration of the identity of isolated compounds with synthetic ones. The availability of authentic synthetic specimens could also serve to establish a reliable method for analyzing the effects of the multi-conjugated bile acids in urine with regard to metabolic disorders in humans, and could serve to clarify the physiological significance of the pathway, the region in which these conjugates are synthesized, the enzyme catalyzing the conjugation, and the dynamics of the conjugates.

We herein report the chemical synthesis of the multi-conjugates of 3β,7β-dihydroxy-5-cholen-24-oic acid and 3β-hydroxy-7-oxo-5-cholen-24-oic acid as authentic reference compounds (Fig. 1), in order to shed light on an unknown abnormality in the bile acid synthesis and metabolism of a patient with NP-C1.

2. Experimental

2.1. Materials and instruments

Methyl hyodeoxycholanoate (methyl 3α,6α-dihydroxy-5β-cholan-24-oate) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was obtained from Tokuyama Co., Ltd. (Tokyo, Japan). 2-Acetamido-1α-chloro-1,2-dideoxy-3,4,6-tri-O-acetyl-D-glucopyranose (α-acetochloroglucosamine) was prepared from N-acetylglucosamine according to a previous paper [9]. Reversed-phase prepacked Sep-Pak® tC_{18} cartridges (adsorbent weight, 5 g) were available from Waters Assoc. (Milford, MA, USA); they were washed with 50 ml methanol and 100 ml distilled water prior to use. All other reagents and solvents used were of analytical grades.

Melting points (m.p.) were determined on a micro hot-stage apparatus and are uncorrected. IR spectra were obtained in KBr discs on a Shimadzu FTIR-8300 spectrometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-EX 270 FT instrument (Tokyo, Japan) at 270 MHz, with $CDCl_3$ or CH_3OD containing 0.1% Me_4Si as the solvent; chemical shifts are expressed as δ ppm relative to Me_4Si. Electron ionization (EI) low-resolution mass spectra (LR-MS) were determined on a JEOL JMS-303 mass spectrometer at 70 eV. High-resolution mass (HR-MS) spectra were measured by using a JEOL JMS-LCmate double-focusing magnetic mass spectrometer equipped with an electrospray ionization (ESI) probe or an atmospheric pressure chemical ionization (APCI) probe under the positive ion mode (PIM) or the negative ion mode (NIM); a mixture of methanol-water (1:1, v/v) was used as the mobile phase. HR-MS spectra were also obtained on a JEOL JMS-700 mass spectrometer with an EI probe under the PIM. Tandem mass spectrometric (MS/MS) analysis was carried out using a LCMS-IT-TOF tandem liquid chromatography-mass spectrometer (LC/ESI-MS/MS) (Shimadzu, Kyoto, Japan) equipped with an ESI-PIM probe; a sample solution was dissolved in methanol-water (1:1, v/v), and a mixed solution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile was used as the mobile phase. Normal-phase (NP) thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (0.25 mm layer thickness; E. Merck, Darmstadt, Germany) using hexane-EtOAc-AcOH mixtures (80:20:1–40:60:1, v/v/v) or EtOAc-methanol-AcOH (8:1:1–7:2:1, v/v/v) as the developing solvent. Reversed-phase (RP) TLC was carried out on pre-coated RP-$18F_{254S}$ plates using methanol-water mixtures (5:5-6:4, v/v) as the developing solvent.

2.2. Synthesis

2.2.1. *Methyl 3α,6α-ditosyloxy-5β-cholan-24-oate (3)*

To a solution of methyl hyodeoxycholate 2 (2.0 g, 4.9 mmol) in dry pyridine (12 ml) a solution of tosyl chloride (2.0 g, 10.5 mmol) in dry pyridine (6 ml) was added, and the mixture was stirred at room temperature for 2 days. Ice chips were added gradually to the mixture, and the precipitated solid was filtered, washed with 10% HCl and water, and recrystallized from benzene-hexane to give the ditosylate 3 in the form of colorless needles: yield, 3.36 g (95%); m.p., 166–167 °C (lit. [10] 165–167 °C). IR, ν_{max} cm^{-1}: 1732 (C=O), 3040 (Ar–H). ^{1}H-NMR ($CDCl_3$), δ: 0.59 (3H, s, 18-CH_3), 0.80 (3H, s, 19-CH_3), 0.88 (3H, d, J = 6.5 Hz, 21-CH_3), 2.17, 2.46 (each 3H, s, 3α- and 6α-$C_6H_4CH_3$), 3.66 (3H, s, –$COOCH_3$), 4.30 (1H, brm, 3β-H), 4.79 (1H, brm, 6β-H), 7.34 and 7.75 (each 4H, m, 3α- and 6α-$C_6H_4CH_3$). LR-MS (EI-PIM), *m/z*: 370 (M–2TsOH, 100%), 355 (M–2TsOH–CH_3, 22%), 255 [M–2TsOH–S.C. (side chain, 115 u), 23%], 249 (33%), 213 [M–2TsOH–S.C.-ring D (42 u), 14%]. HR-MS (APCI-NIM), calculated for $C_{39}H_{53}O_8S_2$ [M–H]$^-$: 713.3182; found, *m/z*: 713.3175.

2.2.2. *Methyl 3β-hydroxy-5-cholen-24-oate (4) and its acetate (5)*

A solution of the ditosylate 3 (3.36 g, 4.7 mmol) and CH_3COOK (360 mg, 3.6 mmol) dissolved in water (3 ml) and N,N'-dimethylformamide (DMF; 20 ml) was refluxed for 24 h. The solution was cooled at room temperature, with ice chips added gradually. The precipitated solid was filtered off and washed with water. The crude solid was recrystallized from aqueous methanol to give an analytical pure sample of the Δ^5 ester 4 in the form of colorless needles: yield, 1.76 g (96%); m.p., 139–141 °C [lit. (10) m.p., 143–144 °C]. IR, ν_{max} cm^{-1}: 1717 (C=O), 3489 (O–H). ^{1}H-NMR ($CDCl_3$), δ: 0.68 (3H, s, 18-CH_3), 0.93 (3H, d, J = 6.5 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 3.52 (1H, brm, 3α-H), 3.66 (3H, s, –$COOCH_3$), 5.35 (1H, m, 6-H). LR-MS (EI-PIM), *m/z*: 388 (M^+, 100%), 370 (M–H_2O, 62%), 355 (M–H_2O–CH_3, 40%), 303 (40%), 277 (70%), 255 (M–H_2O–S.C., 27%), 213 (M–H_2O–S.C.-ring D, 36%). HR-MS (EI-PIM): calculated for $C_{25}H_{40}O_3$ $[M]^+$: 388.5908; found, *m/z*: 388.2977.

The ester (4) was converted into the corresponding acetate (5) by the usual acetic anhydride-pyridine method, which was recrystallized from acetone in the form of colorless thin plates: yield, 1.85 g (95%); m.p., 147–150 °C (lit. [10] m.p., 155–156 °C). IR, ν_{max} cm^{-1}: 1681 (C=C), 1731 (C=O). ^{1}H-NMR ($CDCl_3$), δ: 0.68 (3H, s, 18-CH_3), 0.93 (3H, d, J = 6.2 Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 2.03 (3H, s, 3β-$OCOCH_3$), 3.66 (3H, s, –$COOCH_3$), 4.60 (1H, brm, 3α-H), 5.37 (1H, m, 6-H). LR-MS (EI-PIM), *m/z*: 370 (M–AcOH, 100%), 355 (M–AcOH–CH_3, 15%), 255 (M–AcOH–S.C., 17%), 249 (20%), 213 (M–AcOH–S.C.-ring D, 10%). HR-MS (APCI-NIM), calculated for $C_{27}H_{41}O_4$ $[M-H]^-$: 429.3005; found, *m/z*: 429.2971.

2.2.3. *Methyl 3β-acetoxy-7-oxo-5-cholen-24-oate (6)*

To a magnetically stirred suspension of the 3β-acetoxy-Δ^5 ester 5 (1.8 g, 4.1 mmol), pyridinium dichromate (PDC; 4.6 g, 12 mmol) and Celite (4 g) in dry benzene (35 ml), and 70% *tert*-butylhydroperoxide (*t*-BHPO; 3.5 ml, 27 mmol) were added gradually with ice-bath cooling; the whole mixture was stirred at room temperature for 24 h. After filtration on Celite, the mother liquor was evaporated under reduced pressure to give a dark brown residue, which was passed through a short column on silica gel (10 g). Elution with EtOAc-benzene (95:5, v/v) gave the title compound (6), which was recrystallized from ethanol in the form of colorless needles: yield, 1.3 g (70%); m.p., 167–169 °C. IR, ν_{max} cm^{-1}: 1664, 1735 (C=O). ^{1}H-NMR ($CDCl_3$), δ: 0.69 (3H, s, 18-CH_3), 0.93 (3H, d, J = 6.2 Hz, 21-CH_3), 1.21 (3H, s, 19-CH_3), 2.05 (3H, s, 3β-$OCOCH_3$), 3.67 (3H, s, –$COOCH_3$), 4.72 (1H, brm, 3α-H), 5.71 (1H, brs, 6-H). LR-MS (EI-PIM), *m/z*: 444 (M^+, 2%), 384 (M–AcOH, 100%), 369 (M–AcOH–CH_3, 8%), 269 (M–AcOH–S.C., 18%), 227 [M–AcOH–CH_3–S.C.-part of ring D (27 u), 8%]. HR-MS (EI-PIM), calculated for $C_{27}H_4O_5$ $[M]^+$: 444.6116; found, *m/z*: 444.2876.

2.2.4. *3β-Hydroxy-7-oxo-5-cholen-24-oic acid (7a) and its methyl ester (7b)*

The usual alkaline hydrolysis of the 7-oxo-Δ^5 ester-acetate 6 (810 mg, 1.8 mmol) with 5% methanolic NaOH, followed by acidification with 10% HCl, gave 3β-hydroxy-7-oxo-5-cholen-24-oic acid (7a), which was recrystallized from aqueous methanol in the form of colorless needles: yield, 710 mg (100%); m.p., 237–239 °C. IR, ν_{max} cm^{-1}: 1683 (C=O), 3286 (O–H). ^{1}H-NMR ($CDCl_3$), δ: 0.69 (3H, s, 18-CH_3), 0.95 (3H, d, J = 6.5 Hz, 21-CH_3), 1.20 (3H, s, 19-CH_3), 3.69 (1H, brm, 3α-H), 5.70 (1H, brs, 6-H). LR-MS (EI-PIM), *m/z*: 374 (M–CH_3, 100%), 356 (M–CH_3–H_2O, 65%), 341 (M–H_2O–2CH_3, 51%), 289 (44%), 263 (68%), 255 [M–CH_3–H_2O–S.C. (101 u), 22%], 213 (M–CH_3–H_2O–S.C.-ring D, 29%). HR-MS (EI-PIM), calculated for $C_{24}H_{36}O_4$ $[M]^+$: 388.5492; found, *m/z*: 388.2614.

The acid 7a (1.86 g, 4.8 mmol) was then esterified by the usual *p*-toluenesulfonic acid (*p*-TsOH)-methanol method to give the corresponding methyl ester (7b), which was recrystallized in quantitative yield from aqueous methanol in the form of colorless amorphous solids: yield, 1.92 g; m.p., 138–141 °C. IR, ν_{max} cm^{-1}: 1672 (C=O), 3475 (O–H). ^{1}H-NMR ($CDCl_3$), δ: 0.69 (3H, s, 18-CH_3), 0.93 (3H, d, J=6.2 Hz, 21-CH_3), 1.20 (3H, s, 19-CH_3), 3.60 (1H, brm, 3α-H), 3.67 (3H, s, –$COOCH_3$), 5.67 (1H, brs, 6-H). LR-MS, *m/z*: 402 (M^+, 100%), 369 (M–H_2O–CH_3, 15%), 287 [M–S.C. (115 u), 13%], 269 (M–H_2O–S.C., 5%). HR-MS (EI-PIM), calculated for $C_{25}H_{38}O_4$ $[M]^+$: 402.5762; found, *m/z*: 402.2770.

2.2.5. *Methyl 3β-tert-butyldimethylsilyloxy-7-oxo-5-cholen-24-oate (8)*

To a solution of the 3β-hydroxy-Δ^5 ester 7 (800 mg, 2.0 mmol) in anhydrous DMF (6 ml) and pyridine (0.5 ml), imidazole (1.68 g) and *tert*-butyldimethylsilyl chloride (TBDMSiCl; 840 mg, 5.6 mmol) were added with ice-bath cooling at 10 °C, and the mixture was left to stand at room temperature for 30 min. The reaction product was extracted with CH_2Cl_2 (30 ml), and the combined extract was washed with water, dried with Drierite, and evaporated. The residue was recrystallized from aqueous methanol to give the 3β-*tert*-butyldimethylsilyloxy (TBDMSi) derivative 8 in the form of colorless amorphous solids: yield, 996 mg (97%); m.p., 179–181 °C. IR, ν_{max} cm^{-1}: 1668, 1747 (C=O), 3475 (O–H). ^{1}H-NMR ($CDCl_3$), δ: 0.06 [6H, s, –$Si(CH_3)_2C(CH_3)_3$], 0.68 (3H, s, 18-CH_3), 0.89 [9H, s, –$Si(CH_3)_2C(CH_3)_3$], 0.93 (3H, d, J = 6.8 Hz, 21-CH_3), 1.18 (3H, s, 19-CH_3), 3.60 (1H, brm, 3α-H), 3.67 (3H, s, –$COOCH_3$), 5.67 (1H, brs, 6-H). LR-MS (EI-PIM), *m/z*: 516 (M^+, 2%), 501 (M–CH_3, 4%), 459 [M–$C(CH_3)_3$, 100%], 427 (30%). HR-MS (EI-PIM), calculated for $C_{31}H_{52}O_4Si$ $[M]^+$: 516.8396; found, *m/z*: 516.3635.

2.2.6. *Methyl 3β-tert-butyldimethylsilyloxy-7β-hydroxy-5-cholen-24-oate (9)*

To a freshly prepared solution of $Zn(BH_4)_2$ in Et_2O (18 ml), a solution of the 3β-TBDMSi-7-oxo ester 8 (910 mg, 1.8 mmol) in benzene (9 ml) was added dropwise under N_2. After further stirring at room temperature for 2 h, the mixture was poured into water, and the organic layer was washed with 10% acetic acid and water, dried with Drierite, and evaporated to dryness. The residue was recrystallized from aqueous methanol to give the 3β-TBDMSi-7β-hydroxy ester 9 in the form of colorless amorphous solids: yield, 730 mg (80%); m.p., 92–94 °C. IR, ν_{max} cm^{-1}: 1738 (C=O), 3304 (O–H). ^{1}H-NMR ($CDCl_3$), δ: 0.05 [6H, s, –$Si(CH_3)_2C(CH_3)_3$], 0.69 (3H, s, 18-CH_3), 0.89 [9H, s, –$Si(CH_3)_2C(CH_3)_3$], 0.93 (3H, d, J = 6.8 Hz, 21-CH_3), 1.04 (3H, s, 19-CH_3), 3.49 (1H, brm, 3α-H), 3.67 (3H, s, –$COOCH_3$), 3.83 (1H, brm, 7α-H), 5.24 (1H, brs, 6-H). LR-MS (EI-PIM), *m/z*: 518 (M^+, 11%), 500 (M-H_2O, 9%), 461 (70%), 386 (M–TBDMSiOH, 100%), 369 (M–TBDMSiOH–H_2O, 63%), 327 (29%). HR-MS (EI-

PIM), calculated for $C_{31}H_{54}O_4Si$ [M]$^+$: 518.3806; found, *m/z*: 518.3791.

2.2.7. *Methyl 3β-tert-butyldimethylsilyloxy-7β-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-5-cholen-24-oate (10)*

To a solution of the 3β-TBDMSi-7β-hydroxy ester **9** (500 mg, 0.96 mmol) in anhydrous toluene (25 ml), 2-acetamido-1α-chloro-1,2-dideoxy-3,4,6-tri-O-acetyl-D-glucopyranose (500 mg, 1.4 mmol), freshly prepared $CdCO_3$ (500 mg, 2.9 mmol), and molecular sieves (4 Å, 500 mg) were added, and the resulting suspension was azeotropically refluxed with stirring. After 2 and 4 h, additional amounts of the halosugar (500 mg, 1.4 mmol) and $CdCO_3$ (500 mg) were added in several portions, and the mixture was further refluxed for 8 h. The precipitate was removed by filtration and washed with toluene. The combined mother liquor was evaporated down to dryness under reduced pressure, and the residue was subjected to column chromatography on activated neutral alumina (activity II, 120 g). Elution with EtOAc-benzene (2:8–4:6, v/v) and recrystallization of the eluate from acetone-hexane gave the 3β-TBDMSi-7β-GlcNAc triacetate **10** in the form of colorless amorphous solids: yield, 57 mg (7%); m.p., 207–210 °C. IR, ν_{max} cm^{-1}: 1748 (C=O). ^{1}H-NMR ($CDCl_3$), δ: 0.06 [6H, s, $-Si(CH_3)_2C(CH_3)_3$], 0.64 (3H, s, 18-CH_3), 0.88 [9H, s, $-Si(CH_3)_2C(CH_3)_3$], 0.92 (3H, d, J = 5.9 Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 1.92, 2.02, 2.03, 2.07 (each 3H, s, $-COCH_3$), 3.49 (1H, brm, 3α-H), 3.67 (3H, s, $-COOCH_3$), 3.83 (1H, brm, 7α-H), 4.13 (4H, brm, 2′-, 5′- and 6′-H), 4.78 (1H, d, J = 8.1 Hz, 1′α-H), 5.02, 5.32 (each 1H, m, 3′- and 4′-H), 5.44 (1H, brs, 6-H), 5.47 (1H, brs, NH). LR-MS (EI-PIM), *m/z*: 790 (M–$C(CH_3)_3$, 24%), 517 [M-part of sugar moiety (S.M.) (330 u), 55%], 500 [M–S.M. (346 u)–H, 45%], 369 (M–TBDMSiOH–S.M., 100%), 346 (S.M., 77%), 330 (part of S.M., 84%), 286 (S.M.–AcOH, 38%), 259 (23%). HR-MS (APCI-NIM), calculated for $C_{45}H_{72}NO_{12}Si$ [M–H]$^-$: 846.4824; found: *m/z*, 846.4834.

2.2.8. *Methyl 3β-hydroxy-7β-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-5-cholen-24-oate (11)*

To a solution of the 3β-TBDMSi-7β-GlcNAc triacetate **10** (120 mg, 0.14 mmol) in ethanol (12 ml), *p*-toluenesulfonic acid (100 mg) was added. After being left to stand at room temperature for 1 h, the solution was neutralized with 5% $NaHCO_3$, and the solvent was evaporated. The residue was extracted with $CHCl_3$, and the combined extract was washed with water, dried with Drierite, and evaporated to dryness. Recrystallization of the residue from aqueous methanol gave the title compound (**11**) in nearly quantitative yield in the form of colorless amorphous solids: yield, 103 mg; m.p., 207–210 °C. IR, ν_{max} cm^{-1}: 1747 (C=O), 3290 (O–H). ^{1}H-NMR ($CDCl_3$), δ: 0.65 (3H, s, 18-CH_3), 0.92 (3H, d, J = 5.9 Hz, 21-CH_3), 1.03 (3H, s, 19-CH_3), 1.92, 2.02, 2.03, 2.08 (each 3H, s, $-COCH_3$), 3.49 (1H, brm, 3α-H), 3.67 (3H, s, $-COOCH_3$), 3.83 (1H, brm, 7α-H), 3.66, 3.83, 4.19 (4H, each m, 2′-, 5′- and 6′-H), 4.78 (1H, d, J = 8.1 Hz, 1′α-H), 5.03, 5.33 (each 1H, m, 3′- and 4′-H), 5.46 (1H, brs, 6-H), 5.50 (1H, m, NH). LR-MS (EI-PIM), *m/z*: 386 (M–S.M.–H, 100%), 368 (M–S.M.–H–H_2O, 51%), 253 [M–S.M.–H_2O–S.C. (115 u)–H, 10%], 249 (27%). HR-MS (APCI-NIM), calculated for $C_{39}H_{58}NO_{12}$ [M–H]$^-$: 732.3959; found, *m/z*: 732.3942.

2.2.9. *3β-Sulfooxy-7β-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-5-cholen-24-oic acid disodium salts (1a)*

To a solution of the 3β-hydroxy-7β-GlcNAc ester-triacetate **11** (95 mg, 0.13 mmol) in dry pyridine (5 ml), sulfur trioxide-trimethylamine complex (95 mg, 0.83 mmol) was added, and the mixture was stirred at room temperature for 1 h. The reaction mixture was poured into ice-cooled petroleum ether (25 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 stirred overnight at room temperature. 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-7β-GlcNAc disodium salt (**1a**) was eluted with methanol (20 ml) and recrystallized from methanol-EtOAc in the form of colorless amorphous solids: yield, 78 mg (85%); m.p., 234–236 °C. IR, ν_{max} cm^{-1}: 1637, 1648, 1654 (C=O), 3294 (O–H). ^{1}H-NMR (CD_3OD), δ: 0.70 (3H, s, 18-CH_3), 0.96 (3H, d, J = 6.5 Hz, 21-CH_3), 1.04 (3H, s, 19-CH_3), 3.38–3.88 (6H, brm, 3′-, 4′-, 5′-, 6′-, and 7α-H), 4.15 (1H, brm, 3α-H), 4.52 (1H, d, J = 8.1 Hz, 1′-H), 5.76 (1H, brs, 6-H). LR-MS (ESI-NIM), *m/z*: 672 [M′ (=M–2Na+2H)–H, 100%], 497 (7%), 460 (9%), 389 [M′–SO_3-part of GlcNAc (204 u), 15%], 278 (8%), 97 (HSO_4, 13%). HR-MS (ESI-NIM), calculated for $C_{32}H_{50}NO_{12}S$ [M–2Na+2H–H]$^-$: 672.3054; found, *m/z*: 672.3041.

2.2.10. *3β-Sulfooxy-7β-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-5-cholen-24-oyl glycine disodium salts (1b)*

To a magnetically stirred solution of the 3β-sulfate-7β-GlcNAc disodium salt **1a** (38 mg, 0.054 mmol) in DMF (4 ml), DMT-MM (43 mg, 0.16 mmol) and triethylamine (190 μl) were added, and the mixture was stirred at room temperature for 10 min. Glycine methyl ester hydrochloride (40 mg, 0.32 mmol) was then added to the mixture, which was further stirred for 3 h. After adding 5% methanolic NaOH (4 ml) and stirring for 3 h at room temperature, the alkaline solution was adjusted to pH 8, diluted with water (30 ml), and loaded onto a Sep-Pak® tC_{18} cartridge, which was washed successively with water (20 ml) and 20% methanol (20 ml). Elution with 50% methanol gave the title compound (**1b**), which was recrystallized from methanol-hexane in the form of colorless amorphous solids: yield, 39 mg (93%); m.p., 212–216 °C. IR, ν_{max} cm^{-1}: 1653 (C=O), 3293 (O–H). ^{1}H-NMR (CD_3OD), δ: 0.70 (3H, s, 18-CH_3), 0.98 (3H, d, J = 6.2 Hz, 21-CH_3), 1.04 (3H, s, 19-CH_3), 3.15-3.90 (6H, brm, 3′-, 4′-, 5′-, 6′- and 7α-H), 3.74 (2H, brs, $-CH_2N-$), 4.15 (1H, brm, 3α-H), 4.54 (1H, d, J = 8.4 Hz, 1′-H), 5.77 (1H, brs, 6-H), 7.82 (1H, brm, $-CONH-$). LR-MS (ESI-NIM), *m/z*: 729 [M′ (=M–2Na+2H)–H, 100%], 672 (5%), 631 (M′–H_2SO_4, 6%), 460 (5%), 433 (7%), 410 (M′–H_2SO_4–GlcNAc–H, 8%), 389 (10%), 364 (15%), 254 [M′–H_2SO_4–S.C. (158 u)-GlcNAc, 12%], 97 (HSO_4, 37%). HR-MS (ESI-NIM), calculated for $C_{34}H_{53}N_2O_{13}S$ [M–2Na+2H–H]$^-$: 729.3268; found, *m/z*: 729.3261.

2.2.11. *3β-Sulfooxy-7β-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-5-cholen-24-oyl taurine disodium salts (1c)*

The 3β-sulfate-7β-GlcNAc disodium salt **1a** (33 mg, 0.046 mmol) in DMF (4 ml) was treated with DMT-MM

(36 mg, 0.13 mmol) and triethylamine (160 μl), followed by taurine (33 mg, 0.26 mmol), as described for the preparation of **1b**. After being processed analogously, the reaction mixture was adjusted to pH 10 with 10% aqueous NaOH, then to pH 8 with 10% HCl, diluted with water (30 ml), and loaded onto a Sep-Pak® tC_{18} cartridge, which was washed with water and 10% methanol. Elution with 30% methanol afforded the desired triple-conjugate **1c** as the disodium salt, which was recrystallized from methanol-$CHCl_3$ in the form of colorless amorphous solids: yield, 32 mg (85 %); m.p., 169–175 °C. IR, ν_{max} cm^{-1}: 1654 (C=O), 3285 (O—H). ^{1}H-NMR (CD_3OD), δ; 0.69 (3H, s, 18-CH_3), 0.96 (3H, d, J = 6.5 Hz, 21-CH_3), 1.04 (3H, s, 19-CH_3), 3.16-3.92 (6H, brm, 3′-, 4′-, 5′-, 6′- and 7α-H), 2.98 (2H, t, J = 6.8 Hz, —CH_2S—), 3.59 (2H, t, J = 6.8 Hz, —CH_2N—), 4.26 (1H, brm, 3α-H), 4.53 (1H, d, J = 8.1 Hz, 1′-H), 5.76 (1H, s., 6-H), 7.88 (1H, brm, —CONH—). LR-MS (ESI-NIM), *m/z*: 801 ([M′ (=M−Na+H)−H]$^-$, 60%), 779 (M′−Na+H−H, 15%), 699 (M′−Na+H−H−SO_3, 37%), 680 (M′−Na−H_2SO_4−H, 29%), 490 (20%), 478 (M′−Na−SO_3−GlcNAc−H, 22%), 460 (M′−Na−SO_4−GlcNAc−H, 59%), 389 (100%), 278 (73%), 97 (HSO_4, 57%). HR-MS (ESI-NIM), *m/z*: calculated for $C_{34}H_{54}N_2O_{14}NaS_2$ [M−Na+H−H]: 801.2914; found, *m/z*: 801.2889.

2.2.12. 3β-*Sulfooxy-7-oxo-5-cholen-24-oic acid disodium salts (12a)*

The 3β-hydroxy-7-oxo-Δ^5 ester **7** (580 mg, 1.5 mmol) was treated with sulfur trioxide-trimethylamine complex, followed by 5% methanolic NaOH, as described for the preparation of **1a**. After passing through a Sep-Pak® tC_{18} cartridge and being washed with H_2O and 20% methanol, the crude 3β-sulfate-7-oxo-Δ^5 disodium salt **12a** was eluted with 60% methanol. Recrystallization from methanol-EtOAc gave the analytical pure **12a** in the form of colorless amorphous solids: yield, 512 mg (70%); m.p., 228–232 °C. IR, ν_{max} cm^{-1}: 1542 (C=C), 1671 (C=O). ^{1}H-NMR (CD_3OD), δ: 0.73 (3H, s, 18-CH_3), 0.97 (3H, d, J = 6.2 Hz, 21-CH_3), 1.25 (3H, s, 19-CH_3), 4.25 (1H, brm, 3α-H), 5.69 (1H, brs, 6-H). LR-MS (EI-PIM), *m/z*; 370 [M′ (=M−2Na+2H)−H_2SO_4, 100%], 355 (M′−H_2SO_4−CH_3, 10%), 269 [M′−H_2SO_4−S.C. (101 u), 23%], 227 (M′−H_2SO_4−CH_3−S.C.-part of ring D, 12%). HR-MS (ESI-NIM), calculated for $C_{24}H_{35}O_7S$ [M−2Na+2H−H]$^-$: 467.2103; found, *m/z*: 467.2102.

2.2.13. 3β-*Sulfooxy-7-oxo-5-cholen-24-oyl glycine disodium salts (12b)*

The 3β-sulfate-7-oxo-Δ^5 disodium salt **12a** (100 mg, 0.2 mmol) was treated with DMT-MM (100 mg, 0.37 mmol) and glycine methyl ester hydrochloride (100 mg, 0.82 mmol), followed by 5% methanolic NaOH, as described for the preparation of **1b**. After being processed analogously, the product was passed through a Sep-Pak® tC_{18} cartridge and washed successively with water and 20% methanol. Elution with 60% methanol gave the double-conjugate **12b**, which was recrystallized from methanol-EtOAc in the form colorless amorphous solids: yield, 89 mg (70%); m.p., 239–242 °C. IR, ν_{max} cm^{-1}: 1654 (C=O). ^{1}H-NMR (CD_3OD), δ: 0.73 (3H, s, 18-CH_3), 0.98 (3H, brs, 21-CH_3), 1.23 (3H, s, 19-CH_3), 3.72 (2H, d, J = 6.5 Hz, —CH_2N—), 4.24 (1H, brm, 3α-H), 5.67 (1H, brs, 6-H). LR-MS (EI-PIM), *m/z*: 427 [M′ (=M−2Na+2H)−H_2SO_4, 46%], 409 (19%), 370 (M′−SO_3−glycine, 100%), 352 (M′−H_2SO_4-glycine, 12%), 311 (54%), 269 [M′−H_2SO_4−S.C. (158 u), 40%], 227 (M′−H_2SO_4−S.C.−CH_3-part of ring D, 16%). HR-MS (ESI-NIM), calculated for $C_{26}H_{38}NO_8S$ [M−2Na+2H−H]$^-$: 524.2318; found, *m/z*: 524.2331.

2.2.14. 3β-*Sulfooxy-7-oxo-5-cholen-24-oyl taurine disodium salts (12c)*

The 3β-sulfate-7-oxo-Δ^5 disodium salt **12a** (100 mg, 0.2 mmol) was treated with DMT-MM (100 mg, 0.37 mmol) and taurine (100 mg, 0.81 mmol), followed by 10% aqueous NaOH, as described for the preparation of **1c**. After being processed analogously, the product was passed through a Sep-Pak® tC_{18} cartridge and washed successively with water and 20% methanol. Elution with 60% methanol gave the double-conjugate **12c**, which was recrystallized from methanol-EtOAc in the form of colorless amorphous solids: yield, 109 mg (90%); m.p., 177–181 °C. IR, ν_{max} cm^{-1}: 1543 (C=C), 1666 (C=O). ^{1}H-NMR (CD_3OD), δ: 0.73 (3H, s, 18-CH_3), 0.98 (3H, d, J = 6.2 Hz, 21-CH_3), 1.25 (3H, s, 19-CH_3), 2.95 (2H, t, J = 6.8 Hz, —CH_2S—), 3.59 (2H, m, —CH_2N—), 4.26 (1H, brm, 3α-H), 5.68 (1H, brs, 6-H), 7.87 (1H, brm, —CONH—). LR-MS (EI-PIM), *m/z*: 397 (3%), 370 [M′ (=M−Na+H)−SO_3−taurine−Na, 72%], 355 (M′−SO_4−taurine−CH_3−Na, 8%), 311 (14%), 269 [M′−H_2SO_4−S.C. (230 u), 18%], 227 (M′−H_2SO_4−S.C.−CH_3-part of ring D, 9%). HR-MS (ESI-NIM), calculated for $C_{26}H_{39}NO_9NaS_2$ [M−Na+H−H]$^-$: 596.1964. found; *m/z*: 596.1980.

3. Results and discussion

According to the recent findings of Avelius et al. [8], a patient with NP-C1 excreted abnormal amounts of the multi-conjugates of unusual 7-oxygenated Δ^5-bile acids in urine. These conjugates were shown to have the parent 3β-hydroxy-Δ^5-cholenoic acid structure, and to carry an oxygen-containing function (hydroxy or oxo group) at C-7. The hydroxy groups at the C-3 and -7 positions were sulfated and *N*-acetylamino-glucosylated, respectively, while the carboxyl group at C-24 was either unconjugated or amidated with glycine or taurine (**1a–1c**). The abnormal excretion of the analogous conjugates of nonamidated and glycine- and taurine-amidated 3β-hydroxy-7-oxo-5- cholen-24-oic acid 3-sulfates (**12a–12c**) has also been recorded [8]. Therefore, these new and unusual bile acid multi-conjugates seem to be specific markers of NP-C1. The synthetic outline of the targeted bile acid conjugates is shown in Figs. 2–4.

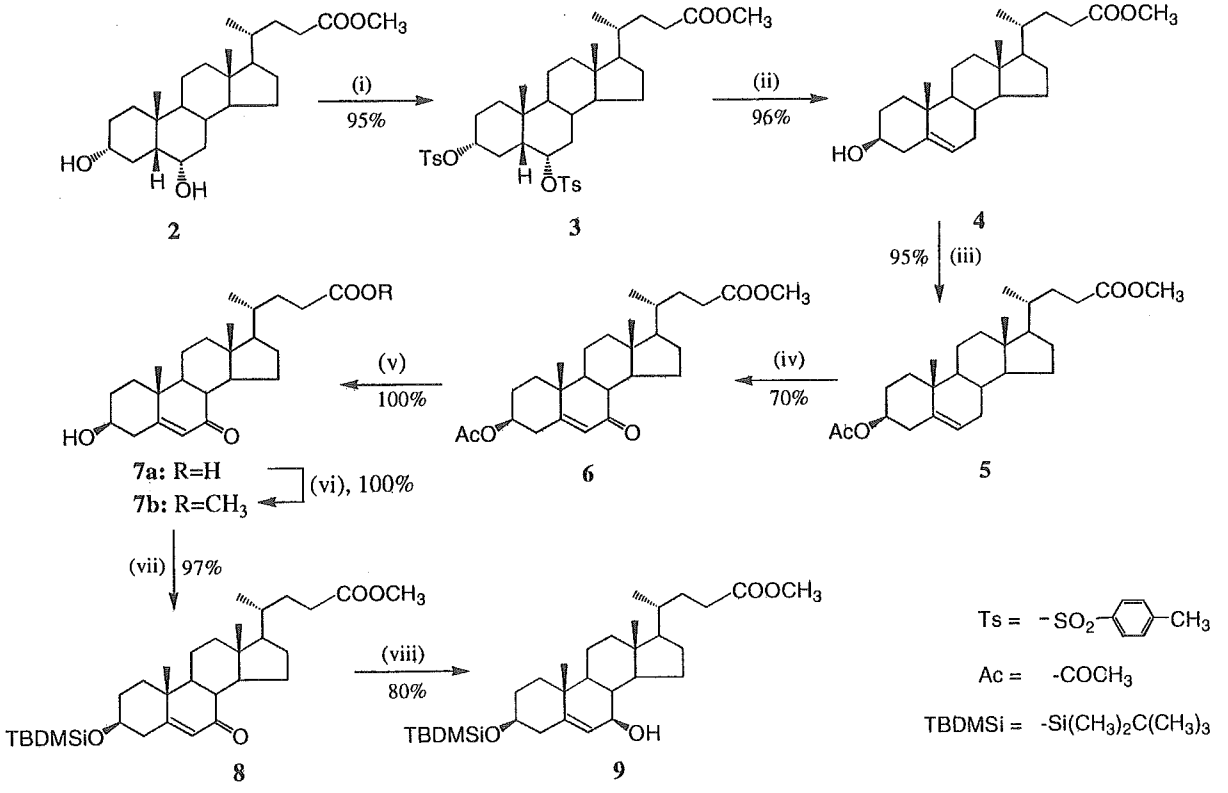
A key intermediate in our synthesis is methyl 3β-TBDMSi-7β-hydroxy-5-cholen-24-oate (**9**), which was prepared from methyl hyodeoxycholate (**2**) in 8 steps (see Fig. 2). Thus, the ester **2** was tosylated with tosyl chloride in pyridine to give the corresponding 3,6-ditosylate (**3**) in nearly quantitative yield. When **3** was treated with boiling DMF in the presence of CH_3COOK for 24 h, simultaneous inversion at the C-3 position and elimination at the C-6 position took place to give methyl 3β-hydroxy-5-cholen-24-oate (**4**) [11], which in turn was acetylated by the usual acetic anhydride-pyridine method to yield the corresponding 3β-acetoxy-Δ^5 ester (**5**). Allylic oxidation of **5** with *t*-BHPO-PDC [12] afforded the corresponding 7-oxo compound (**6**) in good isolated yield. The reaction with the oxidant system proceeded faster and more cleanly than with conventional chromium (VI) complexes [13]. Alkaline hydrol-

ysis of 6 with methanolic KOH, followed by re-esterification of the resulting 3β-hydroxy-7-oxo-Δ^5 acid (7a) with *p*-TsOH in methanol, gave methyl 3β-hydroxy-7-oxo-5-cholen-24-oate (7b). Subsequent protection of the 3β-hydroxy group in 7b with TBDMSiCl and imidazole in DMF gave the corresponding 3β-TBDMSi-7-oxo-Δ^5 ester (8) in nearly quantitative yield.

A preliminary experiment revealed that the reduction of the 7-oxo group in 8 with $NaBH_4$ afforded a mixture of the corresponding epimeric 7α-/7β-ols, accompanied by a small amount of their C-24 hydrolyzed products, from which the desired 7β-hydroxy ester (9) was separated by tedious chromatographic purification. However, when $Zn(BH_4)_2$ in ether [14,15] was used as the reducing agent, the reaction proceeded more cleanly and stereoselectively than with $NaBH_4$, and the 3β-TBDMSi-7β-hydroxy ester (9) was isolated in 77% yield by direct recrystallization, without the need for a chromatographic purification. In addition, less basic $Zn(BH_4)_2$ also left the C-24 ester group intact during the reduction at C-7. The stereochemical configuration at the 7β-hydroxy group in 9 was confirmed by the ^{1}H-NMR signals appearing at 3.83 ppm (7α-H) as a multiplet and at 5.24 ppm (6-H) as a broad singlet [16,17]. The overall yield of the key intermediate (9) based on 2 was 47%.

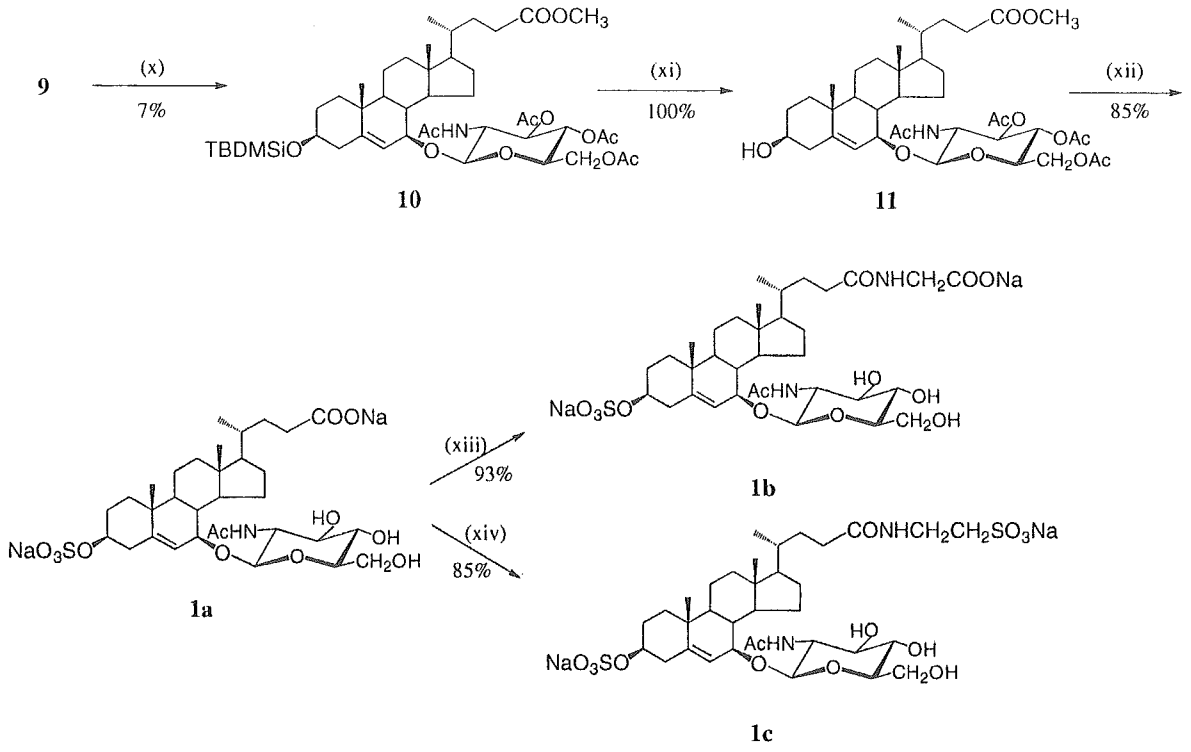
Our next effort was directed at finding a suitable order of conjugation reactions with 9, as well as at removing the protecting groups in both the steroid and sugar moieties. In particular, the order of the conjugations with 9 appeared to be essential for the preparation of the desired bile acid multiconjugates, because of the limited solubility of an intermediary conjugate in many organic solvents. As a result of preliminary experiments, the following order of conjugation reactions with 9 was found to be suitable: (1) N-acetylglucosaminidation at C-7; (2) sulfation at C-3; and (3) amidation at C-24 (see Fig. 3).

At first, β-D-N-acetylglucosaminidation was carried out by the Koenigs-Knorr reaction of 9 with α-acetochloroglucosamine as a glycosyl donor, and a stoichiometric amount of freshly prepared $CdCO_3$ as an activator, in the presence of molecular sieves under azeotropic conditions in refluxing toluene [18,19]. The condensed 3β-TBDMSi-7β-GlcNAc methyl ester-triacetate (10) was isolated in fairly low yield (7%), probably owing to the steric hindrance of the axially-oriented 18- and 19-methyl groups. However, no undesirable by-product was formed at all. Attempting the Koenigs-Knorr reaction using either silver (I) oxide (Ag_2O) or mercury (II) cyanide [$Hg(CN)_2$] [20,21] as an activator also gave 10 in less than 5% yield. Nevertheless, the Koenigs-Knorr condensation with $CdCO_3$ seems profitable, as a mixture of 9 and 10 are easily separated on a short column of silica gel, and 9 can be efficiently recovered. Subsequent deprotection of the TBDMSi group in 10 was attained with *p*-TsOH in methanol at room temperature for 1 h to afford the 3β-hydroxy-7β-GlcNAc methyl ester-triacetate (11) in quantitative yield. The presence of the GlcNAc moiety attached to the Δ^5-steroid nucleus



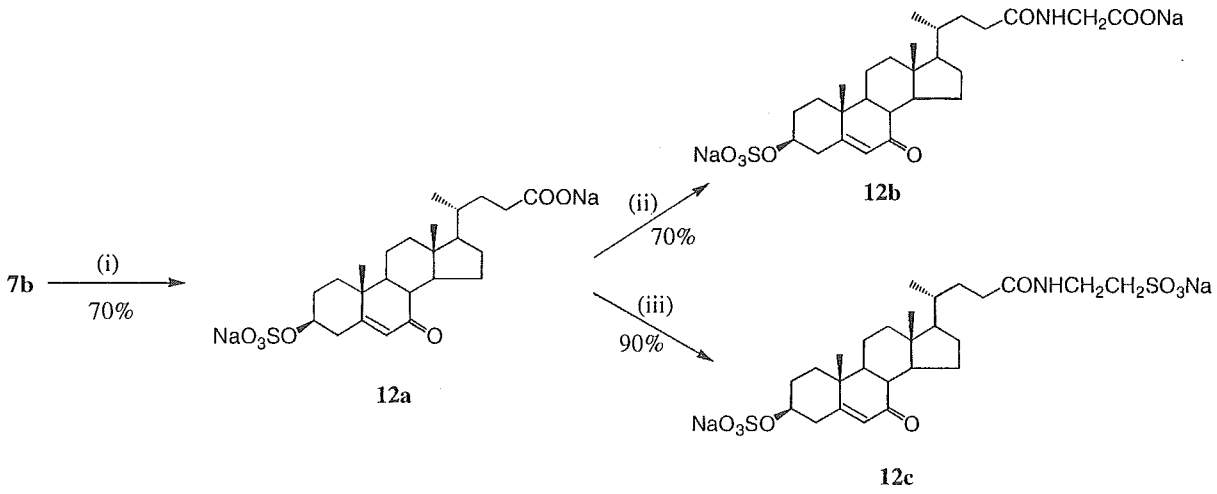
Reagents and conditions; i) tosyl chloride/pyridine, r.t, 2 days. ii) $CH_3COOK/DMF/H_2O$, reflux, 24 h. iii) $(CH_3CO)_2O$/pyridine, r.t, 3 h. iv) PDC/*t*-BHPO/benzene, r.t, 24 h. v) 5% KOH/CH_3OH, r.t, 6 h. vi) *p*-TSA/CH_3OH, r.t, 12 h. vii) TBDMSiCl/imidazole/pyridine/DMF, r.t, 30 min. viii) 1N $Zn(BH_4)_2$/ether, r.t, 2 h.

Fig. 2 – Synthetic route to intermediary methyl 3β-hydroxy-7-oxo-5-cholen-24-oate (7b) and methyl 3β-TBDMSi-7β-hydroxy-5-cholen-24-oate (9) from methyl hyodeoxycholate (2).



Reagents and conditions; x) □-acetochloroglucosamine/$CdCO_3$/toluene, reflux, 14 h. xi) *p*-TSA/CH_3OH, r.t, 1 h. xii) $SO_3^- N^+(CH_3)_3$/pyridine, r.t, 1 h and then 1N NaOH/CH_3OH, r.t, 12 h. xiii) $H_2NCH_2COOCH_3$ HCl/DMT-MM/Et_3N/DMF, r.t, 3 h and then 1N NaOH/CH_3OH, r.t, 3 h. xiv) taurine/DMT-MM/DMF, r.t, 3 h and then 1N NaOH.

Fig. 3 – Synthetic route to nonamidated and glycine- and taurine-amidated 3β-sulfooxy-7β-GlcNAc conjugates of 3β,7β-dihydroxy-5-cholen-24-oic acid (1a–1c) from 9.



Reagents and conditions; i) $SO_3^- N^+(CH_3)_3$/pyridine, r.t, 1 h and then 1N NaOH/CH_3OH, r.t, 12 h.
ii) $H_2NCH_2COOCH_3$ HCl/DMT-MM/Et_3N/DMF, r.t, 3 h and then 1N NaOH/CH_3OH, r.t, 12 h.
iii) taurine/DMT-MM/Et_3N/DMF, r.t, 3 h and then 1N NaOH.

Fig. 4 – Synthetic route to nonamidated and glycine- and taurine-amidated conjugates of 3β-sulfooxy-7-oxo-5-cholen-24-oic acid (12a–12c) from 7b.

by a β-D-glycosidic linkage was confirmed by the ^{1}H-NMR spectra, judging from the signals appearing at 3.83 ppm (7α-H) as a broad multiplet; 4.78 ppm (anomeric 1′α-H) as a doublet (*J*, 8.1 Hz) [22]; 1.92–2.08 ppm (acetyl methyls at C-2′, -3′, -4′ and -6′) as four singlets; and 3.66–5.33 ppm (2′-, 3′-, 4′-, 5′-, and 6′-H) as five multiplets.

The 3β-hydroxy group in **11** was then sulfated with sulfur trioxide-trimethylamine complex in pyridine at room temperature for 3 h [23]. The sulfation reaction proceeded cleanly and rapidly under mild conditions. The crude product was subsequently hydrolyzed with methanolic NaOH to remove the protecting groups at the C-24 methyl ester on the side chain in the aglycone moiety, and the acetoxyl groups at C-3′, C-4′, and C-6′ in the sugar moiety. To purify and isolate the resulting 3β-sulfate-7β-GlcNAc double-conjugate (**1a**) as a crystalline form, the reaction mixture was adjusted to pH 8 with HCl, and loaded onto a Sep-Pak® tC_{18} cartridge for the reversed-phase solid extraction. The cartridge was washed successively with water and 20% aqueous methanol to remove excess reagents, contaminants, and inorganic salts. Elution with methanol gave the crystalline **1a** (as the 3,24-disodium salt) in satisfactory yield (85%).

Finally, the formation of carboxamides at C-24 was carried out by the direct condensation of the carboxylate sodium salt at C-24 in **1a**, and the amino group in glycine or taurine. Exploratory experiments revealed that the direct amidation of the carboxylate (**1a**) with glycine methyl ester hydrochloride in the presence of either *N*-ethylcarbonyl-2-ethoxy-1,2-dihydroquinoline or diethylphosphorylcyanide [24,25] as a coupling agent was unsatisfactory (yield: 60–70%). However, when a versatile coupling agent, DMT-MM [26], was used, the amidation reaction proceeded nearly quantitatively, requiring only the mixing of **1a** (1 eq.), glycine methyl ester hydrochloride (3 eq.), DMT-MM (6 eq.), and triethylamine in DMF at room temperature for 3 h. Activation of the carboxylate salt by DMT-MM and subsequent condensation of the resulting acyloxytriazine with glycine methyl ester hydrochloride proceeded selectively, leading to the formation of the methyl ester derivative of the 24-glyco-3β-sulfooxy-7β-GlcNAc (**1b**), which in turn was hydrolyzed with methanolic NaOH. Since

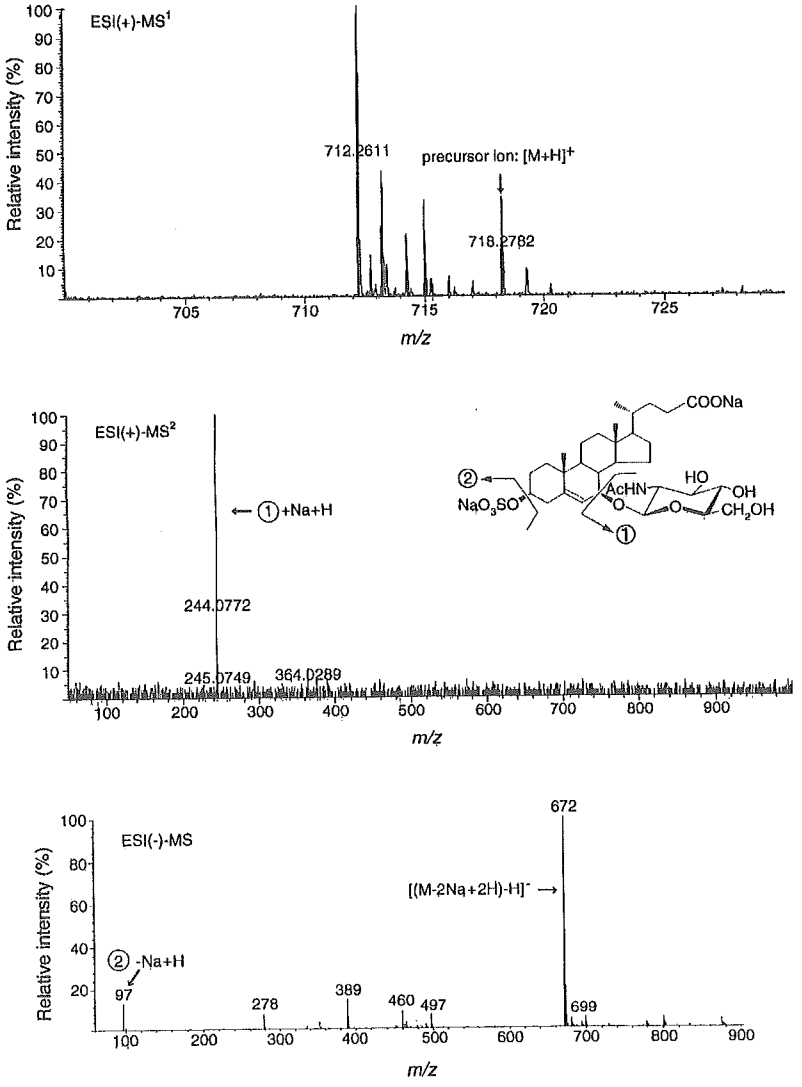


Fig. 5 – LC-MS spectra of 3β-sulfooxy-7β-GlcNAc double-conjugate of 3β,7β-dihydroxy-5-cholen-24-oic acid disodium salt (1a).

the resulting free triple-conjugate was extremely hygroscopic, the hydrolyzed solution was adjusted to pH 8 with HCl, and the mixture was loaded onto a Sep-Pak® tC_{18} cartridge, which upon elution with 50% aqueous methanol gave the desired **1b** as the crystalline disodium salt, in excellent isolated yield (93%).

Transformation of **1a** into the triple-conjugate of the 24-tauro-3β-sulfooxy-7β-GlcNAc disodium salt (**1c**) was similarly achieved by a slight modification of the procedure for **1b**. Thus, **1a** was treated with taurine, DMT-MM, and triethylamine in DMF. After the condensation reaction, the resulting solution was adjusted to pH 8 with aqueous NaOH, and then loaded onto a Sep-Pak® tC_{18} cartridge. Elution with 30% aqueous methanol gave the analytically pure **1c** in an isolated yield of 85%.

By combining of the procedures of sulfation and amidation, as described above, 3β-hydroxy-7-oxo-5-cholen-24-oic acid doubly conjugated with sulfuric acid at C-3, and with glycine (**12b**) or taurine (**12c**) at C-24 (i.e. the major metabolites in the NP-C1 patient [8]), were analogously prepared from the corresponding unconjugate **7b**, via the intermediary 3-monosulfate (**12a**) (see Fig. 4). As expected, the double- and triple-conjugates (**12b–12c** and **1a–1c**) thus prepared were highly hydrophilic and readily soluble in water. However, they were insoluble in many organic solvents, with methanol and DMF as exceptions.

The ^{1}H-NMR data for **1b**, **1c**, **12b**, and **12c** are indicative of the formation of the sulfonate and carboxamide. An appreciable downfield shift (ca. 4.2 ppm) of the 3α-H (broad multiplet) was observed for the sulfo-conjugates by comparison to data for **11** and the 3α-H resonating at 4.15–4.26 ppm [23]. The glyco-conjugates **1b** and **12b** showed the broad singlet signal at 3.74 ppm and the doublet signal at 3.72 ppm, both due to $-CH_2NH-$, and the broad multiplet signal at 7.82 ppm, arising from $-CONH-$ [24,25]. On the other hand, the tauro-conjugates (**1c** and **12c**) were identified by the appearance of two triplet signals at 2.95-2.98 ppm ($-CH_2S-$) and 3.59 ppm ($-CH_2N-$), and by a broad multiplet signal at 7.87–7.88 ppm ($-CONH-$).

The LC/MS spectra with ESI-PIM or ESI-NIM of **1a–1c** (Figs. 5–7) provided confirming evidence for their structures, always giving molecular-related ions as the major fragment

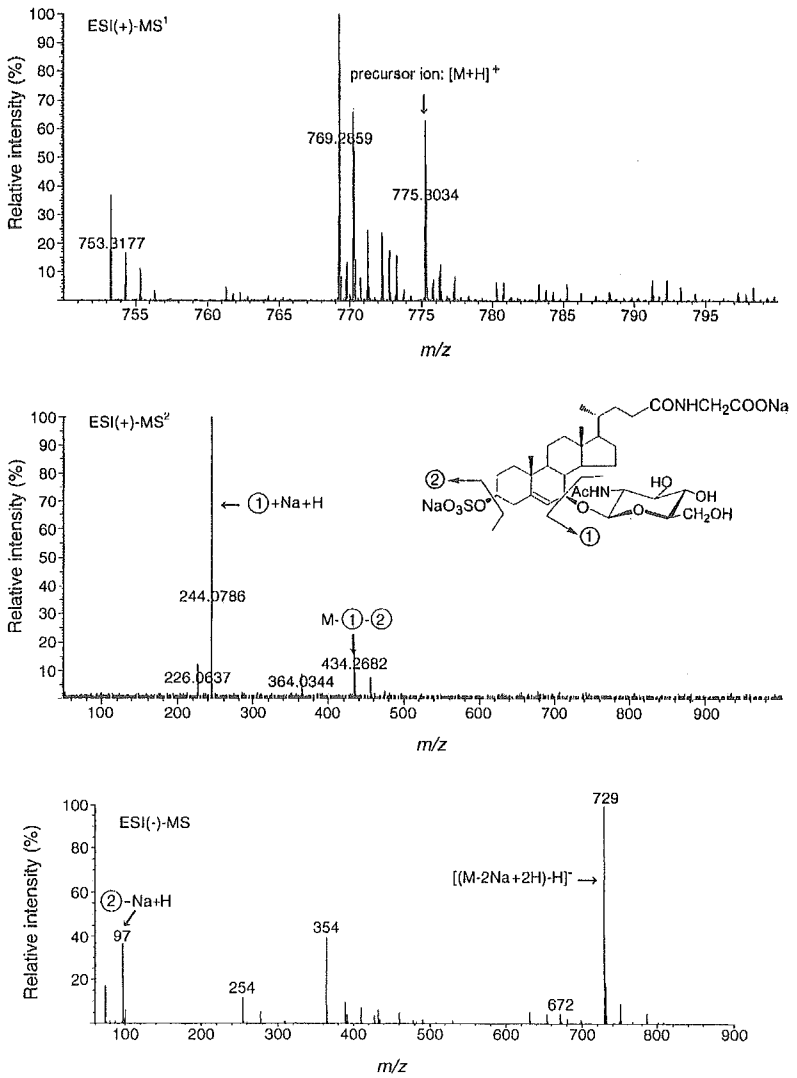


Fig. 6 – LC-MS spectra of 24-glyco-3β-sulfooxy-7β-GlcNAc triple conjugate of 3β,7β-dihydroxy-5-cholen-24-oic acid disodium salt (1b).

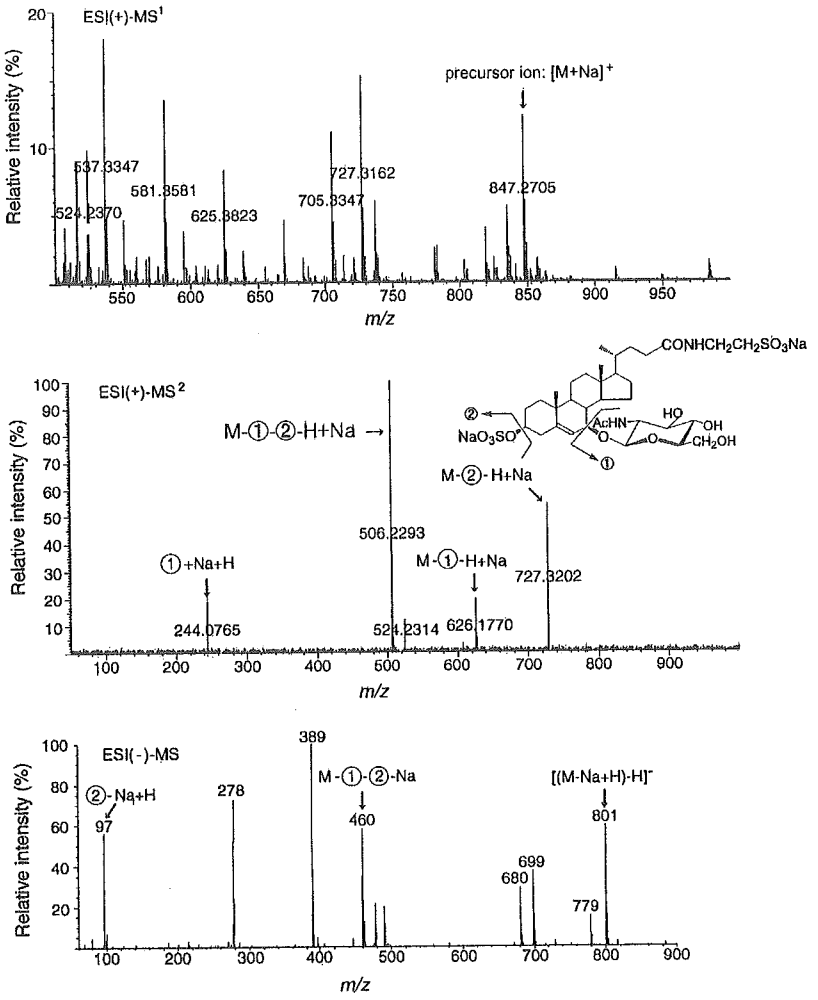


Fig. 7 – LC-MS spectra of 24-tauro-3β-sulfooxy-7β-GlcNAc triple conjugate of 3β,7β-dihydroxy-5-cholen-24-oic acid disodium salt (1c).

ions. In both the ESI-PIM and ESI-NIM, the fragmentation patterns of **1a** and **1b** were similar to each other, while the pattern of **1c** differed. Thus, in the MS/MS with ESI-PIM, the MS^1 spectra of **1a** and **1b** exhibited an intense protonated molecule $[M+H]^+$ at *m/z* 718.2782 and at *m/z* 775.3034, respectively; these ions were selected as precursor ions in the MS^2 spectra, and the occurrence of the base peak at *m/z* 244 in each spectrum indicated the presence of a GlcNAc moiety. On the other hand, the MS^1 spectrum of **1c** showed an adducted molecule $[M+Na]^+$ at *m/z* 847.2705; the selection of the ion as a precursor ion gave the MS^2 spectrum, in which several fragment ions associated with the elimination of GlcNAc and/or OSO_3Na residues from M occurred at *m/z* 727, 626, and 506.

Meanwhile, the ESI-NIM spectra of **1a** and **1b** gave a deprotonated molecule $[(M-2Na+2H)-H]^-$ at *m/z* 672 and at *m/z* 729, respectively, comprising the base peak in each spectrum. The presence of the OSO_3Na moiety was confirmed by the appearance of an ion at *m/z* 97. The ESI-NIM spectrum of **1a** was essentially identical to that measured by the collision-induced dissociation method for the 3-sulfo-7-GlcNAc conjugate of a dihydrocholenoic acid identified in the urine of a NP-C1 patient [8]. On the other hand, the spectrum of **1c** with ESI-NIM exhibited a deprotonated molecule $[(M-Na+H)-H]^-$ at *m/z* 801, accompanied by an ion at *m/z* 97, and a characteristic ion at *m/z* 460 arising from the elimination of the GlcNAc and OSO_3Na residues from M.

The relationship between the formation of the abnormal bile acids and the metabolic defect in NP-C1 is not known. Since a NP-C1 patient with cholestasis excretes significant amounts of the unusual multi-conjugated bile acids **1a–1c** and **12a–12b** in the urine [8], these metabolites are expected to be specific markers of the disease. Further studies on the biological and physiological significance of the unusual metabolites are now being conducted in our laboratory.

Acknowledgments

We would like to express our thanks to Shimadzu Co. for the LC/ESI-MS/MS measurements. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sciences, Sports, and Culture of Japan for 2004-2005 and Nihon University Multidisciplinary Research Grant for 2005–2006.

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Risk Factors for Fatality and Neurological Sequelae after Status Epilepticus in Children

Y. Maegaki[1]
Y. Kurozawa[2]
K. Hanaki[3]
K. Ohno[1]

Abstract

Using multivariate regression analysis, we examined risk factors for fatality and neurological sequelae after status epilepticus (SE) in children. Possible risk factors included sex, age at onset, the cause of SE, pyrexia, asthmatic attack during SE, past history of seizure, predisposing neurological abnormality, seizure duration, type of seizure, and medication with theophylline. Consecutive patients with SE, aged 1 month to 18 years, who were referred to Tottori University Hospital from 1984 to 2002 were reviewed. Of the 234 patients enrolled, 45 patients (19.2%) showed poor outcomes, namely early death in 9 and neurological sequela in 36. Acute neurological insult and progressive neurological disease as the cause of SE were very significantly related to poor outcome (OR = 33.68, p = 0.000). We excluded 21 patients with the etiology of acute neurological insult and progressive neurological disease and then reanalyzed risk factors in the remaining 213 patients. Twenty-nine patients (13.6%) showed poor outcome, namely early death in 6 and neurological sequela in 23. Seizure duration of more than 2 hours (OR = 12.73, p = 0.000) and moderate to severe asthmatic attack (OR = 31.61, p = 0.010) were associated with poor outcome. These results indicate that long-lasting seizure activity and asthmatic attack can exacerbate SE-associated brain injury.

Key words
Status epilepticus · risk factor · prognosis · children

Introduction

Status epilepticus (SE) is a common emergency in infants and children and poses a risk for SE-associated morbidity and mortality. Although SE-associated morbidity and mortality are high in adults, they are relatively low in children: overall mortality in recent pediatric series is 0 – 9% and morbidity is 9 – 34% [3,6,7, 10,19,30]. It is obvious that outcome is mainly associated with the cause of SE, namely acute neurological insults such as meningitis, encephalitis, head trauma, hypoxia, and systemic metabolic disease. After excluding acute neurological insults and systemic metabolic disease, morbidity and mortality are quite low [3,10,19]. Other than the cause of SE, little is known about risk factors for prognosis. Although seizure duration and age at onset may be risk factors, the results are still controversial [3,7,19].

In this retrospective study we sought to find risk factors for outcome after SE during childhood. The risk factors for prognosis after SE have been evaluated simply using χ^2 statistics or t tests in previous studies. These statistical analyses may have failed to extract the risk factors, because a patient commonly may have multiple factors associated with prognosis and various factors correlating with each other. In a study of adult patients, predictors of outcome after SE were different between univariate and multivariate analyses in the same subjects [5]. Univariate analysis seems to overestimate the results, e.g., while univariate statistical analysis revealed a significant correlation between younger age at onset of SE and neurological sequelae; this seemed to be due to a high incidence of acute neurological insults in the

Affiliation
[1] Division of Child Neurology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, Yonago, Japan
[2] Division of Health Administration and Promotion, Faculty of Medicine, Tottori University, Yonago, Japan
[3] Division of Pediatrics and Perinatology, Faculty of Medicine, Tottori University, Yonago, Japan

Correspondence
Yoshihiro Maegaki, M.D. · Division of Child Neurology · Institute of Neurological Sciences · Faculty of Medicine · Tottori University · 36-1 Nishi-machi · Yonago 683-8504 · Japan ·
E-mail: maegaki@grape.med.tottori-u.ac.jp

Received: October 27, 2004 · **Accepted after Revision:** March 25, 2005

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Neuropediatrics 2005; 36: 186 – 192 © Georg Thieme Verlag KG Stuttgart · New York ·
DOI 10.1055/s-2005-865611 ·
ISSN 0174-304X

younger age group [3,19]. Within the same etiology group, age did not significantly affect outcome [19]. In children with SE, there have been only a few studies dealing with various risk factors associated with prognosis using multivariate analysis [29]. Possible risk factors evaluated in this study included sex, age at onset, etiology of SE, pyrexia, asthmatic attack during SE, past history of seizures, predisposing neurological conditions, duration of SE, type of seizure, and proconvulsive drugs. The goal of this study is to identify risk factors other than the cause of SE. Therefore, statistical analysis was performed after the exclusion of patients with an etiology of acute neurological insults and metabolic or progressive disease.

Materials and Methods

Study population and design

Tottori University Hospital serves the western part of Tottori Prefecture and surrounding areas. Most emergency cases involving infants and children are referred to our hospital. Three local hospitals serve as less intensive patient institutions in this area, so intensive-care patients are usually transferred to our hospital. Most neurologically ill infants and children, including those with developmental disorders in this area, are also referred to our hospital. We reviewed the medical records of infants and children aged 1 month to 18 years who were referred to our hospital due to status epilepticus from January 1984 to December 2002. Status epilepticus was defined as any seizure lasting more than 30 minutes or recurrent seizures lasting a total of more than 30 minutes without complete recovery of consciousness. Although treatment for SE varied in each patient, almost all patients received diazepam first. When diazepam failed to stop the seizures, phenytoin or thiopental were then administered.

The etiology of SE was classified using the classification of Maytal et al.: (1) *cryptogenic:* a seizure occurring in the absence of acute precipitating neurological insult or systemic metabolic dysfunction in a patient without preexisting neurological abnormality or neurological insult known to be associated with an increased risk of seizures; (2) *remote symptomatic:* a seizure occurring without acute provocation in a patient with a history of neurological insult known to be associated with an increased risk of convulsions (e.g., history of stroke and meningitis, epilepsy, and developmental disorder such as mental retardation and cerebral palsy); (3) *febrile:* a provoked seizure in which the sole acute provocation was fever (temperature greater than 38.4 °C without history of afebrile seizure. It is important here to distinguish between febrile SE and acute encephalitis. Acute encephalitis was defined as having increased cell count in cerebrospinal fluid (CSF); (4) *acute symptomatic:* a seizure occurring during an acute illness in which there was a known neurological insult or systemic metabolic dysfunction (e.g., meningitis, encephalitis, and head trauma). Severe systemic complications known to cause brain damage were also included (e.g., hypoglycemia, hypoxemia, hypotension); and (5) *progressive encephalopathy:* a seizure occurring in the context of progressive neurological disease [19].

To identify the etiology of SE, routine laboratory data including a complete blood count, serum glucose, serum calcium, urinalysis, CSF, cranial computed tomography or magnetic resonance imaging, and electroencephalogram were collected for each patient, especially after the first SE. After the second or later SEs, laboratory examinations were repeated if necessary. Further metabolic analysis including plasma and urine amino acids, urine organic acids, and blood and CSF lactic acids was performed for diagnosis in patients who showed any neurological sequelae or developmental deterioration after SE. Normal cell count in the CSF is 5/μL or less.

Neurological sequelae were evaluated several days after SE (short-term outcome), usually at discharge from the hospital, and then at the last visit (long-term outcome). If a patient had any neurological sequelae at discharge, follow-up examinations were repeated. Outcomes were assessed at the last follow-up. If a patient was suspected of having any mental abnormality, the Wechsler Intelligence Scale for Children (WISC-R or WISC-III) or the Tanaka-Binet Intelligence scale was applied. Mental retardation was defined as an intelligence quotient of less than 70. If a patient showed a predisposing neurological problem, deterioration of neurological findings after SE was regarded as sequelae. Todd paralysis, which lasts for several hours after seizure with subsequent complete recovery of the patient, was not regarded as sequela. Acute death and neurological sequelae at the last visit were regarded as poor outcome.

To identify risk factors for poor outcome after SE, medical records were reviewed for the following data:

1. Gender.
2. Age at onset of SE: 1 to 12 months, 13 to 24 months, and above 24 months.
3. Etiology of SE: above 5 categories, especially acute symptomatic and progressive encephalopathy.
4. Pyrexia during SE: a body temperature of less than 38.0 °C and greater than 37.9 °C.
5. Asthmatic attack or acute bronchitis during SE. We experienced 2 patients with SE during treatment for respiratory disease with theophylline administration followed by neurological sequelae [17]. The severity of asthmatic attack or respiratory status was classified according to NIH guidelines: mild, moderate or severe [23].
6. Past history of seizures. These include epileptic seizures, febrile seizures, neonatal convulsions and any seizures before SE.
7. A predisposing neurological condition known to be associated with an increased risk of convulsions. This includes a past history of neurological insults (e.g., meningitis, head trauma) and developmental disorders such as mental retardation and cerebral palsy.
8. Duration of SE: less than 1 hour, 1 to 2 hours, or more than 2 hours.
9. Type of seizure: convulsive or non-convulsive.
10. Theophylline. It is well known that theophylline has a proconvulsive effect.

Statistical analysis

Multivariate analysis using a logistic regression model was performed to estimate odds ratios (OR) and 95% confidence intervals for the risk of poor outcome after SE. SAS version 8.2 software (SAS institute, Cary, North Carolina, USA) was used for the

statistical analysis. All results were considered to be significant at the 5% critical level.

Results

Etiology and outcome after SE

Four hundred and seventeen seizures (from 241 patients; 128 boys and 113 girls, aged 2 months to 17 years 9 months, median 2 years 8 months) met the criteria for SE. Two hundred and ten patients (86.8%) were residents in our local area and 32 patients (13.2%) lived outside the area. Three hundred and thirty-eight seizures (81.1%) were treated in our hospital and 79 seizures (18.9%) were initially treated outside. The etiologies of SE were cryptogenic in 35 SE (35 patients), remote symptomatic in 229 SE (85 patients), febrile in 125 SE (118 patients), acute symptomatic in 23 SE (21 patients), and progressive encephalopathy in 5 SE (2 patients). Twenty patients overlapped in etiology, i.e., a patient who had had his first SE during meningitis (acute symptomatic) had a second recurrence (remote symptomatic) as a sequela.

Only short-term outcomes were obtained in 7 live patients (7 SE) and 9 early death patients after SE. The etiologies of the 7 live patients were febrile in 6 and remote symptomatic in 1 and there were no neurological sequelae after SE. Long-term outcomes were all obtained in the remaining 225 patients (401 SE) who were followed-up during a mean of 64.2 months (ranging from 10 days to 225 months). Although most long-term outcomes were the same as the short-term outcomes (393 of 401 SE and 218 of 225 patients), outcomes of 7 patients (8 SE) changed. Motor paralysis improved over several days in 2 patients (3 SE) and mental deterioration returned to the previous level in 2 patients with severe mental retardation (2 SE). Mental retardation became evident at follow-up examinations in 3 patients (3 SE) whose ages at onset of SE were less than 12 months (6 months in 2 and 4 months in 1). Incidentally, it is not always easy to evaluate neurological findings and to give a long-term prognosis in infants.

Overall, 51 patients (21.2%) and 52 of all SEs (12.5%) showed poor outcomes. Nine patients (2.2% of all SEs and 3.7% of all patients) died early after SE. Forty-two of the 232 live patients (18.1%) and 43 of 408 SEs (10.5%) had sequelae after SE: severe mental and motor disability in 19 patients, mental retardation or deterioration in 18, mental retardation along with motor paralysis in 2, motor paralysis in 2, and hemianopsia in 1. As for etiology, poor outcomes after SE were noted in 4 of 35 patients (11.4%) for cryptogenic, 6 of 85 patients (7.1%) for remote symptomatic, 23 of 118 patients (19.5%) for febrile, and 17 of 21 patients (81.0%) for acute symptomatic seizures, and in 1 of 2 patients (50%) for progressive encephalopathy.

One hundred and eighty-four patients had single SE and 57 patients had 2 or more SEs. The number of SEs were 2 in 30 patients, 3 in 10 patients, 4 in 5 patients, 5 in 5 patients, and more than 5 in 7 patients (ranging from 7 to 36). In patients with multiple SEs, the etiologies were cryptogenic and remote symptomatic in 7 patients, remote symptomatic in 30 patients, remote symptomatic and acute symptomatic in 5 patients, febrile in 4 patients, febrile and remote symptomatic in 8 patients, acute symptomatic in 2 patients, progressive encephalopathy in 1 patient. As the outcome was quite good for remote symptomatic etiology, statistical analysis of all 417 SEs would have led to misleading results; i.e., an epilepsy patient had 36 repeated SEs without any sequela but another patient had neurological sequelae after bacterial meningitis-associated SE. Therefore, we assessed one SE for each patient. When a patient experienced 2 or more SEs, the first SE was used for statistical analysis. Because 7 patients could offer no information on the usage of theophylline (5 patients) or the presence of asthmatic attacks in their history (2 patients), we excluded them. Finally, 234 SEs in 234 patients were registered for statistical analysis. Table 1 shows the etiology and outcome. Acute fatality occurred in 9 of 234 patients (3.8%) and neurological sequelae were seen in 36 of 225 live patients (16.0%); a total of 45 patients (19.2%) showed poor outcomes. Poor outcome was frequently observed in acute symptomatic and progressive encephalopathy. As statistical analysis was performed using a multivariate logistic regression model in all patients (Table 2), it was obvious that the etiology of acute symptomatic and progressive encephalopathy were most closely related to poor outcome (OR = 33.68, p = 0.000). Pyrexia, moderate to severe asthmatic attack, and seizure duration of more than 2 hours were statistically associated with poor outcome. Prior neurological disorder was related to good prognosis.

Table 1 Classification of status epilepticus and outcome

Etiology	*No. of patients (%)*	*No. of patients with poor outcome*		
		Death	*Sequelae*	*Total (%)*
Cryptogenic	33 (14.1)	1	2	3/33 (9.1)
Remote symptomatic	66 (28.2)	0	4	4/66 (6.1)
Febrile	114 (48.7)	5	17	22/114 (19.3)
Acute symptomatic	19 (8.1)	3	12	15/19 (78.9)
Progressive encephalopathy	2 (0.9)	0	1	1/2 (50)
Total	234 (100)	9	36	45/234 (19.2)

Risk factors for poor outcome other than the cause of SE

We reanalyzed risk factors after exclusion of patients with an etiology of acute symptomatic and progressive encephalopathy (n = 213). Poor outcome was observed in 29 patients (13.6%) including 6 early death patients (2.8%). Table 3 shows the results. Seizure duration of more than 2 hours was most closely related to poor outcome (OR = 12.73, p = 0.000). Other risk factors for poor outcome were moderate to severe asthmatic attack (OR = 31.61, p = 0.010) and no prior neurological disorder (OR = 0.14, p = 0.034). Prior neurological disorders included mental retardations in 38 patients, mental retardations along with cerebral palsy in 8, brain malformations in 4, chromosomal abnormalities in 4, cerebral palsies in 3, post-brain tumor in 1, post-encephalopathy in 1, and post-cerebral hemorrhage in 1.

Table 2 Odds ratios (OR) with 95% confidential intervals (CI) of various factors for poor outcome after SE in all patients: multivariate analysis

Item	Category	No. of patients	No. of patients with poor outcome (%)	OR	95%CI	p
Sex	male	123	26 (21.1%)	1.00		
	female	111	19 (17.1%)	0.84	0.33 – 2.18	0.723
Age	1 – 12 mo	51	18 (35.3%)	1.00		
	13 – 24 mo	44	9 (20.5%)	0.68	0.19 – 2.45	0.557
	25 mo –	139	18 (12.9%)	0.69	0.20 – 2.39	0.553
Acute symptomatic or progressive encephalopathy	no	213	29 (13.6%)	1.00		
	yes	21	16 (76.2%)	33.68	7.70 – 147.24	0.000
Fever	<38 °C	88	7 (8.0%)	1.00		
	≥38 °C	146	38 (26.0%)	6.66	1.77 – 25.11	0.005
Asthmatic attack	none	214	35 (16.4%)	1.00		
	mild	9	2 (22.2%)	1.65	0.12 – 23.23	0.711
	moderate to severe	11	8 (72.7%)	26.33	2.23 – 310.49	0.009
Seizure duration	<1 h	102	9 (8.8%)	1.00		
	1 – 2 h	73	10 (13.7%)	1.26	0.37 – 4.25	0.708
	≥2 h	59	26 (44.1%)	12.57	3.76 – 41.99	0.000
Prior neurological disorder	none	171	41 (24.0%)	1.00		
	present	63	4 (6.3%)	0.19	0.04 – 0.81	0.025
History of seizure	none	141	40 (28.4%)	1.00		
	present	93	5 (5.4%)	0.34	0.09 – 1.28	0.111
Theophylline	no	201	34 (16.9%)	1.00		
	yes	33	11 (33.3%)	0.96	0.16 – 5.90	0.965
Convulsive seizure	no	32	5 (15.6%)	1.00		
	yes	202	40 (19.8%)	0.97	0.20 – 4.74	0.974

Poor outcome was higher in younger age groups, with pyrexia, with no history of seizure, and theophylline administration, but they were not statistically significant. Serum theophylline concentrations were measured in 13 patients. Only 2 patients had a toxic theophylline concentration (88.5 and 26.2 μg/mL) and showed no sequelae after SE. The theophylline concentrations were within or below the therapeutic range (1.0 – 19 μg/mL) in the other 11 patients. The prognosis after SE was unfavorable in 4 patients whose theophylline concentrations were 19, 17.8, 15.5, and 12.7 μg/mL, respectively. The type of SE, convulsive or nonconvulsive, was not related to prognosis.

Discussion

Major findings in the present study concerning risk factors for poor outcome were seizure duration of more than 2 hours and moderate to severe asthmatic attack after exclusion of patients with an etiology of acute symptomatic and progressive encephalopathy.

Seizure duration

Long-lasting seizure activity directly induces brain damage in animals [21,24]. In mature rats, the longer seizures persist, the more severe and more diffuse is the brain damage that occurs: seizures lasting for 120 minutes induce moderate neuronal necrosis in the neocortex as well as the thalamus and hippocampus [24]. It is well known that immature animals are less vulnerable to neuronal damage and cell loss after prolonged seizures than mature animals [4]. SE does not induce any structural or functional change in the hippocampus in immature rats corresponding to human neonates [4]. Although SE results in hippocampal damage in young rats corresponding to human infants and young children, the changes are milder than in adult rats [4]. This is compatible with the observation that SE-associated morbidity and mortality are lower in children than in adults [3,6,7, 10,19,30]. The mode of cell death from SE is largely necrotic in adult rats, while apoptosis is common in younger rats [32]. These results indicate that neural injury after seizures is highly age-specific. In humans, brain damage is also described after SE [12,20,25,27]. It is not always easy to conclude that brain damage is caused by SE itself, because multiple factors possibly related to outcome coexist in many patients and cannot be excluded in contributing to outcome. Moreover, previously published reports often lack sufficient information about risk factors. Fujikawa et al. pathologically examined 3 patients who died shortly after SE and had no systemic complications underlying brain pathology, such as hypotension, hypoxemia, hypoglycemia, or significant hyperthermia [12]. They found brain damage in these patients similar to that in animal models after SE. In the present

Table 3 Odds ratios (OR) with 95% confidential intervals (CI) of various factors for poor outcome after SE in patients without etiology of acute symptomatic or progressive encephalopathy: multivariate analysis

Item	Category	No. of patients	No. of patients with poor outcome (%)	OR	95%CI	p
Sex	male	111	17 (15.3)	1.00		
	female	102	12 (11.8)	0.89	0.32 – 2.51	0.828
Age	1 – 12 mo	43	12 (27.9)	1.00		
	13 – 24 mo	42	8 (19.0)	0.74	0.20 – 2.73	0.649
	25 mo –	128	9 (7.0)	0.44	0.11 – 1.85	0.263
Fever	< 38 °C	81	4 (4.9)	1.00		
	≥ 38 °C	132	25 (18.9)	3.46	0.79 – 15.21	0.101
Asthmatic attack	none	194	20 (10.3)	1.00		
	mild	9	2 (22.2)	2.06	0.16 – 26.96	0.582
	moderate to severe	10	7 (70.0)	31.61	2.27 – 439.91	0.010
Seizure duration	< 1 h	97	5 (5.2)	1.00		
	1 – 2 h	67	7 (10.4)	1.74	0.46 – 6.58	0.416
	≥ 2 h	49	17 (34.7)	12.73	3.4 – 47.62	0.000
Prior neurological disorder	none	153	27 (17.6)	1.00		
	present	60	2 (3.3)	0.14	0.02 – 0.86	0.034
History of seizure	none	122	25 (20.5)	1.00		
	present	91	4 (4.4)	0.42	0.10 – 1.73	0.23
Theophylline	no	181	19 (10.5)	1.00		
	yes	32	10 (31.2)	0.94	0.15 – 5.95	0.951
Convulsive seizure	no	29	3 (10.3)	1.00		
	yes	184	26 (14.1)	0.77	0.14 – 4.30	0.766

study, adverse systemic factors, except for pyrexia, were classified under the etiology of acute symptomatic and were excluded from the statistical analysis. Nevertheless, subtle systemic factors might have contributed to brain damage in association with long-lasting seizures.

Asthmatic attack during SE

How asthmatic attacks affect SE-associated brain damage is uncertain. Severe hypoxemia, which directly causes brain injury, was regarded as an acute symptomatic etiology and excluded from the statistical analysis in the present study. Mild hypoxemia is common during seizure and may be worsened by asthmatic attacks. In turn, prolonged seizures may induce pulmonary edema which further exacerbates hypoxemia [31]. In our early experience, 2 patients developed SE during treatment for bronchial asthma with theophylline and had severe neurological sequelae [17]. We suspected at first that theophylline-associated SE caused brain damage. SE in association with asthmatic attacks rather than theophylline might contribute to neural damage. Hypoxemia during SE is mentioned in some papers [2,14]. Bahls et al. reported 12 adult patients with theophylline-associated seizures with therapeutic or low toxic serum concentrations whose outcomes were poor [2]. All patients had a history of significant pulmonary disease and 7 of 12 suffered from exacerbation of severe chronic obstructive pulmonary disease at the time of seizure. The influence of mild hypoxemia on SE-associated brain injury has been examined in animals. Mild or transient hypoxemia (arterial pO_2 50 mm Hg), which alone shows no or only minimal brain injury, increases the extent of SE-associated brain injury in mature rats [18,28]. Extracellular glutamate and aspartate do not increase after mild hypoxia alone or seizure alone in the brain of mature rabbits, while they increase after mild hypoxia followed by seizure [35]. The brain injury may be mediated by increased excitatory amino acids.

Theophylline sometimes induces seizures not only in toxic but at therapeutic serum levels [2,8,17]. These theophylline-associated seizures are often prolonged and mortality and morbidity are relatively high [2,8,17]. Yokoyama et al. examined the effect of therapeutic doses of theophylline on electroshock seizures induced in the immature mice and reported that mortality was significantly higher in immature mice than in mature mice [33]. Although theophylline was not related to prognosis in the present study, an adverse effect of theophylline on the brain during SE cannot be completely ruled out. This is because the number of patients who received theophylline was small (33 patients) and asthmatic attacks coincided frequently with theophylline administration. Eight of the 10 patients with moderate to severe asthmatic attacks received theophylline and 6 patients showed poor outcome after SE. Eight of the 9 patients with mild asthmatic attacks received theophylline and 2 patients showed poor outcome after SE. Since theophylline is a popular medication for the treatment of bronchial asthma, further studies are essential

to clarify the effect of theophylline on SE-associated brain damage.

Prior neurological disorder

Patients with prior neurological disorders had a better prognosis than those without prior neurological disorders. The diagnoses of the SE were epileptic seizures or febrile seizures. Only 2 of the 60 patients with prior neurological disorder showed poor outcome and their SEs were very prolonged, namely 10 and 24 hours, respectively. Similarly, only 4 of the 91 patients who had histories of seizure showed poor outcome. The diagnoses of the SE were epileptic seizures or febrile seizures. These results indicate that the prognosis of epileptic and febrile SEs is favorable, unless SEs continue for a long time.

Pyrexia

Pyrexia during SE was one of the risk factors for poor outcome examined in all patients (OR = 6.66, p = 0.005) but was not statistically significant after exclusion of the patients with the etiology of acute symptomatic and progressive encephalopathy (OR = 3.46, p = 0.101). The reason is that acute symptomatic etiology included many pyretic patients such as meningitis and encephalitis: 13 of the 19 patients with acute symptomatic etiology had pyrexia. Nevertheless, pyrexia had a tendency to be associated with poor outcome. In one experiment, hyperthermia aggravated SE-associated brain injury in both mature and immature animals [15,16]. Hyperthermia lowers the seizure threshold and elongates seizure duration [15,16]. Prolonged seizures result in an elevation of body temperature, which may exacerbate seizure-associated brain injury further [31]. In humans, hyperthermia appears to be one of the secondary factors that are associated with poor outcome after acute neurological insults including SE [11]. Hyperthermia-associated brain damage may be mediated by increased cytokine activity [1,13,34] or increased glutamate release [22]. Ichiyama et al. measured pro-inflammatory cytokines in the CSF of patients with prolonged febrile convulsions and acute encephalitis or encephalopathy [1]. They reported that the cytokine levels were elevated in patients with acute encephalitis or encephalopathy whose prognoses were poor, but not in those with prolonged febrile convulsions whose prognoses were unremarkable. Aiba et al. also examined the serum levels of pro-inflammatory cytokines in patients with influenza-associated encephalopathy, and reported that the serum interleukin-6 level correlated with severity [1]. Therefore, the activated cytokines would at least in part mediate SE-associated brain damage.

Overall fatality and neurological sequela

Overall fatality after SE was 2.2% based on seizures and 3.7% based on patients in this study. This was similar to what was reported previously, including those data from population-based prospective studies (2 – 3%) [3,6,7,19,30]. The overall neurological sequelae rates of 10.5% based on seizures and 18.1% based on patients were also similar to that of the previous studies, including population-based prospective studies (20.6%) [3,7,10,19,30]. It was reconfirmed that a major contributor for poor outcome after SE was the cause of SE (Table **2**). Therefore, it is concluded that the outcome after SE is mainly a function of the underlying cause. Fatality and neurological sequelae were very low after exclusion of the etiologies of acute symptomatic and progressive encephalopathy, namely 2.8% and 11.1% based on patients, respectively. On the other hand, prognosis was relatively poor with the etiology of febrile (19.3%), even after the exclusion of the acute symptomatic etiology. Hospital-based studies reveal a relatively high incidence of poor outcome, even when they are performed prospectively [7,10]. The prognosis is quite good in population-based prospective studies [9,26,30]. This difference appears to be due to a difference in the subjects enrolled. A hospital-based study contains many more severe cases than a population-based study and would miss milder cases. Although our study has such a bias in the methodology, this did not interfere with the major findings on risk factors for outcome after SE, because almost all intensive cases were referred to us and almost all cases who had any neurological impairment after SE were followed up for long periods. Nevertheless, to clarify the risk factors for outcome after SE and prevent brain damage from long-lasting seizures, prospective population-based studies are needed.

In conclusion, the present study indicated that the prognosis of SE was related to seizure duration, asthmatic attack, and prior neurological disorder after the exclusion of patients with acute neurological insults and progressive disease. Age at onset, pyrexia, past history of seizure, type of seizure, and theophylline were not related to outcome.

Acknowledgements

This study was supported by the Japan Epilepsy Research Foundation.

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