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Structural characterization of a novel mono-sulfated gangliotriaosylceramide containing a 3-*O*-sulfated *N*-acetylgalactosamine from rat kidney

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A novel mono-sulfated glycosphingolipid based on the gangliotriaose core structure was isolated from rat kidney. The isolation procedure involved extraction of lipids with chloroform/methanol, mild alkaline methanolysis, column chromatographies with anion exchangers and silica beads. The structure was characterized by compositional analysis, FTIR spectroscopy, methylation analysis, ^{1}H-NMR spectroscopy and negative-ion liquid secondary ion mass spectrometry (LSIMS) using the intact glycolipid and its desulfation product. The two dimensional chemical shift correlated spectroscopy provided information on the sugar sequence as well as anomeric configurations, and indicated the presence of a 3-*O*-sulfated *N*-acetylgalactosamine within the molecule. Negative-ion LSIMS with high- and low-energy collision-induced dissociation defined the sugar sequence and ceramide composition, confirming the presence of a sulfated *N*-acetylgalactosamine at the non-reducing terminus. From these results, the complete structure was proposed to be HSO_3-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer (Gg_3Cer III^3-sulfate, SM2b).

Keywords: sulfated glycolipids, gangliotriaosylceramide core, ^{1}H-NMR, liquid SIMS, collision-induced dissociation, rat kidney

Abbreviations: Abbreviations for sulfated glycolipids [17] follow the modifications of the nomenclature system of Svennerholm for gangliosides [37], and the designation of the other glycosphingolipids follows the IUPAC-IUB recommendations [38]. Cer, ceramide; LacCer, lactosylceramide, Galβ1-4Glcβ1-1Cer; Gg_3Cer, gangliotriaosylceramide, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; Gg_4Cer, gangliotetraosylceramide, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; iGb_4Cer, isoglobotetraosylceramide, GalNAcβ1-3Galβ1-3Galβ1-4Glcβ1-1Cer; Gb_4Cer, globotetraosylceramide, GalNAcβ1-3Galβ1-4Galβ1-4Glcβ1-1Cer; SM4s, galactosylceramide sulfate, GalCer I^3-sulfate; SM3, lactosylceramide sulfate, LacCer II^3-sulfate; SM2a, Gg_3Cer II^3-sulfate; SM2b, Gg_3Cer III^3-sulfate; SB2, Gg_3Cer II^3,III^3-bis-sulfate; SM1a, Gg_4Cer II^3-sulfate; SM1b, Gg_4Cer IV^3-sulfate; SB1a, Gg_4Cer II^3,IV^3-bis-sulfate; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry; DQF, double quantum filtered; COSY, chemical-shift-correlated spectroscopy; LSIMS, liquid secondary ion mass spectrometry; CID, collision-induced dissociation; MS/MS, tandem mass spectrometry.

Introduction

Mammalian kidney had been believed to contain only two sulfated glycolipids, GalCer I^3-sulfate (SM4s) [1] and LacCer II^3-sulfate (SM3) [2], until the discovery of a third sulfated glycolipid, Gg_3Cer II^3-sulfate (SM2a) [3] from rat kidney. A series of studies in our laboratory since 1982 has shown that rat kidney contains relatively large amounts (total 240 nmol per g tissue) of a variety of sulfated glycolipids which belong to the gala-series (SM4s), ganglio-series (GlcCer I^3-sulfate [4], SM3, SM2a [3], SB2 [5] and SB1a [6]) and isoglobo-series

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(iGb_4Cer IV^3-sulfate [7] and iGb_5CerV^3-sulfate [8]). Recently, SM2a was found to be synthesized in human endometrial cancer cell lines [9] and in human renal cell carcinoma cells [10], and the accumulation of SB1a was observed in human hepatocellular carcinoma [11]. The ganglio-series sulfated glycolipids seem to be ubiquitous among mammals, whereas iGb_4Cer, which may be the precursor of the biosynthesis of iGb_4Cer IV^3-sulfate, is the species specific glycolipid of rat. In the isolation process of iGb_4Cer IV^3-sulfate and iGb_5CerV^3-sulfate [7, 8], we observed a minor band which migrated a little slower than SM2a on thin-layer chromatography (TLC). In the present studies, we have isolated and determined the complete structure of the isolated unknown glycolipid (designated as Ka). This glycolipid turns out to be a novel mono-sulfated Gg_3Cer, a positional isomer of SM2a, having a sulfate ester at the non-reducing terminus [12].

Materials and methods

Materials

Kidneys (3.9 kg) were obtained from normal Sprague Dawley rats. The mixture of acidic lipids from rat brain and SM3 from human kidney were prepared in this laboratory. SM2a and SB2 were isolated from rat kidney as previously described [3, 5]. Gg_3Cer from guinea pig red blood cells [13] was purchased from Seikagaku Corp., Tokyo. Gg_4Cer was prepared from GM1a ganglioside ($II^3\alpha$NeuAc-Gg_4Cer) by mild acid hydrolysis [14]. All other materials including glycolipids, reference compounds, reagents and chemicals were from the same sources as described in [7] and [8].

Extraction and purification

The isolation of sulfated glycolipids from rat kidney was performed according to standard procedures as described [7]. Briefly, total lipids were extracted from rat kidney with mixtures of chloroform/methanol and treated for 2 h at 37 °C with 0.2 M NaOH in methanol followed by neutralization and dialysis. Sulfated glycolipids were separated from neutral lipids by anion exchange chromatography on DEAE-Sephadex A-25 (acetate form) or DEAE-Toyopearl 650M (acetate form) [8]. After neutral lipids were washed out, sulfated glycolipids were fractionated by a gradient of ammonium acetate in chloroform: methanol:water (5:10:1, v/v/v). The combined fraction containing Ka was first separated by chromatography on an Iatrobeads 6RS-8060 column eluted by a linear gradient with chloroform:methanol (9:1, by v/v) to chloroform:methanol:water (10:10:1, v/v/v) [7, 8]. The fractions containing Ka were combined and purified further by high-performance liquid chromatography (HPLC) using an Iatrobeads 6RS-8005 column (1 × 30 cm, 23.5 ml) with a linear gradient of chloroform:methanol:water (85:15:0.5 to 70:30:3, v/v/v). Final purification used an Iatrobeads 6RS-8005 column (0.6 × 30 cm, 8.5 ml) with a linear gradient of chloroform:methanol (9:1, v/v) to chloroform:methanol:water (75:25:2.5, v/v/v) and total volume of 400 ml.

Analysis

Glycolipids were separated on silica gel 60 high-performance TLC plates (Merck, Darmstadt, Germany) in chloroform:methanol:water (60:40:9, v/v/v) containing 0.2% $CaCl_2$ or 3.5 M NH_4OH and visualized by orcinol reagent [15]. Desulfation of Ka (50 nmol) was performed by hydrolysis with 0.2 ml of 5 mM HCl in DMSO containing 0.5% methanol [7, 8] at 80 °C for 1 h. After HCl and DMSO were evaporated under vacuum over P_2O_5, the product (designated as KaN) was used for further analyses. The compositions of sugar, fatty acid and sphingoid base were determined by their GLC retention time (GC-14A, Shimadzu, Kyoto) and characteristic mass fragmentation patterns on GC-MS (QP-1000, Shimadzu) [4, 16]. The sulfate group was estimated after peracetylation using a cationic dye, azure A [17]. Permethylated glycolipids [18] and partially methylated alditol acetates were prepared and analysed by GLC and GC-MS [7, 8]. FTIR spectroscopy was performed on an FTIR-4200 (Shimadzu) [7]. ^{1}H-NMR spectra were acquired at 400 MHz on a GX-400 spectrometer (Japan Electron Optical Laboratory, Tokyo). One-dimensional spectra with gated supression of water peak and double quantum filtered-chemical shift correlated spectroscopy (DQF-COSY) spectra were obtained at 60 °C in DMSO-d6:D_2O (98:2, v/v) [7]. Negative-ion liquid secondary ion mass spectrometry (LSIMS) was performed on a Concept IH mass spectrometer (Shimadzu/Kratos) fitted with a cesium ion gun [8, 19]. About 0.5 nmol of underivatized Ka in 1 μl chloroform:methanol (1:2, v/v) was mixed with 1 μl triethanolamine as the matrix. Spectra were recorded at an accelerating voltage of 8 kV, with a scan rate of 5 s per decade, and at a resolution of 1000–2000. Linked scan spectra were obtained at constant *B/E* ratio after collision-induced dissociation (CID) had occurred in the first field-free region with air as the collision gas [19]. Negative-ion LSIMS and low-energy CID MS/MS studies were performed on a TSQ 70 triple-stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) with a 10 eV collision energy and 0.2 Pa (1.5×10^{-3} torr) of argon as the collision gas [7, 19, 20].

Results

Isolation of Ka

An unidentified glycolipid, Ka, was eluted after SM2a and simultaneously with iGb_4Cer IV^3-sulfate, iGb_5Cer V^3-sulfate and a large amount of SM4s from the columns of

anion exchangers, DEAE-Sephadex and DEAE-Toyopearl. In order to remove SM4s and other contaminants, the combined fraction containing Ka was first separated by column chromatography using Iatrobeads (6RS-8060). Ka was eluted slightly later than SM2a, with a little overlapping, followed by iGb_4Cer IV^3-sulfate and iGb_5Cer V^3-sulfate. Repeated HPLC on Iatrobeads (6RS-8005) with linear gradients of chloroform:methanol:water resulted in the complete separation of Ka from SM2a. The isolated Ka, which was sensitive to orcinol, but not to resorcinol or Dittmer/Lester reagent, was found to be a homogeneous band on TLC and migrated a little slower than SM2a with both neutral (Fig. 1) and basic solvent systems. The elution position from anion exchangers and staining characteristics on TLC suggested that Ka is a mono-sulfated glycolipid. There was, however, a possibility that Ka may be one of the molecular species of SM2a with different ceramide compositions. The yield of Ka was determined to be 50 pmol per g wet tissue by colorimetric analysis of the sulfate [17].

Desulfation, compositional analysis, and FTIR spectroscopy

After hydrolysis of the sulfate ester at 80 °C for 30 min, visual examination of a TLC separation indicated that more than 50% of Ka was converted to a compound (KaN) which migrated a little slower than Gg_3Cer (Fig. 1). After 1 h, Ka was completely changed into KaN. When subjected to the same hydrolysis conditions, SM2a was almost unchanged after 1 h (data not shown), suggesting that Ka is a sulfated glycolipid with a carbohydrate structure different from SM2a rather than any one of its molecular species.

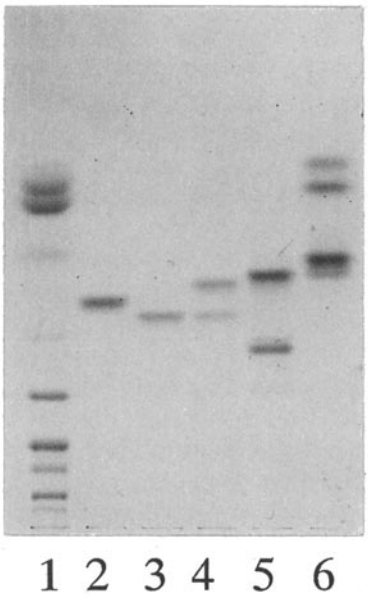

Figure 1. Thin layer chromatogram of isolated Ka and the product of desulfation. The plate was developed in chloroform:methanol:0.2% $CaCl_2$ (60:40:9, v/v/v). Glycolipids spots were visualized with orcinol reagent. Lane 1, the mixture of acidic lipids from rat brain; lane 2, SM2a; lane 3, Ka; lane 4, the products after hydrolysis of Ka for 30 min; lane 5, Gg_3Cer (upper) and Gg_4Cer (lower); lane 6, LacCer (upper two bands) and Gb_3Cer (lower two bands).

Analysis of the sugar constituents of Ka established the presence of 1 mol each of glucose (Glc), galactose (Gal), *N*-acetylgalactosamine (GalNAc) and sulfate ester group. KaN contained Glc, Gal and GalNAc with ratios of 1:1:1 (Table 1). These values indicated that the loss of a sulfate from Ka produced its desulfated derivative, KaN. The relatively facile desulfation as compared with SM2a could indicate that the sulfate is situated at the external sugar of Ka. The FTIR spectrum (data not shown) showed the presence of absorptions at 1240 cm^{-1} (asymmetric S = *O* stretching) and 810 cm^{-1} (C-*O*-S vibration), characteristic of an equatorial sulfate ester of hexose or *N*-acetylhexosamine [7, 8]. The ceramide part mainly contained 4-hydroxysphinganine (t18:0) (75.9%) and saturated fatty acids, 24:0 (31.0%) and 22:0 (21.5%) (Table 1), supporting the fact that KaN has a slightly lower R_f value than Gg_3Cer which mainly has 4-sphingenine (d18:1) and saturated fatty acids [13]. These results were consistent with Ka being a mono-sulfated triglycosylceramide. The fact that the ceramide compositions of Ka and SM2a [3] are similar tends to suggest that Ka is not simply one of the molecular species of SM2a.

Table 1. Compositional analysis of native and desulfated Ka.

Carbohydrate part *Compound*	*Glucose*[a]	*Galactose*	*N-Acetyl galactosamine*	*Sulfate*[b]
	Molar ratio[c]			
Ka	1.0	1.0	0.9	1.0
KaN	1.0	0.9	0.9	

Ceramide part *Fatty acid (% of total*[d]*)*[e]						*Sphingoid base (% of total)*[f]	
16:0	18:0	20:0	22:0	23:0	24:0	d18:1	t18:0
12.1	7.7	11.8	21.5	12.3	31.0	24.1	75.9

Peaks were identified by comparing their GLC retention times with those of standards, and also by GC-MS.
[a]The methyl glycosides were obtained by anhydrous methanolysis and analysed as their trimethylsilyl derivatives.
[b]The sulfate ester was estimated by the peracetylation method using azure A [17].
[c]The molar ratio was calculated with glucose as 1.0.
[d]The values are given as weight per cent.
[e]The fatty acids were analysed as their methyl esters.
[f]Sphingoid bases as their trimethylsilyl derivatives.

^{1}H-NMR spectroscopy

The one-dimensional spectra of the anomeric and ring proton region indicated that Ka (Fig. 2) and KaN contained two β-hexoses and a β-*N*-acetylhexosamine. The chemical shifts of H-1 to H-4 and coupling constants (^3J) indicated that they are a βGlc, a βGal and a βGalNAc. The βGlc was shown to be situated next to ceramide as supported by the coupling of a doublet signal of H-1 at 4.184 ppm ($^3J_{1,2}$ = 7.8 Hz) to a triplet signal of H-2 at 3.036 ppm ($^3J_{2,3}$ = 8.3 Hz) (Table 2). The signals of anomeric and ring protons of KaN (data not shown) were identical to those of Gg_3Cer, suggesting that Ka has the Gg_3Cer core structure, similar to SM2a and SB2. On the other hand, the chemical shifts of these protons in Ka, particularly H-1 and H-3 of βGal and βGalNAc were significantly different from those in SM2a and SB2 (Fig. 2 and Table 2), showing that these protons in Ka are located in different environments from those in SM2a and SB2. From the assignment of chemical shifts based on DQF-COSY spectrum of Ka (Fig. 3), the downfield shifts of H-1 to H-4 of βGalNAc were calculated to be 0.054, 0.167, 0.579, and 0.464 ppm, respectively, as compared with those of KaN or Gg_3Cer (Table 2). The difference in the chemical shifts was the largest (0.579 ppm) with H-3, suggesting that the sulfate group was located on C-3 hydroxyl of the βGalNAc. The downfield shift (0.464 ppm) of H-4 was comparable to that observed for H-4 of βGalNAc of SB2 (0.471 ppm) (Table 2) and iGb_4Cer IV^3-sulfate (0.450 ppm) [7], and significantly larger than that of the vicinal H-4 of 3-*O*-sulfated galactose, e.g. in SM2a (0.308 ppm) (Table 2), and in other sulfated glycolipids [8, 16]. The chemical shifts and coupling constants of the protons at the C-1 to C-4 of sphingoid (Fig. 3, Table 2), which were identical to those of SM2a (Table 2) and iGb_5Cer V^3-sulfate [8], were completely compatible with the t18:0 structure of sphingoid. These results strongly indicated that Ka is an isomer of SM2a having a 3-*O*-sulfated GalNAc instead of a 3-*O*-sulfated Gal.

Methylation analysis

Permethylation analysis of Ka gave three major peaks on GLC (Fig. 4), which co-chromatographed with acetates of 2,3,6-tri-*O*-methylglucitol (-4Glc1-), 2,3,6-tri-*O*-methylgalactitol (-4Gal1-), and 4,6-di-*O*-methyl-2-*N*-methylacetamidogalactitol (-3GalNAc1-), respectively, in approximately equimolar proportion. These peaks were also analysed by GC-MS and identified by comparing their mass spectra with those obtained from Gg_3Cer, SM2a and SB2. Desulfation before permethylation and degradation

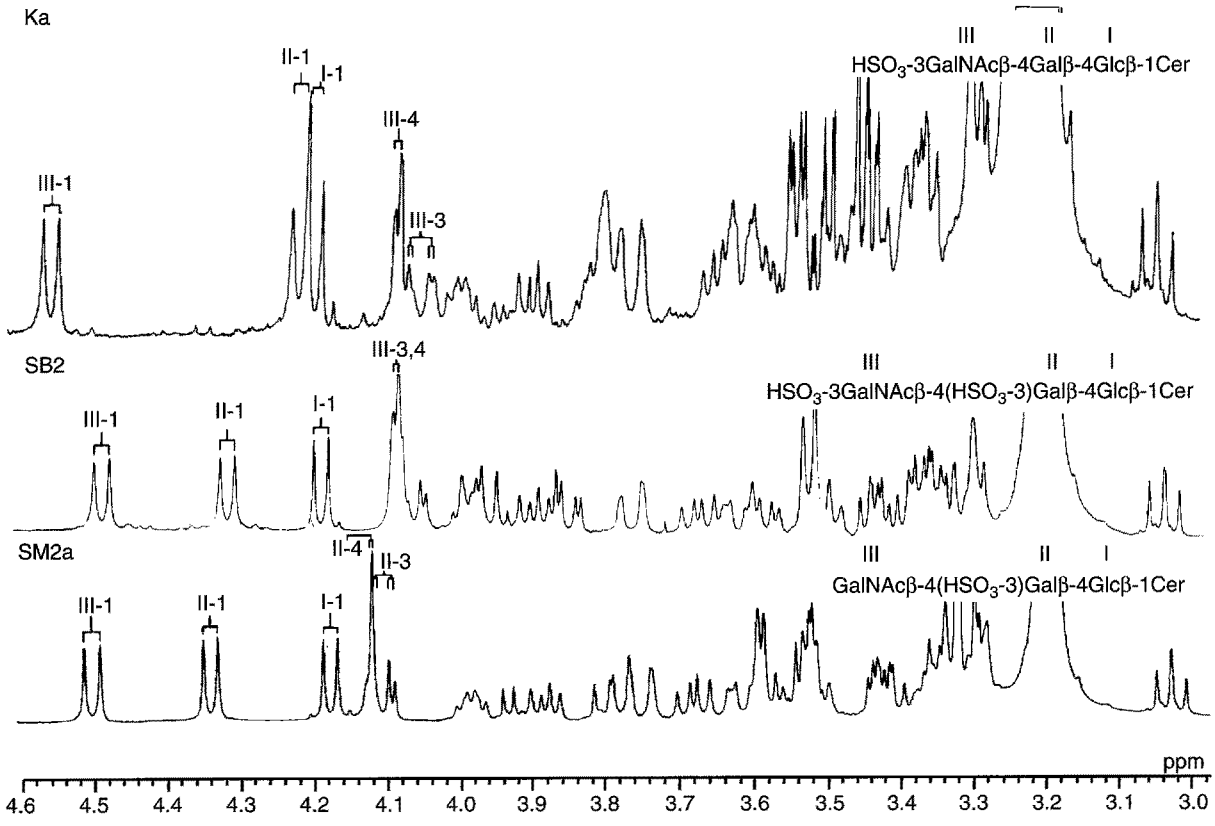

Figure 2. Plot of the anomeric and ring proton region of the one-dimensional ^{1}H-NMR spectra of Ka, SM2a and SB2. Each sulfated glycolipid was dissolved in DMSO-d6:D_2O (98:2, v/v) and submitted to analysis at 60 °C. The sugar residues are specified by Roman numerals as illustrated above the formula. Arabic numbers refer to the proton assignment which is labelled according to its parent carbon.

Table 2. Chemical shifts (ppm from tetramethylsilane) and coupling constants (Hz, in parentheses) for Ka, KaN, SM2a, SB2 and Gg_3Cer.

Compound	*Proton (J)*	*Chemical shift (J)*		
		III GalNAcβ1-4	*II Galβ1-4*	*I Glcβ1-1Cer*
Ka	H1 ($^3J_{1,2}$)	4.545 (8.3)	4.204 (8.3)	4.184 (7.8)
	H2 ($^3J_{2,3}$)	3.788 (10.3)	3.250 (8.7)	3.036 (8.3)
	H3 ($^3J_{3,4}$)	4.034 (3.1)	3.396 (3.2)	3.360 (8.2)
	H4[a]	4.072	3.790	3.29
SM2a	H1 ($^3J_{1,2}$)	4.501 (8.5)	4.338 (7.8)	4.176 (7.8)
	H2 ($^3J_{2,3}$)	3.792 (9.5)	3.341 (8.5)	3.031 (8.2)
	H3 ($^3J_{3,4}$)	3.309 (3.1)	4.104 (3.4)	3.35 (8.5)
	H4[a]	3.592	4.124	3.29
SB2	H1 ($^3J_{1,2}$)	4.480 (8.4)	4.307 (7.9)	4.181 (7.8)
	H2 ($^3J_{2,3}$)	3.966 (9.8)	3.352 (8.7)	3.032 (8.3)
	H3 ($^3J_{3,4}$)	3.842 (3.1)	4.054 (3.2)	3.360 (8.6)
	H4[a]	4.079	4.081	3.29
KaN	H1 ($^3J_{1,2}$)	4.491 (8.5)	4.241 (7.8)	4.182 (7.6)
	H2 ($^3J_{2,3}$)	3.620 (10.1)	3.272 (8.7)	3.034 (8.2)
	H3 ($^3J_{3,4}$)	3.455 (2.7)	3.493 (3.7)	
	H4[a]	3.607	3.815	
Gg_3Cer	H1 ($^3J_{1,2}$)	4.492 (8.3)	4.242 (7.8)	4.167 (7.8)
	H2 ($^3J_{2,3}$)	3.621 (10.7)	3.275 (8.7)	3.050 (8.2)
	H3 ($^3J_{3,4}$)	3.455 (2.7)	3.494 (3.7)	3.35 (8.5)
	H4[a]	3.608	3.816	3.31

Sphingoid	*L1a* ($^2J_{a,b}/^3J_{a,2}/^3J_{b,2}$)	*L1b*	*L2* ($^3J_{2,3}$)	*L3* ($^3J_{3,4}$)	*L4*[a]
Ka	3.578 (−10.4/4.5/5.9)	3.885	3.984 (6.5)	3.434 (5.0)	3.35
SM2a	3.579 (−10.2/4.2/5.7)	3.883	3.984 (6.5)	3.431 (5.4)	3.36

[a]Coupling constants could not be obtained due to overlapping signals, the secondary line shape and/or the limit of resolution.

resulted in the disappearance of -3GalNAc1-; instead a terminal GalNAc (GalNAc1-) appeared (Fig. 4), showing that the sulfate group is linked to the third position of the terminal GalNAc. These results, together with the assignments given by ^{1}H-NMR, supported the hypothesis that Ka is an isomer of SM2a having the Gg_3Cer core structure.

Negative-ion liquid secondary ion mass spectrometry

Figure 5 shows the negative LSIMS spectrum of Ka obtained by using a magnetic sector mass spectrometer. An intense deprotonated molecule $[M–H]^-$ was detected at *m/z* 1273. This is consistent with a structure derived from a glycosphingolipid containing a sulfate ester, an *N*-acetylhexosamine (HexNAc), two hexoses, and a ceramide which corresponds to the combination of the major fatty acid (24:0) plus 4-hydroxysphinganine (t18:0). Less-abundant molecular anions, corresponding to other ceramide compositions, were also detected at *m/z* 1245 (22:0/t18:0) and *m/z* 1217 (20:0/t18:0). These $[M–H]^-$ ions were identical to those obtained from SM2a as well as to $[M–SO_3H]^-$ ions from SB2 [19], suggesting the similarity in ceramide compositions among Ka, SM2a and SB2. The ion at *m/z* 97 corresponds to the hydrogen sulfate anion $[OSO_3H]^-$ [7, 8, 19]. The fragment ions containing ceramide (24:0/t18:0), at *m/z* 666, 828 and 990, which result from the cleavage of the glycosidic bonds between the anomeric carbon and oxygen, showed that Ka has a -O-Hex-O-Hex-O-Cer structure. Furthermore, the presence of the fragment ion (*m/z* 282) corresponding to the sulfated HexNAc (HSO_3-O-HexNAc) and the absence of the ion corresponding to the sulfated lactose plus ceramide (HSO_3-O-Hex-O-Hex-O-Cer, i.e. *m/z* 1070), which is present in the spectra of both SM2a and SB2 [19], confirmed that Ka has a sulfate at the external HexNAc.

The high-energy CID spectrum obtained from the $[M–H]^-$ ion (*m/z* 1273) (Fig. 6A) showed the series of product ions that arose by the sequential cleavage of the glycosidic bonds on either side of the oxygen atom [19]. The peaks at *m/z* 282 and 298 correspond to the terminal

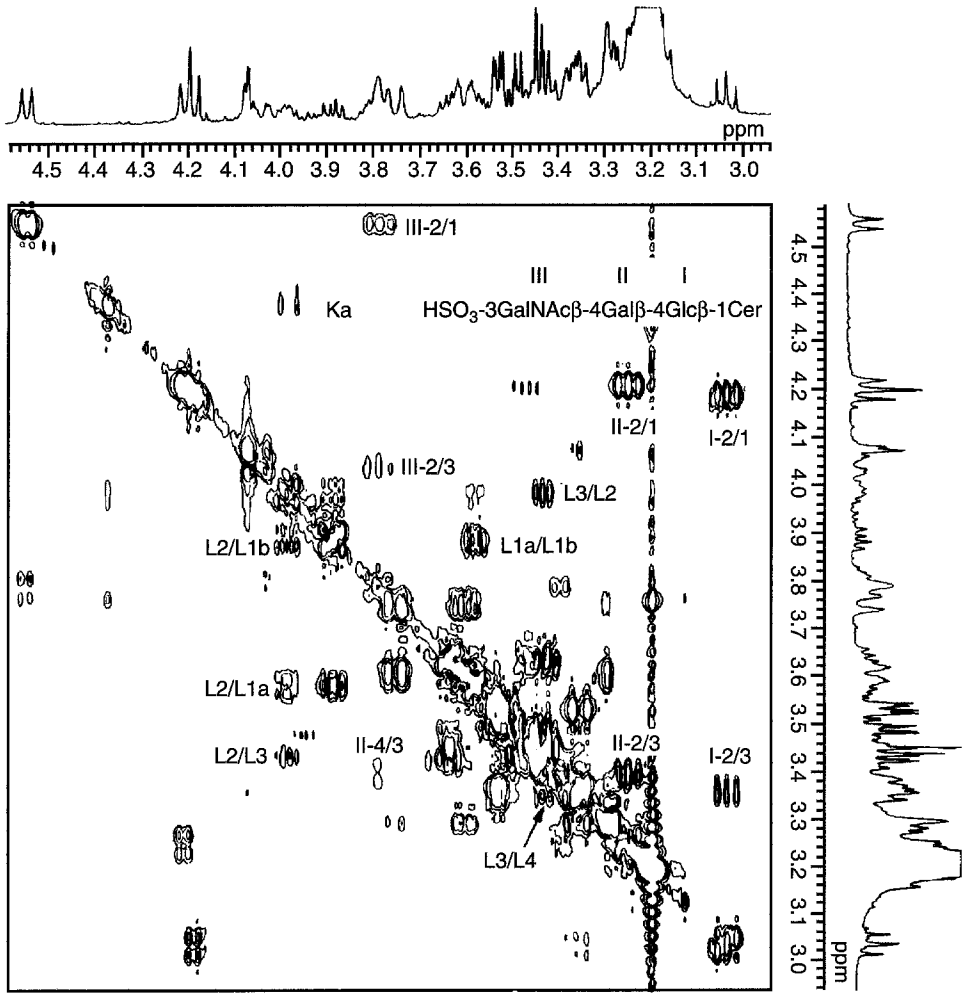

Figure 3. Contour plot of the two-dimensional DQF-COSY spectrum of Ka (ca. 180 nmol) at 60 °C. The spectrum was recorded using 512 increments in t_1 and 96 scans for each t_1 experiment. Prior to the Fourier transformation, the time domain matrix was zero-filled to 2048 points in t_1 and the final matrix of 2K × 2K data points yielded a resolution of 1.0 Hz per point. Spectral widths were 1050 Hz. The spectrum was weighted with Lorenzian-Gaussian functions before processing. The abbreviations labelling each cross-peak correspond to the numbering of sugar residues (Roman numerals), followed by the proton assignment (Arabic numeral). The figure immediately after the Roman numeral denotes the proton whose chemical shift defines the F_2 coordinate (horizontal axis), and the figure next to a slash (/) refers to the proton whose chemical shift is to be read on the F_1 (vertical axis).

sulfated HexNAc. The ions arising from the terminal sulfated disaccharide $[HSO_3\text{-O-HexNAc-O-Hex-(O)}-2H]^-$, were observed at *m/z* 444 and 460. By analogy, the ions at *m/z* 606 and 622 were derived from the sulfated trisaccharide. The ion at *m/z* 665 originated from the complete sugar moiety plus a part of ceramide, $HSO_3\text{-O-Hex-O-Hex-O-CH=CHNH}_2$ [19]. The peaks at *m/z* 356 and 518 (Fig. 6A) could result from the cleavage of the hexose rings [22], linked together by the glycosidic linkages of HexNAc1-4Hex1-4Hex, supporting the linkage assignments given by methylation analysis. The high-energy CID spectrum also contained a series of ions at high mass, that were evenly spaced by 14 amu, as a result of carbon-carbon bond cleavages in the ceramide moiety by charge-remote fragmentation [19, 21, 23]. Figure 6B shows the low-energy CID MS/MS spectrum, which is remarkably different from the high-energy CID spectrum (Fig. 6A). Only one prominent signal corresponding to the sulfated HexNAc, $[(HSO_3\text{-O-HexNAc})-2H]^-$, was produced at *m/z* 282. These results provided clear evidence for the structure of Ka, which is a mono-sulfated gangliotriaosylceramide, HSO_3-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer.

Discussion

We have already reported the presence of a mono-sulfated glycolipid, Gg_3Cer II^3-sulfate (SM2a) [3], and a bis-sulfated glycolipid, Gg_3Cer II^3III^3-bis-sulfate (SB2) [5], based on the Gg_3Cer core from rat kidney. The present results show that the structure of the new sulfated glycolipid (Ka) is HSO_3-3GalNAcβ1-4Galβ1-4Glcβ1-

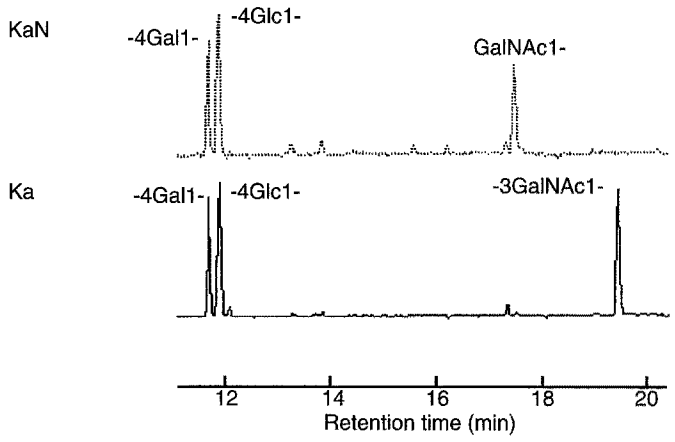

Figure 4. Gas chromatogram of partially methylated alditol acetates derived from Ka and the product of desulfation (KaN). The acetates of partially methylated alditols were analysed by GLC on a CBP1 capillary column. Peaks are acetates of 2,3,6-tri-*O*-methylgalactitol (-4Gal1-), 2,3,6-tri-*O*-methylglucitol (-4Glc1-), 3,4,6-di-*O*-methyl-2-*N*-methylacetamidogalactitol (GalNAc1-), and 4,6-di-*O*-methyl-2-*N*-methylacetamidogalactitol (-3GalNAc1-).

1Cer (Gg_3Cer III^3-sulfate, SM2b), which was found to be an isomer of SM2a. The yield of SM2b was 50 pmol per g wet tissue, which was about 2% of that of SM2a [3].

In the process of structural analysis of Ka (now identified as SM2b), one of the important points had been to rule out the possibility that it is one of the molecular species of SM2a. For this purpose, the ^{1}H-NMR spectra of Ka were compared with those of SM2a and SB2. We have reported that the C2 to C4 ring protons of 3-*O*-sulfated Gal or GalNAc in glycolipids were shifted downfield by 0.1 to 0.6 ppm due to the deshielding effect of the sulfate group. In the spectrum of Ka, the signals of H-2 to H-4 of GalNAc were shifted downfield by 0.168 (0.167), 0.579 (0.579) and 0.465 (0.464) ppm, respectively, compared with those of KaN i.e. desulfated SM2b (downfield shifts from Gg_3Cer are given in parentheses). In contrast, comparison of the H-2 to H-4 signals of the Gal residues in SM2a and KaN, shows downfield shifts of 0.069, 0.661 and 0.309 ppm for the H-2 to H-4 protons in SM2a relative to KaN. SB2 shows downfield shifts for the H-2 to H-4 in both GalNAc and Gal moieties (Table 2). The downfield shift of GalNAcH-3 in SM2b (0.579 ppm) added to that in SM2a (–0.146 ppm) makes 0.433 ppm (0.579–0.146 = 0.433), which was roughly consistent with the downfield shift of GalNAcH-3 in SB2 (0.472 ppm). Similarly, the calculated sum of the downfield shifts for each ring proton in SM2b and that in SM2a approximately equals to the observed value for the ring protons of SB2. This provides further evidence for the hypothesis that SM2b, SM2a and SB2 are homologous glycolipids based on the Gg_3Cer core which differ in the number and position of the sulfate esters.

In high-energy CID spectra, only ions containing sulfate esters were clearly observed, because, in general, the negative charge of sulfated glycolipids is rigidly

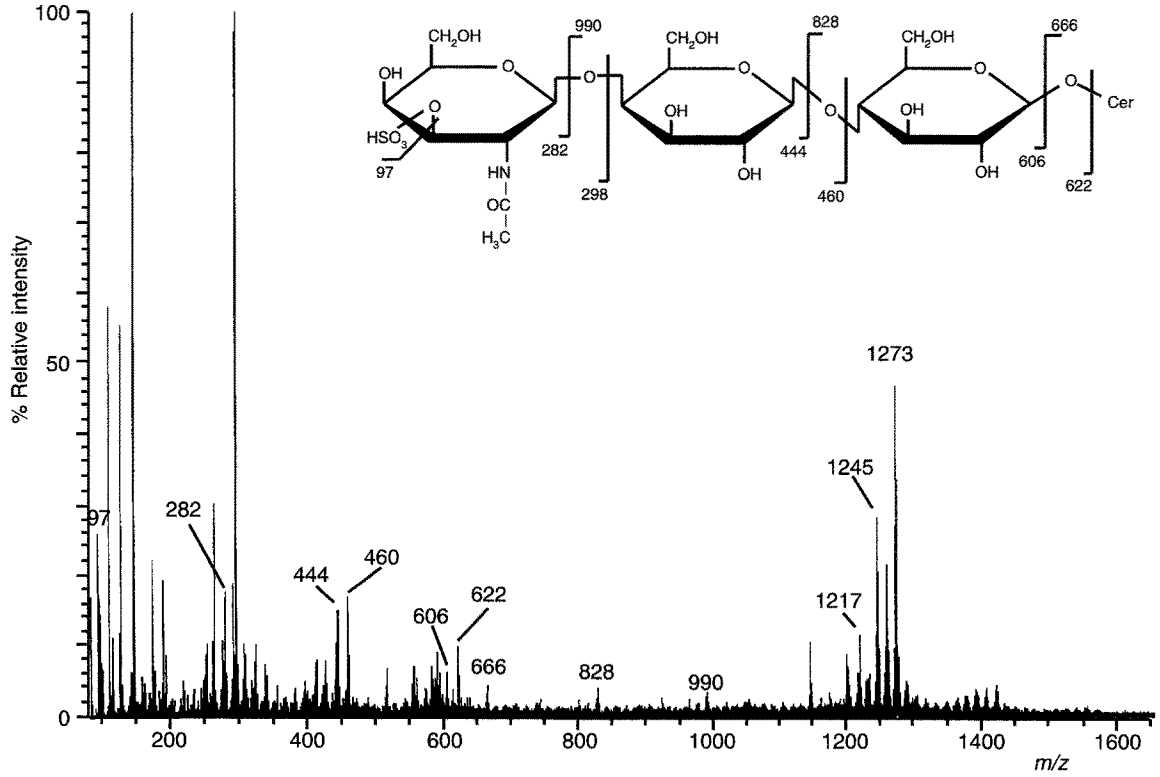

Figure 5. Negative-ion LSIMS spectrum of Ka with its proposed fragmentation scheme (matrix: triethanolamine).

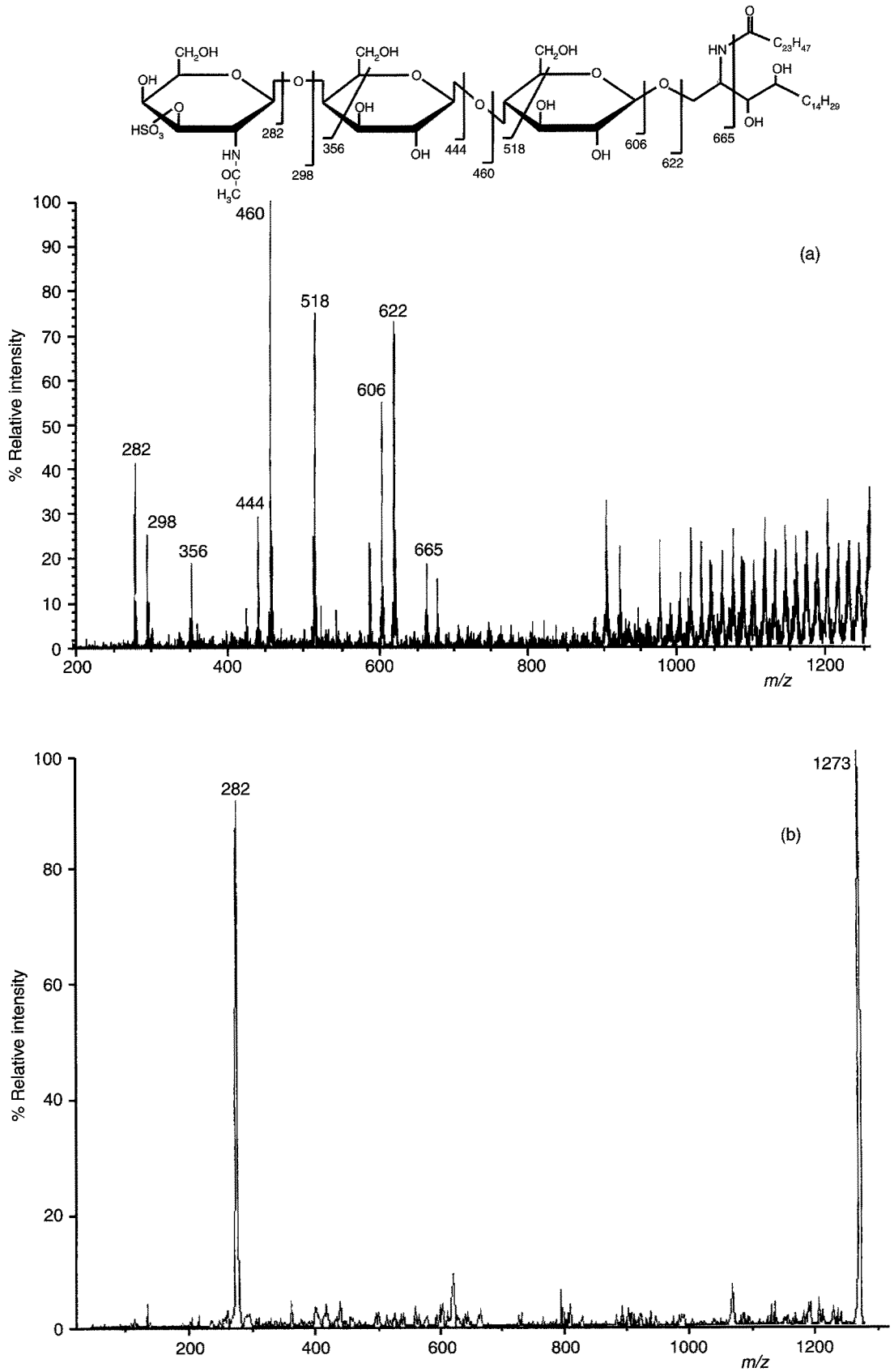

Figure 6. Collision-induced dissociation spectra of Ka with its proposed fragmentation scheme. (A) high-energy CID linked scan spectrum at constant *B*/*E* ratio of the $[M–H]^-$ ion at *m*/*z* 1273. (B) low-energy CID MS/MS product ion spectrum of the $[M–H]^-$ ion at *m*/*z* 1273.

located in the sulfate ester with little possibility of charge migration into the fragmentation portion of the ion [19, 23]. Consequently, the major difference among the high-energy CID spectra of SM2b, SM2a and SB2 was attributed to the intensity of the monosaccharide product ions arising from the non-reducing end [19]. Ions at *m/z* 282 and 298, $[(HSO_3\text{-O-HexNAc-(O)})\text{–}2H]^-$, were observed for SM2b (Fig. 6A) but not for SM2a [19]. The intensity of corresponding ions for SB2 was about 50% of that for SM2b [19]. Although the low-energy CID by itself appeared to be insufficient for structural analysis, it clearly showed the presence of the terminal 3-*O*-sulfated GalNAc (Fig. 6B). In complex carbohydrate analysis, fast atom bombardment mass spectrometry (FAB-MS) or LSIMS is a particularly powerful approach, as reviewed in [24–28]. Recently, several papers dealing with the analysis of sulfated oligosaccharides on FAB-MS have been published [29–33]. Also in the present study, this sensitive strategy, combining LSIMS with CID has proved useful in distinguishing homologous sulfated glycolipids differing in location and number of sulfate esters and confirmed that SM2b has a sulfated GalNAc at the non-reducing terminus.

Although SM2b, SM2a and SB2 have the same Gg_3Cer core structure, the biosynthetic pathway of SM2b should be different from that of SM2a and SB2. SM2a is synthesized by the addition of GalNAc to SM3 [34, 35]. Where SB2 is probably synthesized from SM3 via SM2a, SM2b was recently found to be formed directly from Gg_3Cer by sulfation of its terminal GalNAc in a human renal adenocarcinoma cell line, SMKT-R3 [36]. It was assumed that a single sulfotransferase could contribute to the final sulfation step of the terminal GalNAc in both SM2b and SB2 [36]. Rat kidney further contained two sulfated glycolipids, iGb_4Cer IV^3-sulfate and Gb_4Cer IV^3-sulfate [7], both of which have the terminal 3-*O*-sulfated GalNAc. Further investigations will be necessary to clarify whether or not sulfation of GalNAc in these glycolipids is catalysed by the same sulfotransferase enzyme.

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