Glycoconjugate Journal (1996) **13:** 225–233

Characterization of novel mono-*O*-acetylated GM3s containing 9-*O*-acetyl sialic acid and 6-*O*-acetyl galactose in equine erythrocytes

YOUICHI YACHIDA, KEIKO TSUCHIHASHI and SHINSEI GASA*

Department of Chemistry, School of Medicine, Sapporo Medical University, Sapporo 060, Japan

Received 30 June 1995, revised 12 September 1996

Novel mono-*O*-acetylated GM3s, one containing 9-*O*-acetyl *N*-glycolyl neuraminic acid and another containing 6′-*O*-acetyl galactose, were isolated as a mixture from equine erythrocytes, and the structures were characterized by one- and two-dimensional proton nuclear magnetic resonance (NMR) and fast atom bombardment-mass spectrometry (FAB-MS). The position of the *O*-acetyl residue was identified by the downfield shift of the methylene protons at C-9 of *N*-glycolyl neuraminic acid (9-*O*-Ac GM3) and C-6 of galactose (6′-*O*-Ac GM3) in the NMR spectrum, in comparison to the respective non-acetylated counterparts. To confirm the presence of 6′-*O*-Ac GM3, the *O*-acetylated GM3 mixture was desialylated with *Arthrobacter* neuraminidase, giving 6-*O*-acetyl galactosyl glucosylceramide, the structure of which was estimated by NMR and FAB-MS, together with non-acetylated lactosylceramide with a ratio of 1:1.

Keywords: *O*-acetyl GM3, equine erythrocytes, NMR

Abbreviations: Ac, acetyl; Gc, glycolyl; NeuGc, *N*-Gc neuraminic acid; GM3 (Gc), GM3 containing NeuGc (II^3NeuGcα-LacCer); 4-*O*-Ac GM3 (Gc), GM3 containing 4-*O*-Ac NeuGc; 9-*O*-Ac GM3 (Gc), GM3 containing 9-*O*-Ac NeuGc; 6′-*O*-Ac GM3 (Gc), GM3 containing 6-*O*-Ac Gal; 1D-NMR, one-dimensional nuclear magnetic resonance spectrometry; 2D-COSY, two-dimensional chemical shift-correlated spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; GLC, gas-layer chromatography; GC-MS, gas chromatography-mass spectrometry; TLC, thin-layer chromatography; Ggl, ganglioside; Cer, ceramide; CMH, monohexosylceramide; LacCer, lactosylceramide; 6′-*O*-Ac LacCer, LacCer containing 6-*O*-Ac Gal; Me_2SO-d_6, $^2H_6$-dimethylsulfoxide; CMW, chloroform-methanol-water. Nomenclature and abbreviations of glycosphingolipids follow the system of Svennerholm (*J Neurochem* [1963] **10**: 613–23) and those recommended by the IUPAC-IUB Nomenclature Commission (*Lipids* [1977] **12**: 455–68).

Introduction

O-Acetylation of gangliosides (Ggls), exclusively appearing on neuraminic acid in non-reducing termini, has been found in several mammalian species, including 4-*O*-acetyl (Ac) GM3 from equine erythrocytes [1, 2], 9-*O*-Ac GT1b from mouse brain [3], 9-*O*-Ac GD1a from rat erythrocytes [4], and 9-*O*-Ac GD3 from melanoma cells [5–7]. In particular, 9-*O*-Ac GD3 and non-acetylated GD3 are known to be melanoma-associated Ggl antigens, suggesting the possibility of using Ggls as tumour targets for melanoma immunotherapy [8].

Analysis of the position of labile *O*-Ac residues present in intact glycolipid molecules has been effectively performed by nuclear magnetic resonance spectroscopy (NMR) and fast-atom bombardment-mass spectrometry (FAB-MS). The downfield shift of the proton attached to the carbon bearing an acetoxy group in the NMR spectrum was ascribed to the existence of the Ac residue in the native molecule. In fact, the positions of the Ac esters of 4-*O*-Ac GM3 [2], 9-*O*-Ac GD3 [9, 10], and 7-*O*-Ac GD3 [10, 11], as well as that of the sulfate ester of sulfo-3′-galactosyl ceramide [12], have been clearly identified by these methods.

The Ggls in equine erythrocyte membranes have

*To whom correspondence should be addressed.

0282-0080 © 1996 Chapman & Hall

previously been investigated, resulting in the discovery of GM3 containing *N*-glycolylneuraminic acid (NeuGc) (GM3(Gc)) and its 4-*O*-acetylated derivative, and lactosylceramide (LacCer) as the major components [1, 2, 13]. In the present paper, we further examined minor Ggls in equine erythrocytes to search for other *O*-Ac Ggls, finding two novel mono-*O*-Ac GM3s.

Materials and methods

Chemicals

Silica beads (Iatrobeads) were obtained from Iatron Laboratories (Tokyo). Silica gel thin-layer chromatography (TLC) plates (Silica gel 60) and $^2H_6$-dimethylsulfoxide (Me_2SO-d_6) were from Merck (Germany). Neuraminidase (*Arthrobacter ureafaciens*) was a product of Seikagaku Kogyo Corporation (Tokyo). GM3 (Gc) was isolated from equine erythrocytes as reported in a previous paper [2]. Other standard glycolipids were prepared in this laboratory. All other reagents were of analytical grade.

Isolation of glycolipid

The ratio of the solvent mixture is represented by volume. The Ggl fraction was isolated without alkaline treatment from equine erythrocyte membranes as reported previously [2]. Briefly, 450 g of acetone powder prepared from the erythrocyte membranes obtained from 20 l of whole blood by centrifugation was extracted with $CHCl_3$:CH_3OH:H_2O (CMW) = 4:8:3 (g powder per 5 ml) three times. The crude extract was concentrated *in vacuo* and applied on a DEAE-Sephadex, A-25 (LKB-Pharmacia, Sweden) column (3 × 40 cm), previously equilibrated with CMW = 3:6:1. After the column was thoroughly washed with the equilibration CMW until unbound glycolipids were negative, the acidic glycolipids were eluted with CM:1 M ammonium acetate = 3:6:1. The eluates were concentrated, dialysed to remove salt, evaporated to dryness and applied on an Iatrobeads column (2.5 × 80 cm) with CMW = 90:10:0.5. The ratio of the CMW mixture for elution was changed stepwise from 90:10:0.5, 80:20:2, 70:30:3 to 60:40:4 (1 l each). The crude mono-*O*-Ac GM3 fraction including known 4-*O*-Ac GM3 (Gc), eluted with CMW = 70:30:3 and migrating slightly faster than non-acetylated GM3 (Gc) on TLC, was further chromatographed on repeated silica gel column chromatography with the CMW solvent system as above except for the column scale and elution volume. Fifteen milligrams of an unknown Ggl, abbreviated GL-1, which migrated between 4-*O*-Ac GM3 (Gc) and non-acetylated GM3 (Gc) on TLC, were obtained.

The monohexosyl ceramide (CMH) of the equine erythrocytes was isolated and purified from the above unbound fraction of the extracts on a DEAE-Sephadex column by silica gel chromatography using the CMW solvent system. The CMH fraction obtained was further divided into three components by silica gel column chromatography, each representing one band on TLC (see Fig. 5). The CMH was not exposed to an alkaline solution during preparation.

Alkaline treatment of glycolipid

The purified GL-1 or CMH fraction or a product of neuraminidase-digested GL-1 (approximately 1 mg each) was treated with 1% sodium methoxide in 1 ml of methanol for 2 h at room temperature. After neutralization with acetic acid, the mixture was concentrated and applied on an LH-20 column (0.5 × 10 cm, Pharmacia-LKB, Sweden) with CMW = 60:30:4.5 for desalting. The treated glycolipid was chromatographed on a TLC plate developed with CMW = 60:35:8 followed by staining with an orcinol-sulfuric acid reagent.

Neuraminidase treatment of glycolipid

The purified GL-1, GM3 (Gc) or 4-*O*-Ac GM3 (Gc) (7.5 μg each) was separately digested with 10 or 30 mU of *Arthrobacter ureafaciens* neuraminidase in 0.1 ml of 50 mM acetate buffer, pH 5.0, at 37 °C for 1 h. The mixture was applied on an LH-20 column (0.5 × 10 cm) with CMW = 60:30:4.5 to remove salt, and an aliquot of the lipid product was chromatographed by TLC as described above. GL-1 was further desialylated on a large scale (5.5 mg of GL-1, 1 U of the enzyme in 1 ml of the buffer) for 16 h. After being desalted from the reaction mixture as described above, two products (abbreviated GL-1a and -1b) and unreacted GL-1 were separated from each other by silica gel column chromatography (0.8 × 30 cm) using the CMW sovent system. GL-1a (2.5 mg), GL-1b (2.1 mg) and unreacted GL-1 (0.5 mg) were eluted from the column with CMW = 85:15:1, 80:20:2 and 70:30:3, respectively.

Analyses of lipid and sugar moieties

Fatty acid components, the long chain base and sugar species including sialic acid were separately analysed from the methanolyzates by gas-layer chromatography (GLC) as methyl ester for the fatty acid and *O*-trimethylsilyl derivative for the base and the sugars, respectively, as reported previously [2, 14, 15]. The methylation analysis of the purified GL-1 was performed according to the method of Hakomori [16] and the partially methylated alditol acetates were analysed by gas chromatography-mass spectrometry (GC-MS) as reported previously [15, 17].

Measurement of FAB-MS spectrum

Negative fast atom bombardment-mass spectrometry (FAB-MS) was done on a JEOL JMS-HX100 mass spectrometer equipped with a JMA-DA500 datalizer. The sample in a matrix of triethanolamine was bombarded by

Xe gas at 6 kV (20 mA), and the fragments were accelerated at 5 kV, as described previously [15].

Measurement of NMR spectra

The 500 MHz-NMR spectra of glycolipids (0.5 ~ 1 mg) in 0.4 ml of $^2H_6$-Me_2SO containing 2% ^2H_2O were obtained in the Fourier-transform mode on a Varian JEOL-Alpha500 spectrometer at the High Resolution NMR Laboratory of Hokkaido University as described previously [15]. The chemical shift was indicated by the distance (ppm) from tetramethylsilane as an internal standard. 2D-Chemical shift-correlated spectroscopy (2D-COSY) spectra were obtained as described previously [15], and shown by absolute value representation as contour plots.

Results

Isolation of glycolipid

The Ggl fraction of the equine erythrocytes was composed of GM3 including NeuGc (II³NeuGcα-LacCer) and the 4-*O*-Ac derivative (II³(4-*O*-Ac)NeuGcα-LacCer) as the major Ggl [1, 2, 13]. As a minor component, GL-1 was obtained as one band that migrated between 4-*O*-Ac GM3 (Gc) and non-acetylated GM3 (Gc) as demonstrated in Fig. 1. The purified GL-1 was, however, found to be a mixture of two different acetylated derivatives of GM3 (Gc) from the presence of a heterogeneous Ac group in the FAB-MS and NMR spectra (see below). Some other developing solvents for the TLC of GL-1 such as CMW containing ammonia, or containing $CaCl_2$ and 1-propanol-water, were examined to obtain homogeneous glycolipids, but the attempts were unsuccessful. Similarly unsuccessful were other methods such as high performance liquid chromatography using a spherical silica beads column, reversed-phase column, Amine-column (Pharrmacia-LKB) or Borate column (Pharrmacia-LKB), and the droplet counter current technique. Other very minor components, such as sialylparagloboside (IV³NeuGcα-nLc₄Cer, [13]) as well as unknown mono- and di-*O*-Ac GM3s, were present in the Ggl fraction (the structures of the unknown Ac GM3s will be published elsewhere).

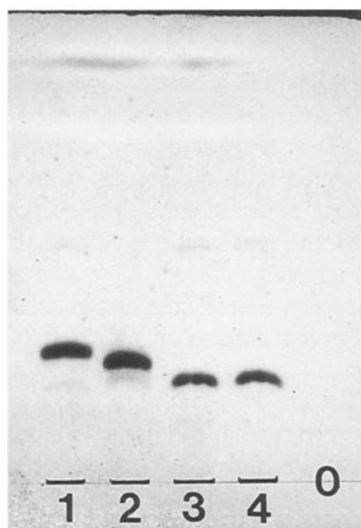

Figure 1. Thin-layer chromatography of equine erthyrocytes GM3 (Gc) and GL-1. Lane 1, 4-*O*-Ac GM3 (Gc); 2, GL-1; 3, GM3 (Gc); 4, alkali-treated GL-1. The Ggls were developed with CMW = 60:35:8 and stained by an orcinol-sulfuric acid reagent. O, origin.

Alkaline treatment of glycolipid

The GL-1 was treated with a mild alkaline solution to remove the alkali-labile group(s), followed by TLC analysis. As shown in Fig. 1, the saponified GL-1 showed an R_F identical to that of authentic GM3 (Gc). In addition, 1D-NMR analysis of the saponified GL-1 yielded a spectrum identical to that of authentic GM3 (Gc) (data not shown), confirming that some alkali-labile group such as *O*-acyl ester bound to the GM3 (Gc) molecule.

Neuraminidase treatment of glycolipid

The purified GL-1, like GM3 (Gc) and 4-*O*-Ac GM3 (Gc), was treated with neuraminidase. As demonstrated in Fig. 2A, the GL-1 was finally desialylated with the enzyme to produce two bands, one of them migrated to the position of LacCer (abbreviated as GL-1b) and the other was converted to a position similar to that of GalCer (GL-1a), whereas GM3 (Gc) changed to LacCer but 4-*O*-Ac GM3 (Gc) did not react. Moreover, the GL-1a was further saponified followed by TLC analysis, revealing conversion to the position of LacCer as shown in Fig. 2B, indicating that the GL-1a possessed an alkali-labile group such as *O*-acyl ester. The GL-1b and saponified GL-1a were finally both characterized as LacCer with their 1D-NMR spectra, which were identical to that of authentic LacCer (data not shown). *Arthrobacter* neuraminidase is reported to cleave internal and external nonreducing terminal *N*-acyl (Ac and Gc) neuraminic acid itself and the C-9-*O*-Ac derivative in the Ggl molecule, but not to cleave the C-4-, C-7- and C-8-*O*-Ac derivatives, and to have no aglycon specificity [18]. Regarding these enzyme properties and the present data, GL-1 was expected to be composed of GM3 containing 9-*O*-Ac NeuGc, which would be changed to LacCer (GL-1b) via the enzyme digestion, and GM3 (Gc) containing an *O*-acylated sugar other than NeuGc, which would be converted to GL-1a. The structure of the GL-1a was analysed by the NMR spectrum as described below.

Lipid and sugar moieties of glycolipid

The fatty acid component, long chain base and sugar species of GL-1 are summarized in Table 1. The major

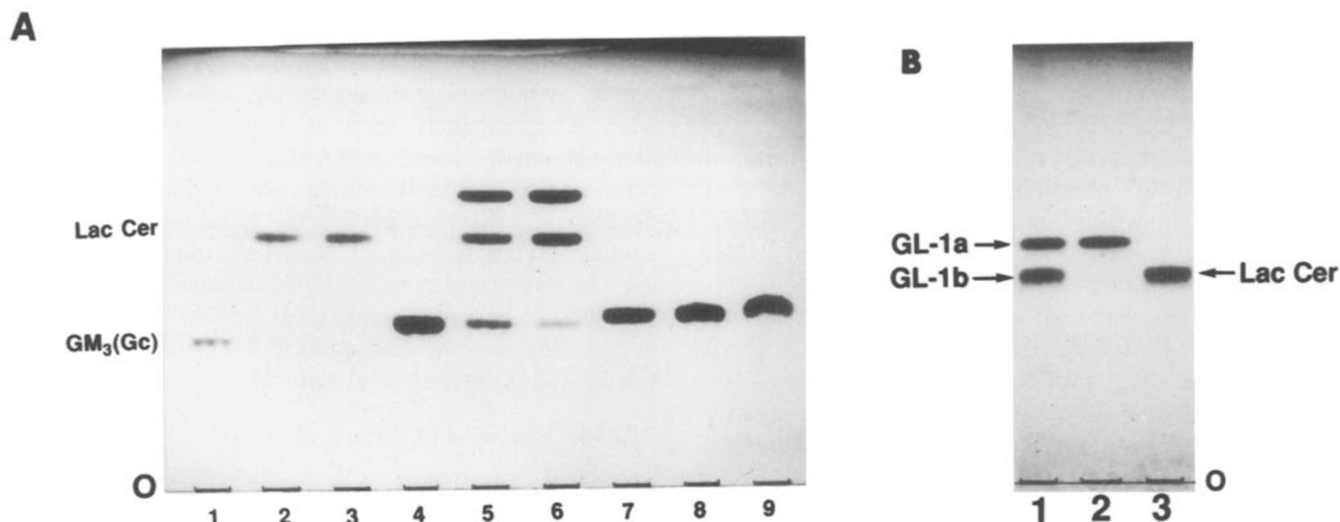

Figure 2. Thin-layer chromatography of neuraminidase-treated equine erythrocytes GM3 (Gc) and GL-1. In panel A, lanes 1, 4 and 7 indicate non-acetylated GM3 (Gc), GL-1 and 4-*O*-Ac GM3 (Gc), respectively; lanes 2, 5 and 8, neuraminidase-digested glycolipids from the Ggls (7.5 μg each) of lanes 1, 4 and 7 with 10 mU of neuraminidase; lanes 3, 6 and 9, digested glycolipids of lanes 1, 4 and 7 with 30 mU of the enzyme. In panel B, lanes 1, 2 and 3 show the neuraminidase-treated GL-1 (lane 6 in panel A), separated GL-1a and saponified GL-1a, respectively.

lipids were C_{24}-fatty acyl and C_{18}-sphingenine, and the sugar moiety was composed of Gal/Glc/NeuGc with a ratio of 1:1:1. The methylation analysis of the GL-1 afforded two partially methylated alditol acetates with 1,3,5-*tri-O*-Ac 2,4,6-*tri-O*-methyl galactitol and 1,4,5-*tri-O*-Ac 2,3,6-*tri-O*-methyl glucitol having similar ratios. These data indicated that the core structure of GL-1 was GM3 containing NeuGc. On the other hand, the lipid moieties of GL-1a and GL-1b were analysed, as in the case of GL-1, revealing that the fatty acid component and the long chain base were almost the same in these glycolipids, as shown in Table 1. In particular, both major fatty acyls, $C_{24:0}$ and $C_{24:1}$ in GL-1a reflected the ionization of the molecule and the fragmentation in the FAB-MS spectrum as described below.

FAB-MS study

The GL-1 was analysed using the negative FAB-MS spectrum with the sequential fragmentation of the sugar moieties. As shown in Fig. 3A, GL-1 was ionized mainly at *m/z* 1321 as a pseudomolecular ion $[M–H]^-$, and fragmented with *m/z* 972 $[\text{hexosylhexosylCer-H}]^-$. This fragment ion indicated at least the presence of a mono-Ac group on the non-reducing NeuGc moiety. Based on the major component of the fatty acid and the long chain base as described above, the ion at *m/z* 1321 was composed of C_{24}-fatty acyl with a saturated bond and C_{18}-sphingenine in the GL-1 structure. In addition, a fragment ion with *m/z* 1014 due to $[\text{Ac-hexosylhexosylCer-H}]^-$ was also observed in the spectrum, while *m/z* 852 due to $[\text{Ac-hexosylCer-H}]^-$ was not, indicating the presence of *O*-acetylated Gal in the GL-1 as an another glycolipid. Other fragment ions with *m/z* 810 and 648 due to $[\text{hexosylCer-H}]^-$ and $[\text{Cer-H}]^-$, respectively, were observed. These FAB-MS data suggested that GL-1 was composed of two different mono-*O*-acetylated GM3s (Gc), one containing *O*-Ac NeuGc and the other containing *O*-Ac Gal.

In addition, the desialylated product from GL-1, GL-1a, yielded pseudomolecular ions with *m/z* 1014 and 1012 (Fig. 3B), in which lipid components were composed of $C_{24:0}$ and $C_{24:1}$ fatty acyls and sphingenine, showing GL-1a to be Ac-hexosylhexosylCer. Thus, observation of these fatty acid components in the FAB-MS spectrum agreed with the results from lipid analysis by GLC described above (Table 1). Additional fragment ions with *m/z* 810 ($C_{24:0}$) and 808 ($C_{24:1}$), as well as *m/z* 648 ($C_{24:0}$) and 646 ($C_{24:1}$), were identified as hexosylCer and Cer, respectively, representing Ac residues located on non-reducing terminal hexose in GL-1a.

NMR study

The 1D-NMR spectrum of the purified GL-1 revealed resonances at δ1.985 and 2.009 ppms due to two *O*-Ac methyl protons, each with almost equal intensities, and amide protons on NeuGc at δ7.42 and 7.45 with equal intensity as demonstrated in Fig. 4A. Similarly, two anomeric protons on β-Gal at δ4.225 (II^1 in the Figure) and δ4.287 ($II^{1'}$: the dashed number is employed in another glycolipid (GM3 containing 6-*O*-Ac Gal)) appeared each with an intensity of half compared to the

Table 1. Lipid and sugar composition of GL-1, GL-1a and GL-1b.

	Lipid composition (%)		
	Long chain base[a]		
	d18:1[b]	*d18:0*[c]	*Unknown*
GL-1	94.7	tr[d]	5.2
GL-1a	98.7	tr	1.2
GL-1b	97.5	tr	2.3

	Fatty acid[e] *(%)*												
	16[f]		18		20		22		24		26		Unknown
	:0[g]	:1[h]	:0	:1	:0	:1	:0	:1	:0	:1	:0	:1	
GL-1	11.4	tr[h]	17.5	1.8	1.1	tr	6.7	tr	28.9	23.1	2.0	2.2	4.0
GL-1a	10.8	tr	16.4	1.9	tr	tr	6.3	tr	32.2	21.2	1.9	2.5	6.1
Gl-1b	12.0	tr	18.0	1.3	tr	tr	5.3	tr	26.8	25.0	2.5	3.2	5.5

	Sugar composition		
	Per trimethylsilyl methylglycoside[i]		
	Glc	*Gal*	*NeuGc*
GL-1	1.0	0.9	0.8
Gl-1a	1.0	0.9	ND[j]
GL-1b	1.0	0.9	ND
	Partially methylated alditol acetate[i,k]		
	0-Gal[l]	3-Gal[m]	4-Glc[n]
GL-1	ND	0.8	1.0
GL-1a	0.8	ND	1.0
GL-1b	0.9	ND	1.0

[a]Measured as trimethylsilyl derivative; [b]sphingenine; [c]sphinganine; [d]trace (1%>); [e]measured as methyl ester; [f]carbon number; [g]saturated; [h]mono-unsaturated; [i]measured by GLC; [j]not detected; [k]measured by GC-MS; [l]1,5-di-*O*-Ac 2,3,4,6-tetra-*O*-methyl galactitol; [m]1,3,5-tri-*O*-Ac 2,4,6-tri-*O*-methyl galactitol; [n]1,4,5-tri-*O*-Ac 2,3,6-tri-*O*-methyl glucitol.

intensity of the anomeric proton on *β*-Glc at δ4.177 (I^1). These heterogeneities of the *O*-Ac residue and anomeric proton indicated that GL-1 was a mixture of two *O*-Ac GM3s (Gc), supporting the result from FAB-MS.

The position of the *O*-Ac group on the GL-1 was analysed by assignment of the ring protons on sugar moieties by the 2D-COSY spectrum. The whole 2D-spectrum of GL-1 enabled us to identify axial H-3 (S^{3ax}), equatorial H-3 (S^{3eq}), H-4 (S^4) and H-5 (S^5) on NeuGc, as well as H-3 (L^3), -4 (L^4) and -5 (L^5) on sphingenine from their cross peaks (Fig. 4B). As shown in the partial 2D-spectrum of GL-1 (Fig. 4C), methylene protons at C-9 on NeuGc and at C-6 on Gal markedly resonated in a lower field with δ3.95 (S^{9a}) and 4.22 (S^{9b}), respectively, assigned by cross peaks with S^{9a-9b}, S^{8-9a} and S^{8-9b}, in the former and with δ4.061 ($II^{6a'}$) and 4.156 ($II^{6b'}$), assigned with $II^{6a'-6b'}$, $II^{5'-6a'}$ and $II^{5'-6b'}$, in the latter, as compared to those of non-acetylated GM3 (Gc) (see Table 2).

These downfield shifts of the protons enabled us to assign the position of the Ac group bound to C-9-*O* on NeuGc in one GM3 (Gc) (9-*O*-Ac GM3 (Gc)) and to C-6-*O* on Gal in the other (6′-*O*-Ac GM3 (Gc)) of GL-1. The downfield shift and splitting pattern of the acetylated C-9 methylene protons on sialic acid agreed with those of 9-*O*-Ac GD3 [9, 10]. These protons and other ring protons of GL-1, assigned by the 2D-spectrum, are summarized in Table 2, together with those of reference GM3 (Gc).

The structure of GL-1a was also analysed with 1D- and 2D-NMR spectra (data not shown). The partial 1D-spectrum demonstrated the presence of an *O*-Ac group

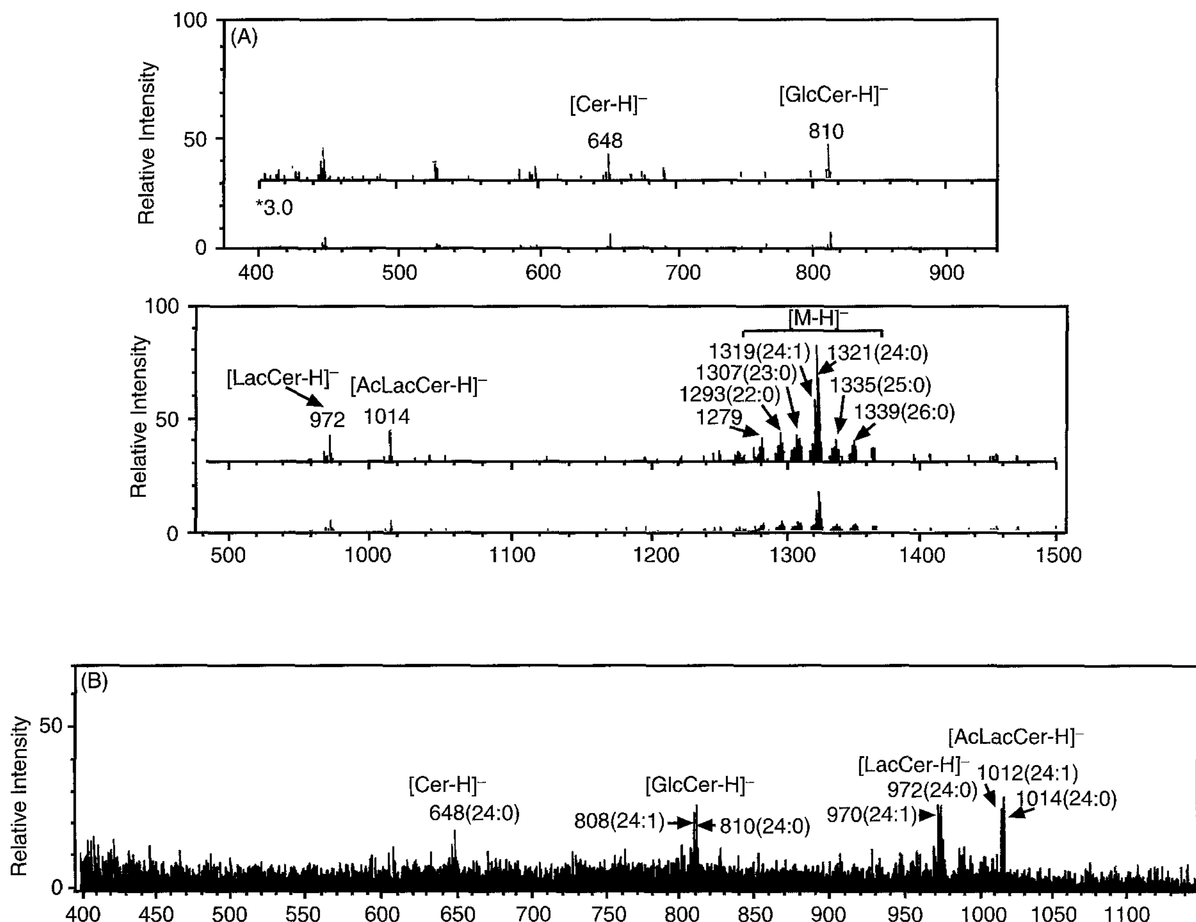

Figure 3. Negative FAB-MS spectra of GL-1 and GL-1a. Panel A, GL-1 (M_r 1322 with $C_{24:0}$); B, GL-1a (M_r 1015 with $C_{24:0}$). In the spectra, the number in parenthesis indicates fatty acid component.

(δ2.050), anomeric protons of β-Gal (δ4.321) and β-Glc (δ4.223), and a significant downfield shift of C-6 methylene protons, assigned with the 2D-spectrum (see Table 2). From these data, GL-1a was identified as acetylated LacCer at Gal C-6-*O*. Judged from the above data, GL-1 isolated from equine erythrocytes was concluded to be a mixture of GM3 containing 9-*O*-Ac NeuGc and GM3 (Gc) containing 6-*O*-Ac Gal.

Discussion

Equine erythrocyte membranes and plasma, as well as the submandibular gland, have been investigated to determine their sialic acid species in detail [19]. These species have been isolated from glycoconjugates with chemical techniques, resulting in the discovery of various mono- and di-*O*-acetylated derivatives, including 9-*O*-Ac NeuGc [20, 21]. However, other Ggls containing *O*-Ac-NeuGc or -NeuAc rather than 4-*O*-Ac GM3 [1, 2] have not yet been found in equine erythrocytes. 9-*O*-Ac NeuAc Monosaccharide has been isolated from several mammalian brains [22] and from mouse erythrocytes [23] and its structure was characterized by means of GC-MS. Ggls containing 9-*O*-Ac NeuAc have been obtained from several mammalian cells or tissues as described above, and from fish [24, 25]; however, these have been di- or tri-sialogangliosides and not monosialoganglioside. In the present paper, GM3 containing 9-*O*-Ac NeuGc (9-*O*-Ac GM3 (Gc)) was obtained for the first time from equine erythrocytes as a mixture with a novel GM3 (Gc) containing 6′-*O*-Ac Gal (6′-*O*-Ac GM3 (Gc)). These mono-*O*-Ac GM3s were hardly separable from each other by any of the chromatographic techniques employed; therefore, their structures, particularly the positions of the *O*-Ac residue, were characterized in the mixed state directly by an undestructive NMR method and indirectly by partial degradation methods using neuraminidase and saponification.

As to the interaction of *O*-Ac GM3 (Gc) identified herein with proteins, the substrate properties of 9-*O*-Ac GM3 (Gc) and 6′-*O*-Ac GM3 (Gc) towards *Arthrobacter* neuraminidase – that is the glycon specificity of 9-*O*-Ac

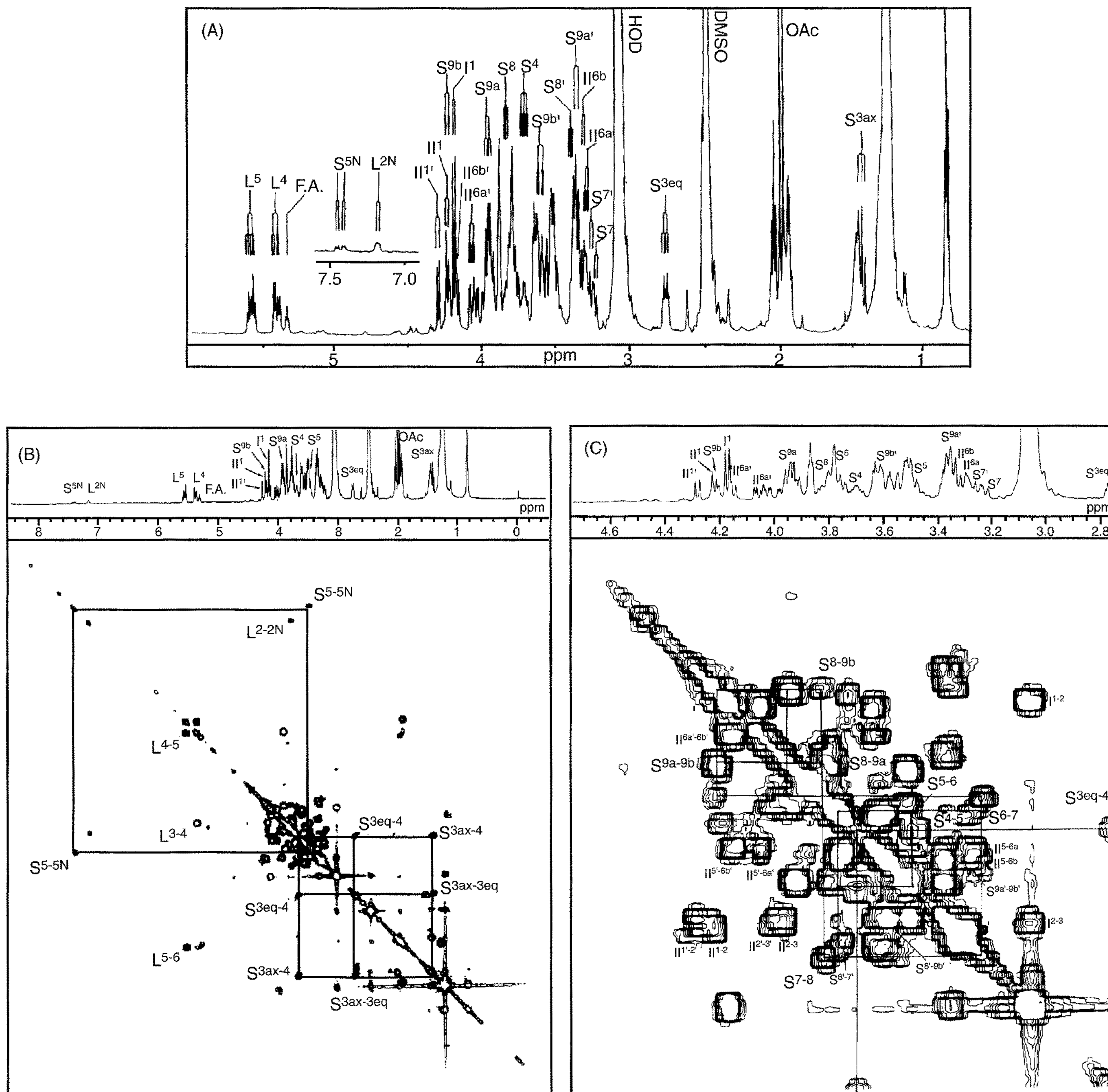

Figure 4. 1D- and 2D-NMR spectra of GL-1. 500 MHz-NMR spectra of GL-1 and GL-1a were measured in a solution of $^2H_6$-Me_2SO containing ^2H_2O at 90 °C. Panels A, B and C indicate whole 1D-, whole 2D- and partial 2D-spectra of GL-1, respectively. In the spectra, L, S, I, II and F.A. show protons on the long chain base, sialic acid, β-Glc, β-Gal and fatty acid, respectively.

NeuGc in the former and aglycon specificity of NeuGc in the latter, were not distinguishable from each other. They were degraded by the enzyme, in a manner similar to non-acetylated GM3 (Gc), although the cleavage rates of these Ggls were slightly lower than that of GM3 (Gc) (Fig. 2A).

It is known that intramolecular-migration of the *O*-Ac group to sterically adjacent hydroxyl groups such as C-7-*O*-Ac to C-9-*O* on the free sialic acid moiety after it has been liberated from the sialoglycoconjugates easily occurs in biological conditions [26], but not on the intact glycoconjugates. Regarding this phenomenon, 6′-*O*-Ac GM3 (Gc) could not be an artificial product from 9-*O*-Ac GM3 with Ac migration. Furthermore, *O*-Ac LacCer,

Table 2. Chemical shifts (δ, ppm) in the NMR spectra of sugar residues of GL-1, GL-1a and reference glycolipids.

	H-1	*H-2*	*H-3(ax)*	*H-3 (eq)*	*H-4*	*H-5*	*H-6a*
GL-1 (9-*O*-Ac GM3 (Gc))							
I	4.177	3.06	3.360	–	3.35	3.37	3.513
II	4.225	3.360	3.98	–	3.786	3.613	3.29
S	–	–	1.435	2.768	3.705	3.50	3.77
GL-1 (6′-*O*-Ac GM3 (Gc))							
I′	4.177	3.06	3.360	–	3.35	3.37	3.513
II′	4.287	3.360	4.032	–	3.786	3.64	*4.061*
S′	–	–	1.435	2.768	3.71	3.485	3.75
GM3 (Gc)[d]							
I	4.175	3.06	3.36	–	3.35	3.36	3.51
II	4.236	3.359	3.998	–	3.788	3.64	3.28
S	–	–	1.450	2.749	3.693	3.50	3.76
GL-1a							
I	4.223	3.06	3.410	–	3.38	3.349	3.656
II	4.321	3.395	3.40	–	3.698	3.77	*4.140*
LacCer[d]							
I	4.173	3.030	3.30	–	3.30	3.30	3.584
II	4.199	3.40	3.30	–	3.613	3.40	3.528

(table cont.)

	H-6b	*H-7*	*H-8*	*H-9a*	*H-9b*	*Gc-CH2*	*9OAc*[a]	*6O′Ac*[b]	*S-5N*[c]
GL-1 (9-*O*-Ac GM3 (Gc))									
I	3.513	–	–	–	–	–	–	–	–
II	3.31	–	–	–	–	–	–	–	–
S	–	3.230	3.82	*3.95*	*4.22*	3.871	1.985 or 2.009	–	7.42 or 7.45
GL-1 (6′-*O*-Ac GM3 (Gc))									
I′	3.513	–	–	–	–	–	–	–	–
II′	*4.156*	–	–	–	–	–	–	–	–
S′	–	3.30	3.39	3.35	3.60	3.874	–	2.009 or 1.985	7.45 or 7.42
GM3 (Gc)[d]									
I	3.52	–	–	–	–	–	–	–	–
II	3.30	–	–	–	–	–	–	–	–
S	–	3.263	3.39	3.36	3.625	3.870	–	–	7.41
GL-1a									
I	3.776	–	–	–	–	–	–	–	–
II	*4.244*	–	–	–	–	–	–	2.050	–
LacCer[d]									
I	3.741	–	–	–	–	–	–	–	–
II	3.584	–	–	–	–	–	–	–	–

[a]9-*O*-Ac NeuGc; [b]6′-*O*-Ac Gal; [c]amide proton on sialic acid; [d]measured at 30 °C [27].

the probable biosynthetic precursor for 6′-*O*-Ac GM3 (Gc), might, to a small degree, be included in the CMH fraction isolated from the extracts of equine erythrocytes, as indicated in Fig. 5, although the Ac position is not known, because of the lack of separation between CMH and *O*-Ac LacCer. The detection of the precursor suggests that 6′-*O*-Ac GM3 could be ascribed to a biologically synthesized product from sialylation of naturally occurring *O*-Ac LacCer as a substrate. Further enzymatic study may support the results reported here.

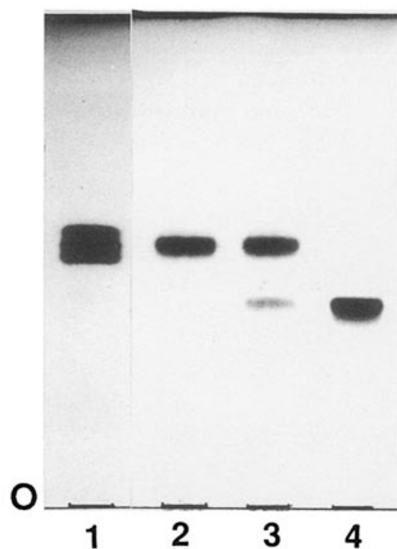

Figure 5. Thin-layer chromatography of CMH fraction after saponification. Lane 1 shows the whole CMH fraction; 2, isolated middle band from the whole CMH fraction; 3, the middle band after saponification; 4, authentic LacCer. O, origin.

Acknowledgements

We are deeply indebted to Mr Kim Barrymore for his help in the preparation of this article, Drs Masahiko Chiba and Yoshihiro Maeda for their technical help in the preparation of glycolipids and Hokkaido Ebetsu Meat Inspection Office for donation of equine blood. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas No. 05274221 from the Ministry of Education, Science and Culture, Japan, and by the Foundation for Research Promotion of Sapporo Medical University.

References

1. Hakomori S, Saito T (1969) *Biochemistry* **8**: 5082–88.
2. Gasa S, Makita, A, Kinoshita Y (1983) *J Biol Chem* **258**: 876–81.
3. Ghidoni R, Sonnino S, Tettamanti G, Baumann N, Reuter G, Schauer R (1980) *J Biol Chem* **255**: 6990–5.
4. Gowda DC, Reuter G, Shukla AK, Schauer R (1984) *Hoppe-Seyler's Z Physiol Chem* **365**: 1247–53.
5. Holgersson J, Karlsson K-A, Karlsson P, Ringquist M, Stromberg N, Thurin J, Błaszczyk M, Herlyn M, Steplewski Z, Koprowski H (1983) In *Proc 7th Int. Symp on Glycoconjugates* (Chester MA, Heinegard D, Lundblad A, Svensson S eds) pp. 856–57. Lund: Rahms.
6. Cheresh DA, Varki AP, Varki NM, Stallcup, WB, Levine J, Reisfeld RA (1984) *J Biol Chem* **259**: 7453–59.
7. Cheresh DA, Reisfeld RA, Varki AP (1984) *Science* **225**: 844–46.
8. Ravindranath MH, Morton DL, Irie RF (1989) *Cancer Res* **49**: 3891–97.
9. Thurin J, Herlyn M, Hindsgaul O, Stromberg N, Karlsson K-A, Elder D, Steplewski Z, Koprowski H (1985) *J Biol Chem* **260**: 14556–63.
10. Ren S, Scarsdale JN, Ariga T, Zhang Y, Klein RA, Hartmann R, Kushi Y, Egge H, Yu RK (1992) *J Biol Chem* **267**: 12632–38.
11. Ren S, Ariga T, Scarsdale JN, Zhang Y, Slominski A, Livingston PO, Ritter G, Kushi Y, Yu RK (1993) *J Lipid Res* **34**: 1565–71.
12. Gasa S, Nakamura M, Makita A, Ikura M, Hikichi K (1985) *Carbohydr Res* **137**: 244–52.
13. Gasa S, Makita A, Yanagisawa K, Nakamura M (1984) In *Advs Exp Biol Med* Vol. 152 (Ledeen RW, Yu RK, Rapport MM, Suzuki K, eds) pp. 111–17. New York: Plenum Press.
14. Yu RK, Ledeen RW (1970) *J Lipid Res* **11**: 506–16.
15. Sako F, Gasa S, Makita A, Hayashi A, Nozawa S (1990) *Arch Biochem Biophys* **278**: 228–37.
16. Hakomori S (1964) *J Biochem* **55**: 205–8.
17. Suetake K, Gasa S, Taki T, Chiba M, Yamaki T, Ibayashi Y, Hashi K (1993) *Biochim Biophys Acta* **1170**: 25–31.
18. Corfield AP, S-Wewer M, Veh RW, Wember M, Schauer R (1986) *Biol Chem Hoppe-Seyler* **367**: 433–39.
19. Schauer R (1978) *Methods Enzymol* **50**: 64–89.
20. Buscher H-P, C-Stenzel J, Schauer R (1974) *Eur J Biochem* **50**: 71–82.
21. Kamerling JP, Vliegenthart JFG, Versluis C, Schauer R (1975) *Carbohydr Res* **41**: 7–17.
22. Haverkamp J, Veh RW, Sander M, Schauer R, Kamerling JP, Vliegenthart JFG (1977) *Hoppe-Seyler's Z Physiol Chem* **358**: 1609–12.
23. Reuter G, Vliegenthart JFG, Wember M, Schauer R, Howard RJ (1980) *Biochem Biophys Res Commun* **94**: 567–72.
24. Waki H, Murata A, Kon K, Maruyama K, Kimura S, Ogura H, Ando S (1993) *J Biochem* **113**: 502–7.
25. Waki H, Masuzawa A, Kon K, Ando S (1993) *J Biochem* **114**: 459–62.
26. Varki A, Diaz S (1984) *Anal Biochem* **137**: 2236–47.
27. Koerner TAW, Prestegard JH, Demou PC, Yu RK (1983) *Biochemistry* **22**: 2676–87.