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Novel di-*O*-acetylated GM3s from equine erythrocytes, one containing 4,9-di-*O*-acetyl-*N*-glycolylneuraminic acid and another containing 4-*O*-acetyl-*N*-glycolylneuraminic acid and 6-*O*-acetyl-D-galactose

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Abstract

A novel GM3 *O*-acetylated at C-4 and at C-9 of *N*-glycolylneuraminic acid (4,9-di-*O*-Ac GM3), together with a second GM3 *O*-acetylated at *O*-4 of the neuraminic acid and *O*-6 of D-galactose (4,6′-di-*O*-Ac GM3) were isolated from equine erythrocytes as a mixture in approximate 1:1 ratio. These two major species were chromatographically inseparable. Their structures, especially the positions of the acetoxy group(s), were determined by means of 1D- and 2D-^{1}H NMR and fast atom bombardment-MS as well as by gas chromatography–MS of partially *O*-methylated *O*-trimethylsilylated monosaccharides derived from the di-*O*-Ac GM3s. In addition, 4-*O*-Ac GM3 was chemically mono-*O*-acetylated with trimethyl orthoacetate under acidic conditions, giving exclusively 4,9-di-*O*-Ac GM3, the NMR and mass spectra of which were used as references to confirm the 4,9-di-*O*-acetylated structure of the naturally-occurring GM3.

Keywords: Equine erythrocytes; GM3, di-*O*-acetylated; NMR spectroscopy

1. Introduction

Of the many species of gangliosides, *O*-acetylated derivatives are most markedly expressed with tissue specificity, with developmental regulation in neuroectodermal tissues, and as oncofetal antigens in some malignancies [1–17]. The major glycolipids in equine erythrocyte membranes have previously been investigated, revealing the existence of GM3 (II^{3}NeuGcα-LacCer) containing *N*-glycolylneuraminic acid (NeuGc) and its 4-*O*-Ac derivative, and lactosylceramide [1,18,19]. Although the *O*-acetylation sites on

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ganglioside molecules were determined to be exclusively the 4-, 7-, 8-, and 9-*O*-positions of the sialic acid moiety in earlier reports, GM3 containing 6-*O*-acetylated Gal was found in equine erythrocytes in our recent study [20]. Analysis of the position of the labile *O*-Ac residue present in intact glycolipids has been effectively performed by ^{1}H NMR and fast-atom bombardment–mass spectrometry (FABMS). The downfield shift of the ^{1}H attached to the carbon bearing an acetoxy group in the NMR spectrum enabled us to assign the position of the *O*-Ac residue in the intact glycolipid in comparison to the spectrum of mother glycolipids such as 4-*O*-Ac GM3 [18], 9-*O*-Ac GD3 [8,10,12], and 7-*O*-Ac GD3 [11,14,15], as well as the position of the sulfate ester of sulfatide [21].

Chemical *O*-acetylation of *N*-acylNeu was investigated earlier at the monosaccharide level, revealing the production of 9-*O*-Ac and 4,9-di-*O*-Ac derivatives under basic conditions using *N*-acetylimidazole [22], and of 9-*O*-Ac and 4-*O*-Ac derivatives under acidic conditions with trimethyl orthoacetate [23]. With respect to the *O*-acetylation of sialoglycoconjugates, ganglioside GD3 was examined following treatment under basic conditions, revealing several products containing *O*-Ac groups in the sugar moieties [12], although the *O*-Ac positions were not determined. In the present paper, we have characterized two novel di-*O*-Ac GM3s, isolated from equine erythrocytes, one having 4,9-di-*O*-Ac NeuGc, the structure of which was compared with that of chemically synthesized 4,9-di-*O*-Ac GM3, and another having 4-*O*-Ac NeuGc and 6-*O*-Ac Gal.

2. Experimental

Chemicals.—GM3 and 4-*O*-Ac GM3 (II^3(4-*O*-Ac NeuGc)α-LacCer) were isolated from equine erythrocytes as reported in a previous paper [18]. Other standard glycolipids were prepared in this laboratory. All other reagents were of analytical grade.

Isolation of glycolipid.—The ganglioside fraction was isolated without alkaline treatment from equine erythrocyte membranes as reported previously [20]. Briefly, acetone powder (450 g) obtained from whole blood (20 L) was extracted three times with 4:8:3 (v:v:v) $CHCl_3$–MeOH–water (CMW) (g powder/5 mL). The combined extract was concentrated and applied to a column (3 × 40 cm) of DEAE-Sephadex, A-25 (acetate form; LKB-Pharmacia) which was previously equilibrated with CMW = 3:6:1. After the column had been thoroughly washed with the equilibration CMW until unbound glycolipids were removed, the acidic glycolipids were eluted with CM–aq 1 M NH_4OAc = 3:6:1 (v:v:v). The eluted fraction was concentrated, dialyzed against water, evaporated to dryness, and applied to a column (2.5 × 80 cm), of latrobeads (Iatron Laboratories) in CMW = 90:10:0.5 (v:v:v). 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 fraction (including new glycolipid abbreviated GL-1) less polar than mono-*O*-Ac GM3 such as 4-*O*-Ac GM3 and 9/6′-*O*-Ac GM3 (a mixture of GM3s containing 9-*O*-Ac NeuGc and 6-*O*-Ac Gal in a ratio of 1:1 [20]) was eluted with CMW = 80:20:2 (v:v:v). The crude fraction was evaporated to dryness and chromatographed further by repeated silica gel column chromatography with the CMW solvent system as before except for the column scale and elution volume, to give 1.2 mg as a homogeneous band on a pre-coated TLC plate (Silica Gel 60, Merck) developed with CMW = 60:35:8 (v:v:v) and visualized on staining with orcinol–sulfuric acid reagent.

O-Acetylation of 4-O-Ac GM3.—Chemical *O*-acetylation was performed under acidic conditions according to the method of Ogura et al. [23], with slight modification. The *O*-acetylation mixture containing 20 mg of well-dried 4-*O*-Ac GM3 in Me_2SO (10 mg glycolipid/1 mL), trimethyl orthoacetate (1 mg glycolipid/1 μL, Wako Pure Chemicals), and a catalytic amount of *p*-toluenesulfonic acid (1 mg/3.3 mg of glycolipid) was incubated for 1 h at 50 °C. After the reaction had been stopped by adding MeOH, the mixture was concentrated and applied to a column (1 × 40 cm) of LH-20 (LKB-Pharmacia) in CMW = 60:35:4.5 (v:v:v) as described already, to remove salt and Me_2SO. The sugar-positive fractions (1 mL per tube) were collected and combined. The *O*-acetylated product was further purified by column (1 × 30 cm) chromatography on latrobeads as already described. The position of the *O*-Ac groups in the products was analyzed by NMR, FABMS, and GC–MS as described later.

Alkaline treatment of glycolipid.—Purified GL-1 and synthesized 4,9-di-*O*-Ac GM3 (~ 0.1 mg each) were separately treated with 1% NaOMe in 1 mL of MeOH for 2 h at room temperature. After neutralization with acetic acid, the mixture was concentrated and applied to an LH-20 column (0.5 × 10 cm) with CMW = 60:30:4.5 (v:v:v) for desalting. The treated glycolipid was analyzed on a TLC plate as has already been described.

GC–MS.—The *O*-Ac positions of GL-1 and synthesized di-*O*-Ac GM3 were determined by GC–MS as reported previously [1,18]. The free hydroxyl groups in di-*O*-Ac GM3 (1 mg) were first ketalized with methyl vinyl ether (1.0 mL) in 1 mL of Me_2SO solution containing *p*-toluenesulfonic acid (1.0 mg) for 6 h at 0 °C. The mixture was concentrated and applied to a column (1 × 30 cm) of LH-20 in $CHCl_3$ for removal of acid and reaction solvents. The sugar-positive fractions excluded from the column were collected, evaporated to dryness, and methylated with 0.2 mL of MeI and $MeSOCH_2^-$ in Me_2SO (1 mL) [24] for 16 h at room temperature. The mixture was applied again to the same LH-20 column, and the sugar-positive fractions were pooled and evaporated to dryness. The ketalized and methylated glycolipid was then methanolyzed with methanolic 0.3 N HCl (1 mL) at 80 °C for 1 h for the sialic acid derivative. After removal of fatty acids from the methanolyzates by extraction with *n*-hexane, the solvent in the residual mixture was evaporated to dryness. The methanolyzed monosaccharide was further derivatized with a mixture of Me_3Si-Cl and *N,O*-bis Me_3Si acetamide (1:9, v:v) at 60 °C for 30 min. The Me_3Si derivatives of the methyl glycosides were analyzed by GC–MS using a JMS-HX100 mass spectrometer (MS and NMR Laboratory of the Faculty of Agriculture, Hokkaido University) equipped with a 25-m capillary column (i.e., 0.25 mm) coated with 2% OV-1 with temperatures programmed from 150 to 280 °C at 5 °C per min.

Analysis of lipid moiety.—The fatty acid and long chain base components were analyzed from the methanolyzates of GL-1 by GLC as their Me esters in the former case and as its Me_3Si derivative in the latter case, using a GC-14A gas chromatograph (Shimadzu) equipped with a 25-m capillary column (i.e., 0.25 mm) coated with 2% DB-5 with temperature programmed from 160 to 280 °C at 5 °C per min as described previously [25].

FAB-mass and NMR spectroscopies.—The negative FAB-mass spectrum of the glycolipids was obtained using a Jeol JMS-HX100 mass spectrometer equipped with a JMA-DA500 Datalizer. The sample in a matrix of $N(CH_2CH_2OH)_3$ was bombarded by Xe gas at 6 kV (20 mA), and the ions were accelerated at 5 kV, as described previously [25–27].

The 500-MHz ^1H 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-Alpha 500 spectrometer at the same laboratory as before, as described previously [26,27]. Chemical shifts are indicated by the distance (ppm) from Me_4Si as an internal standard. 2D-^1H COSY spectra were obtained as already described, and shown by absolute value representation as contour plots.

3. Results

Isolation of glycolipid.—The ganglioside fraction from equine erythrocytes is known to be composed of GM3 containing NeuGc and its 4-*O*-Ac derivative as the major components [1,18,19] and of GM3 containing 9-*O*-Ac NeuGc and 6-*O*-Ac Gal (9/6′-*O*-Ac GM3) as minor ones [20]. Another minor component, a less polar ganglioside, GL-1, was obtained as one band on TLC that migrated faster than 4-*O*-Ac GM3 and 9/6′-*O*-Ac GM3 (Fig. 1). Purified GL-1 was, however, found to be a mixture of two different acetylated species of GM3 from the presence of a heterogeneous Ac group in the FABMS and NMR spectra as well as from the heterogeneity of derivatized sugar compositions in the GC–MS analysis (see below). Some other developing solvents for the TLC of GL-1 such as CMW containing either NH_3, or containing $CaCl_2$ and 1-propanol–water, were examined in an attempt to obtain homogeneous glycolipids, but the attempts were unsuccessful. Similarly, other separation methods, including HPLC using a spherical silica bead column, a reversed-phase column, amine-column (Pharmacia-LKB) and borate

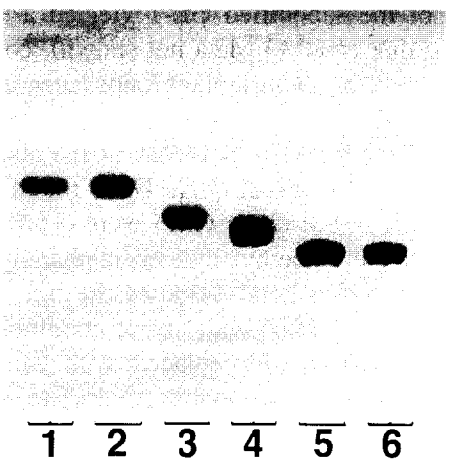

Fig. 1. Thin-layer chromatography of GL-1 and the related reference GM3. Lane 1, GL-1; 2, chemically *O*-acetylated glycolipid generated from 4-*O*-Ac GM3; 3, 4-*O*-Ac GM3; 4, 9/6′-*O*-Ac GM3 (GM3 containing 9-*O*-Ac NeuGc and 6-*O*-Ac Gal in a ratio of 1:1 [20]); 5, saponified GL-1; 6, authentic GM3 containing NeuGc. The glycolipids were developed with CMW = 60:35:8 and stained with orcinol–sulfuric acid reagent.

column (Pharmacia-LKB), and the droplet counter current technique were unsuccessful. The chemical structures of the different components in GL-1 were, therefore, determined for the mixture as described below.

O-Acetylation of 4-O-Ac GM3.—The technique for chemical mono-*O*-acetylation for free neuraminic acid reported by Ogura et al. [23] was applied to 4-*O*-Ac GM3 with the modification of raising the reaction temperature to 50 °C from room temperature in the original report, to yield a di-*O*-acetylated GM3 in high yield, without generation of byproducts. Using this method, 6.0 mg of 4,9-di-*O*-Ac GM3 was obtained from 20 mg of 4-*O*-Ac GM3. The purified, chemically *O*-acetylated 4-*O*-Ac GM3 had an R_f value identical to that of GL-1 on TLC, as shown in Fig. 1. The position of the newly inserted single *O*-Ac group was determined by NMR, FABMS, and GC–MS (see below), revealing *O*-acetylation occurring exclusively at C-9-*O* of 4-*O*-Ac NeuGc, indicating that the reaction proceeded selectively at one position. Elevation of the temperature and/or extension of the reaction time produced several di-*O*-acetylation byproducts (data not shown).

Alkaline treatment of glycolipid.—GL-1 and the chemically *O*-acetylated 4-*O*-Ac GM3 were saponified by mild alkali to remove the alkali-labile group(s), followed by TLC analysis. As shown in Fig. 1, saponified GL-1 or the chemically *O*-acetylated 4-*O*-Ac GM3 showed an R_f value identical to that of authentic GM3. In addition, 1D-NMR analysis of the saponified GL-1 yielded a spectrum similar to that of GM3 (data not shown), confirming the attachment of alkali-labile groups, such as *O*-acyl esters, to GM3.

GC–MS study.—The positions of the alkali-labile group(s) bound to the di-*O*-Ac GM3 found in GL-1 and synthetic 4,9-di-*O*-Ac GM3 were determined by GC–MS of the trimethylsilylated methanolyzates of the glycolipid after ketalization of the free hydroxyl groups and methylation under basic conditions, when alkali-labile groups are replaced with Me groups. GL-1 yielded five peaks, corresponding to monosaccharide derivatives, the retention times and the approximate molar ratio of which are summarized in Table 1 together with those of reference substances. Of the five peaks, two components correspond to the per-Me_3Si derivatives of Me glucoside and Me galactoside, in the approximate molar ratio of 2:1, determined by comparison with the retention times of peaks in the gas chromatogram of the methanolyzates of lactosylceramide (data not shown). The assignments for the fragment ions in the mass spectra of the other three components are summarized in Table 2. From the assignments, these monosaccharides were identified as Me 4-*O*-Me 7, 8, 9-tri-*O*-Me_3Si (*N*-Me *O*-Me_3Si Gc) neuraminoside Me ester, the mass spectrum of which has been published in a previous paper [18], Me 6-*O*-Me 2,3,4-tri-*O*-Me_3Si galactoside (Fig. 2A for the spectrum) and Me 4,9-di-*O*-Me 7,8-di-*O*-Me_3Si (*N*-Me *O*-Me_3Si Gc) neuraminoside Me ester (Fig. 2B) present in an approximate molar ratio of 1:1:1, respectively. The detection of these derivatives reveals the heterogeneity of GL-1 as well as the presence in GL-1 of NeuGc substituted at C-4-*O* or at C-4,9-di-*O* and of Gal substituted at C-6-*O* with alkali-labile groups. The molar ratio of monosaccharides found in GL-1 was concluded to be approximately 1:1:1:1:2 for 4-*O*-Me NeuGc, 4,9-di-*O*-MeNeuGc, 6-*O*-Me Gal, unsubstituted Gal, and unsubsitituted Glc, respectively, from the GC–MS analysis. On the other hand, the chemically *O*-acetylated glycolipid from 4-*O*-Ac GM3 yielded three major monosaccharide derivatives, 4,9-di-*O*-Me NeuGc, Me galactoside, and Me glucoside in the approximate ratio of 1:1:1, from the methanolyzates in the GC–MS analysis (see Table 1). The 6-*O*-Me Gal was negligible (less than 5% of the 4,9-di-*O*-Me NeuGc), indicating that the chemical *O*-acetylation of 4-*O*-Ac GM3 does not occur at the *O*-6 of the Gal moiety.

Analysis of lipid moiety.—The main fatty acids were identified as 24:0 and 24:1 (total 57.7%), and

Table 1
Relative retention times and molar ratios of partially methylated trimethylsilyl derivative of methyl glycoside from GL-1 and synthetic 4,9-di-*O*-Ac GM3 in gas–liquid chromatography [a]

Methyl glycoside (Me_3Si derivative)	Relative retention time [b]	Approximate molar ratio	
		GL-1	Synthesized
Hexosides			
Glc	1.00 [c]	2	1
Gal	0.92	1	1
6-*O*-Me Gal	0.83	1	
N-Me Neuraminoside Me ester			
NeuGc	1.00		
4-*O*-Me NeuGc	0.99	1	
4,9-di-*O*-Me NeuGc	0.96	1	1

[a] Chromatographed on capillary column (25 m) coated with 2% DB-5 from 160 to 280 °C in a rate of 5 °C per min.
[b] The time of methyl glucoside in hexoside and *N*-Me methyl neuraminoside in neuraminoside was normalized to 1.00.

Table 2
Assignment of MS ions of partially *O*-methylated monosaccharide derivatives from GL-1

Assignment			NeuGc [a]	
	6-*O*-Me Gal [b]		4-*O*-Me [c]	4,9-di-*O*-Me
	m/z	Assignment	*m/z*	*m/z*
M^+	(424) [d]	M^+	(669)	611
$M^+ - Me$	409	$M^+ - Me$	654	596
$M^+ - MeO$	393	$M^+ - MeO$	(638)	580
$M^+ - MeOH - Me$	377	$M^+ - (C^9H_2OR$ [e])	566	566
$393 - MeOH$	361	$M^+ - Me - MeOH$	622	564
$M^+ -$ TMSO [f]	335	$M^+ - C^1O_2Me$	610	552
409-TMSOH	319	$M^+ - MeO - MeOH$	(606)	548
409-(OHC^1OMe) − MeOH	317	566-MeOH	534	534
$[TMSOC^2H(TMSO)C^3C^4HOTMS]^+$	305	$M^+ - MeO -$ TMSOH	(548)	490
393-TMSOH	303	$M^+ - (TMSOCH_2CO(Me)NH) - MeOH$	476	418
335-(C^6H_2OMe)	290	$M^+ - (TMSOC^8HC^9H_2OR)$	464	464
$M^+ - (OHC^1OMe) -$ TMSO	275	534-TMSOH	444	444
$M^+ - (OHC^5C^6H_2OMe) -$ TMSOH	260	464-MeOH	432	432
319-$Me_2Si{=}CH_2$	247	$M^+ - (TMSOCH_2CO(Me)NH) -$ TMSOH	418	360
305-$Me_2Si{=}CH_2$	233	$566 -$ TMSOH $- Me - C^1O_2Me$	402	402
$[TMSOC^2HC^3HC^4HOTMS]^+$	217	$M^+ - C^1O_2Me - Me -$ MeOH		
$[TMSOCHCHOTMS]^+$	204	$-(TMSOCH_2CO(Me)NH)$	402	344
$[TMSOSiMe_2]^+$	147	464-2xMeOH	400	400
$[TMSOCHOMe]^+$	133	464-TMSOH	374	374
		$M^+ - (TMSOC^7H(TMSO)C^8HC^9H_2OR)$	362	362
		566-($TMSOCH_2CO(Me)NH$) − TMSOH and/or 362-Me − MeOH and/or 464-C^1O_2Me − TMSOH	315	315
		$362 - Me - C^1O_2Me$	288	288
		362-TMSOH	272	272
		432-($TMSOCH_2CO(Me)NH$)	271	271
		$TMSOCH_2CO(Me)NC^5HC^4HOMe$	217	217
		362-($TMSOCH_2CO(Me)NH$)	201	201

[a] *N*-Me per Me_3Si derivative. [b] Per-Me_3Si derivative. [c] [18]. [d] Value in parentheses was hardly detected. [e] R = Me_3Si in 4-*O*-Me NeuGc and R = Me in 4,9-di-*O*-Me NeuGc. [f] TMSO denotes Me_3SiO.

the major long-chain base was identified as C_{18}-sphingenine, from their GLC analysis (Table 3). These lipid components are similar to those identified in 9/6′-*O*-Ac GM3 [20]. The major lipid components are reflected in the FAB-mass spectrum of GL-1, as described next.

FABMS study.—GL-1 and chemically acetylated 4-*O*-Ac GM3 were analyzed using negative ion FABMS to determine the location of the alkali-labile groups in the former and *O*-Ac residue in the latter. As shown in Fig. 3A, GL-1 yields a major quasi-molecular ion at *m/z* 1363, $[M - H]^-$, corresponding to a component having a fully saturated C_{24}-fatty acyl chain and a $C_{18:1}$-long chain base (sphingenine) as the major lipid components, consistent with the data from the GLC analysis. Further minor molecular ions were observed at *m/z* 1391, 1361, 1335, and 1305 consistent with species with different fatty acid components of $C_{26:0}$, $C_{24:1}$, $C_{22:0}$, and $C_{20:1}$, respectively. These molecular ions informed us of the presence of two additional Ac residues ($2 \times m/z$ 42) on the GM3 molecule (Mr, 1280). The 14 amu difference in the molecular-ion area, *m/z* 1377, 1347, and 1321, might be a result of mass overlapping of di-Ac GM3 molecular species and loss of ketene (42 amu) versus 28 amu (C_2H_4) due to the amide-linked fatty acids in their lipids moiety. Moreover, fragment ions with *m/z* 972 and 970 due to [hexose − hexose − Cer($C_{24:0}$) − H]$^-$ and [hexose − hexose − Cer($C_{24:1}$) − H]$^-$, respectively, which differ from $[M - H]^-$ by 391 amu (di-*O*-Ac NeuGc), were observed, indicating that the two Ac groups are present on the non-reducing NeuGc moiety. A less intense fragment ion is also observed at *m/z* 1014 corresponding to [Ac − hexose − hexose − Cer($C_{24:0}$) − H]$^-$, whereas *m/z* 852 for [Ac − hexose − Cer − H]$^-$ is not, suggesting that GL-1 contains a species bearing *mono-O*-acetylated Gal as a minor glycolipid component,

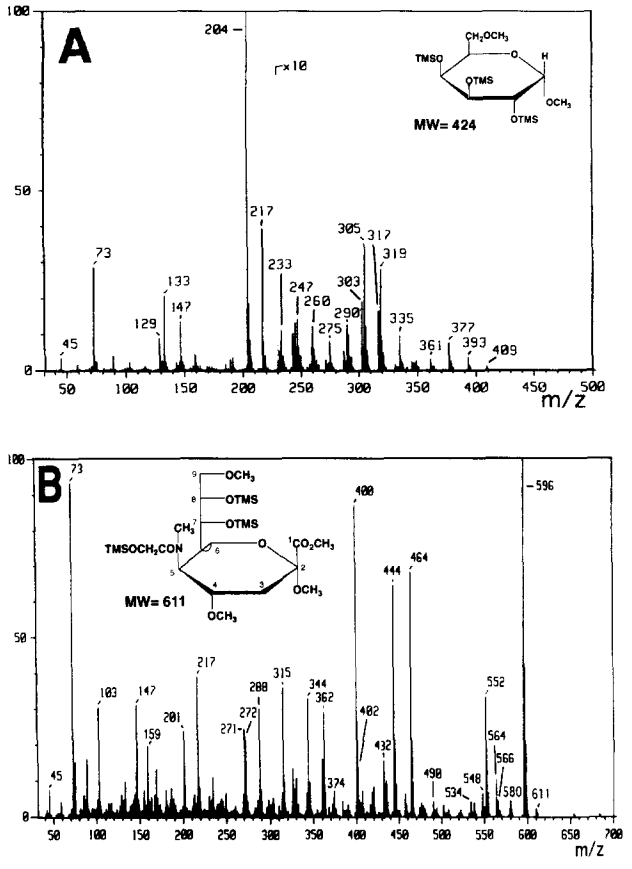

Fig. 2. Mass spectra of partially *O*-methylated monosaccharides derived from GL-1. (A) Methyl 6-*O*-methyl 2,3,4-tri-*O*-Me_3Si galactoside from GL-1; (B) methyl 4,9-di-*O*-methyl 7,8-di-*O*-Me3Si (*N*-methyl *O*-Me_3Si Gc) neuraminoside methyl ester from GL-1 or synthetic 4,9-di-*O*-Ac GM3.

since the data from saponification already described, indicate that GL-1 has a GM3 skeleton. The presence of *O*-Ac Gal is also observed in the GC–MS analysis of the methanolyzates of ketalized and methylated GL-1 (above). Additional fragment ions at *m/z* 810, 808, 648, and 646 for [hexose − Cer($C_{24:0}$) − H]$^-$, [hexose − Cer($C_{24:1}$) − H]$^-$, [Cer($C_{24:0}$) − H]$^-$, and [Cer($C_{24:1}$) − H]$^-$, respectively, are also observed. These ions demonstrate the absence of Ac moieties on the Glc and the Cer in GL-1. Furthermore, ions at *m/z* 366 for [AcNeuGc − H]$^-$, 408 [Ac_2NeuGc − H]$^-$ and 570 [Ac_2NeuGc − hexose − H]$^-$ and/or [AcNeuGc − Ac − hexose − H]$^-$ also appear in the spectrum, although at low intensity. From these FABMS data, GL-1 is assigned as being composed of two different di-*O*-acetylated GM3s, one containing di-*O*-Ac NeuGc and the other containing mono-*O*-Ac NeuGc and mono-*O*-Ac Gal. Furthermore, combining these data with those from the GC–MS analysis, GL-1 appears to be a mixture of 4,9-di-*O*-Ac GM3 and 4,6′-di-*O*-Ac GM3 (undashed and dashed numbers are employed for NeuGc and galactose, respectively) in equimolar ratio.

The negative-ion FAB-mass spectrum of chemically *O*-acetylated 4-*O*-Ac GM3 (Fig. 3B) contains [M − H]$^-$ quasimolecular ions at *m/z* 1363 and 1361 and fragment ions at *m/z* 972, 970, 810, 808, 648, 646, as in the spectrum of GL-1. The ions at 366 and 1014 are, however, of negligible intensity in comparison to those in the spectrum of GL-1 (Fig. 3A), whereas the relative intensity of the ion at *m/z* 408 is stronger than that in the spectrum of GL-1. The variation of these intensities suggests that two Ac residues are localized exclusively on the NeuGc moiety in chemically *O*-acetylated 4-*O*-Ac GM3. The data from the GC–MS analyses identify this residue as 4,9-di-*O*-Ac GM3.

NMR study.—The chemical shifts of sugar ring ^{1}Hs and Ac groups in GL-1 and chemically acetylated 4-*O*-Ac GM3 are summarized in Table 4, together with those of reference 4-*O*-Ac GM3 and unacetylated GM3. The 1D-NMR spectrum of the

Table 3
Compositions of fatty acid and long chain base of GL-1

Long chain base [a] (%)		
d18:1 [b]	d18:0 [c]	Unknown
98.7	tr [d]	1.2

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	
6.0	4.3	4.7	3.1	tr	tr	8.0	tr	44.3	13.4	8.6	2.1	5.5

[a] Determined as trimethylsilyl derivative. [b] Sphingenine. [c] Sphinganine. [d] Trace (1% >). [e] Determined as methyl ester. [f] Carbon number. [g] Saturated. [h] Mono-unsaturated.

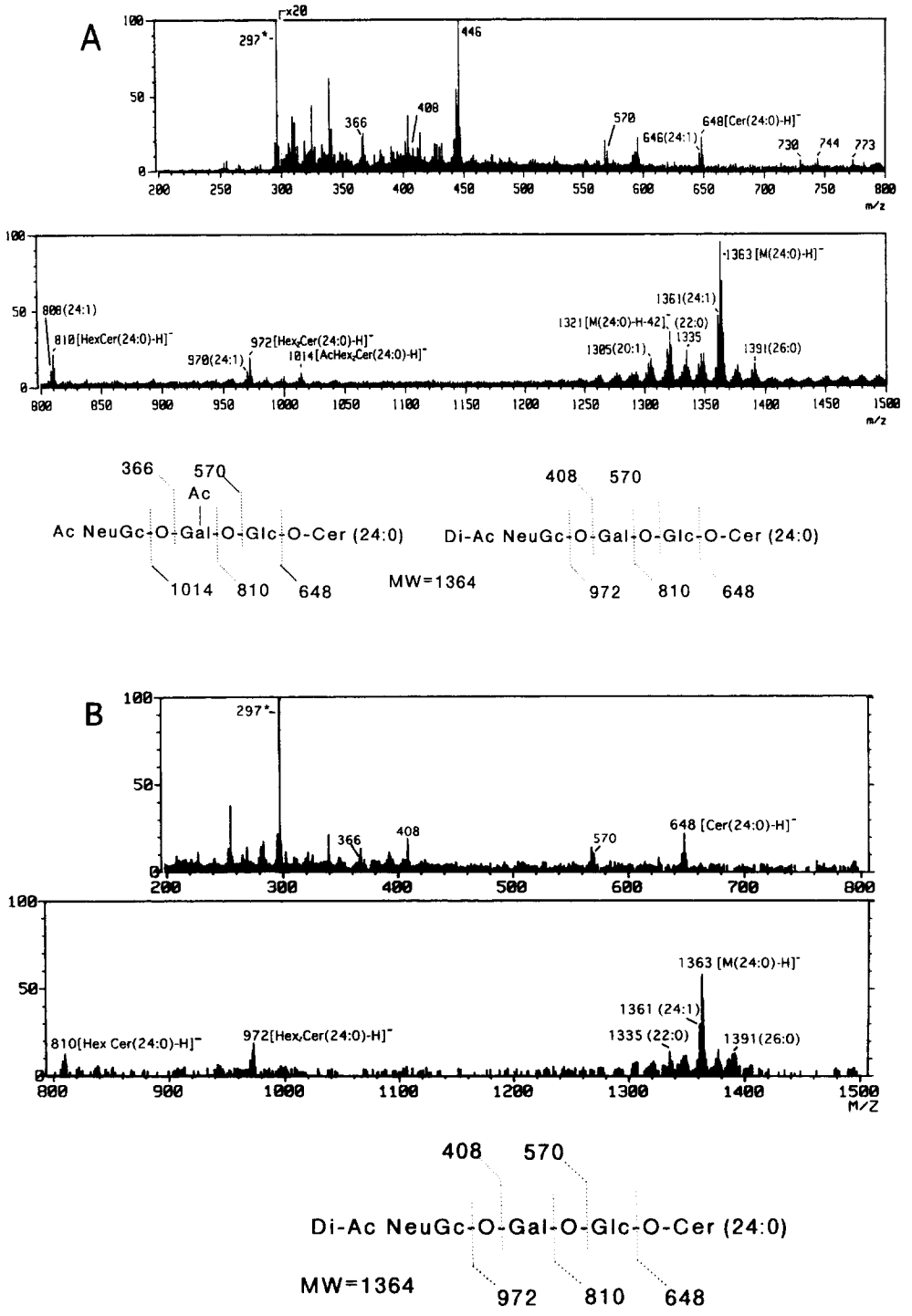

Fig. 3. Negative FAB-mass spectra of GL-1 and synthesized 4,9-di-*O*-Ac GM3. (A) GL-1 (Mr. 1364 with $C_{24:0}$); (B) 4,9-di-*O*-Ac GM3 synthesized from 4-*O*-Ac GM3. In the spectra, the numbers in parentheses indicate fatty acid components and degrees of unsaturation. Asterisked signal indicates ion arising from matrix.

chemically acetylated 4-*O*-Ac GM3 (Fig. 4A) reveals an *O*-Ac methyl signal at δ 1.987 in addition to that at 1.953, observed in the spectrum of 4-*O*-Ac GM3 [18]. Of the two Ac Me signals, the peak at 1.987 could be assigned to a newly inserted Ac residue and the peak at 1.953 to 4-*O*-Ac methyl, since a chemical shift almost identical to the latter is observed in the spectrum of 4-*O*-Ac GM3 (Table 3). The ^{1}Hs at C-3 (two H-3, S^{3ax} and S^{3eq} in the figure), H-4 (S^4), H-5 (S^5) and amide ^{1}H (S^{5N}) on NeuGc were assigned from the cross peaks, $S^{3ax-3eq}$, S^{3ax-4}, S^{3eq-4}, S^{4-5} and S^{5-5N}, respectively in the 2D-COSY spectrum

Table 4
Chemical shifts ($^2H_6$-Me_2SO–^2H_2O = 98:2, 90 °C, Me_4Si internal) for ring protons on sugar residues in NMR spectra of GL-1, synthetic 4,9-di-*O*-Ac GM3 and the reference GM3s

	H-1	H-2 (ax)	H-3 (eq)	H-3	H-4	H-5	H-6a	H-6b	H-7	H-8	H-9a	H-9b	Gc-CH_2	4OAc [a]	9OAc [b]	6′OAc [c]	NAc	S-5N [d]
GL-1 (4,9-di-*O*-Ac GM3 (Gc))																		
I	4.177	3.06	3.37	–	3.36	3.38	3.52	3.54	–	–	–	–	–	–	–	–	–	–
II	4.239	3.357	3.93	–	3.767	3.61	3.29	3.32	–	–	–	–	–	–	–	–	–	–
S [e]	–	–	1.557	2.844	*5.077*	3.81	3.77	–	3.26	3.83	*3.95*	*4.22*	3.808	1.951	1.989	–	–	7.40
GL-1 (4,6′-di-*O*-Ac GM3 (Gc))																		
I′	4.177	3.06	3.37	–	3.36	3.38	3.52	3.54	–	–	–	–	–	–	–	–	–	–
II′	4.300	3.357	4.00	–	3.767	3.63	*4.06*	*4.16*	–	–	–	–	–	–	–	–	–	–
S′	–	–	1.557	2.844	5.077	3.81	3.77	–	3.28	3.39	3.35	3.59	3.808	1.951	–	2.008	–	7.40
Synthesized 4,9-di-*O*-Ac GM3 (Gc)																		
I	4.177	3.06	3.359	–	3.344	3.391	3.516	3.526	–	–	–	–	–	–	–	–	–	–
II	4.244	3.359	3.97	–	3.777	3.618	3.290	3.316	–	–	–	–	–	–	–	–	–	–
S	–	–	1.567	2.844	*5.076*	3.81	3.770	–	3.275	3.83	*3.95*	*4.224*	3.810	1.953	1.987	–	–	7.45
4-*O*-Ac GM3 (Gc)																		
I	4.177	3.06	3.361	–	3.346	3.34	3.52	3.53	–	–	–	–	–	–	–	–	–	–
II	4.247	3.361	3.984	–	3.79	3.622	3.295	3.322	–	–	–	–	–	–	–	–	–	–
S	–	–	1.555	2.836	*5.078*	3.81	3.772	–	3.295	3.383	3.36	3.61	3.810	1.950	–	–	–	7.44
GM3 (Gc)																		
I	4.175	3.06	3.36	–	3.35	3.36	3.51	3.52	–	–	–	–	–	–	–	–	–	–
II	4.236	3.359	3.998	–	3.788	3.64	3.28	3.30	–	–	–	–	–	–	–	–	–	–
S	–	–	1.450	2.749	3.693	3.50	3.76	–	3.263	3.39	3.36	3.625	3.870	–	–	–	–	7.41

[a] Acetyl methyl ^{1}Hs on 4-*O*-Ac NeuGc. [b] Ac on 9-*O*-Ac NeuGc. [c] Ac on 6′-*O*-Ac Gal. [d] amide proton on sialic acid. [e] Sialic acid.

(Fig. 4B). The downfield shift of H-4 (δ 5.076) compared to that (δ 3.693) in the spectrum of GM3 indicates the presence of one Ac residue at C-4-*O* on NeuGc, as reported previously [18,21]. H-6, -7 and -8 of NeuGc were sequentially identified from H-5, assigned as above, with their cross peaks, S^{5-6}, S^{6-7}, and S^{7-8} in the partial 2D-spectrum (Fig. 4C). Based on the downfield shift of H-9a and -9b, the chemical shifts of which were determined from the cross peaks S^{9a-9b}, S^{8-9a}, and S^{8-9b}, and after reference to the spectrum of 9/6′-*O*-Ac GM3 [20], another Ac group was identified at C-9-*O* on the NeuGc. The ring ^{1}Hs, H-1 to H-6 on glucose (I) and galactose (II) as well as ^{1}Hs of the ceramide (data not shown) were also assigned from the respective cross peaks, as demonstrated in Fig. 4C. Combining the data from the NMR analysis with those from the FABMS and GC–MS analyses, the species produced on chemical acetylation of 4-*O*-Ac GM3 was concluded to be 4,9-di-*O*-Ac GM3.

Additionally, the 1D-spectrum of GL-1 revealed a new *O*-Ac moiety at δ 2.008, an anomeric ^{1}H at 4.300 ($II^{1'}$, this dashed proton number was used for 4,6′-di-*O*-Ac GM3 composed of GL-1 only in the NMR analysis for convenience, and differs from that of the 4,'6''-di-*O*-Ac GM3), probably H-1 of a β-galactoside, and two quartet signals at 4.06 and 4.16 as shown in Fig. 5A, as compared to the spectrum of synthetic 4,9-di-*O*-Ac GM3. The two Ac residues giving resonances at 2.008 and 1.989 and two anomeric ^{1}Hs at 4.239 and 4.300 were observed with approximately equal intensities, suggesting that GL-1 was composed of two different di-*O*-Ac GM3s. One of them was identified as 4,9-di-*O*-Ac GM3, since the downfield shifts of H-4 (S^4) at 5.077 and H-9a (S^{9a}) at 3.95 and H-9b (S^{9b}) at 4.22 on NeuGc, which

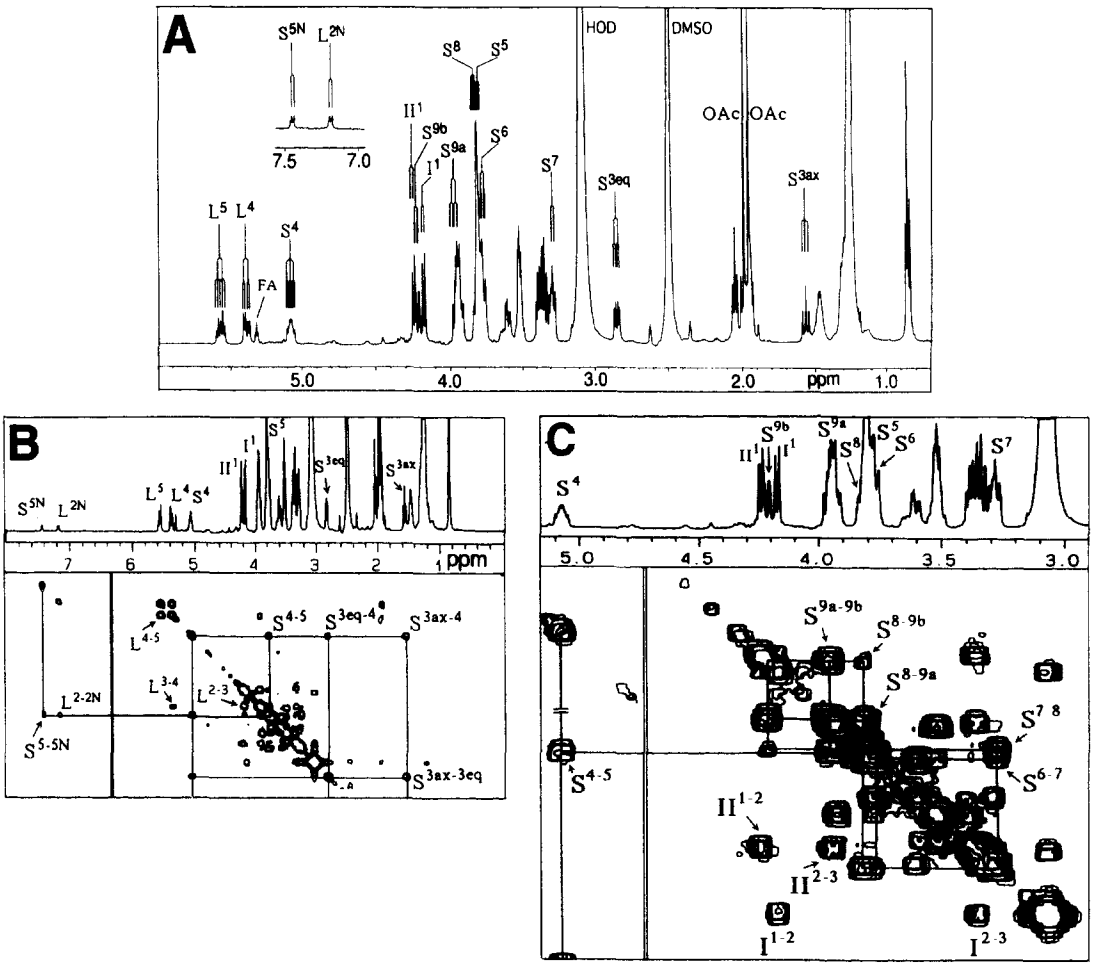

Fig. 4. 1D- and 2D-NMR spectra of synthetic 4,9-di-*O*-Ac GM3. (A) 1D spectrum of the 4,9-di-*O*-Ac GM3; (B) whole 2D-COSY spectrum; (C) partial enlarged spectrum of panel B. L, S, I, II and FA, ^{1}Hs on a long-chain base (sphingenine), sialic acid, β-glucoside, β-galactoside and fatty acid, respectively.

were assigned from the 2D-spectra cross peaks S^{3eq-4} and S^{3ax-4} (Fig. 5B), and S^{8-9a}, S^{8-9b} and S^{9a-9b} (Fig. 5C), indicates the positions of the Ac groups at O-4 and, O-9, similar to those in synthetic 4,9-di-*O*-Ac GM3 already described. The ^{1}Hs at 4.06 and 4.16 were assigned to H-6a ($II^{6a'}$) and H-6b ($II^{6b'}$) on galactoside with the cross peak, $II^{6a'-6b'}$ and the connectivities with H-5 ($II^{5'}$) assigned from $II^{5'-6a'}$ and $II^{5'-6a'}$ in the partial 2D-spectrum (Fig. 5C), as well as by reference to the chemical shifts of 9/6′-*O*-Ac GM3 in an earlier report [20]. The two H-6s of another galactoside resonating at δ3.29 (II^{6a}) and 3.32 (II^{6b}) were assigned with II^{5-6a}, II^{5-6b}, and II^{6a-6b}, being chemical shifts identical to those of galactoside of 4,9-di-*O*-Ac GM3. In comparison between $II^{6'}$ and II^{6}, the downfield shift of $II^{6'}$ also indicates the location of an Ac group at *O*-6 of one galactoside. The slight downfield shift of the anomeric ^{1}H ($II^{1'}$) on the galactoside could be ascribed to a steric effect of the newly inserted 6-*O*-Ac group.

Combining the data from GC–MS, FABMS, and NMR analyses, from migration as a band identical to GM3 on TLC after saponification, and further from comparison of the structure of chemically synthesized 4,9-di-*O*-Ac GM3, GL-1 was concluded to be a mixture of GM3 containing 4,9-di-*O*-Ac NeuGc (4,9-di-*O*-Ac GM3) and 4-*O*-Ac NeuGc and 6-*O*-Ac Gal (4,6′-di-*O*-Ac GM3) in a ratio of approximately 1:1.

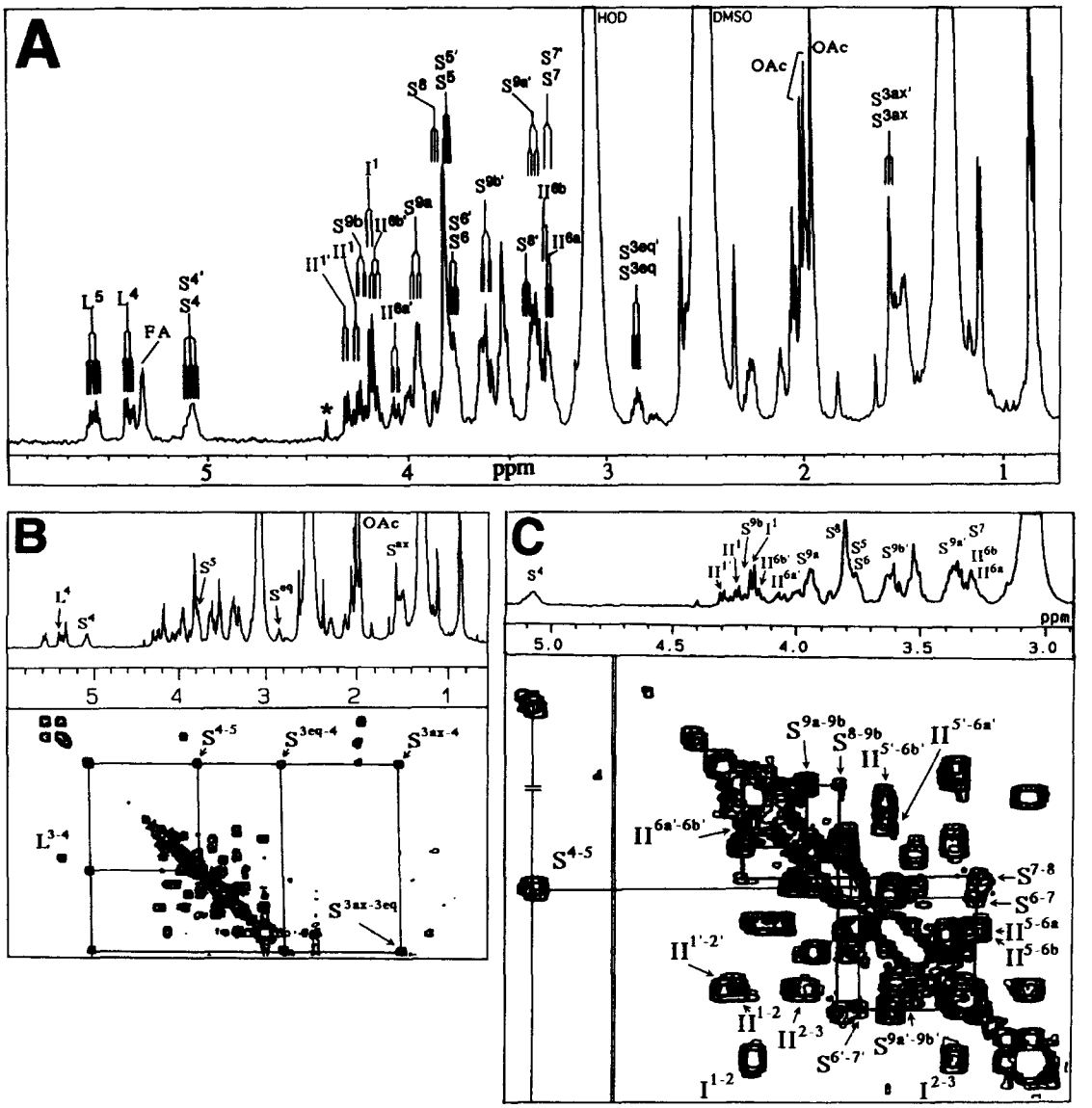

Fig. 5. 1D- and 2D-NMR spectra of GL-1. (A) 1D spectrum of GL-1; (B and C) whole 2D-COSY and the partial 2D-COSY spectra of GL-1, respectively. L, S, I, II, FA and cross peak are as in the legend to Fig. 4. Asterisked peak indicates contaminant.

4. Discussion

A wide variety of *O*-acetylated sialic acid monosaccharides has been isolated from equine erythrocyte membranes by mild acidic hydrolysis, and the structures have been fully characterized as volatile derivatives by GC–MS [28]. Hence, the presence of equine gangliosides containing *O*-acetylated sialic acid such as 9-*O*-Ac GM3 [20] and 4,9-di-*O*-Ac GM3 in the present report was expected; however, the latter is the first report which demonstrates that the sialic acids are bound to the ganglioside. The positions of the Ac residues in gangliosides have been effectively identified from ^{1}H NMR spectra as just described. For a similar purpose, chemical derivatization employing ketalization, methylation under basic conditions, methanolysis and trimethylsilylation has also been employed, followed by GC–MS analysis as reported previously [18]. On degradation, GL-1 yielded three species of partially methylated monosaccharides, 4,9-di-*O*-methyl NeuGc, 4-*O*-methyl NeuGc, and 6-*O*-methyl galactose, the detection of which indicates the presence of *O*-acetylated sugar components in intact GL-1. The possibility that GL-1 is composed of 4,9-di-*O*-Ac NeuGc-(6-*O*-Ac)Gal-Glc-Cer (tri-*O*-Ac GM3) and 4-*O*-Ac NeuGc-Gal-Glc-Cer (4-*O*-Ac GM3) cannot be excluded on the basis of the chemical degradation method or of 2D-NMR analysis. Even homonuclear Overhauser effect spectroscopy using the 2D-NMR technique, which detects the proximity between monosaccharides arising from steric effects between anomeric protons and ring ^{1}Hs, would not be appropriate for characterizing a GM3 mixture such as that described here, since sialic acid has no anomeric protons. However, the possibility of the existence of species bearing more than the Ac groups can be excluded, since (i) the FAB-mass spectra demonstrate the presence of di-*O*-Ac GM3 in GL-1, and (ii) the mobility of the GL-1 on TLC was not identical to that of 4-*O*-Ac GM3, and further that the tri-*O*-Ac GM3 would migrate faster than di-*O*-Ac GM3s, such as synthetic 4,9-di-*O*-Ac GM3 (see Fig. 1), because of the much lower polarity of the tri-*O*-Ac derivative compared to di-*O*-Ac GM3.

Interestingly, a novel di-*O*-acetylated GM3 containing 6-*O*-Ac Gal, 4,6′-di-*O*-Ac GM3, was isolated from the erythrocytes in admixture with 4,9-di-*O*-Ac GM3 in the present experiment. Moreover, a mono-*O*-acetylated GM3 having the 6-*O*-Ac Gal, 6′-*O*-Ac GM3, has previously been obtained from equine erythrocytes [20]. Though several *O*-acylated galactosyl- and glucosyl-Cers in which the acyl group is found on the sugar moieties have been isolated from mammalian brains [29–34], the reports of an *O*-acetylated galactose on a ganglioside could indicate a similarity with equine erythrocytes. Although the enzymatic *O*-acetylation of GM3 in equine tissue or fluids was not investigated in detail, and intramolecular migration of the *O*-Ac group can occur in free sialic acid molecules [35], a small amount of lactosylceramide having the 6-*O*-Ac Gal, which could be a biosynthetic precursor of 4,6′-di-*O*-Ac GM3, has been detected in a neutral glycolipid fraction from equine erythrocytes (Yachida, unpublished results). This phenomenon suggests that biosynthesis of Ac GM3 from *O*-Ac lactosylceramide could take place.

With respect to interactions with protein, GL-1 was not desialylated by several neuraminidases employed, confirming the substrate specificity of these enzymes, which have been shown to be inactive to 4-*O*-substituted sialic acid on sialoglycoconjugates [36]. Similarly, GL-1 was not recognized by a monoclonal antibody raised against 9-*O*-Ac GD3, and recognizing 9-*O*-Ac NeuAc.

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