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Preparation and characterization of four new variously deacetylated lysogangliosides, breakdown products of GM_1

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ABSTRACT

Four new deacylated lysogangliosides were obtained through alkaline hydrolysis of either C_{18} or C_{20} sphingosine homologues of GM_1. By this procedure, both the fatty acids residue and the *N*-acetyl group of sialic acid were removed to give mono-*N*-acetyl-lysoGM$_1$ (C_{18} and C_{20}); the additional loss of the *N*-acetyl group of the acetylgalactosamine moiety gave de-*N*-acetyl-lysoGM$_1$ (C_{18} and C_{20}) with three free amino groups. The structures of four deacetylated lysogangliosides were unambiguously characterized by chemical analysis and ^1H and ^{13}C NMR spectroscopy as well as by negative ion FABMS. The aim of this study was to isolate pure breakdown products of gangliosides, enabling the evaluation of the mechanism of action of glycosphingolipids through their cleavage and identification of structures of potential pharmacological activity. These new substances were prepared as candidates to influence eicosanoid production and the mechanisms dependent on protein kinase C and phospholipase A_2.

INTRODUCTION

Gangliosides are amphipathic glycosphingolipids consisting of sugar and ceramide moieties. They are widely distributed in the body, particularly in the central nervous system, liver, spleen, and blood cells. They act as membrane antigen in some tumours[2], and as receptors for viruses and bacteria and their toxins[3]. Recently, they became known as mediators of cell-to-cell relations, and cell-to-microrganism and cell-adhesion protein interaction[4]. They are known to inhibit PLA_2, (ref 5) and are degraded in the body, by ceramidases and deacylases, into the large family of lysogangliosides characterized by the loss of a fatty acid chain[6].

Lysogangliosides are present in normal[7] and in pathological conditions[8–10] in various cells and tissues. Unlike their parent gangliosides, they inhibit growth factor action and the response of immune cells to phorbol esters, and modulate receptor function. They have been shown to block protein kinase C[11]. Although

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their physiological role has not been completely clarified, they are considered to be modulators of cell growth[6] and of the signal transduction system[12].

The present study was aimed at mimicking, through alkaline hydrolysis, the cellular enzymes responsible for ganglioside–lysoganglioside conversion, and to isolate by-products in a state of purity.

RESULTS AND DISCUSSION

Starting from the raw GM_1* mixture, this paper reports the preparation of four lysoGM$_1$ derivatives varying in the length of their sphingosine chain and in the number of *N*-acetyl groups in the glycan chain. The purified GM_1 mixture, a compound of two constituents with different sphingoid moieties (C_{18} or C_{20} sphingosine), was separated by HPLC to give GM_1 C_{18} and C_{20} homologs, in a high degree of purity (99%). Successive production of mono-*N*-acetyl-lysoGM$_1$ (**3** and **4**) and the corresponding de-*N*-acetyl-lysoGM$_1$ (**5** and **6**) was accomplished by a modified version of the procedure described by Tayot et al.[13]. Owing to the acid lability of glycosidic linkages, the hydrolysis reaction of GM_1 C_{18} or C_{20} components was performed under alkaline conditions in butan-1-ol, this solvent being more effective than water or methanol. Generally, a reaction time of 3 h at 100°C was required to split only two amide bonds of GM_1, whereas removal of the remaining *N*-acetyl group of *N*-acetylgalactosamine might sometimes require longer reaction times. Purification of **3** and **5** or **4** and **6** from the hydrolysis mixture was accomplished by column chromatography on silica gel to give products of high purity.

The structures assigned to **3–6** were confirmed by combined ^1H–^{13}C NMR spectroscopy further supported by negative ion FABMS.

^1H NMR spectroscopy. — All assignments of proton resonances of lysogangliosideGM_1 derivatives were made by comparison with the experimental gangliosideGM_1 parent and with ^1H NMR data previously reported by Koerner et al.[14] and Neuenhofer et al.[10].

The comparison of ^1H NMR spectra of the homologous mono-*N*-acetyl-lysoGM$_1$ pair (C_{18} and C_{20} sphingosine; **3** and **4**) with those of GM_1 (**1** or **2**) revealed the following differences as key features (Fig. 2, Table I). (*1*) The presence of a unique methyl resonance (III-8) of the GalNAc residue at 1.82 ppm in **4**, instead of the two present in GM_1 (**2**) at 1.80 (III-8) and 1.90 ppm (A-11); (*2*) the appearance of

* Abbreviations: GM_1, II^{3}NeuAcGgOse$_4$Cer * = *O*-β-D-Gal(1 → 3)-*O*-β-D-GalNAc-(1 → 4)-[*O*-α-Neu5Ac-(2 → 3)]-*O*-β-D-Gal-(1 → 4)-*O*-β-D-Glc-(1 → 1)-Cer; mono-*N*-acetyl-lysoGM$_1$, II^{3}Neu-GgOse$_4$Sph* = *O*-β-D-Gal-(1 → 3)-*O*-β-D-GalNAc-(1 → 4)-[*O*-α-Neu-(2 → 3)]-*O*-β-D-Gal(1 → 4)-*O*-β-D-Glc-(1 → 1)-Sph; d-*N*-acetyl-lysoGM$_1$, 11^3-Neu11^{4}GalNGgOse$_3$Sph * = *O*-β-D-Gal-(1 → 3)-*O*-β-D-GalN-(1 → 4)-[*O*-α-Neu-(2 → 3)]-*O*-β-D-Gal-(1 → 4)-*O*-β-D-Glc-(1 → 1)-Sph. Those marked with an asterisk follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1977)[1]; Cer, ceramide; Sph, sphingosine.

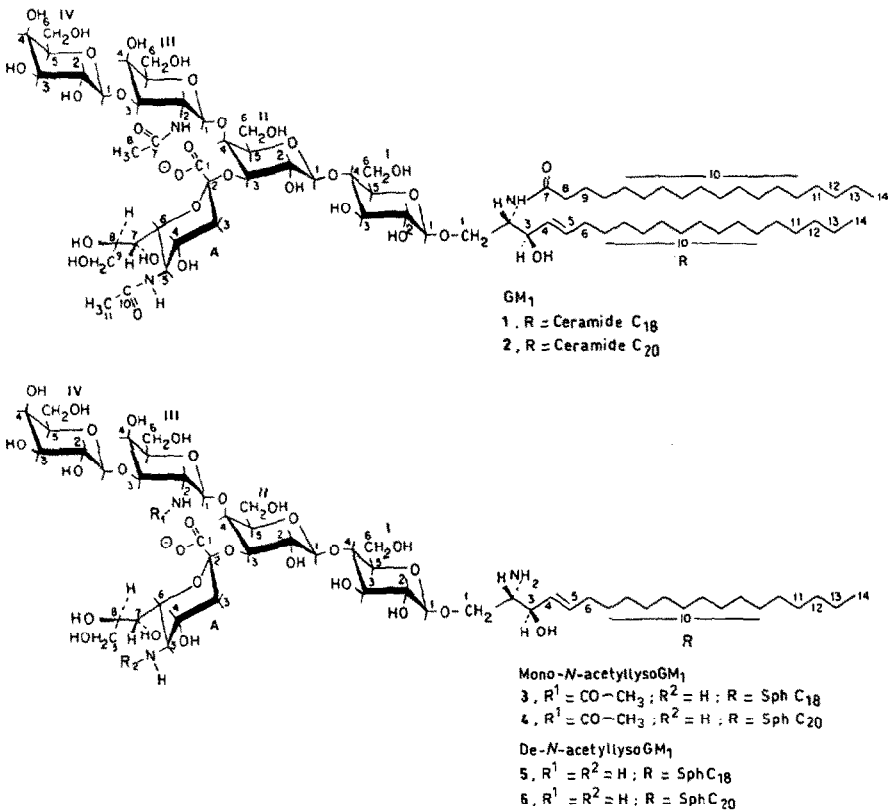

Fig. 1. Structures, symbolism, and numbering of GM_1 C_{18} and C_{20} (**1** and **2**), mono-*N*-acetyl-lysoganglioside GM_1 C_{18} and C_{20} (**3** and **4**), and de-*N*-acetyl-lysoGM$_1$ C_{18} and C_{20} (**5** and **6**). The hexopyranoside residues are indicated as follows: I, D-glucose; II, D-galactose; III, *N*-acetyl-D-galactosamine or D-galactosamine; and IV, D-galactose; the sialic acid residue is denoted as A (Neu5Ac or Neu; *N*-acetylneuraminic acid or neuraminic acid)

a sole acetamido doublet at 7.54 ppm (III-7) in **4** (data not shown) instead of the three NH–CO doublets (III-7, A-10, and R-7) present in GM_1 (**1** and **2**); (*3*) the anomeric proton region showed four doublets, two of them well resolved at 4.77 and 4.22 ppm, and the other two partially overlapped at 4.33 and 4.30 ppm; (*4*) the absence in **4** of the α-carbonyl methylene protons R8, present at 2.03 ppm in GM_1 (**2**), demonstrated the lack of the fatty acid chain of the ceramide residue of GM_1 (**2**); (*5*) the significant upfield shift of 0.87 ppm observed for methine proton signal R2 of the sphingosine moiety at 2.89 ppm in **4**, with respect to that of GM_1 (**2**) at 3.76 ppm, confirmed the cleavage of the amido bond of the ceramide residue and its conversion into a free primary amino group; (*6*) the value of the integration ratio III-8/R14 of 0.99 found in **4** [between the $NCOCH_3$ signal (III-8) of the GalNAc residue at 1.82 ppm and the triplet (R14) at 0.85 ppm] compared with that (0.50) of the corresponding signals for the intact GM_1 parent (III-8/R14 and AII/R14) further confirmed the loss of the fatty acid moiety; (*7*) the upfield shift

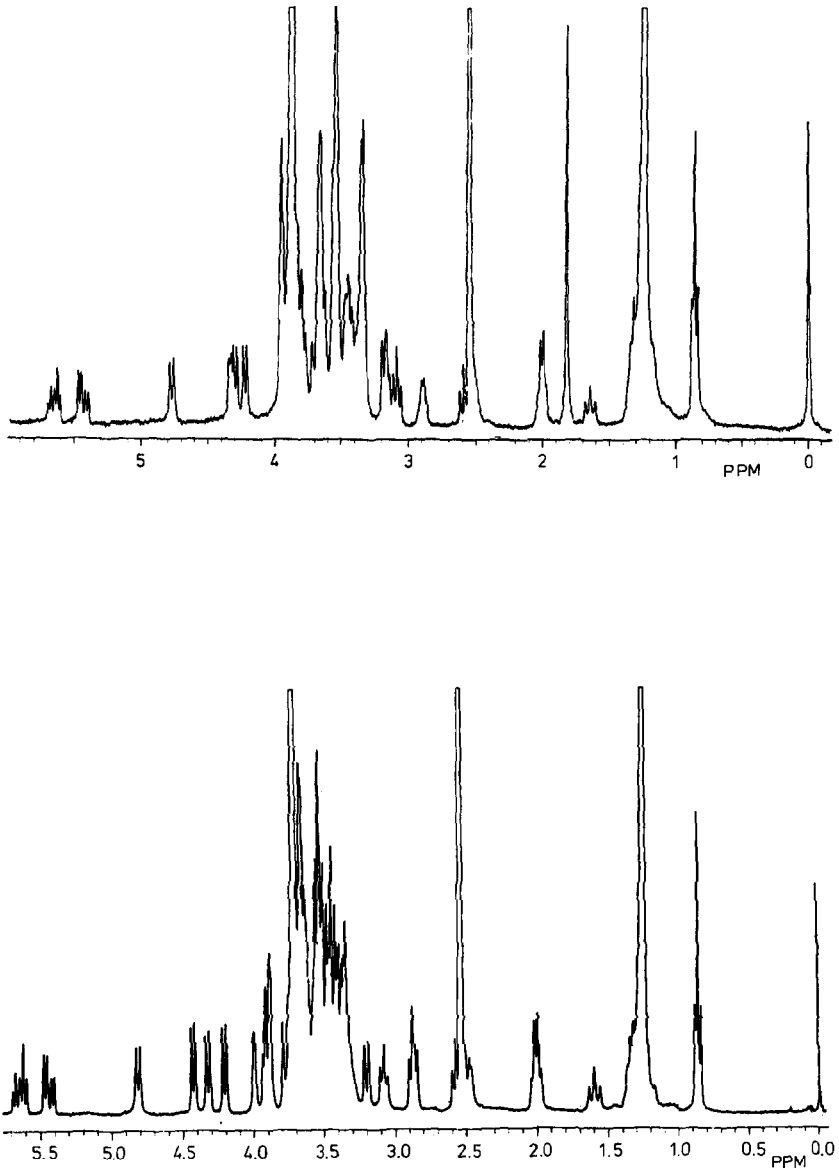

Fig. 2. Proton NMR spectra of mono-*N*-acetyl-lysoGM$_1$ C_{20} (upper panel) and of de-*N*-acetyl-lysoGM$_1$ C_{20} (lower panel) obtained at 300 MHz and 39.3°C; Me_4Si as internal reference, 0.00 ppm.

of 0.79 ppm for methine proton A-5 of the sialic acid residue, from 3.37 ppm in GM$_1$ (**2**) to 2.58 ppm in **4**, indicated the conversion of the acetamido group into free primary amino group; (*8*) the small downfield shift of 0.12 ppm observed for the R3 oxymethine resonance of **4**, with respect to that of R-3 of GM$_1$, caused a chemical shift reversal of protons R-3/III-3 with respect to III-3/R-3 of the GM$_1$ parent; the inversion was induced by loss of the fatty acid unit of ceramide, which

TABLE I

^{1}H Chemical shifts [a] of ganglioside GM_1 C_{20} (2) parent, and of mono-*N*-acetyl-lysoGM$_1$ C_{20} (4) and de-*N*-acetyl-lysoGM$_1$ C_{20} (6) derivatives

Proton	GM_1	Mono-*N*-acetyl-lysoGM$_1$	De-*N*-acetyl-lysoGM$_1$
R-5	5.54, dt	5.63, dt	5.63, dt
	$J_{4,5}$ 15.5	$J_{4,5}$ 15.5	$J_{4,5}$ 15.5
	$J_{5,6}$ 6.8	$J_{5,6}$ 6.8	$J_{5,6}$ 6.6
R-4	5.34, dd	5.43, dd	5.43,dd
	$J_{3,4}$ 7.1	$J_{3,4}$ 6,8	$J_{3,4}$ 6.8
III-1	4.80, d	4.77, d	4.81, d
	$J_{1,2}$ 8.4	$J_{1,2}$ 8.5	$J_{1,2}$ 8.4
II-1	4.31, d	4.30, d	4.32, d
	$J_{1,2}$ 7.8 [b]	$J_{1,2}$ 8.1	$J_{1,2}$ 8.1
IV-1	4.31, d	4.33, d	4.42 d
	$J_{1,2}$ 7.1 [b]	$J_{1,2}$ nm [c]	$J_{1,2}$ 7.1
I-1	4.18, d	4.22, d	4.20, d
	$J_{1,2}$ 7.7	$J_{1,2}$ 7.8	$J_{1,2}$ 7.8
R-3	3.87 [e], t	3.99 [e], t	3.93 [e], t
	$J_{2,3} = J_{3,4} = 7.8$	$J_{2,3} = J_{3,4} = 6.3$	$J_{2,3} = J_{3,4} = 6.3$
A-6	3.20, d	3.18, d	3.20, d
	$J_{5,6}$ 9.6	$J_{5,6}$ 10.0	$J_{5,6}$ 10.0
II-2	3.18, dd	3.16, dd	nm [c]
	$J_{2,3}$ 9.2	$J_{2,3}$ 8.9	
I-2	3.08, dd	3.08, dd	3.08 [d], dd
	$J_{2,3}$ 8.3	$J_{2,3}$ 8.1	$J_{2,3}$ 8.1
III-2	3.94 [e], dd	3.91 [e], dd	2.86 *d*, dd
	$J_{2,3}$ 11.2	$J_{2,3}$ 10.4	$J_{2,3}$ 10.2
R-2	3.76 [e], pq	2.89, pq	2.87 [d], pq
	J 6.5	*J* 6.5	*J* 6.5
A-5	3.37 [e], t	2.58, t	2.57 [d], t
	J 9.6	$J_{5,6}$ 9.7	$J_{5,6}$ 9.7
A-3e	2.58, dd	2.49, dd	2.48 [d], dd
	$J_{3a,3e}$ 12.5	$J_{3a,3e}$ 12.4	$J_{3a,3e}$ 12.3
	$J_{3e,4}$ 4.8	$J_{3e,4}$ 4.0	$J_{3e,4}$ 4.0
R-6	1.93, q	1.99, q	199.q
	J 6.5	*J* 6.5	*J* 6.6
R-8	2.03	lacking	lacking
	J 7.3, s	lacking	lacking
A-11	1.90, s	lacking	lacking
III-8	1.80, s	1.82, s	lacking
A-3A	1.69, t	1.64, s	1.60, s
	$J_{3a,3e}$ 12.5	$J_{3a,3e}$ 12.4	$J_{3a,3e}$ 12.3
	$J_{3a,4}$ 10.8	$J_{3a,4}$ 10.8	$J_{3a,4}$ 10.8
R-10	1.23, bs	1.24, bs	1.23, bs
R-14	0.85, t	0.85, t	0.85, t
	J 6.9	*J* 6.9	*J* 6.9

[a] obtained at 300 MHz at 39.2°C in Me_2SO-d_6–D_2O (ratio ca. 99:1), referenced to internal Me_4Si (0.00 ppm); signal multiplicities: s, singlet; bs, broad signal; d, doublet; dd, double doublet; t, triplet; dt, doublet of triplets; q, quartet; pq, pseudoquartet. [b] Values obtained at 22°C. [c] nm, Not measurable, [d] Signal assigned by spin decoupling. Data obtained or confirmed from a COSY 45° study. Several chemical shifts assignments were supported by spin decoupling and COSY 45° experiments (unpublished results).

was converted into sphingosine. All of the remaining chemical shift assignments of **3** and **4** were in agreement with the expected structures of mono-*N*-acetyl-lysoGM$_1$ C_{18} and C_{20}.

Finally, comparison of the spectra of the other homologous pair, de-*N*-acetyl-lysoGM$_1$ **5** and **6**, with those of the corresponding mono-*N*-acetyl-lysoGM$_1$ pair (**3** and **4**) and also with GM$_1$ (**1** and **2**) showed the following characteristic chemical shift differences. (*1*) The disappearance in **6** of the $NCOCH_3$ protons of the GalNAc (III-8) and the sialic acid (A-11) residue and also the absence of the corresponding amido protons, A10 and III-7; (*2*) the remarkable upfield shift of 1.05 ppm observed for methine triplet III-2, from 3.91 ppm in **4** to 2.86 ppm in **6**, confirmed the deacetylation of the GalNAc moiety to a free primary amino group, geminal to the III-2 proton (this assignment was well supported by spin decoupling and COSY 45° experiments); (**3**) the R2 and A5 methine signals, both geminal to a free primary amino group showed, as already found for the same signals of **4**, the same pronounced upfield shifts. No significant shift differences between signals of each single homologous pair **1** and **2**, **3** and **4** and **5** and **6** each differing only in the nature of their sphingoid moiety (C_{18} and C_{20} sphingosine), were observed, except for small shift variations of III-1, III-2, R-2, A-5 and A-3 methine protons caused by different degrees of hydrogen bonding and small structural (C_{18} or C_{20} sphingosine) and conformational variations.

The significant upfield shifts observed for methine protons R-2 and A-5 in the mono-*N*-acetyl-lysoGM$_1$, and R-2, A-5 and also the more remarkable one observed for III-2 in the de-*N*-acetyl-lysoGM$_1$, with respect to the corresponding protons of GM$_1$, permit generalization of the correlation between substituent effect and chemical shift of the methine protons involved in the conversion of an amido group to a free primary amino group ($CH–NHCOCH_3 \rightarrow CH–NH_2$). Removal of the fatty acid residue of the ceramide unit, which simplified to sphingosine, occurred in the case of methine R-2 during alkaline hydrolysis. It should be noted that, besides the upfield shifts of the III-3 and A-5 methine protons, the disappearance of both $NCOCH_3$ signals of the GalNAc and Neu5Ac residues was also experienced.

^{13}C NMR spectroscopy.—Comparison of the ^{13}C NMR spectra of mono-*N*-acetyl-lysoGM$_1$ C_{20} (**4** or **3**) with those corresponding to GM$_1$ (**1** or **2**), and with those reported by Sillerud et al.[15], showed the following characteristic shift differences (Fig. 3, Table II). (**1**) For **4**, the presence of a unique $NCOCH_3$ carbon resonance (III-8) of the GalNAc residue at 23.30 ppm, in contrast with two resonances for **2** at 23.36 (III-8) and 22.43 ppm (A-11); (**2**) the lack of R8, R9, and R7 carbon resonances confirmed, as previously found in the corresponding ^1H NMR spectra, the loss of the fatty acids moiety of the ceramide residues.

The ^{13}C NMR spectra of the de-*N*-acetyl-lysoGM$_1$ C_{20} or C_{18} (**6**) (Fig. 3) compared with those of mono-*N*-acetyl-lysoGM$_1$ **4** showed the following significant differences. (**1**) The disappearance of the $NCOCH_3$ carbon resonance (III-8) of the GalNAc residue of **4**; (*2*) the same lack of carbon resonances R8, R9, and R7

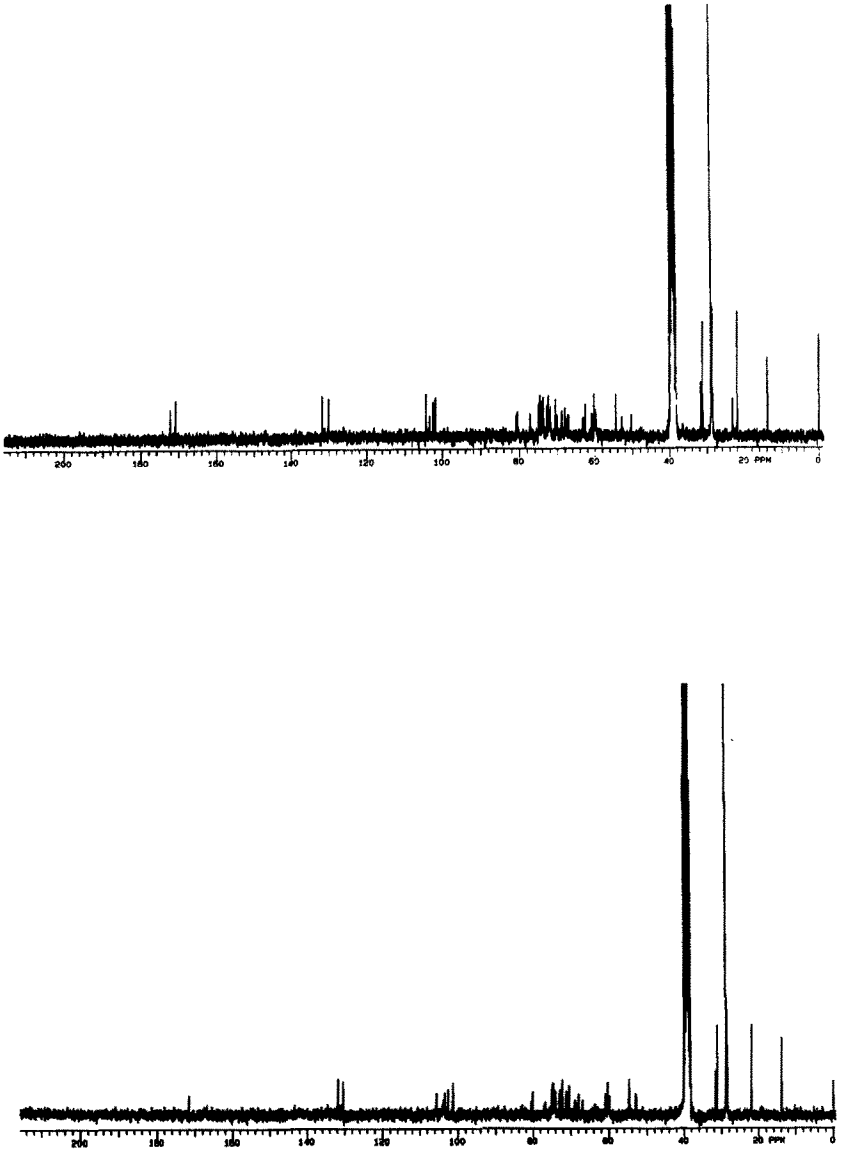

Fig. 3. ^{13}C NMR spectra of mono-*N*-acetyl-lysoGM$_1$ C_{20} (upper panel) and de-*N*-acetyl-lysoGM$_1$ C_{20} (lower panel) recorded at 75.4 MHz and 22°C; Me_4Si as internal standard.

as observed in the spectra of mono-*N*-acetyl-lysoGM$_1$ **4**; (*3*) the presence of a unique carbon resonance at 171.53 ppm, unambiguously assignable to the carboxyl carbon resonance (A-1) of the sialic acid moiety; in the same region, the mono-*N*-acetyl-lysoGM$_1$ **4** showed two carbon resonances at 172.26 (III-7) and 170.88 (A-1); by contrast GM$_1$ showed three carbonyl resonances and a fourth resonance assignable to the unique carboxyl carbon resonance; (*4*) the remaining carbon

TABLE II

^{13}C Chemical shifts * of ganglioside GM_1C_{20} (**2**) and of mono-N-acetyl-lysoGM$_1$ C_{20} (**4**) and de-*N*-acetyl-lysoGM$_1C_{20}$ (**6**) derivatives

Carbon	GM_1	Mono-*N*-acetyl-lysoGM$_1$	De-*N*-acetyl-lysoGM$_1$
R4	131.29 [b]	131.93	131.62
R5	131.29 [b]	130.19	130.54
IV-1	104.40	104.25	105.53
III-1	102.39 [c]	102.31 [c]	102.52 [c]
II-1	103.61	103.34	103.62
I-1	103.41 [c]	102.47 [c]	103.41 [c]
A2	101.85	101.88	101.35
III-3	81.05	80.89	81.32
I-4	80.68	80.60	80.25
II-4	77.43	77.34	76.76
II-3	75.36	75.01	75.15
R2	52.87	54.59	54.65
III-2	52.45	52.92	52.95
A5	50.65	50.43	52.81
R9	25.29	lacking	lacking
R8	35.47	lacking	lacking
R7	171.24	lacking	lacking
R6	31.67	31.62	31.62
R10	28.96	28.88	28.65
R11	28.79	28.75	28.76
R12	31.19	31.16	31.16
R13	21.99	21.98	21.98
R14	13.83	13.85	13.85
A11	22.43	lacking	lacking
III-8	23.36	23.30	lacking
A-10	172.12 [d]	lacking	lacking
III-7	171.67 [d]	172.26	lacking
A-1	170.51	170.88	171.53

[a] Spectra were recorded at 75.4 MHz, and referenced to internal Me_4Si (0.00 ppm). [b] These signals are separated at 39.2°C. [c,d] Signals within any vertical column may be reversed. Carbon resonance assignments were also compared with chemical shift values reported by Sillerud et al.[15].

resonances occurred at a field position similar to those found for the mono-*N*-acetyl-lysoGM$_1$ (**3** and **4**).

Thus, the ^{13}C NMR data are completely in agreement with the ^1H NMR data and permit the unambiguous assignment to **4** of the structure mono-*N*-acetyl-lysoGM$_1$ C_{20}, and to **6** of the structure de-*N*-acetyl-lysoGM$_1$ C_{20}.

Mass spectrometry.—To corroborate the NMR data, the four lysoganglioside derivatives (**3–6**) were subjected to FABMS. Four characteristic, prominent pseudomolecular ions (M − 1) were found at m/z 1236 and 1264 for the homologous mono-*N*-acetyl-lysoGM$_1$ C_{18} and C_{20} pair (**3** and **4**), and at m/z 1194 and 1222 for the corresponding de-*N*-acetyl-lysoGM$_1$ pair (**5** and **6**), see Table III and Fig. 4.

The high relative intensity of (M − 1) negative peaks confirmed their composition as the sialooligosaccharide and sphingoid moiety, indicating that each com-

TABLE III

Characteristic negative ions of lysoganglioside GM_1 derivatives in FAB Mass Spectra [a]

m/z	LysoGM$_1$ [b]		Mono-*N*-Ac-lysoGM$_1$ [c]		De-*N*-Ac-lysoGM$_1$ [c]	
	C-18	C-20	C-18	C20	C-18	C-20
M−1	1278, nd	1306, s	1236, s	1264, s	1194, s	1222, s
(M−1)-NeuAc	987, nd	1015, w	987, w	1015, w	945, w	973, w
A1	298, nd	326, nd	298, w	326, w	298, w	326, w
A2	460, nd	488, w	460, w	488, m	460, m	488, m
A3	913, nd	941, w	871, w	899, m	871, w	899, m
A4	1116, nd	1144, w	1074, m	1102, m	1032, m	1060, m
B1	179, w	179, w	179, w	179, w	179, w	179, w
B2	382, w	nd	364, w	382, w	340, w	340, w
B3	835, w	833, m	791, m	791, m	749, m	749, m
B4	997, w	995, w	955, w	955, w	913, w	913, m

[a] The relative intensities of the negative ions are listed as follows: s, strong; m, medium; w, weak; nd, not detected. [b] Data as reported by Neuenhofer et al.[10]. *c* Experimental data.

pound was at a high state of purity and contained exclusively C_{18} or C_{20} sphingosine. In contrast, the mass data of the lysoGM$_1$ parent[10] reflected a mixture of both C_{18} and C_{20} sphingosine constituents.

Comparison of the fragmentation patterns of the two homologous pairs with that of the parent lysoGM$_1$ showed corresponding fragments of types A and B, of comparable intensity, at the expected mass-to-charge values, demonstrating unambiguously their lysogangliosideGM_1 structures. During FABMS analysis, the glycosidic bonds are readily split according to the fragmentation scheme shown in Fig. 5 (ref 10). Fragments of type A systematically contained the sphingoid C_{18} and C_{20} moiety of lysogangliosides, whereas fragments of type B contained terminal sugar units.

In addition, negative ions of detectable intensity were found at *m/z* 1218 and 1246 (**3** and **4**), and *m/z* 1176 and 1204 (**5** and **6**), each pair differing by 18 mass units with respect to the corresponding pseudomolecular ion, and arising from the latter through loss of water. Furthermore, all four derivatives preferentially lost the neuraminic acid fragment, giving rise to a negative ion at *m/z* 266 and a daughter ion at *m/z* 248 by loss of water from the former. Both these ions were rather intense, particularly those at *m/z* 248 (base peak). Also, the ions at *m/z* 622 and the corresponding homologues at *m/z* 650, differing by 28 mass units, could presumably be due to loss of the neuraminic acid residue from fragments of type A3.

The fragments B1, of detectable relative intensity, each found at *m/z* 179, derived from cleavage of the Gal-*O* residue-IV during fragmentation. In the case of B3 and B4 fragments of lysoGM$_1$ C_{18} and C_{20} (data taken from ref 10), the *m/z* value for each pair should be the same; the difference of 2 mass units observed for each fragment relative to the C_{20} homologue was due to loss of two hydrogen atoms.

The characteristic ions of the lysoganglioside derivatives (**3–6**) and those of parent lysoGM$_1$ are listed in Table III. Comparison between the fragments of the homologous mono-*N*-acetyl-lysoGM$_1$ pair (**3** or **4**) with those of the de-*N*-acetyl-lysoGM$_1$ pair (**5** or **6**), differing by 42 amu, unambiguously indicated the loss of the *N*-acetyl residue of the acetamido group of GalNAc (III-8). Finally, comparison of

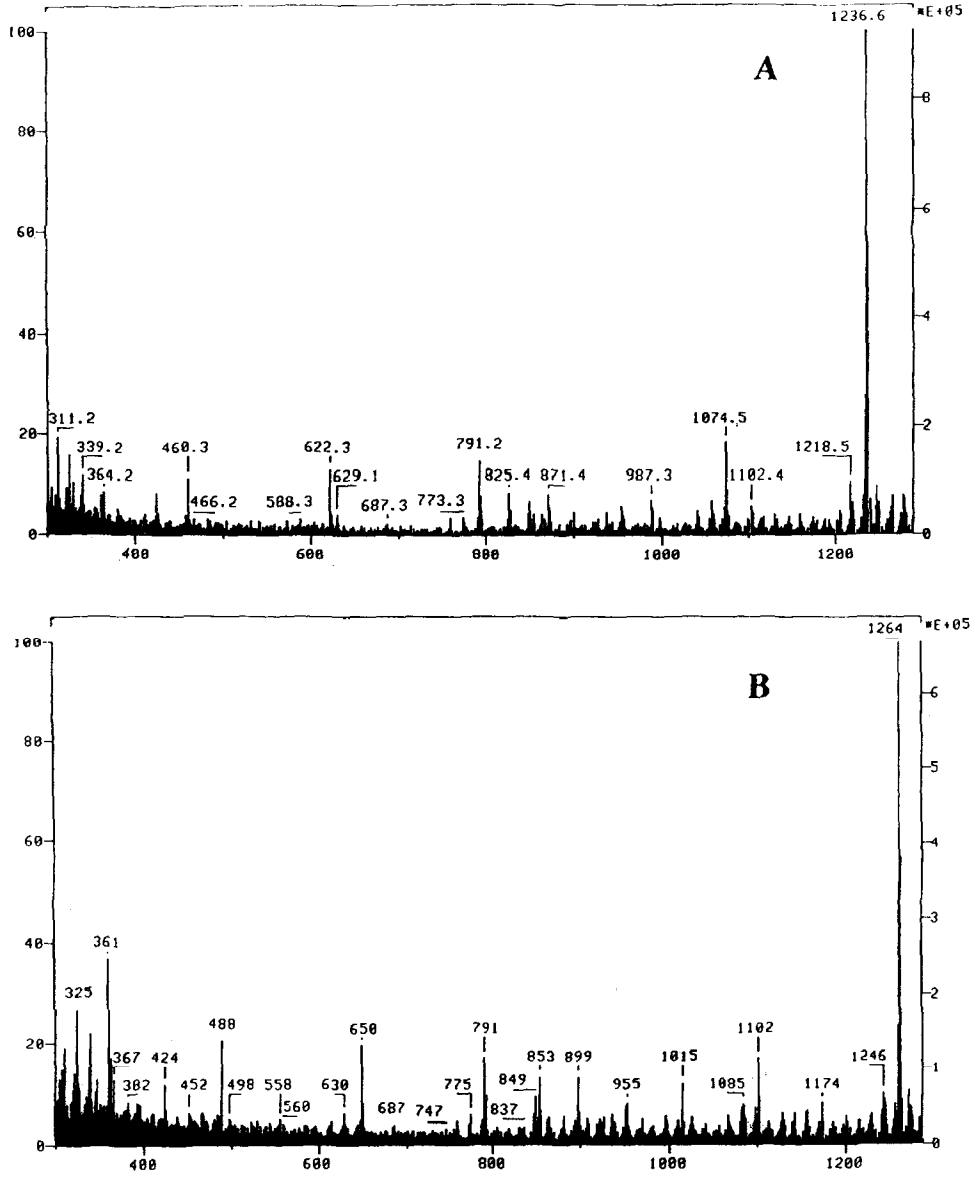

Fig. 4. FAB mass spectra of lysoganglioside derivatives; mono-*N*-acetyl-lysoGM$_1$ C_{18} (A) and C_{20} (B); de-*N*-acetyl-lysoGM$_1$ C_{18} (C), and C_{20} (D).

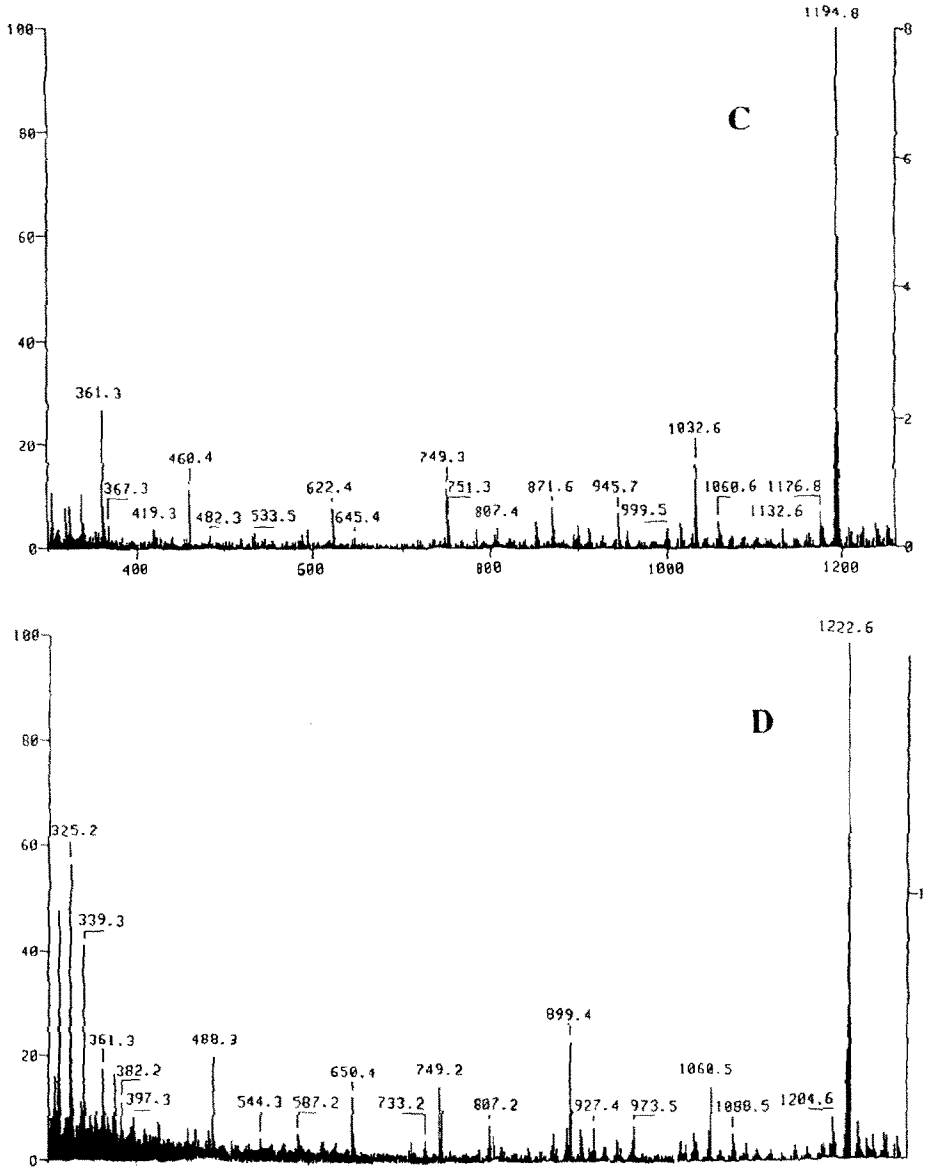

Fig. 4. (continued)

the corresponding fragments of each homologous pair containing C_{18} or C_{20} sphingosine revealed series of pairs of ions differing by 28 amu.

In conclusion, we have obtained four new, variously deacetylated lysogangliosides through the alkaline hydrolysis of either the C_{18} or C_{20} sphingosine homologue of the monosialoganglioside GM_1. Following this procedure, both the fatty

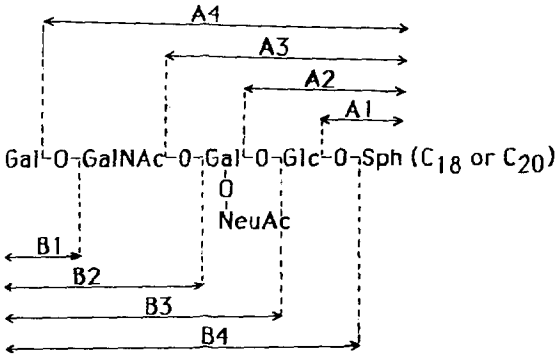

Fig. 5. Mass fragmentation scheme of lysogangliosideGM_1 derivatives (**3–6**) by FABMS.

acid moiety and the *N*-acetyl group of the sialic acid units of GM_1 were removed completely, to give the separated mono-*N*-acetyl-lysoGM$_1$, composed of C_{18} and C_{20} sphingosines, respectively: a part of GM_2 (**1** and **2**) was found to have also lost the last *N*-acetyl group of the galactosamine moiety, giving rise to de-*N*-acetyl-lysoGM$_1$ C_{18} (**5**) and C_{20} (**6**) with three free primary amino groups.

The compounds reported here, having all lost the fatty acid chain, belong to the family of lysoganglioses and are expected to modify membrane expression of PKC and of PLA_2. Having also lost the acetyl groups, they are, moreover, presumed to share the characteristics of *N*-deacetylated gangliosides, which are reported to trigger positive or negative transmembrane signals[16].

EXPERIMENTAL

Isolation of the GM_1 ganglioside (method).—Ganglioside GM_1 was prepared from a mixture of raw bovine brain according to the tetrahydrofuran–NaH_2PO_4 buffer (pH 6.8) procedure reported by Tettamanti et al.[17].

GM_1 was isolated from the total ganglioside extract by sequential application of HPLC on 200 g of Lichoprep Si 60 Merck (40–63 μm) silica gel with a Jobin–Yvon Chromatospac Prep 100 apparatus. Usually, the columns were eluted with 4:4:1 $CHCl_3$–MeOH–H_2O. Fractions (250 mL) containing the GM_1 ganglioside, identified by TLC, were pooled separately, dialyzed, and freeze-dried to obtain an amorphous mass. The degree of purity of isolated GM_1 was judged to be ca 99.5% by TLC (Kieselgel 60/F_{254} plate) and via ^1H/^{13}C NMR data.

Separation of two GM_1 molecular species (C_{18} and C_{20} sphingosine).—The isolated ganglioside GM_1 mixture was composed of two constituents differing only in the nature of their sphingosine moiety (C_{18} and C_{20} sphingosine). Both the GM_1C_{18} (**1**) and GM_1C_{20} (**2**) sphingosine species were separated by preparative HPLC on 1000 g of Silica C_{18} Matrix (Amicon, 50 μm, 60 atm) with a Jobin–Yvon Chromatospac Prep 1000 apparatus fitted with a Gilson 115 UV detector at 204

nm. Ordinarily, an aliquot of GM_1 (5 g) was dissolved in 100 mL of mobile phase and applied to the Chromatospac column, which was eluted (80 mL/min) with 5 L of 83:1:16 MeOH–THF–H_2O followed by 10 L of 86:2:12 MeOH–THF–H_2O, to give 1.5 g of GM_1C_{18} (**1**) and 1.2 g of GM_1C_{20} (**2**), both as pure amorphous materials.

*Preparation of mono-*N*-acetyl-lysoGM$_1$ and of de-*N*-acetyl-lysoGM$_1$ (homologous pairs with C_{18} and C_{20} sphingosine residues).*—To obtain mono-*N*-acetyl-lysoGM$_1$ and de-*N*-acetyl-lysoGM$_1$, a modified version of the procedure described by Tayot et al.[13] was used (**1** or **2**, ca. 150 mg) was dissolved 1:9 10 M KOH–butan-1-ol (10 mL) and stirred for differing periods at various temperatures, in a screw-capped vial. Typical hydrolysis conditions were 4 h at 110°C: yield, **3** or **4**, 25%; **5** or **6**, 55%; referred to C_{18} or C_{20} GM_1 prior to purification. After cooling, distilled water (10 mL) was added to the hydrolysis solution and the carefully mixed solution placed in a separation funnel overnight. The lower aqueous phase containing the mono-*N*-acetyl-lysoGM$_1$ (**3** or **4**) and the de-*N*-acetyl-lysoGM$_1$ (**5** or **6**) as main compounds was collected, concentrated under vacuum to eliminate butan-1-ol, adjusted to pH 9 with concd HCl, and successively clarified by centrifugation at room temperature at 3000 RPM. The clear supernatant solution was dialyzed in a Visking tube against distilled water for two days to remove the salts, then freeze-dried.

The mono-*N*-acetyl-lysoGM$_1$-C_{18} (**3**) and -C_{20} (**4**) and the de-*N*-acetyl-lysoGM$_1$-C_{18} (**5**) and -C_{20} (**6**) were purified from the hydrolysis mixture by column chromatography on 100 g of Lichoprep Si 60 (Merck, 40–63 μm) with a Jobin–Yvon apparatus. The column was run with 65:25:3 $CHCl_3$–MeOH–1 M NH_3OH (500 mL), 65:25:4 $CHCl_3$–MeOH–1M NH_3OH (500 mL), and finally 55:45:10 $CHCl_3$–MeOH–2.5 M NH_3OH (1 L).

Fractions (250 mL) were collected and the elution profile was monitored by TLC. Fractions containing mono-*N*-acetyl-lysoGM$_1$-C_{18} and -C_{20} and de-*N*-acetyl-lysoGM$_1$-C_{18} and -C_{20} were dried under vacuum. The purity of **3** and **4**, and **5** and **6** was tested by TLC (Kieselgel 60/F_{254}, 20 × 20 cm plates, Merck, Darmstadt). The purity of **3**–**6** was analyzed by eluting with 50:40:10 $CHCl_3$–MeOH–0.25 M NH_3OH. Detection was effected with resorcinol–HCl or with aq 50% H_2SO_4, and the presence of NH_2 groups was detected by a trinitrobenzenesulphonic acid reagent[18]. Typical R_f values of mono-*N*-acetyl-lysoGM$_1$$C_{18}$/$C_{20}$ and de-*N*-acetyl-lysoGM$_1$$C_{18}$/$C_{20}$ were 0.10 and 0.05, respectively. The overall yield referred to GM_1 was ca. 30 mg (20%) of **3** or **4**, and 66 mg (44%) of **5** and **6**.

^{1}H NMR spectroscopy.—Spectra of the samples were obtained with a Varian XL 300 WB Superconducting Spectrometer, operating at 300 MHz. All of the samples were freeze-dried three times with D_2O (99.95%), and the resulting residue (5 mg) was dissolved in 0.6 mL of freshly prepared Me_2SO-d_6 (99.95%), adding 2 or more drops of D_2O (ratio ca. 99:1) until removal of residual exchangeable protons. Chemical shifts a reported in ppm downfield from the proton resonance of internal Me_4Si (δ 0.00 ppm), and coupling constants (J) in Hz. The spectra were recorded

at 22 and 39.2°C (the latter temperature for best resolution). Deuterated solvents were of highest purity (E. Merck, Darmstadt, FRG).

^{13} NMR spectroscopy.—The corresponding ^{13}C NMR spectra of the four lysogangliosideGM_1 derivatives were recorded with a Varian XL 300-MHz Superconducting Spectrometer, operating at 75.4 MHz; the amount of sample was doubled (10 mg in 0.7 mL) to increase the sensitivity and decrease the total time of accumulation). The probe temperature was 21 ± 1 and 39°C. Chemical shifts were references to internal Me_4Si (δ 0.00 ppm). The carbon resonance assignments were confirmed by the degree of protonation obtained by means of the attached proton test (APT) technique.

Mass spectrometry.—FABMS[19] was carried out with a Finnigan Model MAT-90, reverse-geometry mass spectrometer fitted with an FAB source and an Ion Tech atom gun. The target was bombarded with a Xe neutral atom beam accelerated to 5.5–6 keV energy. Spectra were obtained for a full mass scan from 1450 to 80 mass units. The samples were directly analyzed with glycerol/hexamethylphosphoramide matrix system (ratio 1:1) which is useful for molecular weight determination, as well as for elucidating the carbohydrate sequence[20].

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