Stereochemical structures of synthesized and natural plasmalogalactosylceramides from equine brain

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Abstract **Modified galactosylceramide with a long-chain cyclic acetal at the sugar moiety, plasmalogalactosylceramide, was isolated from equine brain. To identify the isomeric stereostructure of the natural product, the plasmalo derivative was chemically synthesized from galactosylceramide through acetalization. The presence of cyclic acetal linkage, the linked position and length of the acetal chain of the synthesized and natural products were determined by proton nuclear magnetic resonance spectroscopy and fast-atom bombardment–mass spectrometry, as well as gas chromatography–mass spectrometry and gas–liquid chromatography. The orientation of the acetal chain linked to galactoside was characterized by connectivity between the cyclic acetal proton and ring proton(s) on the sugar moiety using the homonuclear Overhauser effect. This revealed that, of the two positional isomers of the acetal linkage with 4,6-*O*-acetal and 3,4-*O*-acetal derivatives obtained from the acetalization reaction, the former positional isomer, separated into two spots, was identified to 'endo'- and 'exo'-type acetal chains. In comparison to the NMR data of the synthesized derivative, equine brain acetalized lipid was found to be an 'endo'-type 4,6-*O*-acetal derivative.—**Yachida, Y., M. Kashiwagi, T. Mikami, K. Tsuchihashi, T. Daino, T. Akino, and S. Gasa. **Stereochemical structures of synthesized and natural plasmalogalactosylceramides from equine brain. *J. Lipid Res.* 1998.** 39: **1039–1045.**

Supplementary key words plasmaloglycolipid • acetalization • galactosylceramide • NMR • fatty aldehyde

Glycosphingolipids (GSLs) play roles in intercellular recognition and transmembrane signaling (1, 2). Some of these GSLs are modified in normal and tumor tissues, such as *O*-fatty acylated galactosylceramide (GalCer) in several mammalian (3–5) and fish (6) brains, *O*-fatty acylated glucosylceramide in mammalian epidermis (7), *O*-acetyl GD3 in melanoma cells (8, 9), *O*-acetyl GM3 in horse erythrocytes (10–14), and *O*-acetylated GM3 at the Cer moiety in glioma (15). PlasmaloGSLs, which are conjugated with a long-chain fatty aldehyde at the sugar moiety and reported to form a cyclic acetal linkage exclusively on galactose, have recently been isolated from normal human brain as novel modified GSLs (16, 17). Modification with fatty acetal was reported to occur at 3,4-*O* and 4,6-*O* on Gal of GalCer (16) and Gal sphingosine (psychosine) (17), and their chemical structures were characterized mainly with mass spectra using fast-atom bombardment–mass spectrometry, and by methylation analysis. The presence and chain length of fatty aldehyde have also been determined by gas chromatography–mass spectrometry (GC–MS) as an enol methyl ether derivative. In particular, plasmalopsychosines have been chemically synthesized from psychosine with acetalization, giving acetal structures identical to naturally occurring plasmalolipid (18). However, the stereostructure of natural plasmaloGalCer has not yet been characterized. In the present paper, we describe isolation of plasmaloGalCer from equine brain, its synthesis and identification of its stereochemical structure.

MATERIALS AND METHODS

Chemicals

DEAE-Sephadex, A-25 and LH-20 were purchased from Pharmacia-LKB (Sweden). Iatrobeads (8060) were from Iatron (Tokyo). Precoated thin-layer chromatography (TLC)-plates (Silica gel 60) and pyridine-d_5 were obtained from Merck (Germany). Other reagents were of analytical grade.

Isolation of plasmaloGalCer

The ratio of solvent mixtures is expressed by volume. Whole horse brain (457 g wet weight) was homogenized with acetone (1 g/9 ml) to yield an acetone powder with a dry weight of 104 g. The glycolipids were extracted three times from the powder with chloroform–methanol–water (CMW, 4:8:3) at room temperature. The neutral glycolipid fraction was isolated from the combined and concentrated extracts with a DEAE-Sephadex, A-25 (acetate form) column (2.5 × 30 cm) by elution with CMW

Abbreviations: CMW, chloroform–methanol–water; GalCer, galactosylceramide; GC–MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; GSL, glycosphingolipid; NMR, nuclear magnetic resonance spectroscopy; NOESY, homonuclear Overhauser effect spectroscopy; TLC, thin-layer chromatography.

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(40:60:10). The total neutral glycolipids were chromatographed on a silica-gel (Iatrobeads) column (2.5 × 50 cm) by stepwise elution with CM from 95:5 to 90:10, 85:15, 80:2, 75:25, and 70:30 each with 1,000 ml. The fraction that consisted of several less polar glycolipids was eluted with CM, 95:5 and 90:10, and the combined eluates were further chromatographed on a silica-gel column with a smaller column size (1.2 × 40 cm) by stepwise elution with the above CM, and chromatography was repeated to obtain homogenous glycolipid. The purified glycolipids were chromatographed on a TLC plate, developed with CMW (90:10:0.5), and visualized by spraying with orcinol–sulfuric acid reagent, for estimation of the purity.

Acetalization of galactosylceramide

The chemical acetalization of GalCer was performed according to the method of Sadozai et al., with a slight modification, utilizing synthesis of plasmalo-psychosine (18) and -methyl galactoside (19). Briefly, equine brain GalCer (30 mg containing non-hydroxy fatty acid, co-purified as described above) in dimethylformamide (2 ml) was incubated with *p*-toluene sulfonic acid (5 mg) and 1,1-dimethoxyhexadecane (30 mg). The reaction mixture was applied to an LH-20 column (1 × 50 cm) with chloroform to remove dimethylformamide and *p*-toluene sulfonic acid. The acetalized products were further purified on a silica-gel column (1 × 40 cm) by stepwise elution with CM from 98:2 to 96:4, 94:6, 92:8 and 90:10 each with 300 ml. An aliquot of the fraction was chromatographed by TLC as above.

Analysis of lipid and sugar moieties

The fatty acid and fatty aldehyde of synthesized and natural plasmaloGalCer were analyzed from the methanolyzates of the purified GSL using a gas–liquid chromatography (GLC) apparatus (GC-14A, Shimadzu) equipped with a capillary column (0.25 mm × 50 m) coated with 1% of DB-5, with programmed temperatures from 150 to 250°C at 5°C per min. The methanolysis of the glycolipid (0.1 mg) was carried out with 1 ml of 1 N HCl in anhydrous methanol at 80°C for 16 h, followed by extraction three times with 1 ml of *n*-hexane. After concentration of the extracts under N_2 gas to approximately 20 μl, an aliquot of the extracts was subjected to GLC. The GLC peaks were characterized using a GC–MS (JEOL JMS-OISG-2) with electron impact ionization, equipped with a capillary column (0.25 mm × 50 m) coated with 1% of OV-1 and the same programmed temperatures as the above GLC, at the NMR–MS Laboratory of the Faculty of Agriculture in Hokkaido University.

The sugar species and the substituted sites of the synthesized and natural plasmaloGalCers were determined by GC–MS as above with partially methylated alditol acetate derived from permethylated GSL in basic conditions following acetolysis/hydrolysis, reduction with $NaBH_4$, and peracetylation as reported previously (20).

NMR spectroscopy

One (1-D)- and two-dimensional (2-D) proton nuclear magnetic resonance spectroscopy (NMR) spectra of the glycolipids (approximately 1 mg) in 0.3 ml of pyridine-d_5 containing 2% D_2O were obtained at 90°C in a Fourier-transform mode on a Bruker AMX-500 spectrometer at the above laboratory, as described previously (13, 15). Chemical shifts (δ, ppm) were measured using tetramethylsilane as an internal standard.

RESULTS

Isolation of plasmaloGalCer

The TLC profiles of the GSLs purified from equine brain are presented in **Fig. 1**, together with *O*-acylated- and unacylated-GalCers. Of these less-polar GSLs, the lipid abbreviated as N-1 was a plasmaloGalCer (see below) with a yield of 2.3 mg, and co-purified with 6-*O*-fatty acyl GalCer (12 mg yield) and 2-*O*-acyl GalCer (8 mg) as well as unmodified GalCer. The mobility of N-1 did not change on TLC analysis after saponification with 0.2 N sodium methoxide in methanol (data not shown), by which an *O*-acyl group was released from the *O*-acylated GalCers, indicating no modification with an alkali-labile group as in *O*-acylation. The structures of N-1 and *O*-acylated GalCers were determined by NMR, MS, and GLC as described below (MS and GLC data not shown for the N-1).

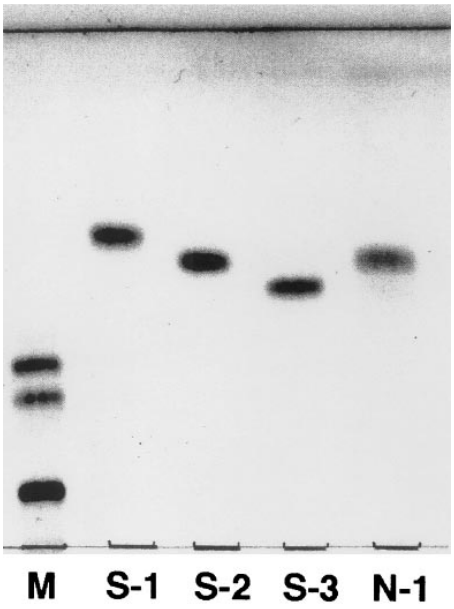

Fig. 1. Thin-layer chromatography of chemically synthesized and natural plasmaloGalCers. Lane M shows a mixture of 6-*O*-acyl- (upper), 2-*O*-acyl- (middle), and non-acyl (lower)-GalCers obtained from equine brain. The plate was developed with chloroform–methanol–water 90:10:0.5, and visualized by staining with an orcinol–sulfuric acid reagent.

Acetalization of GalCer

To identify the structure of naturally acetalized GalCer (N-1) from equine brain, GalCer from the brain was chemically acetalized with a 1,1-dimethoxy derivative of hexadecanal. Three products, abbreviated as S-1, -2, and -3, were obtained from the reaction followed by purification. S-1 (0.9 mg, 3% yield) eluted faster with CM, 98:2; S-2 (22.5 mg, 75%) eluted later with 98:2; and S-3 (2.4 mg, 7%) eluted with 96:4 from the silica-gel column. Of these chemically acetalized GalCers, S-2 showed an R_f identical to N-1 as demonstrated in Fig. 1.

Analysis of lipid and sugar moieties of plasmaloGalCer

The lipid moieties of the above plasmaloGalCers were analyzed by GLC and GC–MS after methanolysis with anhydrous methanolic HCl, which converted the fatty acid to the methyl ester; the fatty aldehyde was derivatized to dimethyl acetal as a major component and an enol methyl ether as a minor one. Such conversions of fatty aldehydes

TABLE 1. Composition of fatty acids[a]

Carbon Chain Length	S-2[b]		N-1	
	Saturated	Monounsaturated	Saturated	Monounsaturated
	%		%	
16	3	trace	trace	trace
18	11	trace	34	2
19	trace	trace	trace	trace
20	12	trace	6	2
21	12	trace	1	trace
22	trace	trace	7	1
23	trace	trace	8	trace
24	44	7	24	trace
25	8	trace	5	1
26	1	trace	4	2
Unknown		2		3

[a]Measured as methyl ester by gas–liquid chromatography. Trace, <0.4%.
[b]Lipid composition was identical to those of S-1 and S-3.

under an anhydrous acidic condition were confirmed by analysis of the methanolyzates of authentic hexadecanal. The starting fatty aldehyde itself was detected at a level of less than 0.1%. The lipid data are summarized in **Tables 1–4.** From the sum of the respective areas with aldehyde and fatty acid derivatives in the gas chromatograms, synthesized and natural GalCers were estimated to have both lipid components with approximately similar ratios. The saturated fatty aldehydes were detected in N-1 with hexadecanal and octadecanal at 33% and 44%, respectively, together with their monounsaturated aldehydes as a minor component. The composition and ratio of the lipid moiety released from S-1 and S-3 by methanolysis were similar in these lipids, indicating that they were stereoisomers of the acetal ring and/or positional isomers of S-2. The lipid compositions of S-1, -2, -3, and N-1 were alternatively confirmed by positive ion fast-atom bombardment–mass spectrometry (data not shown).

The substituted sites of Gal on S-1, -2, -3 and N-1 with fatty acetal were further analyzed by GC–MS of the partially methylated alditol acetates, in which acetalized positions in the intact sugar moiety were replaced with *O*-acetates. S-1, S-2, and N-1 gave only 2,3-*di*-*O*-methyl-1, 4, 5, 6-*tetra*-*O*-acetyl galactitol, indicating these GSLs to be substituted at C-4,6-*O* on the Gal, whereas S-3 gave 2, 6-*di*-*O*-methyl-1, 3, 4, 5-*tetra*-*O*-acetyl galactitol, substituted at C-3,4-*O* on Gal (Table 4). Combined data from this methylation analysis and GLC analysis of the lipid moiety suggested that S-1 and S-2 were diastereomeric isomers of each other for the cyclic acetal ring and that S-3 was the positional isomer of S-1 and S-2.

TABLE 2. Composition of fatty aldehydes

Carbon Chain Length	S-2	N-1
	%	%
16:0		
Aldehyde	trace	1
Enol ether	4	1
Acetal	96	32
16:1		
Aldehyde	—	trace
Enol ether	—	3
Acetal	—	8
18:0		
Aldehyde	—	trace
Enol ether	—	7
Acetal	—	37
18:1		
Aldehyde	—	trace
Enol ether	—	trace
Acetal	—	11

Trace, <0.4%.

TABLE 3. Composition of long chain base

Long Chain Base	S-2	N-1
	%	%
Sphingenine d18:1	97	95
Sphinganine d18:0	trace	1
Unknown	3	4

Trace, <0.4%.

NMR study

The partial 1-D proton NMR spectra of S-1, -2, -3 and N-1 are shown in **Fig. 2**, together with that of GalCer as a reference. The assignment of proton signals inserted in the figure was performed with the 2-D chemical shift-correlated spectroscopy spectra (data not shown), and the data are summarized in **Table 5**. The triplet signal H-1′′ (peak 1′′ in Fig. 2; the carbon number in each group is shown in Fig. 4) at δ5.291 ppm with one proton from the relative integration newly appeared in the 1-D spectrum of S-1 in comparison with the spectrum of GalCer, and the H-1′′ was coupled to an upper field signal H-2′′ (1.75 ppm) having two-proton intensity, which was further coupled to H-3′′ (1.48 ppm) with two protons in the 2-D spectrum. The chemical shifts, splitting pattern, and integrations of these proton signals suggested the presence of a sequence of dioxomethine–methylene–methylene, and H-1′′ was consequently identified as a methine proton of the acetal group.

The 1-D spectrum of S-2 also showed a new triplet H-1′′ signal at δ4.665 ppm (Fig. 2), and the proton was coupled with H-2′′ (1.74 ppm) and the H-2′′ further coupled with H-3′′ (1.48 ppm) in the 2-D spectrum, indicating H-1′′ to be an H-1 of the acetal as in the case of S-1. The S-1 and S-2 were, therefore, each configurational isomers at C-1′′ of the acetal with an asymmetric center having C-4-*O*, C-6-*O*, H-1′′ of the acetal and a hydrocarbon chain. These isomers were each characterized by 2-D homonuclear Over-

TABLE 4. Composition of saccharides

Saccharide	Partially Methylated Alditol Acetate[a]
S-1	1,4,5,6-tetra-*O*-acetyl 2,3-di-*O*-methyl galactitol
S-2	1,4,5,6-tetra-*O*-acetyl 2,3-di-*O*-methyl galactitol
S-3	1,3,4,5-tetra-*O*-acetyl 2,6-di-*O*-methyl galactitol
N-1	1,4,5,6-tetra-*O*-acetyl 2,3-di-*O*-methyl galactitol

[a]Analyzed by gas chromatography–mass spectrometry.

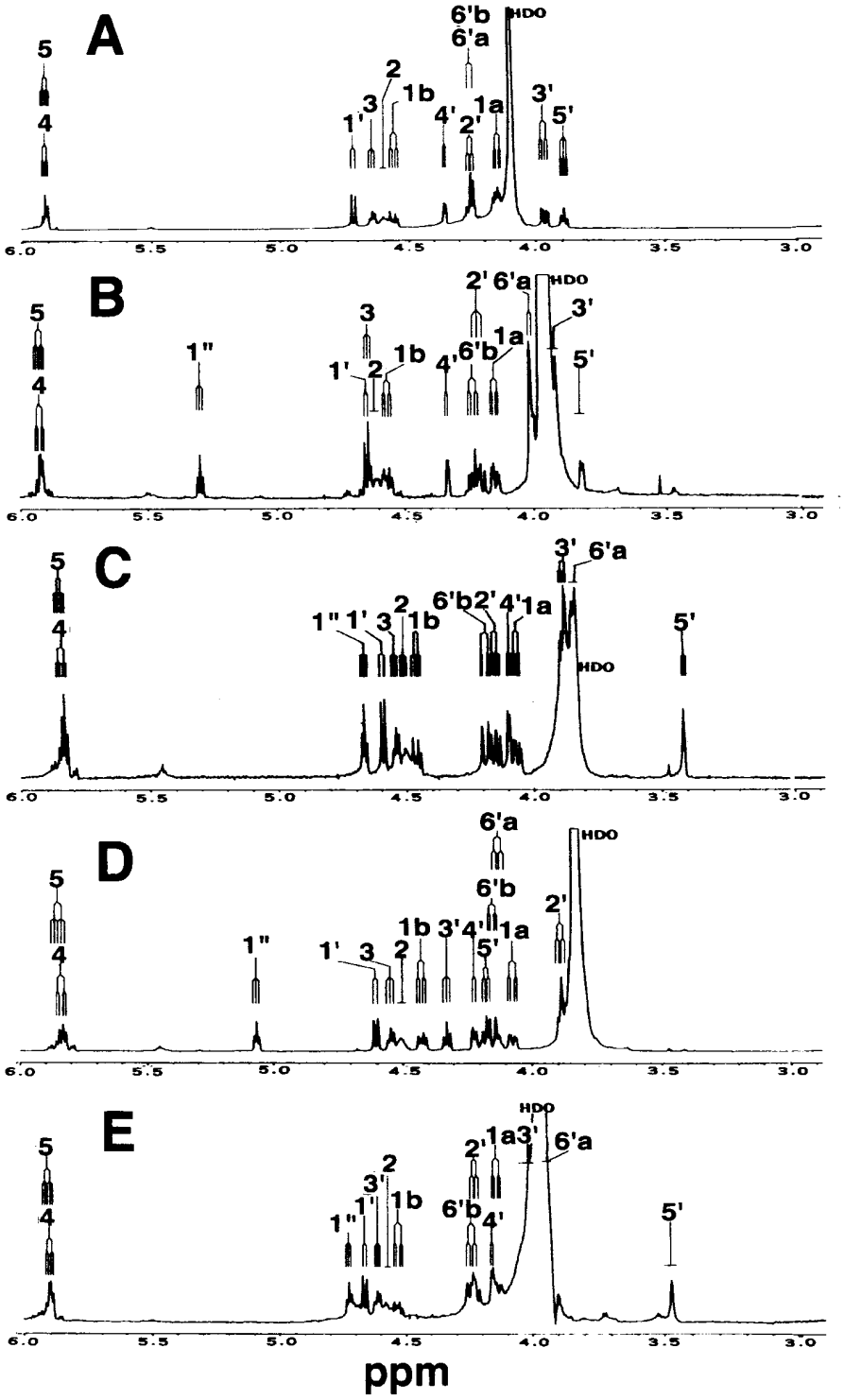

Fig. 2. Partial 1-D proton NMR spectra of plasmaloGalCers and GalCer. The NMR spectra were measured at 90°C in pyridine-d_5 containing 2% D_2O. Panels, A, B, C, D, and E indicate the spectra of GalCer, S-1, S-2, S-3, and N-1, respectively. The proton signal assigned was labeled with the numbering of the carbon atom demonstrated in Fig. 4.

hauser effect spectroscopy (NOESY). The H-1′′ of the acetal of S-1 was positioned close to H-2′ on Gal based on the appearance of an NOE peak (1′′–2′), and acetal H-2′′ nearby H-4' and H-6'a on Gal from peaks 2′′–4′ and 2′′–6′a, respectively, as shown in **Fig. 3A**. These NOE peaks involved a stereostructure of a cyclic acetal ring having an 'exo'-type hydrocarbon chain and consequently an equatorial orientation of H-1′′ of the acetal on a six-membered ring (1,3-dioxane ring) as illustrated by structure *1* in **Fig. 4**. On the other hand, H-1′′ of the acetal of S-2 had NOE peaks toward H-4′ and –6′a (Fig. 3B), indicating proximity of acetal H-1′′ and Gal H-4′ and H-6′a, and consequently

TABLE 5. Chemical shifts and coupling constants (Hz) of ring proton on galactoside and acetal proton of plasmaloGalCers and reference GalCer

	Chemical Shift (δ, ppm)									
	Galactoside							Acetal		
PlasmaloGalCer	H-1′	H-2′	H-3′	H-4′	H-5′	H-6′a	H-6′b	H-1′′	H-2′′	H-3′′
S-1	4.644	4.203	3.91	4.330	3.818	4.008	4.230	5.291	1.75	1.48
S-2	4.589	4.146	3.875	4.094	3.413	3.87	4.187	4.665	1.74	1.48
S-3	4.602	3.884	4.325	4.219	4.166	4.148	4.17	5.066	1.74	1.48
N-1	4.620	4.183	3.95	4.117	3.430	3.857	4.203	4.682	1.79	1.62
GalCer	4.705	4.240	3.951	4.341	3.882	4.226	4.238			
	Coupling Constant (Hz)									
	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{5',6'a}$	$J_{5',6'b}$	$J_{6'a,6'b}$	$J_{1'',2''}$		
S-1	7.4	7.9	3.5	<1.5	<1.5	4.9	11.8	5.4		
S-2	7.9	8.0	4.0	<1.5	<1.5	<1.5	12.3	4.9		
S-3	7.9	6.6	6.4	<1.5	4.0	3.5	10.3	4.5		
N-1	7.9	7.9	4.0	<1.5	ND	<1.5	12.3	4.9		
GalCer	7.4	10.0	3.5	<1.5	6.9	5.9	eq			

ND, not determined; eq, equivalent.

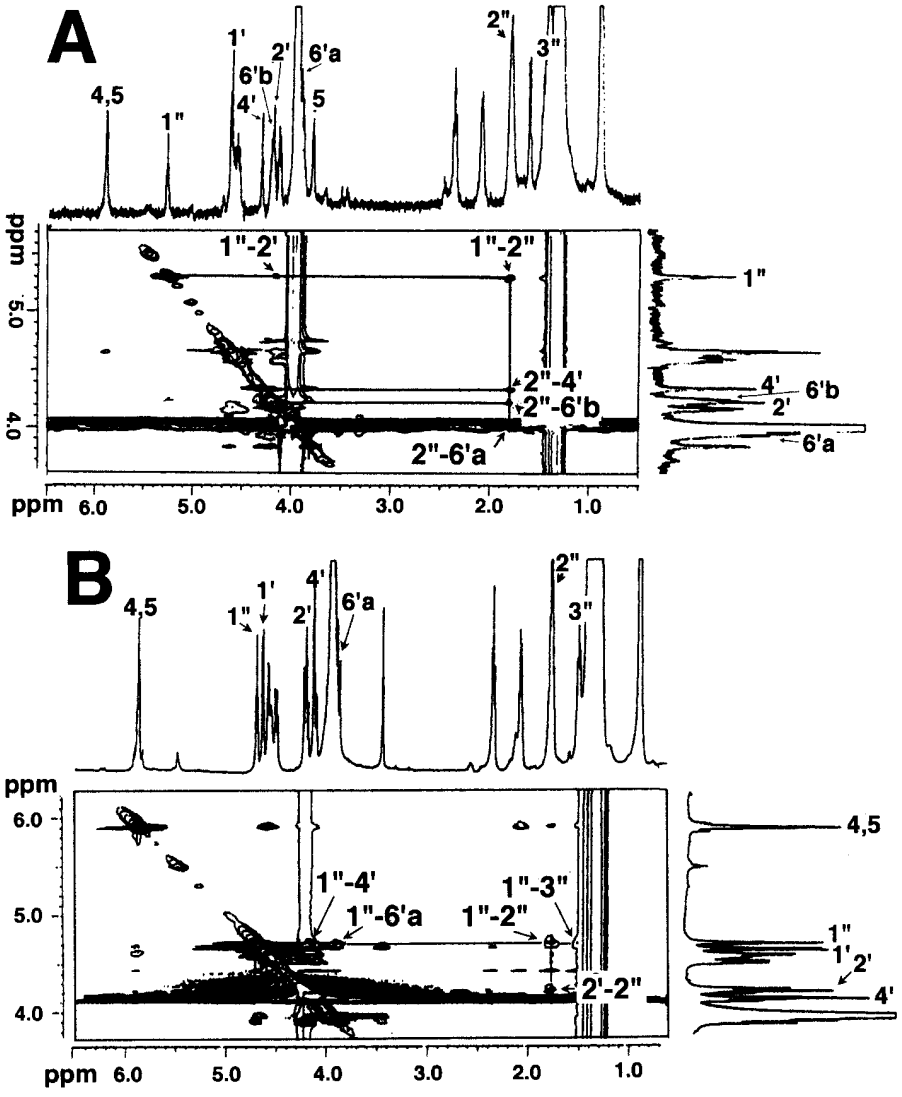

Fig. 3. Partial 2-D NOESY spectra of plasmaloGalCers. Panels A and B indicate spectra of S-1 and S-2, respectively.

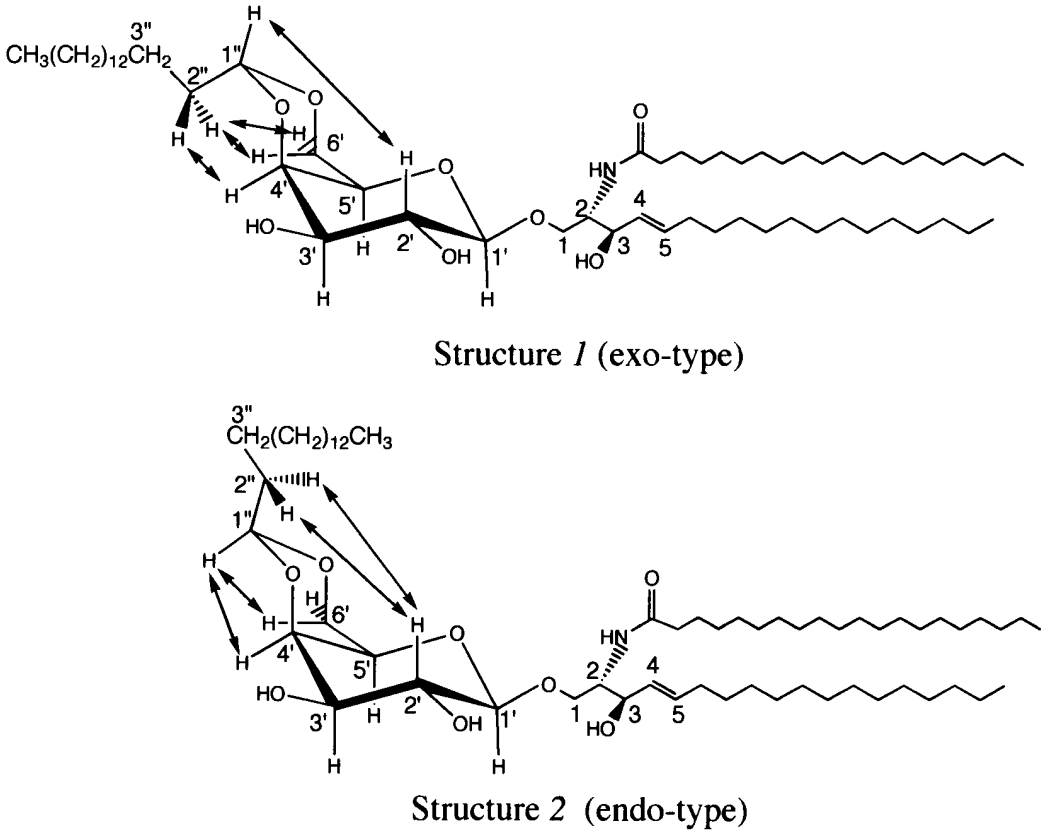

Fig. 4. Stereostructure of "exo"- and "endo"-type plasmaloGalCers. The NOE peak was observed in the 2-D spectra (Fig. 3) between protons indicated by arrow.

S-2 was an "endo"-type stereoisomer of the hydrocarbon chain having an axial H-1′′ of the acetal (structure *2* in Fig. 4). Of the two stereoisomers, S-1 and S-2, the 1-D and 2-D spectra of S-2 were most similar to those of N-1, indicating that N-1 and S-2 had isomeric structures identical to that of structure *2*.

The 1-D spectrum of S-3 demonstrated the presence of H-1′′ of the acetal (Fig. 2), coupled with H-2′′ and the H-2′′ sequentially with H-3′′ as observed in the 2-D spectrum (data not shown), just as in the cases of S-1 and S-2. The stereochemical assignment of the "endo"- or "exo"-type hydrocarbon chain, of S-3 with a cyclic acetal ring having a five-membered ring was, however, unsuccessful, as the NOE spectrum was very complex.

DISCUSSION

In the present paper, plasmaloGalCer having 4,6-*O*-hexadecylidene and -octadecylidene and their unsaturated derivatives was isolated from equine brain, and the stereoisomeric structure was characterized by NMR study as structure *2* with the "endo"-type in comparison to the stereoisomeric structures of chemically synthesized plasmaloGalCers. The synthesized 4,6-*O*-hexadecylidene derivative was chromatographically separated into two diastereomeric isomers of the structures *1* and *2*, S-1 and S-2, respectively, with long hydrocarbon chains of fatty aldehyde, forming the 1,3-dioxane structure of the cyclic acetal ring. Though the two stereoisomers of 3,4-*O*-hexadecylidene derivatives should also occur, one of these isomers was barely detected on TLC analysis, probably because of the much lower yield of the isomer. The configuration at acetal carbon, the asymmetric center of the synthesized 4,6-plasmaloGalCers, was effectively characterized by the NOESY spectrum as above, revealing the acetal proton (H-1′′) to be equatorial in S-1 and axial in S-2 in the 1,3-dioxane ring (see Fig. 4). These assignments were supported by previous data with different chemical shifts of H-1 of the acetal between equatorial and axial protons on a 1,3-dioxane ring, in which the equatorial proton resonated in a field lower than δ4.8 ppm whereas the chemical shift of the axial proton was δ4.5 to 4.7 ppm (22). The chemical shifts of the axial H-1 of the acetal of other plasmalo derivatives have also been observed comparably in the upper field with δ4.582 ppm (observed in $CDCl_3$–CD_3OD) in synthesized plasmalopsychosine (18) and with δ4.500 ppm ($CDCl_3$) of per-*O*-acetyl methyl β-galactopyranoside 4,6-*O*-hexadecylidene (19), in accordance with the above observations.

With respect to biological activity of plasmaloGSL, plasmalopsychosine has been reported to have a weak inhibitory effect on protein kinase C activity, and an enhancements of $p140^{trk}$ phosphorylation, mimicking activity of nerve growth factor on rat pheochromocytoma cells (23). These phenomena indicate that plasmalopsychosine has

important biological roles in the nervous system, though the biosynthetic pathway of the plasmalolipids is not yet known.

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REFERENCES

1. Hannun, Y. A., and R. M. Bell. 1989. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science.* **243:** 500–507.
2. Hakomori, S. 1990. Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J. Biol. Chem.* **265:** 18713–18716.
3. Klenk, E., and J. P. Lohr. 1967. On the ester cerebrosides of brain. *Hoppe-Seylers Z. Physiol. Chem.* **348:** 1712–1714.
4. Kishimoto, Y., M. Wajda, and N. S. Radin. 1968. 6-acyl galactosyl ceramides of pig brain: structure and fatty acid composition. *J. Lipid Res.* **9:** 27–33.
5. Tamai, Y. 1968. Further study on the faster running glycolipid in brain. *Jpn. J. Exp. Med.* **38:** 65–73.
6. Tamai, Y., K. Nakamura, K. Takayama-Abe, K. Uchida, T. Kasama, and H. Kobatake. 1993. Less polar glycolipids in Alaskan pollack brain: isolation and characterization of acyl galactosyl diacylglycerol, acyl galactosyl ceramide, and acyl glucosyl ceramide. *J. Lipid Res.* **34:** 601–608.
7. Gray, G. M., R. J. White, and J. R. Majer. 1978. 1-(3′-O-acyl)-beta-glucosyl-N-dihydroxypentatriacontadienoylsphingosine, a major component of the glucosylceramides of pig and human epidermis. *Biochim. Biophys. Acta.* **528:** 127–137.
8. Cheresh, D. A., R. A. Reisfeld, and A. P. Varki. 1984. O-acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant. *Science.* **225:** 844–846.
9. Thurin, J., M. Herlyn, O. Hindsgaul, N. Stromberg, K. A. Karlsson, D. Elder, Z. Steplewski, and H. Koprowski. 1985. Proton NMR and fast-atom bombardment mass spectrometry analysis of the melanoma-associated ganglioside 9-O-acetyl-GD3. *J. Biol. Chem.* **260:** 14556–14563.
10. Hakomori, S., and T. Saito. 1969. Isolation and characterization of a glycosphingolipid having a new sialic acid. *Biochemistry.* **8:** 5082–5088.
11. Gasa, S., A. Makita, and Y. Kinoshita. 1983. Further study of the chemical structure of the equine erythrocyte hematoside containing O-acetyl ester. *J. Biol. Chem.* **258:** 876–881.
12. Gasa, S., A. Makita, K. Yanagisawa, and M. Nakamura. 1984. Glycosphingolipids of equine erythrocytes membranes: complete characterization of a fucoganglioside. *Adv. Exp. Med. Biol.* **174:** 111–117.
13. Yachida, Y., K. Tsuchihashi, and S. Gasa. 1996. Characterization of novel mono-O-acetylated GM3s containing 9-O-acetyl sialic acid and 6-O-acetyl galactose in equine erythrocytes. *Glycoconj. J.* **13:** 225–233.
14. Yachida, Y., K. Tsuchihashi, and S. Gasa. 1997. Novel di-*O*-acetylated GM3s from equine erythrocytes one containing 4,9-*di*-*O*-acetyl *N*-glycolyl neuraminic acid and another containing 4-*O*-acetyl *N*-glycolyl neuraminic acid and 6-*O*-acetyl galactose. *Carbohydr. Res.* **298:** 201–212.
15. Suetake, K., K. Tsuchihashi, K. Inaba, M. Chiba, Y. Ibayashi, K. Hashi, and S. Gasa. 1995. Novel modification of ceramide: rat glioma ganglioside GM3 having 3-O-acetylated sphingenine. *FEBS Lett.* **361:** 201–205.
16. Levery, S. B., E. D. Nudelman, and S. Hakomori. 1992. Novel modification of glycosphingolipids by long-chain cyclic acetals: isolation and characterization of plasmalocerebroside from human brain. *Biochemistry.* **31:** 5335–5340.
17. Nudelman, E. D., S. B. Levery, Y. Igarashi, and S. Hakomori. 1992. Plasmalopsychosine, a novel plasmal (fatty aldehyde) conjugate of psychosine with cyclic acetal linkage. Isolation and characterization from human brain white matter. *J. Biol. Chem.* **267:** 11007–11016.
18. Sadozai, K. K., J. K. Anand, E. D. Nudelman, and S. Hakomori. 1993. Synthesis of plasmalopsychosines A and B two novel lysosphingolipids found in human brain. *Carbohydr. Res.* **241:** 301–307.
19. Sadozai, K. K., S. B. Levery, J. K. Anand, and S. Hakomori. 1996. Model compounds from plasmaloglycolipids: preparation of long chain cyclic acetals of methyl beta-d-galactopyranoside and determination of their regio- and stereochemistry by proton NMR. *J. Carbohydr. Chem.* **15:** 715–725.
20. Sako, F., S. Gasa, A. Makita, A. Hayashi, and S. Nozawa. 1990. Human blood group glycosphingolipids of porcine erythrocytes. *Arch. Biochem. Biophys.* **278:** 228–237.
21. Budzikiewicz, H., J. I. Brauman, and C. Djerassi. 1965. Massenspektrometrie und ihre anwendung auf strukturelle und stereochemische probleme-LXVII. *Tetrahedron*. **21:** 1855–1879.
22. Jackman, L. M., and S. Sternhell. 1969. Applications of nuclear magnetic resonance spectroscopy in organic chemistry. Pergamon Press, London.
23. Sakakura, C., Y. Igarashi, J. K. Anand, K. K. Sadozai, and S. Hakomori. 1996. Plasmalopsychosine of human brain mimics the effect of nerve growth factor by activating its receptor kinase and mitogen-activated protein kinase in PC12 cells. Induction of neurite outgrowth and prevention of apoptosis. *J. Biol. Chem.* **271:** 946–952.