Glycoconjugate J (1987) 4: 119-127

Ganglioside Lactones: ^{1}H-NMR Determination of the Inner Ester Position of G_{D1b}-Ganglioside Lactone Naturally Occurring in Human Brain or Produced by Chemical Synthesis

DOMENICO ACQUOTTI[1], GIOVANNI FRONZA[2], LAURA RIBONI[1], SANDRO SONNINO[1]* and GUIDO TETTAMANTI[1]

[1]*Study Center for the Functional Biochemistry of Brain Lipids, Department of Biological Chemistry, The Medical School, University of Milan, Via Saldini 50, 20133 Milano, Italy*
[2]*CNR Study Center for Natural Organic Substances, Department of Chemistry, Polytechnic of Milan, Milano, Italy*

Received January 16/March 13, 1987.

Key words: ganglioside lactones, NMR

The complete definition of the chemical structure of G_{D1b}-ganglioside (G_{D1b}) lactone isolated from human brain has been given by means of spectrometric and spectroscopic analyses. G_{D1b} lactone contains a single ester linkage involving the external sialic acid carboxyl group and the C-9 hydroxyl group of the internal sialic acid unit. A synthetic lactone of G_{D1b} prepared treating G_{D1b} with glacial acetic acid characterized in the same way showed an identical chemical structure.

Gangliosides are sialic acid-containing glycosphinglipids that reside in the outer leaflet of the plasma membranes of vertebrate cells [1]. They are assumed to play a role in a variety of surface events such as specific recognition of external ligands and biotransduction of membrane mediated information [2]. The sialic acid residue(s) of gangliosides may play a fundamental role in these events.

The sialic acid carboxyl groups are dissociated at physiological pH [3]. The presence of the negative charge enhances the hydrophilic character and introduces additional

Abbreviations: Ganglioside nomenclature is according to Svennerholm [16] and the IUPAC-IUB Recommendations [17]. G_{M1}, G_{M1}-ganglioside, II^{3}NeuAc-GgOse$_4$Cer, Galβ1-3GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glcβ1-1'Cer; G_{D1b}, G_{D1b}-ganglioside, II^3(NeuAc)$_2$GgOse$_4$Cer, Galβ1-3GalNAcβ1-4[NeuAcα2-8NeuAcα2-3]Galβ1-4Glcβ1-1'Cer; G_{D1b} lactone, G_{D1b}-L, Galβ1-3GalNAcβ1-4[NeuAc(1-9)α2-8NeuAcα2-3]Galβ1-4Glcβ1-1'Cer; Cer, ceramide; FAB-MS, fast atom bombardment-mass spectrometry; ^{1}H-NMR, proton nuclear magnetic resonance; 1D-NMR, one dimensional NMR; 2D-COSY, two dimensional correlated spectroscopy; DMSO-d_6, deuterated dimethylsulfoxide.

*Author for correspondence.

dipole moments in the ganglioside molecule [4], with consequences for both the steric conformation and the exposure of the oligosaccharide portion, which tends to protrude more from the membrane surface [5]. The negative charges may also be essential in the cation binding capacity [6], as well as in other interactions of gangliosides with either extramembrane ligands or intramembrane components. Under physiological conditions the negative charge of gangliosides could be eliminated with no change of pH or removal of sialic acid units, by means of a simple process of (enzyme-assisted?) lactonization. This would require one or more sialic acid carboxyl groups to form inner ester(s) with hydroxyl groups belonging to the same ganglioside molecule.

Ganglioside lactones have been shown to occur naturally in the nervous system of rodents [7] and in human brain [8], where they are relatively abundant. An inner ester of the disialoganglioside G_{D1b} (G_{D1b} lactone, G_{D1b}-L), in which the external sialic acid carboxyl group esterifies with one of the hydroxyl groups of the internal sialic acid was isolated and characterized [8] from the brains of 50 to 70 years-old human subjects. In this paper we demonstrate, by the use of conventional 1D-NMR and 2D-NMR spectroscopy, that the lactone ring of native G_{D1b} lactone involves the primary C-9 hydroxyl group of the internal sialic acid unit. Description of the chemical synthesis of G_{D1b}-L from G_{D1b} and of its characterization is also given.

Materials and Methods

Materials

All commercial chemicals were of the highest grade available and solvents were redistilled before use. Silica gel precoated thin layer plates (HPTLC, Kieselgel 60, 250 μm thick, 10 cm × 10 cm) were from Merck GmbH, Darmstadt, W. Germany. G_{D1b} was extracted from calf brain [9], purified to above 99% and structurally characterized as previously described [8]. *Vibrio cholerae* sialidase was purchased from Behringwerke, Marburg, W. Germany.

Extraction and Purification of G_{D1b} Lactone from Human Cerebral Cortex

Native G_{D1b} inner ester (in which the external sialic acid carboxyl group esterifies one of the hydroxyl groups of the internal sialic acid unit [8]), was isolated from the total ganglioside mixture obtained from specimens of normal human cerebral cortex. These specimens were obtained at the time of surgery from patients, aged 50-70 years and operated for different intracranial diseases. The characterization of brain specimens and the extraction, purification and chemical characterization of G_{D1b} lactone were performed as described previously [8]. 180 μg of purified compound were obtained from 3.1 g of cerebral tissues.

Preparation of G_{D1b} Lactone by Chemical Synthesis

Synthetic G_{D1b} lactone was prepared from G_{D1b} according to the procedure of McCluer and Evans [10]. Briefly, 5 mg portions of G_{D1b} were solubilized in chloroform/methanol, 2/1 by vol. The solutions were dried under vacuum at 37°C and maintained overnight

under high vacuum. Each sample was treated with 0.5 ml of glacial acetic acid, and allowed to stand at room temperature for 12 h. Then it was frozen and lyophilized. Each of these preparations was used for different chemical analyses, which gave the following results: a) this derivative behaved as a monosialoganglioside on ion-exchange chromatography, indicating that one sialic acid carboxyl group was free and dissociated; b) the reaction product was resistant to sialidase action, showing that the carboxyl group vicinal to the α-ketosidic linkage was not free; c) alkaline treatment (with NaOH) made the compound susceptible to sialidase treatment with the liberation of sialic acid and G_{M1}; d) ammonia treatment of the reaction product produced a compound containing sialic acid and sialic acid-amide in a molar ratio of 1:1, as shown by GLC-MS analysis, which means that the synthetically-obtained G_{D1b} derivative underwent ammoniolysis involving only one sialic acid residue, which was transformed in the corresponding amide and indicates that only one inner ester is present in the molecule; e) the FAB-MS spectrum showed pseudomolecular ions M-1, corresponding to the molecular species that contain C18 and C20 sphingosine, at m/z 1817 and 1845, 18 units below those of G_{D1b} measured under identical conditions, and the ions m/z 581 and 537 deriving from the disialosyl residue minus 18 (H_2O) and to 581 minus CO_2, respectively. Moreover, in the spectrum there are hardly any significant peaks at 308 and 290 m/z, corresponding to sialic acid. All these results are exactly the same as those obtained with natural G_{D1b}-L which have already been published [8]. For these reasons they have simply been summarized. They indicate that the product of G_{D1b} treatment with glacial acetic acid is a derivative containing a single inner ester between the carboxyl group of external sialic acid and a hydroxyl group of the internal sialic acid.

Analytical Methods

Thin layer chromatography, ion-exchange column chromatography, sialidase treatment, alkaline treatment, GLC-MS analysis and FAB-MS analysis were performed as previously described [8].

For NMR measurements, samples were carefully dried under high vacuum and then dissolved in DMSO-d_6, or DMSO-d_6/^2H_2O, 20/1 by vol.

The spectra in DMSO-d_6 for the identification of the amide protons were obtained at 300 MHz on a Bruker CXP 300 instrument equipped with an Aspect 2000 computer and operating in the Fourier transform mode with quadrature detection; the assignment of these protons was performed by using spin decoupling difference spectra (SDDS) and Nuclear Overhauser Effect (NOE) difference experiments.

Spectra after deuterium exchange were obtained on a Varian VXR 400. The conventional one dimensional spectrum of natural G_{D1b}-L, 180 μg, was obtained at 25-35°C, with 2000 transients. The two dimensional scalar correlated ^{1}H-NMR spectra, COSY, were recorded at 400 MHz using the 90°-t-90° pulse sequence, with a spectral width of 2200 Hz, an acquisition time of 233 ms and with an FT size of 1K × 1K.

Results and Discussion

We recently isolated from human cerebral cortex a lactone derivative of G_{D1b}-ganglioside (G_{D1b}-L), containing an inner ester linkage between the carboxyl group of

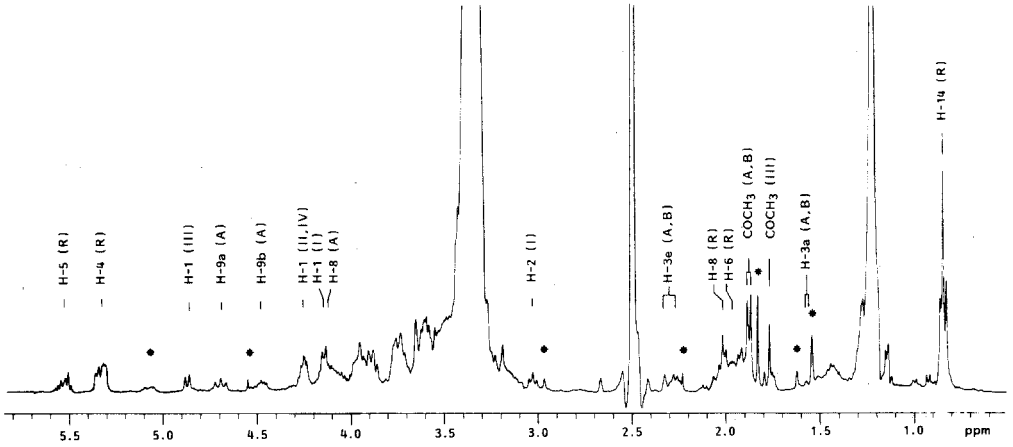

Figure 1. 400 MHz ^{1}H-NMR spectrum of natural G_{D1b}-L dissolved in DMSO-d_6:$^{2}H_2O$ and recorded at 35°C. Asterisks indicate signals deriving from contaminants. Proton designation, according to [15], is reported in Fig. 4.

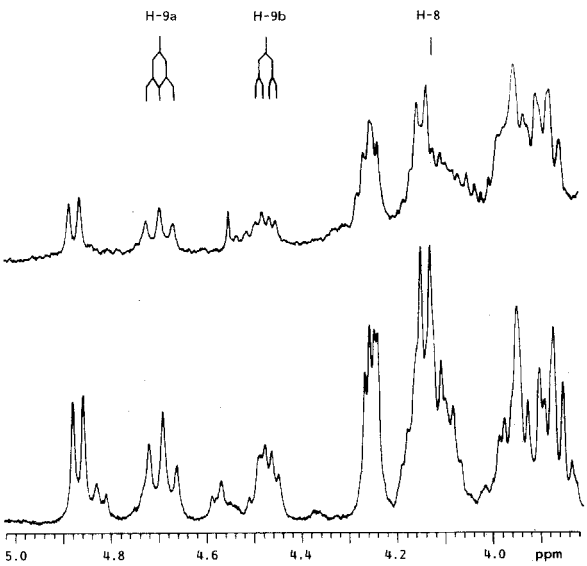

Figure 2. Comparison of the region between 3.8 and 5.0 ppm of a) natural G_{D1b}-L and b) semisynthetic G_{D1b}-L dissolved in DMSO-d_6:$^{2}H_2O$ at 35°C. Asterisks indicate signals due to a minor component in the solution of the semisynthetic G_{D1b}-L.

the external sialic acid and one of the hydroxyl groups of the internal sialic acid unit [8]. The linkage position remained to be established. This was accomplished with the present work using nuclear magnetic resonance spectroscopy.

The ^{1}H-NMR spectrum of natural G_{D1b}-L is shown in Fig. 1. In the upfield region of the spectrum the signals of the side-chain methyl and methylene protons at 0.85 and 1.23 ppm, the acetyl protons of *N*-acetylgalactosamine at 1.78 and of sialic acids at 1.88 and 1.89 ppm, and the α-carbonyl and the broad allylic methylene protons at 2.03 and 1.95 ppm, are clearly recognized. The H-3 methylene protons of the sialic acid at 1.55, 1.59 and 2.25, 2.32 ppm are partially obscured by the presence of impurities. In the low field part of the spectrum, besides the crowded region of the protons of the oligosaccharide-core between 3 and 4 ppm, the olefinic hydrogens at 5.34 and 5.53 ppm and the anomeric protons of *N*-acetylgalactosamine, of the galactose units and of glucose, at 4.87, 4.26, 4.24 and 4.14 ppm respectively, are found. In the region between 4 and 5 ppm, which is characteristic for the anomeric protons for all gangliosides, two new signals appear, a triplet and a doublet of doublets at 4.70 and 4.48 ppm, respectively. Since the acylation of a hydroxyl group deshields the α-proton(s) by 0.6-1.2 ppm [11-13] the new signals observed in the anomeric region should be due to the inner ester linkage present in G_{D1b}-L.

The spectrum contains several peaks deriving from contaminants not completely eliminated during ganglioside preparation. It is known that ganglioside lactones are very unstable. Therefore, purification of native G_{D1b}-L was carried out in the shortest way, omitting in particular the time consuming column-rechromatographic steps. This method protected the inner ester linkage from hydrolysis, but could not eliminate all the contaminants. In fact when G_{D1b}-L was carefully purified by repeated column-chromatography, the NMR peaks from contaminating impurities completely disappeared with a concomitant drastic decrease of the peak intensity corresponding to the inner ester linkage.

In order to obtain a detailed analysis of the spectrum of the lactonised part of the molecule and to remove the uncertainties due to contaminants we analyzed also the synthetic G_{D1b}-L, obtained from pure G_{D1b}. After 12 h treatment with glacial acetic acid G_{D1b} was completely converted into a major derivative (more than 90%) and a minor one. The most abundant derivative showed the same TLC behavior as natural G_{D1b}-L. Chemical analyses also indicated a similar structure (see the Methods section). Fig. 2 shows the region between 3.8 and 5.0 ppm of the ^{1}H-NMR spectra of the natural and synthetic G_{D1b}-L. In both spectra the signals deriving from the presence of the lactone ring show the same chemical shifts and coupling constants, which when also taking into account the chemical data presented in the Methods section, showed that the two compounds are identical. Some minor differences between the two spectra are recognizable and are due probably to the presence in the synthetic G_{D1b}-L sample of a second compound (less than 10%). It is known [10] that prolonged glacial acetic acid treatment of G_{D1b} leads to the formation of the dilactone derivative, which should give rise to the minor peaks present in the spectrum of synthetic G_{D1b}-L.

The proton-proton interconnections have been deduced from the two dimensional homonuclear chemical shift correlated spectrum shown in Fig. 3. Due to the complexity of the molecule, the region between 3 and 4 ppm is particularly crowded and only a limited number of chemical shifts, listed in Table 1, could be assigned. As shown in

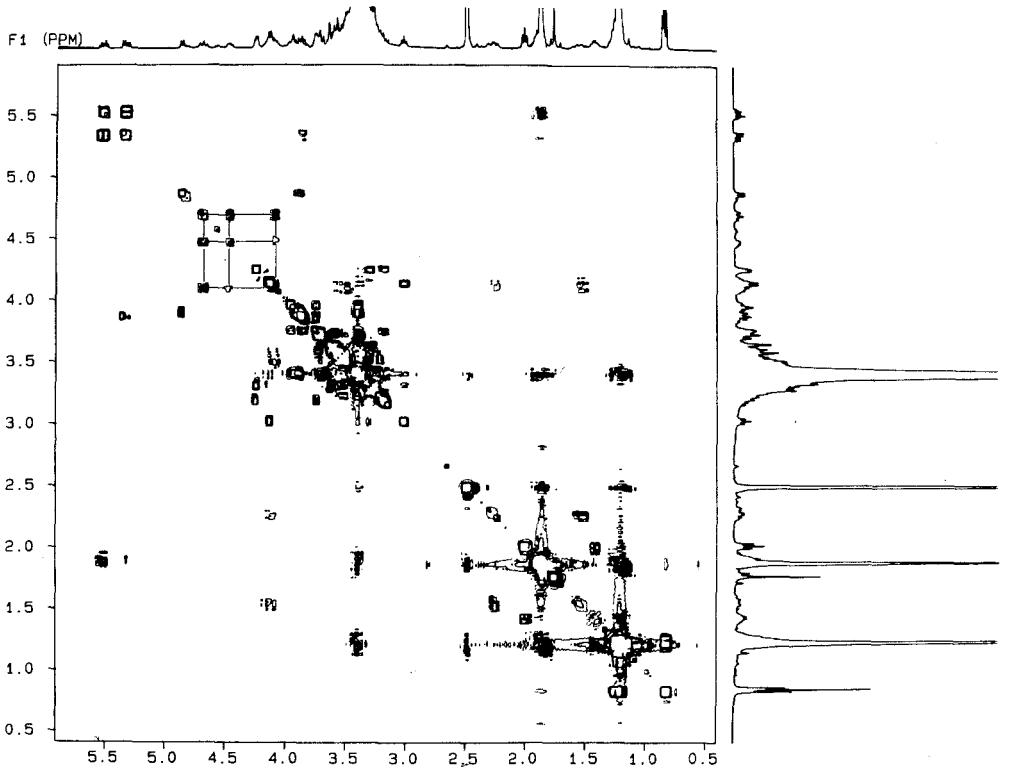

Figure 3. 400 MHz ^{1}H-NMR shift correlated 2D-NMR spectrum of the semi-synthetic G_{D1b}-L in DMSO-d_6:^2H_2O at 35°C. The strong peak at 1.87 ppm is due to a residue of sodium acetate. The solid lines indicate the interaction between the three spin system of the lactone ring.

Fig. 3, the new signals at 4.70 and 4.48 ppm are mutually coupled (J = 11.5 Hz) and are correlated with a third proton, occurring at 4.12 ppm with coupling constants of 11.5 and 6.0 Hz. This analysis clearly indicates that the two protons occurring at low fields belong to a primary alcoholic function. Since FAB-MS analysis showed (see also [8]) that the ester linkage involved the carboxyl group of external sialic acid and a hydroxyl group of internal sialic acid, the conclusion can be drawn that lactonization involves the hydroxyl group at position 9 of the internal sialic acid. This type of lactone linkage is the same already found in carbodiimide treated colominic acid [14].

The chemical shifts of CH_2OH groups for gangliosides dissolved in DMSO-d_6 usually range from 3.3 to 3.7 ppm [15]; thus lactonization induces a deshielding of about 1 ppm for these protons, which exceeds the low field effect observed for primary alcohols by acetylation [11] (about 0.5 ppm). This difference may be due to a change of several long-range interactions following the ring formation and the conformational rearrangement of the molecule. The values of the vicinal coupling constants of the lactone ring protons indicate that the hydrogen H-8 (A) and H-9a (A) (see Fig. 4) are in a trans diaxial orientation. Therefore, if we assume that the lactone ring has a chair conformation, and that

Table 1. Chemical shifts (ppm from tetramethylsilane) and coupling constants (Hz) for GD1b lactone in DMSO-d_6[a]. Proton designation, according to [15], is reported in Fig. 4.

Reporter group	Chemical shift	Coupling constants (J)	
H-3 (R)	3.85	J (3,4)	6.7
H-4 (R)	5.34	J (4,5)	15.5
H-5 (R)	5.53	J (5,6)	6.5
H-6 (R)	1.95	J (8,9)	7.2
H-8 (R)	2.03	J (2,NH)	8.8
H-10 (R)	1.23		
H-14 (R)	0.85		
NH (R)	7.50		
H-1 (I)	4.14	J (1,2)	7.6
H-2 (I)	3.03	J (2,3)	7.6
H-3 (I)	3.33		
H-1 (II)	4.24	J (1,2)	7.3
H-2 (II)	3.20		
H-1 (III)	4.87	J (1,2)	8.9
H-2 (III)	3.90	J (2,NH)	9.5
NH (III)[b]	9.16	J (2,3)	9.5
$COCH_3$ (III)	1.78		
H-1 (IV)	4.26	J (1,2)	7.5
H-2 (IV)	3.33		
H-3a (A or B)[c]	1.55, 1.59	J (3a, 3e)	13.0, 11.5
H-3e (A or B)[c]	2.25, 2.32	J (3a, 4)	11.5, 11.5
H-4 (A or B)[c]	4.14, 4.14	J (3e, 4)	5.0, 5.5
H-5 (A or B)[c]	3.70, 3.58	J (9a, 9b)	11.5
H-8 (A)	4.12	J (8, 9a)	11.5
H-9a (A)	4.70	J (8, 9b)	6.0
H-9b (A)	4.48	J (5,NH)	8.0, 8.5
NH (A or B)[b,c]	8.26, 8.05		
$COCH_3$ (A or B)[c]	1.88, 1.89		

[a] Estimated error ±0.01 ppm for chemical shifts and ±0.6 Hz for coupling constants.
[b] The chemical shifts of the amide protons of GalNAc and of sialic acid residues depend strongly on the water content of the solution.
[c] Resonances which were not assigned to A or B (see Fig. 4) sialic acid residues.

two chair conformations are theoretically possible, only that with an equatorial orientation of substituents at the C-8 (A) carbon applies to G_{D1b}-L.

In conclusion, this work gives complete definition of the structure of G_{D1b}-lactone found to occur in human and possibly other animal brain. The simple procedure for chemically preparing the same compound from G_{D1b} makes it easier to plan and carry out studies aimed at ascertaining the physico-chemical and behavioral changes undergone by a ganglioside like G_{D1b}, upon lactonization.

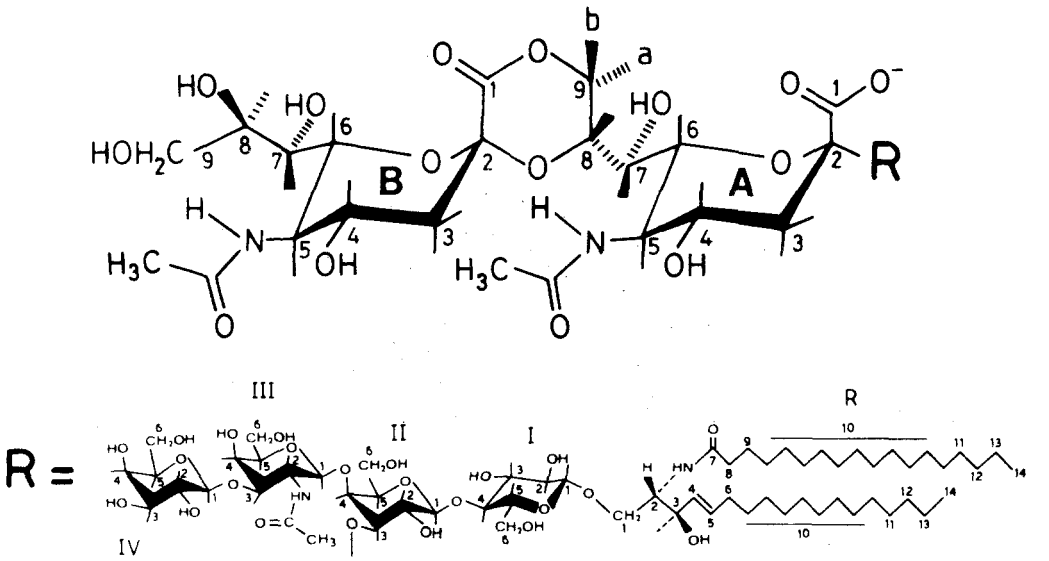

Figure 4. Proposed chemical structure for the G_{D1b} inner ester from human brain. The plane of the lactone ring is perpendicular to that of the two sialic acid units.

Acknowledgements

This work was supported in part by grants from the Italian Ministry of Education (MPI, 40% funds) and from the C.N.R. (Progetto Finalizzato Medicina Preventiva e Riabilitativa; Sottoprogetto Basi Molecolari Malattie Ereditarie, 86.00108.51). One of us (D.A.) is pleased to thank Fondazione Villa Rusconi, Varese, Italy, for financial support.

References

1 Wiegandt H (1982) Adv Neurochem 4:149-223.
2 Brady RO, Fishman PH (1979) Adv Enzymol 50:303-23.
3 Blix G, Lindberg E, Odin L, Werner I (1956) Acta Soc Med Upsalien 61:1.
4 Maggio B, Cumar FA, Caputto R (1975) Biochem J 175:1113-18.
5 McDaniel RV, McLaughlin A, Winiski AP, Eisenberg M, McLaughlin S (1984) Biochemistry 23:4618-24.
6 Behr JP, Lehn JM (1973) FEBS Lett 31:297-300.
7 Gross SK, Williams MA, McCluer RH (1980) J Neurochem 34:1351-61.
8 Riboni L, Sonnino S, Acquotti D, Malesci A, Ghidoni R, Egge H Mingrino S, Tettamanti G (1986) J Biol Chem 261:8514-19.
9 Tettamanti G, Bonali F, Marchesini S, Zambotti V (1973) Biochim Biophys Acta 296:160-70.
10 McCluer RH, Evans JE (1972) Adv Exp Med Biol 19:95-102.

11 Culvenor CCJ (1966) Tetrahedron Lett 1091.
12 Sonnino S, Kirschner G, Fronza G, Egge H, Ghidoni R, Acquotti D, Tettamanti G (1985) Glycoconjugate J 2:343-54.
13 Yu RK, Koerner TAW, Ando S, Yohe H, Prestegard JH (1985) J Biochem (Tokyo) 98:1367-72.
14 Lifely MR, Gilbert AS, Moreno C (1981) Carbohydr Res 94:193-203.
15 Koerner TAW, Prestegard JH, Demov PC, Yu RK (1983) Biochemistry 22:2676-87.
16 Svennerholm L (1964) J Lipid Res 5:145-55.
17 IUPAC-IUB Commision on Biochemical Nomenclature (1977) Lipids 12:455-68; (1982) J Biol Chem 257:3347-51.