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SYNTHESIS AND STRUCTURAL CHARACTERIZATION OF THE DILACTONE DERIVATIVE OF GD1a GANGLIOSIDE

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ABSTRACT

Treatment of GD1a {α-Neu5Ac-(2→3)-β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer} with dicyclohexylcarbodi-imide in anhydrous methyl sulfoxide affords 94–98% of GD1a-dilactone. The involvement of the carboxyl groups of the two sialic acid residues in the lactone rings was proved by ammoniolysis and reduction experiments, which gave ganglioside derivatives containing the amide of sialic acid and *N*-acetylneuraminulose, respectively. ^{1}H-N.m.r. spectroscopy showed that the lactone rings involved position 2 of each galactose residue in the ester linkages.

INTRODUCTION

Gangliosides, glycosphingolipids characterized by the presence of one or more residues of sialic acid, are normal components of the external lipid layer of plasma membranes and are abundant in the nervous system[1] where they may contribute to the process of signal transduction through the membrane[2]. Sialic acid and/or its carboxyl group may play a fundamental role in the above process. At physiological pH, the dissociation of the carboxyl group[3] increases the hydrophilic character and introduces additional dipole moments in the ganglioside molecule[4]. The negative charges are essential for the cation-binding capacity[5] and for other ganglioside interactions with extra-membrane ligands or intra-membrane components. Reduction in the number of negative charges of a ganglioside may modulate the expression of function. Under physiological conditions, the negative

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charge of gangliosides can be modified by lactonization without removal of sialic acid residues. Gangliosides occur naturally as lactones in the nervous system[6]. They are present in small amount in the brain of rodents[7,8], and are relatively abundant in some cerebral areas of aged patients[6]. Ganglioside lactones are unaffected by sialidase[6] and when used in the therapy of neurological diseases (see refs. 9 and 10 for reviews on the bio-medical potential of gangliosides) exert specific pharmacological effects[11,12].

The availability of ganglioside lactones is essential for *in vitro* and *in vivo* experiments to clarify the significance of lactonization. The isolation of ganglioside lactones by chromatography from the total lipid extract has disadvantages mainly due to their paucity in the tissue and lability in the media used for their extraction and purification[6,13]. We have reported[14] a chemical synthesis of the lactone of monosialoganglioside GM1 {β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer} and now report on the synthesis of a GD1a derivative which contains two lactone rings.

EXPERIMENTAL

Methyl sulfoxide and methanol were dried by distillation from calcium hydride and magnesium, respectively. Precoated thin-layer plates of Kieselgel 60 (Merck) were used for h.p.t.l.c., and Silica Gel 100 (0.063–0.2 mm, 70–230 mesh, ASTM) was used for column chromatography. Ion-exchange chromatography was carried out on G50W-8 (H^+) resin (100–200 mesh) (Bio-Rad). GD1a ganglioside was extracted from calf brain[15], purified to >99%, and characterized as described[16].

Preparation of GD1a dilactone (GD1a-2L). — A solution of GD1a (200 mg) in methyl sulfoxide (3 mL) was passed through a column (50 × 4 cm) of G50W-8 (H^+) resin, equilibrated and eluted with methyl sulfoxide. The elutate was stirred with dicyclohexylcarbodi-imide (60 mg) overnight at room temperature, then filtered, and treated with cold acetone (15 mL). A solution of the precipitate in chloroform–2-propanol (1:1, 1 mL) was diluted with cold acetone (15 mL), and the product was collected and dried under high vacuum.

Reactions on GD1a-2L. — (*a*) A solution of GD1a-2L (5 mg) in water (100 μL) was mixed immediately with aqueous sodium borohydride (1.75 mL, 60 mg/mL). After 10 min at room temperature, the solution was dialyzed, frozen, and lyophilized to give GD1a-2ol, R_{GD1a} 1.6 (t.l.c.; chloroform–methanol–acetic acid, 20:65:15).

(*b*) Dry ammonia was bubbled through a solution of GD1a-2L (5 mg) in dry chloroform–2-propanol (5 mL, 1:1) for 1 h at room temperature under continuous stirring. The mixture was then concentrated under high vacuum to give GD1a-amide, R_{GD1a} 1.4 (t.l.c. as above).

(*c*) Solutions of GD1a-2L, GD1a-2ol, and GD1a-amide severally in methanolic 0.05M hydrogen chloride[17] (1 mL) were kept for 1 h at 80° and then concentrated. A solution of each residue in trimethylsilylimidazole (30 μL) was

kept for 20 min at 60°, and then analyzed for the trimethylsilylated derivatives of *N*-acetylneuraminic acid, *N*-acetylneuraminamide, and *N*-acetylneuraminulose by g.l.c.–m.s. on a 25-m OV1 capillary column at 240° in a Varian Mat 112S instrument coupled to a PDP 11/34 data system.

^{1}H-N.m.r. spectroscopy. — 500-MHz spectra were recorded on solutions of dry samples in $(CD_3)_2SO$ or $(CD_3)_2SO/D_2O$ (20:1) at 35° with a Bruker AM instrument operating in the F.t. mode. Resonances were assigned using spin decoupling difference spectroscopy (SDDS) and 2D correlated spectroscopy (COSY).

T.l.c. and densitometry. — Gangliosides were analyzed by t.l.c., using chloroform–methanol–acetic acid (20:65:15) and detection with *p*-dimethylaminobenzaldehyde for 15 min at 120° (ref. 18). GD1a-2ol was identified by using the diphenylamine reagent[19] for 3–5 min at 90°. Quantification was effected by densitometry[20]. The molar absorption coefficient of the *p*-dimethylaminobenzaldehyde derivative of *N*-acetylneuraminulose was determined on GD1a-2ol purified by dialysis and column chromatography using chloroform–methanol–water (60:35:4). GD1a-2ol was quantified gravimetrically and by g.l.c. of the fatty acid content, using behenic acid[21] as the internal standard.

Colorimetric procedures. — GD1a- and GD1a-2L-bound sialic acid was determined by the resorcinol–HCl method[22,23].

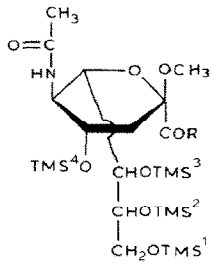
RESULTS AND DISCUSSION

The dilactone derivative of GD1a ganglioside was prepared in yields of 94–98% by treatment of the free acid form with dicyclohexylcarbodi-imide. The yield was based on analysis (t.l.c. and densitometry) of the stable product (GD1a-2ol) of borohydride reduction. This procedure was chosen since, during t.l.c. using chloroform–methanol–acetic acid (20:65:15) and several systems which contained methanol, GD1a-2L was partially hydrolyzed to GD1a and also converted into methyl ester derivatives.

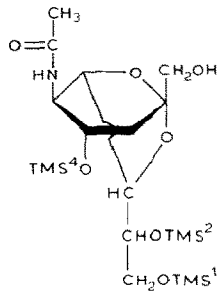
Methanolysis of GD1a-2L followed by trimethylsilylation and g.l.c.–m.s. revealed the presence of *N*-acetylneuraminic acid. Similar treatment of GD1a-2ol and GD1a-amide (obtained by ammoniolysis of GD1a-2L) revealed sialic acid derivatives with retention times of 1.75 and 1.9, respectively, relative to that of trimethylsilylated Neu5Ac. The main mass-spectral fragmentation pattern of these compounds confirmed data already reported[14,24], and were consistent with the *N*-acetylneuraminamide and *N*-acetylneuraminulose structures shown in Fig. 1. Thus, it is concluded that both the sialic acid carboxyl groups present in GD1a-2L are involved in lactone rings.

The position of the two lactone rings was established by n.m.r. spectroscopy, since conventional methylation analysis is not suitable for ganglioside lactones because of the strong alkaline conditions involved.

The region 3.0–4.0 p.p.m. of the n.m.r. spectra of gangliosides, which



1 R = OCH_3
2 R = NH_2



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m/z	R = OCH_3	R = NH_2	m/z	
M^+	625	610	M^+	565
$M^+ - [-CH_3]^+$	610	595	$M^+ - [-CH_3]^+$	550
$M^+ - [-COR]$	566	566	$M^+ - TMSOH$	475
$M^+ - [-CHOTMS^2 \mid CH_2OTMS^1]$	420	405	$M^+ - [-CH_2OTMS^1]$	462
$M^+ - [-CH_2OTMS^1, TMS^3OH, TMS^4OH]$	342	327	$M^+ - [-CHOTMS^2 \mid CH_2OTMS^1]$	360
$M^+ - [205, CH_3OH, TMS^4OH]$	298	283	$M^+ - [205, TMSOH]$	271
$M^+ - [-CHOTMS^3 \mid CHOTMS^2 \mid CH_2OTMS^1, -NHCOCH_3]$	259	244	TMS = Me_3Si	

Fig. 1. Main mass-spectral fragmentation pattern of trimethylsilyl derivatives of *N*-acetylneuraminic acid (**1**), *N*-acetylneuraminamide (**2**), and *N*-acetylneuraminulose (**3**), released from GD1a-2L, GD1a-amide, and GD1a-2ol, respectively, by mild methanolysis.

contains resonances for the ring protons other than anomeric, is crowded and difficult to analyze. Where the hydroxyl groups are esterified, the protons are deshielded by 0.6–1.4 p.p.m.[13,14,25,26] and appear in the region 4.0–5.0 p.p.m., which is more amenable to analysis.

The ^{1}H-n.m.r. spectra of GD1a and GD1a-2L are shown in Fig. 2, and the chemical shifts and coupling constants are reported in Table I. The assignments

TABLE I

^{1}H-N.M.R. DATA[a] FOR GD1a AND GD1a-2L (CHEMICAL SHIFTS IN P.P.M. FROM Me_4Si, J IN Hz)

Atom	*GD1a*			*GD1a-2L*			$\Delta\delta$[b]
	δ	J		δ	J		
H-1(I)[c]	4.15	$J_{1,2}$	7.7	4.15	$J_{1,2}$	7.7	
H-2(I)	3.03	$J_{2,3}$	8.5	3.03	$J_{2,3}$	9.0	
H-3(I)	3.35			3.37			
H-1(II)	4.26	$J_{1,2}$	8.0	4.61	$J_{1,2}$	7.7	0.35
H-2(II)	3.16	$J_{2,3}$	9.6	4.54	$J_{2,3}$	10.6	1.38
H-3(II)	3.75			4.08			0.33
H-1(III)	4.75	$J_{1,2}$	8.5	4.82	$J_{1,2}$	8.3	0.08
H-2(III)	3.93	$J_{2,3}$	10.0	3.54			−0.39
H-3(III)	3.55	$J_{3,4}$	2.5				
NH(III)	7.12	$J_{2,NH}$	9.5	7.52[d]			0.40
$COCH_3$(III)	1.76			1.91			0.15
H-1(IV)	4.29	$J_{1,2}$	7.8	4.52	$J_{1,2}$	7.6	0.23
H-2(IV)	3.27	$J_{2,3}$	9.8	4.64	$J_{2,3}$	10.4	1.37
H-3(IV)	3.91	$J_{3,4}$	3.0	3.88	$J_{3,4}$	2.5	−0.03
H-3*a*(A)	1.62	$J_{3e,3a}$	12.0	1.51[f]	$J_{3e,3a}$	13.4	−0.11
H-3*e*(A)	2.59	$J_{3e,4}$	5.0	2.35	$J_{3e,4}$	5.0	−0.24
H-4(A)	3.67	$J_{3a,4}$	11.5	~4.11	$J_{3a,4}$	11.0	0.34
H-5(A)	~3.40	$J_{NH,5}$	7.8	3.79			0.39
NH(A)	8.04[e]			8.04			
$COCH_3$(A)	1.87			1.87			
H-3*a*(B)	1.39	$J_{3e,3a}$	12.0	1.58[f]	$J_{3e,3a}$	13.4	0.19
H-3*e*(B)	2.71	$J_{3e,4}$	4.8	2.35	$J_{3e,4}$	5.0	−0.36
H-4(B)	3.53	$J_{3a,4}$	12.0	~4.11	$J_{3a,4}$	11.0	0.57
H-5(B)	~3.40	$J_{NH,5}$	8.0	3.57	$J_{NH,5}$	8.5	0.17
NH(B)	8.10[e]			8.15			0.05
$COCH_3$(B)	1.87			1.88			
H-2(R)	3.77			3.78			
H-3(R)	3.87	$J_{3,4}$	7.0	3.86	$J_{3,4}$	7.0	
H-4(R)	5.33	$J_{4,5}$	15.2	5.32	$J_{4,5}$	15.4	
H-5(R)	5.53	$J_{5,6}$	6.5	5.52	$J_{5,6}$	6.4	
H-6(R)	1.91			1.92			
H-8(R)	2.02			2.03			
H-10(R)	1.23			1.23			
H-14(R)	0.85			0.85			
NH(R)	7.49	$J_{NH,2}$	8.0	7.49	$J_{NH,2}$	9.0	

[a]For solutions in $(CD_3)_2SO$; estimated error ±0.01 p.p.m. for $\Delta\delta$ and ±0.6 Hz for J. [b]Differences (>0.02 p.p.m.) between the values for GD1a-2L and GD1a. [c]See formula **4** for designation of the units. [d]Broad signal. [e,f]Chemical shifts may be interchanged.

were based on the results of SDDS and COSY experiments, and comparison with data for simpler gangliosides and ganglioside lactones[14,26,27]. Thus, H-1 of GalNAc and H-2 of Glc (see **4** for proton nomenclature) resonate at ~4.8 and ~3.03 p.p.m., respectively. Assignment of the resonances belonging to the two Gal residues and the two Neu5Ac residues is more difficult. Koerner *et al.*[27] found, for the GM4 and GM3 gangliosides [α-Neu5Ac-(2→3)-β-Gal-(1→1)-Cer and α-Neu5Ac-(2→3)-β-

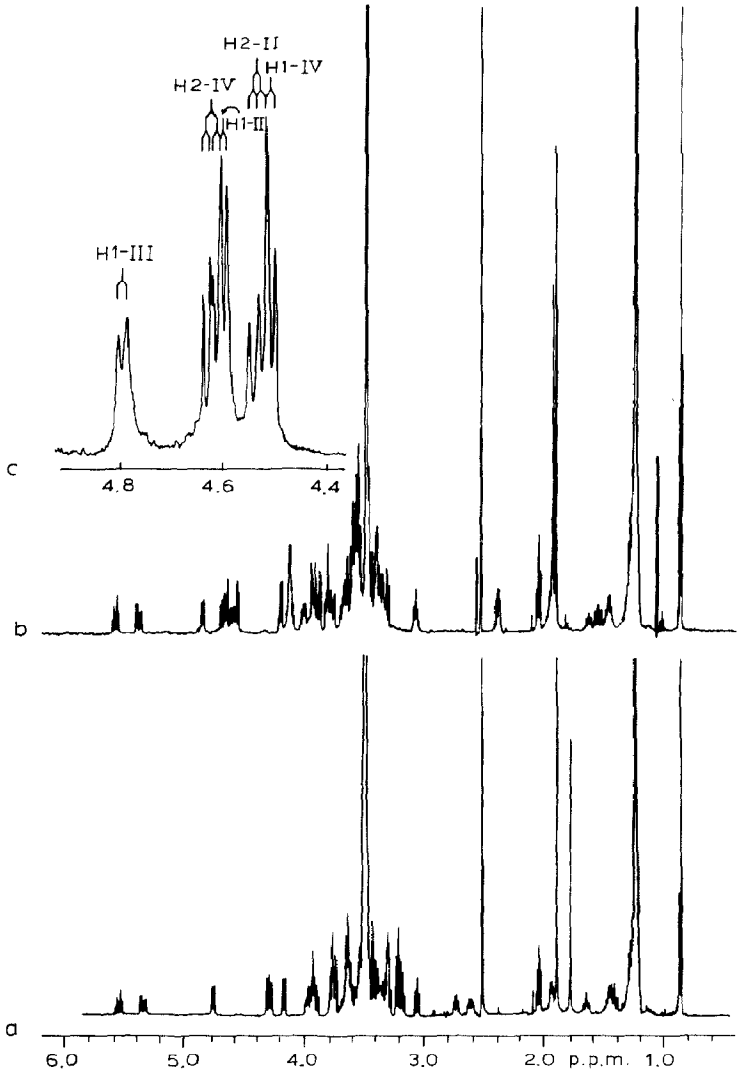


Fig. 2. 500-MHz ^{1}H-n.m.r. spectra of solutions of GD1a (a) and GD1a-2L (b) in $(CD_3)_2SO$–D_2O (20:1) at 35°. The inset (c) shows the region between 4.4–4.9 p.p.m. for GD1a-2L.

Gal-(1→4)-β-Glc-(1→1)-Cer, respectively], that the α-Neu5Ac residue induced a downfield shift of ~0.6 p.p.m. for the H-3 resonance of the β-Gal. This shift was reduced to ~0.2 p.p.m. when the Gal was linked to GalNAc as in GM2 {β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer} and GM1. Since GD1a can be derived by substitution of HO-3 of the terminal Gal (residue IV) by a sialic acid residue, the signal of H-3 of Gal-IV should be shifted downfield by 0.6 p.p.m. for GD1a with respect to GM1, as observed (3.31 p.p.m. for GM1 and 3.91

p.p.m. for GD1a). This assignment was confirmed by the observation that resonances of H-1,2,3 of Gal-II have practically the same chemical shifts in GM1 and GD1a. The resonances of H-3*a*,3*e*,4 of Neu5Ac are shifted significantly when it is linked to the terminal compared with the internal Gal[27] (*e.g.*, 2.53, 1.63, and 3.74 p.p.m. for GM1, and 2.75, 1.36, and 3.55 p.p.m. for GM3). As GD1a shows similar sets of chemical shifts, the inner (A) and external (B) Neu5Ac residues can be distinguished. The region 4.0–5.0 p.p.m. in the ^{1}H-n.m.r. spectrum of GD1a-2L contained signals for four anomeric protons and two new signals (dd) at 4.54 and 4.64 p.p.m., which must be due to the protons adjacent to the esterified hydroxyl groups. The COSY spectrum presented in Fig. 3 indicates that these protons are coupled to two anomeric protons (4.61 and 4.52 p.p.m., respectively). Moreover, the resonances of the anomeric protons of the Glc and GalNAc are identified at 4.15 and 4.82 p.p.m., respectively, and are correlated with those (3.03 and 3.54 p.p.m.) of H-2 belonging to the same sugar, showing that these two residues are not involved in lactone formation. Thus, the two hydroxyl groups involved in lactonization are located at position 2 of the inner and terminal Gal residues, the signals at 4.54 and 4.64 p.p.m. are due to H-2 of these residues, and the structure **4** is assigned to GD1a-2L.

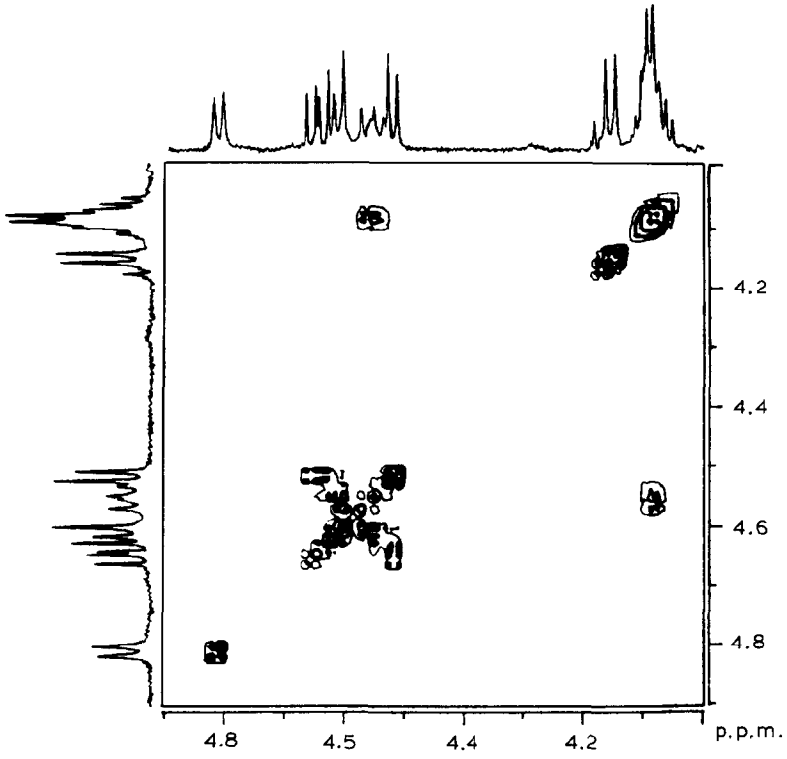
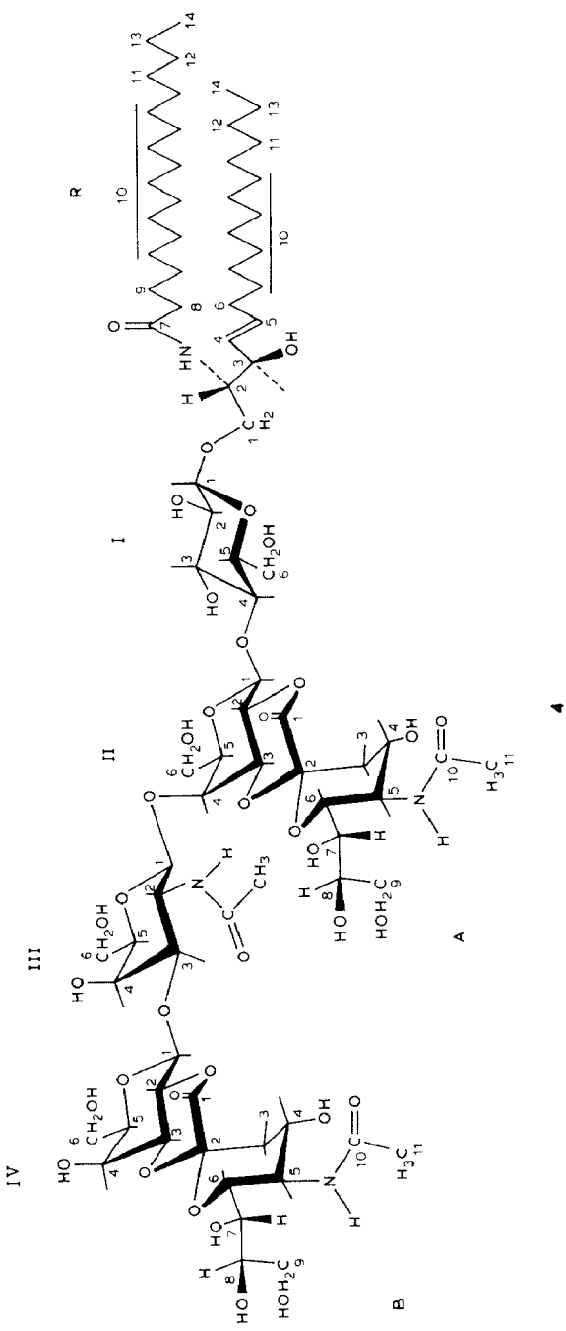


Fig. 3. The 4.0–4.9 p.p.m. region of the 500-MHz ^{1}H-n.m.r. shift-correlated spectrum of a solution of GD1a-2L in $(CD_3)_2SO$–D_2O (20:1) at 35°.



The assignment of the resonances to a specific galactose residue was based on a comparison with data for simpler ganglioside lactones, such as the synthetic GM3-L[26], GM1-L[14], and GD1b-L, the natural monolactone of GD1b found in human brain[13]. GM3-L and GM1-L have the same kind of lactone linkage found in GD1a-2L. Examination of the published data revealed that the lactonization shift displayed by H-3 is sufficiently different to distinguish between Gal-II and Gal-IV. After lactonization, the H-3(II) of GM1 (internal residue) is deshielded by ~0.33 p.p.m., whereas the H-3(II) of GM3 (terminal residue) is unaffected. Similar behaviour is observed for H-3 of the two Gal residues in GD1a, thus allowing, through the COSY experiments, the internal and terminal Gal residues to be distinguished. The resonances of the Neu5Ac residues of GD1a-2L were similar: only those of H-5 differed by 0.22 p.p.m. The lower-field resonance at 3.79 p.p.m. was similar to that (3.75 p.p.m.) for H-5 in the GM1 inner ester (3.75 p.p.m.) and the higher-field resonance at 3.57 p.p.m. was similar to that (3.53 p.p.m.) found for GM3 lactone. On this basis, the protons of inner and external Neu5Ac were distinguished tentatively.

Several studies[27,28,29] have established the existence of a through-space interaction of the GalNAc and Neu5Ac residues in the most complex gangliosides. Such a secondary structure is stabilized by van der Waals forces between the two saccharides[29] and produces variations in the chemical shift of ^{1}H and ^{13}C resonances of the same sugar residue. These effects greatly facilitate the distinction between the two Neu5Ac residues in GD1a ganglioside.

The lactonization is expected to induce major modifications of the secondary structure due to the reorientation of the Neu5Ac residue. The $\Delta\delta$ values reveal that the resonances of the protons of GalNAc are shifted markedly, although this residue is not modified structurally. Moreover, the proton chemical shifts of the two Neu5Ac residues of GD1a-2L are much more similar than that found in GD1a. These observations indicate that the through-space interactions originally present in GD1a are modified considerably in the lactone derivative. Determination of the presence or absence of different through-space interactions in the lactone structure requires complete assignment of the spectrum of GD1a-2L.

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