SYNTHESIS, STRUCTURE, AND CONFORMATION OF THE DILACTONE DERIVATIVE OF GD1b GANGLIOSIDE

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ABSTRACT

Treatment of GD1b, β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→8)-α-Ncu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer, with dicyclohexylcarbodi-imide in anhydrous methyl sulfoxide affords 95–98% of GD1b-dilactone. The carboxyl groups of the two sialic acid units are involved in ester linkages, as proved by ammoniolysis and reduction which gave derivatives containing the amide of sialic acid and *N*-acetylneuraminulose, respectively. ^{1}H-N.m.r. spectroscopy showed that the lactone rings involved position 9 of the inner sialic acid and position 2 of the inner galactose and that the disialosyl chain is extended toward the -β-Gal-(1→4)-β-Glc- portion of the ganglioside moiety.

INTRODUCTION

Gangliosides, glycosphingolipids characterized by the presence of one or more units 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

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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 charge of gangliosides can be modified by lactonization. Gangliosides occur as lactones in the nervous system[6], in small amount in the brain of rodents[7,8], and relatively abundantly in some cerebral areas of aged patients[6]. A small amount has been found in melanoma cells[9]. Ganglioside lactones are unaffected by sialidase[6] and, when used in the therapy of neurological diseases[10,11], exert specific pharmacological effects[12,13].

The availability of ganglioside lactones is essential for *in vitro* and *in vivo* experiments in order 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 their lability in the media used for their extraction and purification[6,14]. We have reported chemical syntheses of the lactone of monosialoganglioside GM1[15], β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer, of the dilactone of GD1a[16], 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, and the monolactone of GD1b[14], and now report on the synthesis of a GD1b derivative that contains two lactone rings.

EXPERIMENTAL

Methyl sulfoxide and methanol were dried by distillation from CaH_2 and Mg, respectively. H.p.t.l.c. was performed on Kieselgel 60, and column chromatography on Silica Gel 100 (0.063–0.2 mm, 70–230 mesh, ASTM, Merck). Ion-exchange chromatography was carried out on G50W-8 (H^+) resin (100–200 mesh, Bio-Rad). GD1b ganglioside was extracted from pig brain[17], purified to >99%, and characterized as described[18].

Preparation of GD1b dilactone (GD1b-2L). — A solution of GD1b (100 mg) in methyl sulfoxide (1.5 mL) was passed through a column (50 × 4 cm) of G50W-8 (H^+) resin, equilibrated, and eluted with methyl sulfoxide. The eluate was stirred with dicyclohexylcarbodi-imide (30 mg) at room temperature overnight, then filtered, and treated with cold acetone (10 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. Yield, 98%.

Reactions on GD1b-2L. — (*a*) A solution of GD1b-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 GD1b-2ol, R_{GD1b} 1.88 (t.l.c., chloroform–methanol–aq. 0.2% $CaCl_2$, 50:42:11).

(*b*) Dry ammonia was bubbled through a stirred solution of GD1b-2L (5 mg) in dry chloroform–2-propanol (5 mL, 1:1) at room temperature for 1 h. The mixture was then concentrated under high vacuum to give GD1b-amide, R_{GD1b} 1.64.

(c) Solutions of GD1b-2L, GD1b-2ol, and GD1b-amide severally in methanolic 0.05M hydrogen chloride[19] (1 mL) were kept for 1 h at 80°, then concentrated. A solution of each residue in trimethylsilylimidazole (30 μL) was kept at 60° for 20 min, 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 OV-1 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 40°, using a Bruker AM instrument operating in the F.t. mode. The assignment of the resonances was determined by COSY, long-range COSY, and triple quantum-filtered COSY experiments (the latter for the protons in $CHCH_2OH$ groups). Dipolar connections were determined by ROESY experiments performed using the sequence $90–t_1–90–\Delta–(90–SL–90)–\Delta–90–acq(t_2)$, where SL is a train of short pulses for spin lock and Δ is a z-filter to avoid scalar coupling cross-peaks[20]. The spin-lock length was 240 ms, the RF carrier frequency was offset away from the region of sugar proton resonances during the spin-lock time, and the RF power was lowered (2980 Hz) to avoid HOHAHA coherence transfer artifacts. Higher RF field-strength and RF offset in the middle of the spectrum before and after mixing time were used to maximize spectral resolution and to avoid resonance offset effects. The spectrum was recorded with a spectral width of 3000 Hz and 80 transients for each t_1 value. A shifted-sine bell ($\pi/4$) was applied in both directions. The ROESY method is particularly suitable for molecules that have a motional correlation time τ_c near the condition $\omega\tau_c = 1$, where ω is the angular Larmor frequency. The laboratory-frame n.O.e. effect is then near zero, whereas the rotating-frame n.O.e. is always positive and increases for increasing values of τ_c[21]. Moreover, ROESY avoids spin diffusion which, in the conventional NOESY experiments, produces cross-peaks that simulate non-existent spatial interactions and allows cross-peaks due to proton exchange to be recognized since they have opposite phase with respect to direct n.O.e.'s.

T.l.c. and densitometry. — Gangliosides were analyzed by t.l.c., using chloroform–methanol–aq. 0.2% $CaCl_2$ (50:42:11) and detection[22] with p-dimethylaminobenzaldehyde for 15 min at 120°. GD1b-2ol was identified using the diphenylamine reagent[23] for 3–5 min at 90°. Quantification was effected by densitometry[24]. The molar absorption coefficient of the p-dimethylaminobenzaldehyde derivative of N-acetylneuraminulose was determined on GD1b-2ol purified by dialysis and column chromatography, using chloroform–methanol–water (60:35:5). GD1b-2ol was quantified gravimetrically and by g.l.c. of the fatty acid content, using behenic acid[25] as the internal standard.

Colorimetric procedures. — GD1b- and GD1b-2L-bound sialic acid were determined by the resorcinol–HCl method[26,27].

RESULTS AND DISCUSSION

Treatment of gangliosides with dicyclohexylcarbodi-imide in anhydrous methyl sulfoxide, an effective procedure for the production of lactones[15,16], gave 96–98% of the dilactone of GD1b ganglioside. Yields were based on analysis (t.l.c. and densitometry) of the stable product (GD1b-2ol) of borohydride reduction. This procedure[16,28] was necessary since, in t.l.c. with several solvent systems that contain water and methanol, GD1b-2L was partially hydrolyzed to GD1b-L and GD1b and also converted into methyl ester derivatives.

Methanolysis of GD1b-2ol and GD1b-amide (obtained by ammoniolysis of GD1b-2L) followed by trimethylsilylation and g.l.c.–m.s. revealed N-acetyl-neuraminulose and N-acetylneuraminamide[15,16,28,29]. The N-acetylneuraminic acid was present only in traces and was derived probably from contamination with GD1b. Thus, both the sialic acid residues in GD1b-2L are involved in lactone rings.

The ¹H-n.m.r. and the COSY data of GD1b-2L are shown in Table I. Studies of gangliosides and ganglioside lactones[14–16,30,31] provided data on H-1 of Glc, Gal, and GalNAc residues, and H-3a,3e of the Neu5Ac residues. The H-3a protons of Neu5Ac (see Fig. 1 for the proton code) of GD1b-2L resonate at 1.30 and 1.53 p.p.m. The latter can be assigned to the inner Neu5Ac residue, since its chemical shift is similar to that of the H-3a resonance of GM1-L[15]. Moreover, esterification produces a marked downfield shift (1.0–1.5 p.p.m.) of the resonance of the proton α to the esterified hydroxyl group[32]. Thus, the resonances of these protons, in GD1b-2L, are expected to move from 3–4 p.p.m. to 4–5 p.p.m., thereby allowing an easy identification of the protons belonging to the lactone rings.

The data in Table I indicate that, besides H-1, the resonances of H-2 of Gal-II and H-9a,9b of the Neu5Ac-A residues have chemical shifts in the region 4.1–4.9 p.p.m. These resonances are expected to occur at 3.1–3.6 p.p.m. for native gangliosides[30]. Thus, the strong deshielding must be due to the two lactone rings. The formation of lactones involving the COOH-(B) and OH-9(B), COOH-(A) and
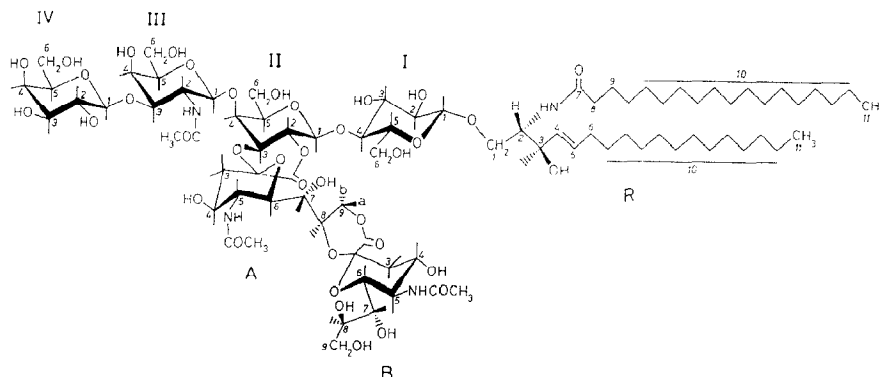

Fig. 1. Proposed structure for GD1b-2L.

OH-9(B), and COOH-(B) and OH-2(II) is probably energetically and kinetically unfavoured with respect to the δ-lactones, and implies marked variations of the conformation of the sialic acid side-chain, not found in GD1b-2L (see below).

TABLE I

^{1}H-N.M.R. DATA FOR GD1b-2L[a]

	Glc (I)	Gal (II)	GalNAc (III)	Gal (IV)	Neu5Ac (A)	Neu5Ac (B)	Cer (R)
Chemical shifts (p.p.m.)							
H-1	4.15	4.59	4.85	4.18			
H-1a							3.44
H-1b							3.99
H-2	3.04	4.51	3.44	3.32			3.78
H-3	3.32	4.11	4.05	3.26	1.53(*a*) 2.38(*e*)	1,30(*a*) 2.35(*e*)	3.89
H-4	3.43	4.08	3.84	3.63	4.18	4.18	5.36
H-5	3.29	3.56	3.39	3.45	3.67	3.52	5.54
H-6					3.47	3.32	1.93
H-6a	3.73	*b*	3.50	*b*			
H-6b	3.54	*b*	3.60	*b*			
H-7					3.45	*b*	1.32
H-8					4.03	3.34	2.03
H-9a					4.74	3.60	1.46
H-9b					4.57	3.42	
NH			7.17		8.04	8.16	7.43
COCH₃			1.88		1.91	1.88	
H-10							1.23
H-11							0.85
Coupling constants (Hz)							
$J_{1,2}$	8.0	7.8	8.0	7.5			
$J_{1a,2}$							3.7
$J_{1b,2}$							4.2
$J_{1a,1b}$							10.2
$J_{2,3}$	8.2	10.5	10.5	9.5			7.5
$J_{2,NH}$			8.0				
$J_{3,4}$	*b*	3.0	3.2	3.0	11.0(*a*, 4) 5.5(*e*, 4)	11.0(*a*, 4) 5.5(*e*, 4)	7.0
$J_{3a,3e}$					13.5	13.0	
$J_{4,5}$	*b*	<1.2	<1.2	<1.2	10.0	10.0	15.2
$J_{5,6a}$	<2.0	*b*	6.0	*b*			6.5
$J_{5,6}$					10.0	10.0	
$J_{5,NH}$					8.5	8.5	
$J_{5,6b}$	6.0	*b*	6.0	*b*			6.5
$J_{6a,6b}$	11.5	*b*	11.0	*b*			*b*
$J_{6,7}$					1.8	1.5	*b*
$J_{7,8}$					9.0	10.0	*b*
$J_{8,9a}$					11.5	2.0	*b*
$J_{8,9b}$					5.0	6.0	*b*
$J_{9a,9b}$					11.5	11.0	*b*

[a]Internal Me₄Si, J ±0.6 Hz, (CD₃)₂SO–D₂O (98:2), 40°. [b]Not determined.

Therefore, the COOH group of the inner Neu5Ac and OH-2 of the inner Gal, and the COOH of the outer Neu5Ac and OH-9 of the inner Neu5Ac are involved in the lactones. The same type of linkage has been found for the GM3[31], GM1[15], and GD1b[14] monolactones, and for GD1a[16] and GD3[33] dilactones.

The J values and n.O.e. effects reveal conformational features of the oligo-saccharide moiety of GD1b-2L. The pyranose rings in Glc, Gal, and GalNAc exist in the $^4C_1(D)$ conformation, and in the $^2C_5(L)$ form for the Neu5Ac. For the inner Neu5Ac residue, the $J_{6,7}$ and $J_{7,8}$ values of 1.8 and 9.0 Hz, respectively, are similar to those reported for the native gangliosides, so that lactonization does not effect the conformation of the C-6,7,8 moiety. The conformation of the Neu5Ac side-chain has been studied extensively[34], and, on the basis of the J values and n.O.e. effects, it was shown that H-7 is in close proximity to H-5 and makes a dihedral angle of 90° with H-6.

The J values give little information on the conformation of the lactone rings since only two carbons are protonated. Additional information can be obtained from the n.O.e. effects. For δ-lactones, the atoms C–CO–O–C tend to be coplanar and the ring has a preference for the half-chair conformation[35–37], but can adopt boat[38,39] or skew[40] conformations.

Inspection of Dreiding stereomodels reveals that, for a half-chair conforma-tion for the inner lactone ring of the GD1b-2L, H-3(II) and H-3e(A) are in close proximity (<2 Å). Thus, there should be a strong n.O.e. effect, which was not found. However, there was a n.O.e. between H-2(II) and H-8(A), which, in the above conformations, are separated by ~5 Å.

Provided that H-2(II) and H-3(II) are *trans*-diaxial, only three conforma-tions, in addition to the chair and half-chair mentioned above, are allowed for the lactone ring with distances between H-8(A) and H-2(II) of 2–3 Å, namely, the $B_{2(II),2(A)}$ (**1**), $^{1(A)}S_{2(II)}$ (**2**), and $^{3(II),1(A)}B$ (**3**). The present qualitative approach does not predict the preferred conformation.

$B_{2(II),2(A)}$ $^{1(A)}S_{2(II)}$ $^{3(II),1(A)}B$

1   **2**   **3**
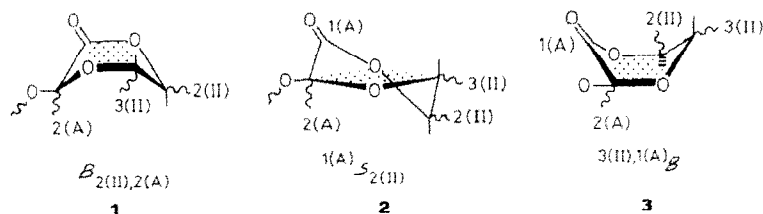
Similar considerations can be applied to the lactone ring which links the two Neu5Ac residues. Thus, H-8(A) and H-9a(A) must be *trans*-diaxial, as dictated by the $J_{8,9a}$ value of 11.5 Hz. In the $^{9(A)}C_{2(B)}$ and the half-chair $^{O1(B)}H_{8(A)}$ conformations, H-8(A) and H-3e(B) are in close proximity but there was no n.O.e. However, there was a n.O.e. effect between H-9a(A) and H-6(B). The other possible confor-mations, which leave H-9a(A) and H-8(A) *trans*-diaxial, and in which H-9a(A) and

H-6(B) are sufficiently close to give a n.O.e. effect, are the $^{9a(A),2(B)}B$, $B_{8(A),1(B)}$, and $^{9(A)}S_{1(B)}$ forms.

The above information on conformations indicates that the disialyl chain in GD1b-2L is extended toward the -β-Gal-(1→4)-β-Glc- portion.

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