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Nuclear Overhauser effect investigation on GM1 ganglioside containing *N*-glycolyl-neuraminic acid (II^3Neu5GcGgOse$_4$Cer)

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The conformational properties of the oligosaccharide chain of GM1 ganglioside containing *N*-glycolyl-neuraminic acid, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Gc-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer, were studied through NMR nuclear Overhauser effect investigations on the monomeric ganglioside in dimethylsulfoxide, and on mixed micelles of ganglioside and dodecylphosphocholine in water. Several interresidual contacts for the trisaccharide core -β-GalNAc-(1-4)-[α-Neu5Gc-(2-3)]-β-Gal- were found to fix the relative orientation of the three saccharides, while the glycosidic linkage of the terminal β-Gal- was found to be quite mobile as the β-Gal-(1-3)-β-GalNAc- disaccharide exists in different conformations. These results are similar to those found for two GM1 gangliosides containing *N*-acetyl-neuraminic acid and neuraminic acid [1].

Keywords: gangliosides, *N*-glycolyl-neuraminic acid, NMR, conformation, nOe

Abbreviations: Ganglioside nomenclature is in accordance with Svennerholm [23] and the IUPAC-IUB Recommendations [24]. GM3(Neu5Ac), II^3Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM3(Neu5Gc), II^3Neu5GcLacCer, α-Neu5Gc-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1(Neu5Ac), II^3Neu5AcGgOse$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1(Neu5Gc), II^3Neu5GcGgOse$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Gc-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1(Neu), II^3NeuGgOse$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD1a, IV^3Neu5AcII^3Neu5AcGgOse$_4$Cer, 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; GalNAc-GD1a, IV^4GalNAcIV^3Neu5AcII^3Neu5AcGgOse$_4$Cer, β-GalNAc-(1-4)-[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; Neu, neuraminic acid; Neu5Ac, *N*-acetyl-neuraminic acid; Neu5Gc, *N*-glycolyl-neuraminic acid; Cer, ceramide.

Introduction

Gangliosides [2], sialic acid containing glycosphingolipids, are components of mammalian cell plasma membranes and form a heterogeneous family of compounds that differ in their sialic acid-, neutral oligosaccharide chain- and ceramide structures. They appear to play a role in specific interaction processes and in regulating membrane enzyme activities and cell functions [3–4]. The varying degree of ganglioside involvement is probably due to specific ganglioside-protein interaction within the membrane itself. It should, however, be noted that fundamental parameters, like the geometrical properties of the ganglioside molecule, play a role in the microdomain organization of the membrane [5–6]. In fact the oligosaccharide chain bulky structure, tied to the primary and secondary structures, determines the spatial arrangement of the molecule in the hydrophilic layer [7].

Four main sialic acid structures have been found in gangliosides: the *N*-acetyl-, *N*-glycolyl, 9-*O*-acetyl-*N*-acetyl- and 4-*O*-acetyl-*N*-glycolyl- derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-glicero-D-galacto-nonulosonic acid) [8]. Gangliosides containing *N*-acetyl- and *N*-glycolyl-neuraminic acid are widespread in

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mammals but those containing *N*-glycolyl-neuraminic acid (Neu5Gc) have never been found in healthy human tissue [9], only in cancer tissue [10].

The purpose of the present work was to investigate the effect of the glycolic group of Neu5Gc on the conformational properties of the GM1 oligosaccharide chain [11], and make a comparison with what is already known about GM1(Neu5Ac) and GM1(Neu) conformations [1].

High resolution NMR investigations of the three dimensional (3D) structure of the GM1(Neu5Gc) oligosaccharide chain were made by evaluating nOe effects in both dimethylsulfoxide and water solution.

To overcome the difficulty of the high molecular mass of ganglioside aggregates the gangliosides were inserted into perdeuterated dodecylphosphocholine (DPC) micelles in a low molar ratio of one-ganglioside per micelle. This model system allowed the ganglioside to be kept in water solution and, moreover, mimics the natural phospholipid environment of membrane gangliosides. The DPC model system has already been used for detailed studies on the molecular properties of a number of gangliosides, GM1(NeuAc) [1], GD1a [12, 13], GalNAc-GD1a [13] and GM3 [14].

Materials and methods

Materials

Deuterated dimethylsulfoxide (DMSO-d_6), >99.95% isotopically pure, was purchased from Merck (FRG) and dodecylphosphocholine-d_{38} was from MSD (Canada). Chelex 100 (100–200 mesh, sodium form) was from Biorad (USA). Ganglioside GM1 containing *N*-glycoyl neuraminic acid, GM1(Neu5Gc), was prepared by a semisynthetic procedure from GM1(Neu5Ac) [11, 15, 16].

After chromatographic purification GM1(Neu5Gc) was >99% pure. The structural characterization was confirmed by NMR as reported below.

NMR spectroscopy

GM1(Neu5Gc) was purified from cation contamination by passing a water solution of the ganglioside through a Chelex 100 cation exchange resin column.

Mixed micelles were prepared by dissolving the dried ganglioside and the dodecylphosphocholine (molar ratio 1:40) in deuterated potassium phosphate buffer (50 mM, 0.5 ml, pH 6) [17]. ^{1}H-NMR and ^{13}C-NMR spectra were respectively performed at 500 and 125 MHz, on a Bruker AM500 spectrometer, and analysed on a X32 Bruker satellite station equipped with the standard Bruker UXNMR software.

The chemical shift assignments were obtained by 1D- and 2D-HOHAHA, and ^{1}H-^{13}C heterocorrelated HSQC experiments [1, 12]. The nOe investigation was realized by 2D-ROESY experiments using an off-resonance spin lock procedure to avoid scalar transfer, as described elsewhere [1, 12, 13, 18].

2D ^{1}H-^{1}H and ^{1}H-^{13}C experiments were respectively acquired with 512 and 256 t_1 increments, and 128 scans were collected for each t_1. After zero filling and appropriate window function multiplication, the time domain spectra were transformed to give 2048 × 1024 point matrixes. Distance evaluation was based on the hypothesis of proportionality $V_{ij} \propto 1/r_{ij}^6$, where V_{ij} is the cross-peak volume and r_{ij} the proton-proton distance for proton pair H_i-H_j [19]. The intraresidual fixed distances between syndiaxial (0.25 nm) and transdiaxial (0.31 nm) protons were used as internal references. For the DMSO sample five experiments were performed at different temperatures, varying the mixing time and the off-resonance lock frequency; the nOe distances derived from the five spectra were averaged to give the reported distances. Four experiments were performed on the D_2O sample and the results averaged.

Molecular mechanics calculations were performed on a Silicon Graphics IRIS 4D35GT workstation. Molecular modelling was carried out with an INSIGHT/DISCOVER package. The computation of the minimum energy conformations of the oligosaccharide moiety was performed by restrained molecular mechanics calculations using CVFF [20] with a dielectric constant ε of 80 [13]. The nOe restraints were defined as harmonic forcing potentials.

Results and discussion

The complete proton and carbon chemical shift assignments for GM1(Neu5Gc) ganglioside in dimethylsulfoxide and water solution are reported in Tables 1 and 2, respectively.

The conformation of the trisaccharide fragment -β-GalNAc-(1-4)-[α-Neu5Gc-(2-3)]-β-Gal- is of particular interest since all the molecules investigated till now, GM1(Neu5Ac) [1], GD1a [12, 13] and GalNAc-GD1a [13], show this fragment to be fairly rigid. As a preliminary the potentially flexible glycerol sialic acid side chain, which shows a number of nOe interactions with the GalNAc residue, was analysed. It was found that vicinal coupling constants (Table 3) and nOe interactions along the side chain are in accordance with previous results for Neu5Ac linked to several gangliosides [1, 12, 13, 18] and Neu5Gc in GM3 ganglioside [14]. This suggests a rigid 3D structure for the glycerol sialic acid chain. Thus it can be deduced that neither substitution at the amino group, nor different aglicone structures, influences the sialic acid glycerol chain 3D structure. The trisaccharide fragment -β-GalNAc-(1-4)-[α-Neu5Gc-(2-3)]-β-Gal- is characterized by several inter-residual nOe contacts (Table 5).The GalNAc residue has

Table 1. ^{1}H and ^{13}C chemical shifts (ppm) of GM1(Neu5Gc) in DMSO-d_6 solution at 300 K.

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*11*
Glc	H	4.15	3.04	3.30	3.28	3.34	3.63				
(I)							3.74				
	OH		5.04				4.47				
	C	103.5	73.2	72.7	74.8	74.3	60.3				
Gal	H	4.27	3.13	3.74	3.94	3.74	3.38a				
(II)							3.63b				
	OH		4.91				4.23				
	C	103.7	68.9	74.0	77.4	71.9	59.8				
GalNAc	H	4.90	3.92	3.50	3.73	3.64	3.47				
(III)							3.47				
	OH				4.23		4.51				
	NH		7.53								
	C	102.5	50.8	81.0	67.3	74.1	61.0				
Gal	H	4.22	3.30	3.27	3.60	3.35	3.52				
(IV)							3.52				
	OH		3.67	4.66	4.30		4.82				
	C	104.5	70.8	80.4	68.3	75.4	60.6				
Neu5Gc	H			1.64ax	3.84	3.42	3.22	3.17	3.50	3.32R	3.86
(A)				2.56eq						3.62S	3.86
	OH				4.81			4.60	5.99	4.67	5.44
	NH					7.65					
	C			37.5	67.5	52.3	72.9	68.4	74.1	63.5	61.2
Sph	H	3.97	3.77	3.89	5.45	5.53	1.93				
		3.43									
	OH			4.78							
	NH		7.41								
	C	69.1	53.0	70.7	131.4	131.3	31.7				

Table 2. ^{1}H and ^{13}C chemical shifts[a] (ppm) of GM1(Neu5Gc) inserted in a micelle of dodecylphosphocholine in D_2O at 310 K.

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*11*
Glc	H	4.48	3.37	3.66	3.63	3.62	3.82				
(I)							4.01				
	C	103.0	72.70	72.71	79.17	74.76	59.20				
Gal	H	4.56	3.39	4.19	4.17	3.78	3.79[b]				
(II)							3.79[b]				
	C	102.70	70.01	74.42	77.40	72.28	60.66[b]				
GalNAc	H	4.83	4.08	3.83	4.19	3.76	3.85[b]				
(III)							3.85[b]				
	C	102.7	50.80	81.59	67.71	74.81	60.32[b]				
Gal	H	4.58	3.55	3.68	3.95	3.72	3.80[b]				
(IV)							3.80[b]				
	C	104.80	70.70	72.65	68.44	74.77	60.42[b]				
Neu5Gc	H			1.97ax	3.92	3.93	3.65	3.63	3.80	3.66R	4.15
(A)				2.71eq						3.90S	4.15
	NH					7.65					
	C			36.91		51.40	74.56	67.90	74.21	62.60	61.0
Sph	H	4.20	3.94	4.10	5.35	5.77	2.02				
		3.75									
	C	69.86	53.20	70.63	130.37	133.75	32.65				

[a]Proton and carbon chemical shifts were respectively referenced to the typical sphingosine olefinic H4 resonance at 5.45 ppm and to Gal(IV) C1 resonance at 104.8 ppm.
[b]exchangeable.

Table 3. ^{3}J Coupling constants (Hz) for the glycerol chain (C6-C7-C8-C9) of sialic acid in GM1(Neu5Gc) and GM1(Neu5Ac) in DMSO-d_6.

	6,7	*6,OH7*	*7,8*	*8,OH8*	*8,9S*	*8,9R*	*9R,9S*	*9,OH9*
GM1(Neu5Gc)	<2	3.0	8.9	2.5	<2	5.7	11.5	6.0
GM1(Neu5Ac)	<2	3.7	8.7	3.0	2.1	5.9	11.5	6.1

strong nOe interactions with the internal Gal (III1/II4); III-NH/II2); the sialic acid ring has a very strong nOe with the internal Gal (A3a/II3) and a number of interactions with the GalNAc (A-OH8/III1; A8/III1; A-OH9/III-OH6). This net of interresidual contacts gives a fairly rigid structure to the GalNAc-(Neu5Gc-)Gal trisaccharide. This particular feature has already been found in GM1(Neu) and GM1(Neu5Ac) [1], GD1a [12, 13], GalNAc-GD1a [13], GM2 [21] and discussed in detail. This rigid and constant conformation probably depends on a favourable hydrogen bond between GalNAc-NH and the sialic acid carboxylic group, as suggested by the low field chemical shift and the low temperature coefficient for GalNAc-NH in DMSO-d_6 (Table 4). On the contrary, the terminal β-Gal-(1-3)-β-GalNAc- glycosidic linkage is characterized by high flexibility, as indicated by the presence of the IV1/III2, and IV1/III4 nOe interactions in D_2O, and by the IV1/III2, IV1/IIINH and IV1/III4 interactions in DMSO-d_6, which cannot be simultaneously satisfied by a single rigid Gal-GalNAc glycosidic linkage. Similar results were also obtained for GM1(Neu5Ac).

The nOe investigation of the fully deuterated dodecylphosphocholine/GM1(Neu5Gc) mixed system in D_2O results in fewer detected interresidual contacts than in the organic solvent, due to the lack of labile hydroxyl and amide protons (Table 5). Furthermore the crowding of the ring protons spectral region led to strong signal overlapping and prevented an accurate evaluation of a number of cross peak volumes, particularly that of the III1/A8 contact (Fig. 1). The III1/A8 distance was probably underestimated as there was partial overlapping with the strong III1/III5 intraresidual contact. In any case, compared with the nOe contacts in DMSO solution, no substantial differences were found nor were there any further interactions for fixed protons.

Table 4. Coupling constant J (Hz) and temperature coefficients dδ/dT (-10^{-3} ppm per °C) for amide and hydroxy protons of GM1(Neu5Gc) and GM1(Neu5Ac) in DMSO-d_6 solution.

Residue		*GM1(Neu5Gc)*		*GM1(Neu5Ac)*	
		J	*dδ/dT*	*J*	*dδ/dT*
Glc	OH2	3.6	6.0	3.7	5.19
(I)	OH3	1.5	2.5	5.2	2.84
	OH6	5.7	4.5	5.8	5.39
Gal	OH2	5.0	8.0	5.2	7.51
(II)	OH6	5.9	3.0	6.4	3.37
Neu5Gc	OH4	5.6	4.0	5.2	4.49
(A)	NH5	7.8	5.5	8.5	4.80
	OH7	3.4	4.0	3.7	3.27
	OH8	<2.5[a]	0.5	3.0	<1
	OH9	6.2	12.0	6.1	10.67
	OH11	6.0	5.0		
GalNAc	NH2	9.5	2.0	8.2	2.88
(III)	OH4	4.5	7.5	4.9	7.42
	OH6	5.9	5.5	5.2	4.38
Gal	OH2	<2	ND	<2	<1
(IV)	OH3	5.3	7.5	5.2	7.56
	OH4	4.4	6.0	5.2	5.62
	OH6	5.8	4.5	6.1	3.11
Sph	OH3	5.6	5.0		
	NH2	9.2	5.5		

[a]broad signal.
ND, not determined.

Table 5. Interresidual contacts and nOe distances for GM1 (Neu5Gc) in DMSO-d_6 solution and inserted into a micelle of dodecylphosphocholine in D_2O.

Monomers of GM1(Neu5Gc) in DMSO-d_6		*Mixed micelles of GM1(Neu5Gc)/DPC in D_2O*	
Contact	*Distance (Å)*	*Contact*	*Distance (Å)*
IV-1/III-2	3.5	IV-1/III-2	3.5
IV-1/III-3	2.2	IV-1/III-3	2.2
IV-1/III-4	3.1	IV-1/III-4	2.7[a]
IV-1/III-NH	3.8		
A-3a/II-3	2.3	A-3a/II-3	2.6
III-1/II-4	2.2	III-1/II-4	2.3
III-NH/II-2	4.2		
III-1/A-OH8	3.0		
IIIOH6/AOH9	3.0		
III-1/A-8	3.1	III-1/A-8	2.7[a]
II-1/I-4	2.3	II-1/I-4	[b]
II-1/I-6	3.1	II-1/I-6	[b]
II-OH2/I-6	2.9		
II-OH2/I-6′			
II-1/I-OH3	3.2		
II-OH2/I-OH6	3.9		

[a]Distance results underestimated because of cross peak overlapping.
[b]Values are not attributed because of strong overlapping.

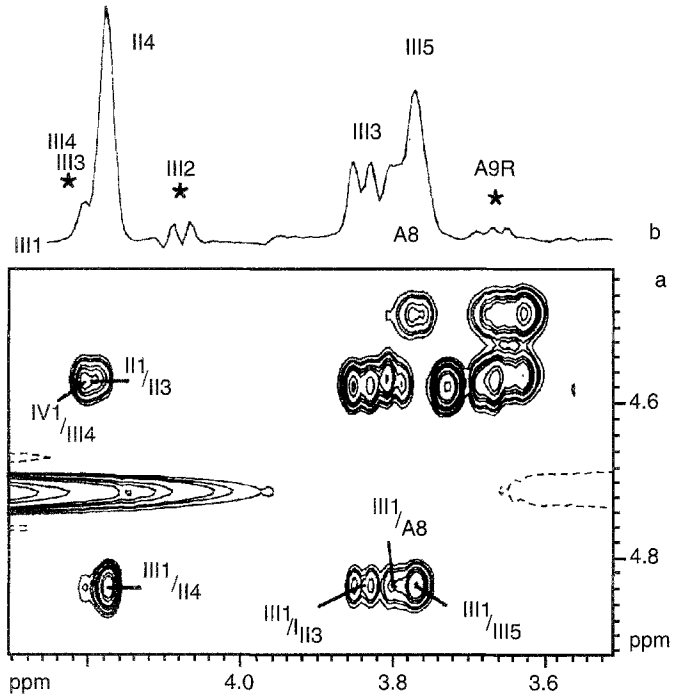

Figure 1. 500 MHz ^{1}H-NMR partial 2D ROESY spectrum (150 ms mixing time) of GM1(Neu5Gc) in fully deuterated DPC/D_2O solution at 310 K. (a) The connectivities of the anomeric protons are shown. The overlapping of III1/A8 interaction with III1/III5 intraresidual contacts is highlighted. (b) Projection of the III1 row at 4.83 ppm. Signals marked with an asterisk are affected by Hartmann-Hahn magnetization transfer (Tocsy transfer). For the III2 peak both rOe and Tocsy effects are present. The A9R peak is entirely due to Tocsy transfer from A8: protons III1 and A9R are too far away to give nOe contact. A9S is not observed since the A8,9S J coupling constant is low. This indirectly confirms the III1-A8 interaction.

The nOe distances were introduced as initial constraints in molecular mechanics calculations to obtain the minimum energy conformations of the pentasaccharide moiety of the ganglioside. Identical minimum energy calculations using the same procedure and dielectric constant were performed for GM1(Neu5Ac) using the nOe data already presented [13]. As expected, given the similarity of the nOe results for the two molecules, the simulations gave the same minimum energy 3D structures for the two compounds. Table 6 shows the glycosidic angles defining those conformations. The glycosidic bond IV-III manifests its typical flexibility, occupying two conformations. The nOe interactions for the II-I linkage are too few to fix a single conformation, nor do they give any indication of bond flexibility. The values given in Table 6 correspond to the minimum energy conformation for this linkage. Furthermore it is possible to verify that all the pairs of Ψ and Φ values fall in the sterically allowed areas of the corresponding nOe distance maps drawn for GM1(Neu5Ac) [13].

Table 6. Torsional angles (degree) of glycosidic linkages for GM1(Neu5Gc) related to the minimum energy conformations as obtained by CVFF molecular mechanics calculations with $\varepsilon = 80$. A comparison with results for GM1(Neu5Ac) is outlined.

Glycosidic bond	*GM1(Neu5Gc)*		*GM1(Neu5Ac)*	
	Φ	Ψ	Φ	Ψ
IV–III	46,	7 or	42,	11 or
	37,	−29	36,	−30
III–II	33,	18	33,	15
II–I	39,	−5	47,	−1
A–II	−161,	−29	−163,	−28

Table 7. The molecular mass m (kDa), the aggregation number N, the hydrodynamic radius R_h (Å), the axial ratio R_a/R_b of the ganglioside aggregate, the surface area a_0 (Å^2) and the packing parameter P of the monomer in the aggregate.

	m	N	R_h	R_a/R_b	a_0	P
GM1(Neu5Gc)	576	365	62.4	2.5	93.8	0.437
GM1(Neu5Ac)	470	301	58.7	2.3	95.4	0.428

Conclusion

Although very minor conformational differences between GM1(Neu5Gc) and GM1(Neu5Ac) are not excluded, our results indicate that the conformational properties of the GM1 ganglioside and, in particular, those of the trisaccharide Gal-(sialic acid)-GalNAc, are independent of the sialic acid structure.

However GM1(Neu5Gc) and GM1(Neu5Ac) were shown to display different aggregate physical parameters (Table 7) [11]. Any changes in aggregative properties were usually related to large changes in primary structure (increasing-decreasing of the sugar residue number) or else to the different conformational properties of the ganglioside oligosaccharide chain [7].

As no differences were found in the 3D structures the higher packing property shown by GM1(Neu5Gc) remains unexplained. Our proposal is that some water molecules could be excluded from the micelle hydrophilic layer due to the presence of a further hydroxyl group in the GM1(Neu5Gc) polar head. This would favour the formation of new intermolecular hydrogen bonds between the ganglioside oligosaccharide chains and promote a greater packing of the ganglioside molecules in the aggregate structure, in agreement with an earlier observation on glycosphingolipids containing 2-hydroxy fatty acid [7, 22].

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