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^{1}H-NMR study of G_{M2} ganglioside: evidence that an interresidue amide–carboxyl hydrogen bond contributes to stabilization of a preferred conformation

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Several properties of the exchangeable amide protons of the ganglioside G_{M2} were studied in detail by ^{1}H-NMR spectroscopy in fully deuterated dimethylsulfoxide [$^{2}H_{6}$]DMSO/2% H_2O, and compared with data obtained for the simpler constituent glycosphingolipids G_{A2} and G_{M3}. In addition to chemical shifts, $^{3}J_{2,HN}$ coupling constants, and temperature shift coefficients, the kinetics of NH/^{2}H chemical exchange were examined by following the disappearance of the amide resonances in [$^{2}H_{6}$]DMSO/2% $^{2}H_{2}O$. The results included observation of an increase in half-life of the *N*-acetylgalactosamine acetamido HN by more than an order of magnitude in G_{M2} compared to G_{A2}, attributable to the presence of the additional *N*-acetylneuraminic acid residue. Additional one-dimensional dipolar cross relaxation experiments were also performed on nonexchangeable protons of G_{M2}. The results of all of these experiments support a three-dimensional model for the terminal trisaccharide in which a hydrogen bond is formed between the *N*-acetylgalactosamine acetamido NH and the *N*-acetylneuraminic acid carboxyl group. The interaction is proposed to be of the π-acceptor type, a possibility which has not yet been explored in the literature on carbohydrates. The proposed model is discussed in comparison with that of Sabesan *et al.* (1984, *Can J Chem* **62**:1034–45), and the models of G_{M1} proposed more recently by Acquotti *et al.* (1990, *J Am Chem Soc* **112**:7772–8) and Scarsdale *et al.* (1990, *Biochemistry* **29**:9843–55).

Keywords: G_{M2}, ganglioside, ^{1}H-NMR, three dimensional structure, hydrogen bonding.

Ganglio-series gangliosides, containing the glycosphingolipid core structures GalNAcβ1-4Galβ1-4Glcβ1-Cer ($Gg_3Cer \equiv G_{A2}$) or Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer ($Gg_4Cer \equiv G_{A1}$), usually with an *N*-acetylneuraminic acid (NeuAc) linked α2-3 to the internal galactose residue (G_{M2} and G_{M1}, respectively), have been the subject of numerous ^{1}H and ^{13}C NMR studies designed to simplify elucidation of their primary structures, as well as probe their secondary structures and dynamics [1–12]. Considerable interest has focused on the interaction between the NeuAc and *N*-acetylgalactosamine (GalNAc) residues in the core tetrasaccharide, GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-. In earlier work, Koerner *et al.* [8] considered a number of possible secondary structural elements, consistent with observed chemical shift data for a whole series of monosialo- and asialoganglio structures in fully deuterated dimethylsulfoxide ([$^{2}H_{6}$]DMSO), which might provide stabilizing interactions for these two residues: (1) a dipole–charge interaction between the GalNAc acetamide and NeuAc carboxylate; (2) hydrophobic interactions including the GalNAc amide methyl and NeuAc C-3 methylene groups; (3) hydrogen bonding interactions, such as between the GalNAc amide and NeuAc C-4 hydroxyl; (4) a counterion effect. No particular conformation was proposed, at that time. On the other hand, based on HSEA minimum energy conformational calculations and their own set of NMR data, Sabesan *et al.* [9] proposed a three-dimensional structure stabilized primarily by van der Waals forces. More recently, while this manuscript was in preparation, two extensive studies of the three-dimensional structure of G_{M1} ganglioside have appeared [11, 12]. In the study by Acquotti *et al.* [11], a distance mapping procedure, incorporating dipolar correlations between amido, hydroxy, and C-linked protons, was followed by molecular mechanics calculations. In this study it was suggested that the allowed conformations for the GalNAcβ1-4(NeuAcα2-3)Galβ1- trisaccharide in G_{M1} are favourable for formation of a hydrogen bond between the GalNAc NH and the NeuAc carboxylate group [11]. In the study by Scarsdale *et al.* [12], distance constraints based on dipolar correlations between C-linked protons, treated as pseudo-energies, were incorporated into molecular mechanics calculations that included electrostatic and hydrogen bonding interactions. Although somewhat different conclusions were reached regarding the allowed

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conformations of the GalNAcβ1-4(NeuAcα2-3)Galβ1- trisaccharide in G_{M1}, the formation of a similar type of H-bond was proposed to stabilize the structure [12]. Presented here is evidence, based primarily on a comparative analysis of ^{1}H-NMR data for the exchangeable amide protons of G_{M2} and two constituent glycosphingolipids (G_{A2} and G_{M3}), that such a hydrogen bonding interaction exists, and could contribute to the stabilization of a single conformer only slightly different from that proposed by Sabesan *et al.* [9] and Acquotti *et al.* [11].

Materials and methods

^2H_2O and [$^2H_6$]DMSO (both 99.96 atom %) were purchased from Aldrich (Milwaukee, WI, USA) and Cambridge Isotope Laboratories (Woburn, MA, USA), respectively. All other solvents used were of HPLC grade. Gangliosides G_{M3} and G_{M2} were purchased from Sigma (St. Louis, MO, USA) and Boehringer-Mannheim (Indianapolis, IN, USA), respectively. Neutral G_{A2} was prepared by stepwise degradation of G_{M1} (Sigma) as follows: (1) desialosylation in 1% acetic acid at 100°C for 2 h, with removal of released sialic acid and unreacted ganglioside by passage through NaOAc activated DEAE Sephadex (Sigma) in $CHCl_3/MeOH/H_2O$ (30/60/8 by vol); (2) hydrolysis of the resulting G_{A1} with β-galactosidase from bovine testis (Sigma) in 0.1 M sodium citrate buffer, pH 4.5, containing an equal amount by weight of sodium deoxytaurocholate (Sigma). The product was purified by a sequence consisting of solid phase extraction on a C_{18} silica cartridge (Analytichem International, Harbor City, CA, USA), passage through DEAE Sephadex (to remove deoxytaurocholate), and a second solid phase C_{18} silica extraction (to remove salt released from the DEAE Sephadex).

The identity and purity of all glycosphingolipids were checked by comparison of one-dimensional (1D) ^{1}H-NMR spectra ([$^2H_6$]DMSO/2% ^2H_2O, 303 K) of deuterium-exchanged samples to published spectra [7].

^{1}H-NMR spectroscopy

All spectra were recorded on a Bruker (Karlsruhe, Germany) AM-500 Fourier transform spectrometer/Aspect 3000 data system, using quadrature detection. The sweep width was 5000 or 7000 Hz, collected over 16 K data points. The residual HO^2H resonance was suppressed by a presaturation pulse during the preparatory delay (PD = 2 s).

Transient NOE studies

One-dimensional transient nuclear Overhauser effect spectra [13] were obtained by selective inversion recovery in the difference mode (SIR-ΔNOE) [14]. Six mixing times from 10 to 300 ms were used.

Temperature shift studies

For 1D ^{1}H-NMR spectra of non-deuterium-exchanged samples, the sweep width was expanded from 5000 to 7000 Hz, in order to include amide HN protons. Samples (0.5–0.75 mg) were dissolved in [$^2H_6$]DMSO/2% (0.45 ml), and spectra obtained at nominal temperatures from 303–328 K at 5 deg intervals. Each spectrum was recorded only after temperature equilibration in probe for at least 10 min. Although the probe temperatures were not calibrated, the stability at each point was judged better than ±0.5 deg, all samples were run on the same day, and spectra of all samples were obtained at a given temperature before going on to the next point.

Deuterium exchange kinetics

For these studies, samples (0.50–0.75 mg) were dissolved initially in pure [$^2H_6$]DMSO (0.45 ml). Samples were equilibrated in probe (nominal temperature 305 ± 2 K) for 10–15 min, then exchange was initiated by addition of 9 μl ^2H_2O, followed by brief (5 s) agitation. After replacement of the sample in probe, spectrum acquisition was initiated as soon as re-shimming could be accomplished (≈3–4 min). Spectra were acquired once every hour for 36–48 h, with 256 transients collected per spectrum (≈9.5 min total acquisition time). The extent of $^2H/^1H$ exchange over time was measured by the decrease in amplitude of HN resonances (using the intensity of nonexchangeable anomeric protons as internal standards).

Conformational energy calculations

Minimum energy conformational calculations were performed using the GESA version [15] of the hard sphere/exo-anomeric effect (HSEA) program [16], kindly provided by Dr. Bernd Meyer of the UGA Complex Carbohydrate Research Center, Athens, GA, USA. Further manipulations were performed using the Alchemy II and Sybyl molecular modelling software packages (Tripos Associates, St. Louis, MO, USA).

In defining glycosidic torsional angles, ϕ and ψ, the common non-IUPAC system is used; that is, for neutral α- or β-D-pyranosides, ϕ is the dihedral angle defined by H-1′—C-1′—O-1′—C-*x* and ψ is the dihedral angle defined by C-1′—O-1′—C-*x*—H-*x*; for α-D-NeuAc, ϕ is the dihedral angle defined by C-1′—C-2′—O-2′—C-*x* and ψ is the dihedral angle defined by C-2′—O-2′—C-*x*—H-*x*.

Results

Amide proton chemical shifts

In Table 1 are listed the chemical shifts and coupling constants obtained for amide protons of glycosphingolipids in [$^2H_6$]DMSO/2% H_2O at 308 K. The chemical shifts were similar to those reported by Gasa *et al.* [17] in pure [$^2H_6$]DMSO, and were assigned accordingly, except that the assignment of the GalNAcH-N in G_{M2} was confirmed by decoupling from the vicinal H-2 resonance. Comparing data for G_{A2} and G_{M2}, it is apparent that the presence of

Table 1. Chemical shifts (ppm from tetramethylsilane) and three bond coupling constants, $^3J_{2,HN}$ (Hz) of glycosphingolipid amide protons in [$^2H_6$]DMSO/2% H_2O at 308 ± 2 K.[a]

Compound[a]	*NeuAc* δ	*NeuAc* J	*GalNAc* δ	*GalNAc* J	*Cer* δ	*Cer* J
G_{M3}	8.006	(6.9)			7.474	(9.5)
G_{A2}			7.752	(6.9)	7.472	(9.5)
G_{M2}	7.953	(7.7)	7.232	(9.5)	7.475	(9.5)

[a] Abbreviations: GM_3=NeuAcα2-3Galβ1-4Glcβ1-1Cer; GA_2=GalNAcβ1-4Galβ1-4Glcβ1-1Cer; GM_2=NeuAcα2-3(GalNAcβ1-4)Galβ1-4Glcβ1-1Cer; Cer=ceramide=*N*-acylsphingosine.

the NeuAcα2-3 unit in G_{M2} causes a pronounced upfield shift ($\Delta\delta = -0.52$ ppm) of the GalNAcβ1-4 acetamido HN resonance. By contrast, the G_{M3} NeuAcα2-3 acetamido HN undergoes only a small shift change ($\Delta\delta = -0.053$ ppm) on addition of the vicinal GalNAcβ1-4 to form G_{M2}. The upfield shift of the GalNAc HN resonance is accompanied by a significant increase in its $^3J_{2,HN}$ coupling constant.

Temperature dependence of amide proton chemical shifts

Chemical shifts of amide protons were plotted as a function of temperature over a 25 deg range in the same solvent (Fig. 1). It is clear from these data that the behaviour of the HN of GalNAc in G_{M2} is radically different from that of all others measured, being considerably smaller in magnitude and opposite in sign (Table 2).

Deuterium exchange of amide protons

$N^1H/^2H$ exchange rates were measured directly by addition of 2% ^2H_2O to glycosphingolipid samples equilibrated initially in pure [$^2H_6$]DMSO. To monitor reduction in intensity of the HN resonances with time, NMR spectra were recorded at one hour intervals. It was observed that, in G_{M2}, the GalNAc acetamido HN resonance persisted long after the NeuAc HN resonance disappeared (Fig. 2). Comparison with the simpler constituent triglycosylceramides showed that, while the exchange rates of NeuAc acetamido protons were virtually the same in G_{M2} as in G_{M3}, there was a 34-fold increase in the half-life of the GalNAc HN in G_{M2} compared with G_{A2} (Table 3).

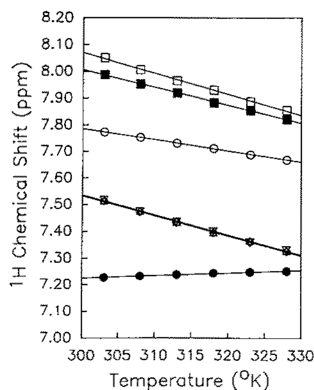

Figure 1. Plot of amide proton chemical shifts (ppm) versus temperature (K) for G_{A2}, G_{M3}, and G_{M2} in [$^2H_6$]DMSO/2% H_2O. Symbols used are: □, NeuAc of G_{M3}; ■, NeuAc of G_{M2}; ○, GalNAc of G_{A2}; ●, GalNAc of G_{M2}; ▽, Cer of G_{M3}; ◇, Cer of G_{A2}; △, Cer of G_{M2}.

Table 2. Temperature dependencies[a] of amide proton chemical shifts (in ppb deg^{-1}) for glycosphingolipids in [$^2H_6$]DMSO/2% H_2O.

Compound	*NeuAc*	*GalNAc*	*Cer*
G_{M3}	−7.84		−7.49
G_{A2}		−4.20	−7.49
G_{M2}	−6.66	+0.99	−7.45

[a] Calculated slope from linear least squares analysis of six data points from 303–328 K.

Dipolar cross relaxation (nOe and T_1)

Dipolar cross relaxation rates obtained by the selective inversion–recovery difference nOe method were used to estimate some interproton distances in G_{M2}. Results obtained from inversion of NeuAc H-3_{ax} (1.62 ppm) are illustrated in Fig. 3. Cross relaxation was observed almost instantaneously with NeuAc H-3_{eq} and Gal H-3, while other nOes (NeuAc H-4 and 6/7 [tightly coupled pair]; Gal H-1 and 4; GalNAc H-1) became apparent at about 100 ms. An expected nOe to NeuAc H-5 was unobservable due to interference from

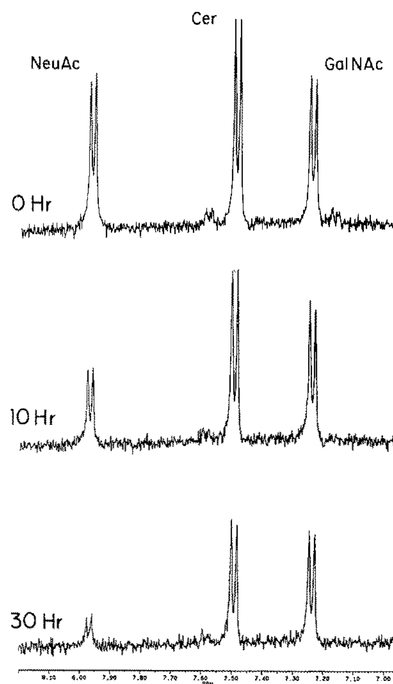

Figure 2. Decay of amide proton resonances of G_{M2} during deuterium exchange. 2% ^2H_2O was added to G_{M2} in pure [$^2H_6$]DMSO at 305 K.

Table 3. Half-lives[a] (h) for deuterium exchange of amide protons of glycosphingolipids in [$^2H_6$]DMSO/ 2% ^2H_2O at 305 K.

Compound	*NeuAc*	*GalNAc*	*Cer*
G_{M3}	14.7		77.4
G_{A2}		10.8	80.0
G_{M2}	14.5	368	78.9

[a] Calculated inverse slope from decay of HN resonance over 15 h, fitted to single exponential.

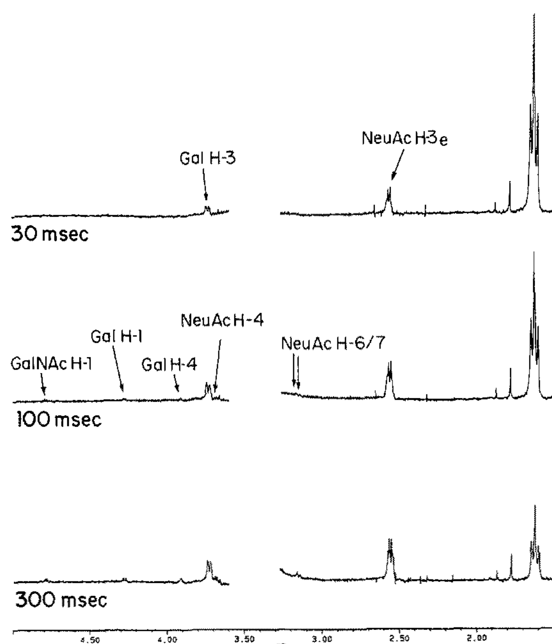

Figure 3. Transient 1D dipolar crossrelaxation following selective inversion of H-3_{ax} of G_{M2} at 1.628 ppm. The 40 ms inverting pulse was followed by variable mixing delays indicated prior to the 90° detection pulse.

the residual HO^2H peak, which is imperfectly cancelled. The greater part of the later developing nOes is most likely secondary in origin, following logical spin diffusion pathways originating from H-3_{ax} (NeuAc H-3_{eq} → H-4 → H-6/7; NeuAc H-5 → H-7/6; Gal H-3 → H-1; Gal H-3 → H-4 → GalNAc H-1). Their assignment as secondary nOes is supported by the large magnitudes observed for the primary correlations. Thus, even though some protons might be close enough to H-3_{ax} (≈4.0 Å) for a direct nOe to be detectable in principle (e.g., NeuAc H-6, Gal H-1), the secondary transfer pathway may be more rapid [18–20]. Both mechanisms may operate simultaneously in the case of cross relaxation with NeuAc H-4.

Inversion of NeuAc H-3_{eq} (not shown) produced no observable inter-residue cross relaxation in the first 100 ms, at which time a small secondary enhancement of Gal H-3 could be seen adjacent to the NeuAc H-4 enhancement. In

Table 4. Comparison of interproton distances (Å) in G_{M2} derived from calculated minimum energy conformations and from 1D nOe experiments in [$^2H_6$]DMSO/2% ^2H_2O at 305 K.

	Lit. (HSEA)[a]	*Recalc. (GESA)*	*Experimental*
A-3_{ax} → A-3_{eq}	1.79	1.79	[1.80][b]
A-3_{ax} → II-3	2.05	2.10	2.1 (±0.1)
III-1 → III-5	2.41	2.41	[2.41][b]
III-1 → II-4	2.46	2.36	2.2 (±0.1)

[a] From Sabesan *et al.* [9].
[b] Fixed calibration distance (using standard geometry).

similar experiments with NeuAc H-3_{ax} and H-3_{eq} of G_{M3} (not shown), no clear inter-residue enhancements were observed from either proton, through mixing times of 300 ms.

In consideration of the arguments outlined in the paragraph before last, values of σ were obtained from nOe buildup in the first 100 ms following inversion, so that a linear approximation could be made, enabling the use of the simple relationship

$$\left[\frac{r_{ik}}{r_{ij}}\right]^6 = \frac{\sigma_{ij}}{\sigma_{ik}}$$

where r_{ij} could be a calibrating intra-residue distance. The distances were compared with those obtained for G_{M2} from HSEA-type minimum energy conformational calculations [9] (see Table 4). Interestingly, while the distance A-3_{ax} → II-3 was found to be approximately the same as that predicted by the HSEA calculation of Sabesan *et al.* [9], the distance III-1 → II-4 was significantly shorter than that predicted. Identical results were reported by Acquotti *et al.* [11] for these distances in G_{M1} (2.1 and 2.2 Å, respectively). Note that in Scarsdale *et al.* [12], somewhat different distances were estimated for G_{M1} (2.3 and 2.4 Å, respectively).

Cross relaxation rates obtained for the intra-residue proton pair H-3_{eq} → H-3_{ax} were used to estimate the effective correlation time, τ_c, for reorientation of the internuclear vector in a number of gangliosides, using the equation

$$\sigma_{ij} = \frac{K^2}{10} \frac{1}{[r_{ij}]^6} \left[\tau_c - \frac{6\tau_c}{1 + (2\omega\tau_c)^2} \right]$$

[20, 21].[1] Assuming a moderately rigid saccharide ring, such values should represent approximately the overall correlation time of the NeuAcα2-3 residue in these gangliosides. The results are listed, along with T_1 values for H-3_{eq}, in

[1] Although this equation applies strictly to the case of an isotropically tumbling molecule, a more exact treatment would require detailed knowledge of the geometry of the glycosphingolipid molecules under consideration. Since the shapes of these molecules in solution is still a subject for debate, the simplified equation is used here to obtain estimated values of τ_c for comparative purposes only.

Table 5. Estimated correlation times (τ_c, ns) for reorientation of the H-3_{eq} → H-3_{ax} vector[a], along with selective H-3_{ax} spin–lattice relaxation times (T_1, ms), for gangliosides in [$^2H_6$]DMSO/2% ^2H_2O at 305 K.

Compound	τ_c	T_1
G_{M3}	1.23	449
G_{M2}	1.73	315
$IV^3NeuAcnLc_4Cer$	1.32	396

[a] Calculated from initial cross-relaxation rate σ_{ij}, assuming r_{ij} = 1.80 Å.

Table 5. The difference in correlation time for the NeuAcα2-3 residue observed between G_{M3} and G_{M2} is clearly greater than that between G_{M3} and $IV^3NeuAcnLc_4Cer$, which differs from G_{M3} by extension with an internal *N*-acetyllactosaminyl unit. Since the latter increment can be attributed solely to the effects of increase in molecular weight and saccharide length on the overall tumbling rate of the ganglioside, resulting from addition of two sugars in a linear array, it can be inferred from these data that a significant reduction in conformational mobility of the NeuAcα2-3 residue of G_{M3} results from addition of the vicinal β-GalNAc residue to make G_{M2}.

Conformational analysis

Calculations on the GalNAcβ1-4(NeuAcα2-3)Galβ1- trisaccharide, using the GESA version of the HSEA program, gave a single minimum energy conformation with glycosidic torsion angles ϕ, ψ for the NeuAcα2-3Galβ linkage, −162°, −18°, and for the GalNAcβ1-4Galβ linkage, 46°, 13°. The latter result differed somewhat from the HSEA conformation reported by Sabesan *et al.* [9], 55°, 10°. This variance is doubtless due to the fact that, in the earlier work [9], the conformation of this linkage was calculated for the GalNAcβ1-4Galβ disaccharide, and was not recalculated after attachment and minimization of the NeuAc residue, but was assumed to be unchanged.

As can be seen from the results listed in Table 4, both HSEA-type calculations yielded an overestimate for the inter-residue III-1 → II-4 distance compared with the result calculated from experiment. A more compatible set of torsion angles for the GalNAcβ1-4Galβ linkage was found to be in the vicinity of 30°, 10°. By incorporating this change, the distance between the GalNAc acetamido nitrogen and the nearest NeuAc carboxyl oxygen atom, which was 4.60 Å in the conformation of Sabesan *et al.* [9], and 4.25 Å in the newly calculated GESA minimum, was reduced to 3.60 Å. The cost for this change, in terms of the GESA potential functions, was +1.24 kcal mol^{-1}. Further conformational changes to the G_{M2} trisaccharide were tried, with a view towards reducing this distance still further, to test whether an H-bond between the GalNAc acetamido HN and the NeuAc carboxyl group could be attained with a reasonable cost in hard-sphere and exo-anomeric potential energies. A rotation of the NeuAc carboxyl group about the C-1—C-2 bond, from +80/−100° (relative to the C-2—O-2 bond) [9] to +60/−120°, reduced the distance to 3.23 Å, while a small change in the NeuAcα2-3Galβ torsion angles (within the presumed error limits [±5°] for the GESA calculation) to −167°, −23°, reduced it further, to 3.10 Å. The cost was +1.23 kcal mol^{-1} for the first alteration, and negligible (<0.01 kcal mol^{-1}) for the second. The distances A-3_{ax} → II-3 and III-1 → II-4 in this conformation were 2.00[2] and 2.17 Å, respectively. In this way, a crude trial structure was obtained, similar to those proposed by Sabesan *et al.* [9] and Acquotti *et al.* [11], incorporating a significant hydrogen bonding interaction between the GalNAc acetamido HN and the NeuAc carboxyl group. Logically, such a hydrogen bond would have to be in the range from −2 to −3 kcal mol^{-1} to be compatible with the proposed conformational changes. The proposed model is illustrated in Fig. 4.

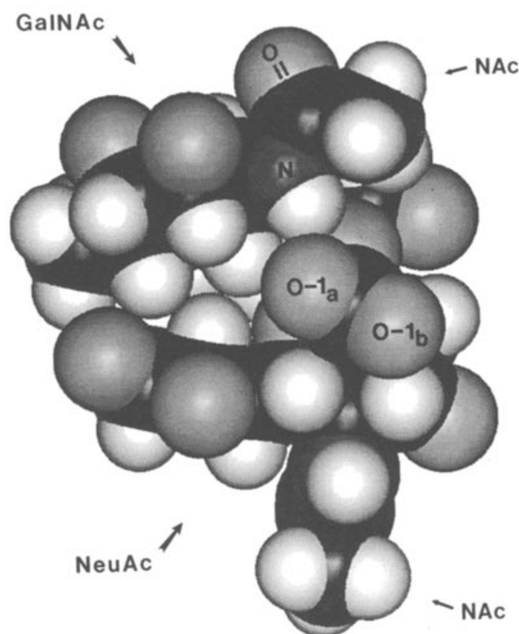

Figure 4. Proposed model of G_{M2} terminal trisaccharide incorporating H-bonding between GalNAc acetamido HN and NeuAc carboxyl O-1_a. The view is from the nonreducing end.

Discussion

Amide proton data

The upfield shift of the GalNAc HN in G_{M2} is consistent with the three-dimensional structure proposed by Sabesan *et al.* [9], which places the GalNAc HN in the shielding

[2] This distance is at the van der Waals limits for two non-bonded protons, and just within the lower error limit set for the nOe calculation.

zone above the plane of the anisotropic NeuAc carboxyl group. A close examination of this model suggested that a further consequence of such a juxtaposition might be formation of a hydrogen bond with the carboxyl π-system as proton acceptor. Generally, π-systems are considered weak H-bond acceptors [22, 23]. Such estimations arise from studies of inter- and intramolecular interactions with neutral acceptors such as unsaturated or aromatic hydrocarbons, ketones, nitriles, etc. [23–31]. Examination of some studies suggests that, at least with intramolecular systems, hydrogen bonds with π-acceptors can cover a much wider part of the normal energy range, depending on the geometry of the interaction [23, 32]. In the case of G_{M2}, the intramolecular acceptor also carries a full negative charge; in addition, the more polarizable carboxyl group should be capable of redistributing its electron density to increase the partial charge on the oxygen closer to the H-bonded proton, thus increasing the strength of the interaction. Optimization of this interaction can be achieved with a moderate alteration of the HSEA-calculated conformation.

As one test of this possibility, temperature shift coefficients were measured for all HN resonances of the three glycosphingolipids for comparison in the same solvent. A reduction in temperature susceptibility has been commonly accepted as an indicator of reduced interaction with solvent, due either to intramolecular H-bonding, steric hindrance (crypticity), or both, in proteins and polypeptides [33–38], as well as carbohydrates [39–42], although Buffington *et al.* [43] have cautioned that reliance solely on the conventional interpretation of temperature shift coefficients can lead to erroneous results (see below for further discussion of this point). In G_{M2}, the small positive slope for this proton is consistent with an H-bonding interaction with a π-acceptor, which weakens with increasing temperature. It is also possible to explain this effect as a result of purely steric factors: physical shielding of the proton from solvent interaction by the carboxyl group, and a small change in their relative orientation with increased temperature, resulting in a downfield shift for the HN.

Similar chemical shift and temperature shift coefficient arguments were used to support an inter-residue hydrogen bonding network proposed to stabilize the conformations of chondroitin 4-sulfate and hyaluronate [40, 44]. This network included hydrogen bonds between glucuronate carboxyl groups and amide protons of 2-acetamido-2-deoxy-hexopyranose units. In these cases, however, the acetamido HN signals of GalNAc or *N*-acetylglucosamine (GlcNAc) units were shifted downfield upon glycosidic linkage to glucuronate, indicating interaction in the plane of the carboxyl groups, and therefore the more usual lone pair acceptor type.

The observation of a substantial decrease in exchange rate for the GalNAc HN in G_{M2} is a second result consistent with either H-bonding, crypticity, or both. As mentioned above, Buffington *et al.* have noted [43], in studies of α- and β-anomers of 2-acetamido-2-deoxyhexopyranoses, that NH/^{2}H exchange rates correlated well with other spectroscopic data indicating a hydrogen bonding interaction between the acetamido HN and the glycosidic oxygen in α- but not β-anomers, while reliance on temperature shift correlations for HN resonances gave inconsistent results. For example, in four solvent systems examined, the amide proton of β-GlcNAc exchanged faster than that of α- while, in three of the four solvents, the β-anomer exhibited a smaller temperature shift coefficient than the α-. Similar effects are aptly demonstrated by the data in Tables 2 and 3. It can be seen that for G_{A2}, the GalNAc HN chemical shift is already less susceptible to temperature change than those of the NeuAc residues or ceramides. Furthermore, inspection of a molecular model of G_{A2} shows a clear possibility of an H-bonding interaction of the GalNAc HN with O-3 of the Gal residue. Yet the exchange of this proton was the most rapid among all that were measured. On the other hand, although the temperature susceptibilities of the ceramide and NeuAc HN resonances were of comparable magnitude, the half-lives for HN exchange were roughly five times longer for those on ceramide than for those of NeuAc residues. Nevertheless, in the case of G_{M2}, the consistent indication from both criteria is that a secondary structure is present which shields the GalNAc acetamido HN from interaction with solvent.

One additional result may be interpreted as evidence for an interaction of the GalNAc acetamido with the NeuAc carboxyl group in G_{M2}: the increased splitting of the GalNAc HN resonance in G_{M2} vs G_{A2} (Table 1). Although Gasa *et al.* [17] have commented on the difficulty of correlating the $^3J_{2,HN}$ coupling constants with structural features in glycosphingolipids, these are doubtless interpretable in terms of a Karplus-type relationship [45] similar to that applied in polypeptide conformational analysis [19, 46, 47]. Such reasoning has been applied to the problem of glycosaminoglycan secondary structure [48], based on the Karplus-type expression developed by Bystrov *et al.* [49]. More recently, an attempt was made to develop this treatment further [50], allowing for partitioning between populations of stable rotamers suggested by PCILO calculations on GalNAc [51]. This was used as part of a comprehensive NMR and computational methodology applied to the secondary structure of glycosphingolipids such as globoside [50] and G_{M1} ganglioside [11].

The value of 9.5 Hz for $^3J_{2,HN}$ in G_{M2} is close to the maximum reported $^3J_{HN,\alpha}$ for antiperiplanar conformations of amino acid residues in bovine pancreatic trypsin inhibitor (BPTI) [47]. On this basis, one could propose an enforced dihedral angle close to 180° ($\Phi_{IUPAC} = -120°$)[3] between

[3] Φ_{IUPAC} are given for dihedral angles H-N—C-2—H-2 of the GalNAc 2-*N*-acetamido group in analogy with the IUPAC–IUB convention angle for amino acid residues in peptides [58]. These relate to the dihedral angles θ by the formula $\theta = |\Phi - 60°|$, and are quoted for the purpose of relating this work to studies in which Karplus-type functions have been derived primarily for peptide conformational analysis [19, 46, 47].

HN and H2 of GalNAc in G_{M2}. Acquotti *et al.* [11] reached a similar conclusion for the GalNAc acetamido group of G_{M1} (also pointing out that the acetamido group may oscillate through a range of approximately $\pm 30°$ around this value). On the other hand, the value of 6.9 Hz measured for the same proton coupling in G_{A2} corresponds either to a stable dihedral angle closer to $\pm 140°$ [47], or to averaging over two or more low energy rotamers [11]. The PCILO calculations of Yadav and Luger [51] on GalNAc indicated that the conformation of lowest energy was to be found with a HCNH torsion angle of 160° ($\Phi_{IUPAC} = -140°$), stabilized by a hydrogen bond between the acetamido carbonyl and HO-3. This angle would still require $^3J_{2,HN}$ in the neighbourhood of 9 Hz. In G_{A2}, there would be an opportunity for an additional inter-residue hydrogen bonding interaction between the acetamido HN and O-3 of the aglyconic β-Gal residue, as discussed previously. This could produce a further deviation from the antiperiplanar conformation for this proton, to approximately 140° ($\Phi_{IUPAC} = -160°$), consistent with the smaller vicinal coupling constant. The change in this coupling constant, on sialosylation of G_{A2} to produce G_{M2}, is then consistent with a realignment of the acetamido group due to an attractive interaction between HN and the NeuAc carboxyl group.

A consideration of the effects of hydrogen bonding of polarizable groups such as amide and carboxyl side chains may shed light on glycosylation-induced shift changes observed in carbon and proton NMR spectra of gangliosides [5–7, 9], some of which are otherwise difficult to explain. In attempting to rationalize such changes for nuclei of sugars disposed vicinally to a newly attached residue, attention has usually been paid to the effects of anisotropic groups of the new residue, conformational adjustments of sugar rings induced by the new residue, steric crowding, and, to a lesser extent, changes in orientation of anisotropic side chains relative to their pyranose rings. More attention may need to be paid to the last mentioned contribution, and certainly to the redistribution of electronic density that is proposed to take place upon engagement of an amide group in a hydrogen bond [33, 43, 52]. For example, an increased polarization across the HNCO group is a consequence which would be expected to bring a greater negative charge density to the amide oxygen, which could therefore explain, in part, the rather remote deshielding effect of sialosylation on H-2 of GalNAc [7].

Dipolar crossrelaxation data

Although Sabesan *et al.* previously conducted extensive dipolar crossrelaxation experiments on G_{M1}OS and related oligosaccharides [9], it seemed appropriate to pursue similar experiments for the following reasons: (1) since no nOe data were given for G_{M2}OS, application of their results to this compound must include an assumption that the additional galactose unit has no conformational or dynamic effect on the remainder of the molecule, an assumption which may not be valid; (2) the steady state nOe difference experiment [53] used by Sabesan *et al.* is not optimal for extracting accurate distance information in macromolecules; furthermore, their ability to obtain some distances, even approximately, was hindered by spectral overlap; (3) it was desired to compare correlation times for the same monosaccharide units in different glycosphingolipids under closely similar experimental conditions, to get an idea of possible dynamic effects these units might have on each other. In the time since this study was initiated, more extensive studies of the conformational properties of gangliosides, including G_{M1}, have appeared [11, 12]. Since G_{M2} was not studied by either of these groups, the same reservations regarding the possible effects of the terminal galactose residue can be raised. It is worth noting that the effect of sialosylation on the temperature dependency of the GalNAc HN chemical shift was found to be more dramatic with G_{M2} than was apparent for G_{M1} [11, 17]. Finally, the two latest studies do not appear to be in agreement regarding the conformation of G_{M1} [11, 12].

Interestingly, although perhaps not surprisingly, it was found in the present study that a significant reduction in conformational mobility of the NeuAcα2-3 residue of G_{M3} results from addition of the vicinal β-GalNAc residue to make G_{M2} (Table 5). Similar conclusions were drawn by Acquotti *et al.* [11] concerning the NeuAc residue in the ganglioside G_{M1}. Consistent with this are observations of clear inter-residue dipolar crossrelaxation between NeuAc and galactose, particularly between NeuAc H-3_{ax} and Gal H-3, which could not be observed for G_{M3} in this study. Studies made by other groups indicate that for the terminal unbranched NeuAcα2-3Galβ1- sequence, such as occurs in G_{M3}, the NeuAc appears to rotate flexibly between two or three possible positions in conformation space [54–56]. The occurrence in G_{M2} of stabilizing interactions between the vicinally linked GalNAc and NeuAc residues is the most probable reason for the observed differences from G_{M3} [7, 9, 11, 12]. However, the finding that the apparent distance between GalNAc H-1 and Gal H-4 is shorter in the oligosaccharide of G_{M2} than that obtained either by Sabesan *et al.* using the HSEA calculation, or independently using GESA, implies that inter-residue interactions other than van der Waals forces contribute to the three-dimensional structure of G_{M2}. The molecular geometry for the proposed hydrogen bond was improved by adjusting the conformational preference of the GalNAc residue to account for this shorter distance. As always, the caveat must be added that the results obtained in this work may relate substantially to the nonphysiological solvent system used. One should be cautious in comparing data obtained in different solvents, especially dimethylsulfoxide and water, since the latter may compete more strongly with intramolecular hydrogen bond formation. Nevertheless, it was observed by H. Eaton and Hakomori (unpublished results), in NMR spectroscopic measurements made on gangliosides dispersed in detergent

micelles, that the NeuAc residue has a consideraby slower correlation time in G_{M1} than in G_{M3}.

The data and conformational calculations obtained in this study are clearly insufficient to support a detailed discussion of internal motion or the influence of solvent. The possible significance of conformational transitions and 'virtual conformations' are also difficult to evaluate. Nevertheless, the observation of strong dipolar interactions between residues, along with the indication, from estimates of τ_c, of restricted motion of the potentially more flexible NeuAc-Gal linkage, seem to support the hypothesis that this particular trisaccharide system is characterized by a relatively low level of conformational flexibility, although contributions from significantly less populated conformers cannot be ruled out. This appears to be the concensus from most studies published so far [7, 11, 12]. It would be desirable to treat this system using a full mechanical force field, with explicit inclusion of solvent molecules, and molecular dynamics simulations [57]; at the present time, however, there are insufficient computational facilities available in this laboratory to undertake such studies. With regard to application of a more appropriate force field, a precise consideration of the geometrical constraints associated with π-H bonding may be necessary in order to judge the strength of this interaction in gangliosides.

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