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Conformational analysis of sialyloligosaccharides*

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ABSTRACT

The conformational properties of several sialyloligosaccharides present as terminal sequences in N- and O-linked carbohydrate groups of glycoproteins, have been analyzed based on the n.m.r. data of selected sialosides. The compounds examined include representatives of the α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc, α-D-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc, α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc, and α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GalNAc series. Two deuterated sialosides were prepared by enzymic sialylation of 6-deuterated galactose derivatives of methyl β-D-galactopyranoside and lactoside. These were useful for the unambiguous establishment of the "*gt*" orientation of the flexible C-6 methylene unit of the galactose through ^1H–^1H coupling constants. Of all the (2→6) sialosides examined, only the deuterated di- and tri-saccharide afforded useful nuclear Overhauser enhancement data that could be used to evaluate the global minimum-energy conformations. Hard-sphere exoanomeric effect calculations estimated the glycosidic torsion angles for the global minimum-energy conformer of α-D-NeuAc-(2→6)-β-D-Gal linkages to be −163/−132/61° (θ, ψ, and ω, respectively). However, the potential energy well surrounding this global minimum was very shallow and indicated a broad population distribution of conformers. These are illustrated by the isoenergy contour maps. The observation of n.O.e. between the H-3*ax* and H-6*R* of the galactose in two deuterated (2→6) sialosides, indeed supported the presence of one of the global minimum-energy conformers. The conformational analysis carried out for the di- and tri-saccharide [α-D-NeuAc-(2→6)-β-D-Gal-OMe and α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-Glc-OMe respectively] was then extended to sialoside linkages of other tri- and penta-saccharides by comparison of their ^1H- and ^{13}C-n.m.r. chemical shifts. HSEA calculations for the (2→3) sialosides indicated the potential energy well containing the global minimum energy-conformer (θ, ψ = −160 ±4, −11 ±2°) was deeper than the one estimated for the (2→6) sialosides. The n.O.e. data are consistent with the distribution of the majority of conformers around the lowest-energy one in solution. CPK models highlighting the topographical differences between the lowest-energy conformations of α-(2→6) and α-(2→3) sialosides are presented.

INTRODUCTION

Sialic acid, a C_9 acidic ketopyranose, is found as a terminal sugar in a diverse group of carbohydrates present in glycoproteins and glycolipids, which mediate a

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variety of biological processes[1]. Cell-surface sialyloligosaccharides serve as receptor determinants for influenza and other viruses[2,3], for mycoplasma[4,5], for bacterial toxins[1], for blood-group and tumor-specific antibodies[1,6], for interferon[7], for recirculating lymphocytes seeking capillary entry in to the lymph system[8], and for a variety of plant and animal lectins[9–11]. In addition, they appear to be responsible for the developmental modulation of the activity of the neural cell-adhesion molecule[12] and can induce differentiation of lymphoid stem cells to macrophages[13]. Many of these processes involve a carbohydrate-binding protein, which exhibits a high degree of specificity toward sialyloligosaccharides of defined sequence. A complete understanding of the binding specificity of these proteins will ultimately require knowledge of the three-dimensional structures of both the proteins and the various sialyloligosaccharides in an aqueous environment. To date, two sialic acid-binding proteins have been crystallized and their three-dimensional structures determined, namely, influenza virus sialidase and the influenza virus hemagglutinin[3,14,15]. In both cases, the sialic acid-binding sites have been identified. However, little is known about the solution conformations of the sialyloligosaccharides to which they bind[16].

Through the combined use of high-field n.m.r. spectroscopy and molecular modeling techniques, it is now possible to obtain information regarding the secondary structures of oligosaccharides defined by the rotation around the interunit glycosidic bonds connecting the sugar residues[17–30]. In general, this information is suitable to evaluate the three-dimensional solution structure of an oligosaccharide, as the pyranose rings of sugar residues appear to have fairly rigid conformations in solution. Thus, the solution conformations of many biologically relevant neutral oligosaccharides have been determined, allowing a detailed analysis of their interactions with antibodies and lectins[31].

Examination of the sialyloligosaccharide sequences of glycoproteins and glycolipids reveals that sialic acid is found attached to three neutral disaccharide sequences. These are β-D-Gal-(1→3)-β-D-GlcNAc, β-D-Gal-(1→4)-β-D-GlcNAc, and β-D-Gal-(1→3)-β-D-GalNAc, commonly referred to as type 1, type 2, and type 3 sequences, respectively[32]. Sialic acid is found attached in α-D-NeuAc-(2→3)-β-D-Gal linkage in all the three sequences, in the α-D-NeuAc-(2→6)-β-D-Gal linkage in the type 2 sequence, and in the α-D-NeuAc-(2→6)-β-D-GlcNAc linkage in the type 1 sequence. In addition, sialic acid is found in α-D-NeuAc-(2→6)-α-D-GalNAc sequences of O-linked glycoproteins and in α-D-NeuAc-(2→8)-α-D-NeuAc-(2→3)-β-D-Gal sequence attached to type 1 and type 3 sequences[33]. Although the primary structures of several sialosides have been investigated using sialyloligosaccharides derived from natural sources[34–36], the difficulty in obtaining sufficient quantities of most of the sialosides has hampered a systematic analysis of their conformational properties.

Recently, it has been demonstrated that sialyloligosaccharides can be prepared in quantities sufficient for n.m.r. investigations through the combined use of chemical and enzymic procedures[37], and using these methods we have prepared a variety of sialosides containing α-D-NeuAc-(2→6)-β-D-Gal and α-D-NeuAc-(2→3)-β-D-Gal linkages[37,38]. In this report, we present a detailed n.m.r. analysis of the synthetic compounds together with HSEA calculations to evaluate their conformational properties. These results

provide a basis for further refinement of sialyloligosaccharide conformations in aqueous solutions. Moreover, since these sequences are commonly found to terminate the carbohydrate groups of glycoproteins and glycolipids, such information will aid the development of working molecular models to better understand protein–sialyloligosaccharide interactions involved in biological systems.

EXPERIMENTAL

General methods. — All solvents and reagents were purified according to the standard procedures[39]. CMP-NeuAc and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis). Methyl 6 (*S*)-β-D-(6-^{2}H)galactopyranoside (**1b**) was prepared from 6 (*S*)-2,3,4, tri-*O*-acetyl-1,6-anhydro-β-D-(6-^{2}H)galactopyranose[40] by converting it into the corresponding acetylated glycosyl bromide followed by condensation with methanol[41]. *O*-deacetylation afforded methyl 6 (*S*)-β-D-(6-^{2}H)galactopyranoside (**1b**). Methyl 6 (*S*)-β-D-(6-^{2}H)lactoside was prepared as described by Bock and Refn[41]. β-D-Gal-(1→4)-3-deoxy-β-D-GlcNAc-$OCH_2CH_2CH_3$* (**3**) was kindly provided by Professor R. U. Lemieux of The University of Alberta (for the preparation of the corresponding methyl glycoside see ref. 42). The Galβ1,4GlcNAc α-(2→6) sialyltransferase (EC 2.4.99.1) was purified as described previously[43].

^{1}H-N.m.r. spectra were recorded on a Bruker WM-500 (500 MHz) spectrometer with 32K data points (Aspect 2000 computer). The ^{1}H spectra were measured at 296 K and the proton chemical shifts for compounds in deuterium oxide are expressed relative to the HOD signal (4.81 p.p.m. at 296 K), which sets the chemical shift of acetone at 2.223 p.p.m. ^{13}C-N.m.r. spectra were measured at 304 K with the foregoing instrument operating at 125.76 MHz. A relaxation delay of 1 s was introduced between 90° pulses and a line-broadening factor of 2 Hz was introduced prior to the Fourier transformation of the free-induction decay. The carbon chemical shifts are expressed[16,44] relative to external Me_4Si using the deuterium lock of the spectrometer, which sets the ^{13}C chemical shift of 1,4-dioxane in D_2O at 66.88 p.p.m. at 304 K.

Methyl (5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-*2-nonulopyranosylonic acid)-(2→6)6(S)-β-D-(6-^{2}H)galactopyranoside* (**5b**). — Methyl 6 (*S*)-β-D-(6-^{2}H)galactopyranoside[41] (**1b**, 18.7 mg, 95.8 μmol) and CMP-NeuAc (22 mg; 35 μmol) were dissolved in 0.1M sodium cacodylate buffer (pH 6.5, 1 mL) containing Triton-CF 54 (0.1%) and bovine serum albumin (2 mg). The Galβ1,4GlcNAc α-(2→6) sialyltransferase (400 mU) was added and the solution was incubated for 24 h at 37°. The mixture was then diluted to 13 mL and applied on a column (1.5 × 9 cm) of Dowex 1-X2 (HPO_4^{2-} form, 200–400 mesh). The column was washed with distilled water (175 mL) and then eluted with 5mM sodium phosphate buffer (pH 6.8). Fractions (5 mL) were assayed for sialic acid by the periodate–resorcinol procedure[45]. The fractions containing the product, which eluted before free sialic acid, were pooled and evaporated to a dry

* 2-Acetamido-2,3-dideoxy-D-*ribo*-hexopyranose is trivially abbreviated as 3-deoxy-D-GlcNAc in this article.

residue. The product was then redissolved in 2 mL of water and applied on a column (1.6 × 24 cm) of Sephadex G-15 (Sigma) equilibrated and eluted with water. The fractions (1.5 mL) containing the sialyloligosaccharide, as evidenced by the periodate–resorcinol procedure[45], were measured for conductivity to exclude contamination by salts, pooled and lyophilized; yield 10.6 mg; ^{1}H-n.m.r. (see Fig. 1b); ^{13}C-n.m.r. (see Table IV).

*Methyl (5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosylonic acid)-(2→6)-6(S)-β-*D*-(6-^{2}H)galactopyranosyl-(1→4)-β-*D*-glucopyranoside* (**5c**). — The deuterated lactoside **1c**[41] (32.0 mg) was incubated (37°) with CMP-NeuAc (25 mg) in a sodium cacodylate (100mM) buffer (1 mL, pH 6.5) containing Galβ1,4GlcNAc α-(2→6) sialyltransferase (400 mU), bovine serum albumin (2 mg) and Triton CF-54 (0.1%) for 24 h. The product from this mixture was purified as described for **5b**; yield 12.5 μmol (based on sialic acid assay). The complete hydrogen chemical-shift assignments for this compound was made by 1D-total correlation spectroscopy using Gaussian shaped selective pulses[46]; ^{1}H-n.m.r. see Table III; ^{13}C-n.m.r. see Table IV.

*Propyl (5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosylonic acid)-(2→6)-β-*D*-galactopyranosyl(1→4)-2-acetamido-2,3-dideoxy-β-*D*-ribo-hexopyranoside* (**8**). — β-D-Gal-(1→4)-3-deoxy-β-D-GlcNAc-$OCH_2CH_2CH_3$[42] (**3**; 8 mg, 19.5 μmol based on wt.) and CMP-NeuAc (25 mg, 39 mol) were dissolved in 1 mL aq. 0.1M sodium cacodylate, pH 6.5, containing 2 mg bovine serum albumin. The Galβ1,4GlcNAc α-(2→6) sialyltransferase (400 mU) was added and the solution was incubated for 24 h at 37°. The isolation of the product was carried out as described earlier; yield 14.1 mol (based on sialic acid assay); ^{1}H-n.m.r. δ*: 4.493 (d, 1 H, *J* 8.5 Hz, H-1), 4.434 (d, 1 H, *J* 8.2 Hz, H-1′), 3.823 (t, 1 H, H-5″), 3.495 (dd, 1 H, *J* 8.2, 9.7 Hz, H-2′), 2.710 (dd, 1 H, *J* 4.5, 11.9 Hz, H-3″*eq*), 2.514 (m, 1 H, H-3*eq*), 2.025, 2.003 (2 × S, 6 H, 2 × CH_3-CONH-), 1.687 (m, 1 H, H-3*ax*), 1.577 (t, 1 H, *J* 11.9 Hz, H-3″*ax*), 0.838 (t, 3 H, CH_3-CH_2CH_2-O-); ^{13}C-n.m.r. see Table IV.

*(Methyl 5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosylonate)-(2→6)-β-*D*-galactopyranosyl(1→4)-2-acetamido-2-deoxy-β-*D*-glucopyranosyl-(1→3)-β-*D*-galactopyranosyl(1→4)-*D*-glucopyranose, (LSTc methyl ester* **9a**). — LSTc[31] (**9**) was dissolved in Me_2SO (5 mL) containing MeI (0.5 mL). The solution was stirred for 3 h at room temperature by which time all the starting material disappeared. The solution was diluted with water (10 mL) and lyophilized to obtain LSTc methyl ester in near-quantitative yield; ^{13}C-n.m.r. see Table IV.

Hard-sphere exoanomeric (HSEA) effect calculations. — The HSEA effect calculations (Table II) were performed following the procedure of Lemieux *et al.*[17,18]. The three-dimensional coordinates for the neutral sugar residues were obtained from the appropriate X-ray or neutron-diffraction data[47,48]. For the α-sialic acid residue, these parameters were synthesized from those of β-D-NeuAc according to the reported

* (*a*) The pyranose residues are numbered from the reducing end. Thus, in **8**, the GlcNAc hydrogens are denoted from 1–6, the galactose hydrogens 1′–6′ and sialic acid hydrogens 3″–9″. (*b*) In α-D-NeuAc-(2→3,6)-β-D-Gal disaccharides, the NeuAc unit atoms are denoted by C-1′–C-9′ and the galactose unit atoms by C-1–C-6.

TABLE I

List of sialyloligosaccharides and related asialo-compounds used in the n.m.r. studies

Asialo compounds	α-D-*NeuAc*-(2→6)-β-D-*Gal glycosides*	α-D-*NeuAc*-(2→3)-β-D-*Gal glycosides*	α-D-*NeuAc*-(2→6)-β-D-*GlcNAc glycosides*
β-D-Gal-OMe, **1a**	α-D-NeuAc-(2→6)-β-D-Gal-OMe, **5a**	α-D-NeuAc-(2→3)-β-D-Gal-O$(CH_2)_8$ CO_2Me, **10a** β-D-NeuAc-(2→3)-β-D-Gal-O$(CH_2)_8$ CO_2Me, **10b**	
6 (*S*)-β-D-(6-^{2}H)Gal-OMe, **1b**	α-D-NeuAc-(2→6)-6(*S*)-β-D-(6-^{2}H) Gal-OMe, **5b**		
6 (*S*)-β-D-(6-^{2}H)Gal-(1→4)-β-D-Glc-OMe, **1c**	α-D-NeuAc-(2→6)-6(*S*)-β-D-(6-^{2}H) Gal-(1→4)-β-D-Glc-OMe, **5c**		
β-D-Gal-(1→4)-β-D-GlcNAc-OMe, **2**	α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-OR, R = Me, **6a** R = $(CH_2)_8CO_2Me$, **6b** β-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-O$(CH_2)_8$-CO_2Me, **7**	α-D-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-OMe, **11** α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc-OMe, **12**	
β-D-Gal-(1→4)-3-deoxy-β-D-GlcNAc-O-$CH_2CH_2CH_3$, **3**	α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-3-deoxy-β-D-GlcNAc-O-$CH_2CH_2CH_3$, **8**	α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GalNAc-O$(CH_2)_5COOCH_3$, **13**	α-D-NeuAc-(2→6)-β-D-GlcNAc-O$(CH_2)_8COOCH_3$, **16**
β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-Glc, LNNT, **4**	α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-Glc, LSTc, **9** Me-α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-Glc, **9a**	α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, LSTa, **14** α-D-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, LSTd, **15**	β-D-Gal-(1→3)-[α-D-NeuAc-(2→6)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, LSTb, **17** α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-[α-D-NeuAc-(2→6)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, DSL, **18**

procedure[16,49a]. The energy minimization procedures for the orientation of the carboxylate group of the sialic acid indicated two energy minima separated by 0.43 kcal, which corresponded to torsion angles of −92.2° (0.4 kcal/mol) and 74.7° (−0.03 kcal/mol) with the glycosidic oxygen. Both the orientations were included in the HSEA calculation of each sialoside. Since both the orientations predicted near identical values for the torsion angles around the glycosidic linkages, the orientation of the carboxylate unit (74.7°) with lower energy was assumed to represent the most stable conformer (Table II). The definition of the torsion angles for the glycosidic linkages are as follows: α-D-NeuAc-(2→3)-β-D-Gal: φ, C-1′–C-2′–O2′–C-3); ψ, C-2′–O-2′–C-3–H-3; and α-D-NeuAc(2→6)βDGal/GlcNAc (see footnote on p. 30): φ, C-1′–C-2′–O-2′–C-6; ψ, C-2′–O-2′–C-6′–C-5; and ω, O-6–C-6–C-5–O-5. The orientation of the carboxylate group is defined by the torsion angles: O-1′A–C-1′–C-2′–O-2′.

SYNTHESIS OF SIALOSIDES

Table I shows the list of sialyloligosaccharides that were used in the n.m.r. studies. The syntheses of all these compounds except **5b**, **8**, and **9a**, have been published[37]. The 6 (*S*)-deuterated methyl galactoside (**1b**) was prepared from 6 (*S*)-1,6-anhydro-β-D-(6-^{2}H) galactopyranose[40] via the glycosyl bromide, and the corresponding deuterated methyl lactoside was prepared as described by Bock and Refn[41].

Even though both the deuterated galactoside **1b** and lactoside **1c** were poor substrates, these could be sialylated readily by using excess CMP-sialic acid and Galβ1,4GlcNAc α-(2→6) sialyltransferase. Similarly, while the 3-deoxy-*N*-acetyllactosamine derivative **3** exhibited diminished binding affinity to the enzyme by 10 fold as compared to the natural substrate *N*-acetyllactosamine (K_m = 10mM), it was still a good substrate to undergo quantitative sialylation. All the sialylated products were readily isolated in highly pure form by ion-exchange chromatography. The structural identities of these sialosides were established by both ^{1}H- and ^{13}C-n.m.r. spectroscopy (See Tables III and IV). As shown in Fig. 1, the ^{1}H-n.m.r. spectra of the two key disaccharides, **5a** and **5b**, were sufficiently well resolved at 500 MHz that all the proton signals could be readily assigned. As expected, the deuteration of the C-6 of the galactose residue simplified the signals for both H-5 and H-6 of the galactose to doublets with spacing of ~8.5 Hz. For the complete assignment of hydrogen chemical-shifts in the trisaccharides **5c** and **6b** (Table III), 1D-TOCSY experiments using selective Gaussian shaped pulses were used according to the reported methods[46]. For the disaccharides and trisaccharides, the ^{13}C chemical-shift assignments could be readily made by comparison with the asialo materials **1b**, **1c**, and **3**, respectively (Table IV).

HARD SPHERE EXOANOMERIC EFFECT CALCULATIONS OF SIALOSIDES

Estimation of the conformational properties for sialosidic linkages was begun with the glycosidic torsion angles for the three neutral disaccharides sequences, β-D-Gal-(1→3)-β-D-GlcNAc (type 1: φ,ψ = 55,20°), β-D-Gal-(1→4)-β-D-GlcNAc (type 2;

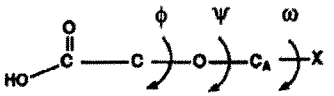
TABLE II

The minimum energy conformation as estimated by the hard sphere exoanomeric (HSEA) effect calculations[17,18] for glycosidic linkages involving the sialic acid and the aglycon. The torsion angles[a] (φ,ψ,ω)[17] represent the calculated values for the sialosidic linkage shown in bold letters

Compound	*Glycosidic torsion angles (°)*	*Energy (kcal/mol)*
α-D-*NeuAc-(2→6)-β-D-Gal glycosides*		
α-D-NeuAc-(**2→6**)-β-D-Gal-OMe, **5a**	−163/−132/61	2.35
α-D-NeuAc-(**2→6**)-β-D-Gal-(1→4)-β-D-GlcNAc-OMe, **6a**	−164/−131/71 (*gt*)	−1.83
	−170/−116/174 (*tg*)	0.75
α-D-NeuAc-(**2→6**)-β-D-Gal-(1→4)-β-D-3-deoxy-GlcNAc-$OCH_2CH_2CH_3$, **8**	−162/−128/60	−2.57
α-D-NeuAc-(**2→6**)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-Glc, LSTc, **9**	−160/−131/60	−5.71
α-D-*NeuAc-(2→3)-β-D-Gal glycosides*		
α-D-NeuAc-(**2→3**)-β-D-Gal-O-$(CH_2)_8CO_2Me$, **10a**	−160/−13	−0.49
α-D-NeuAc-(**2→3**)-β-D-Gal-(1→4)-β-D-GlcNAc-OMe, **11**	−163/−13	−2.20
α-D-NeuAc-(**2→3**)-β-D-Gal-(1→3)-β-D-GlcNAc-OMe, **12**	−163/−12	−3.11
α-D-NeuAc-(**2→3**)-β-D-Gal-(1→3)-β-D-GalNAc-O$(CH_2)_5CO_2Me$, **13**	−163/−11	−3.46
α-D-NeuAc-(**2→3**)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, LSTa, **14**	−163/−11	−7.35
α-D-NeuAc-(**2→3**)-β-D-Gal-(1→3)-[α-D-NeuAc-(2→6)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, DSL, **18**	−156/−16	−7.65
α-D-*NeuAc-(2→6)-β-D-GlcNAc glycosides*		
β-D-Gal-(1→3)-[α-D-NeuAc-(**2→6**)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, LSTb, **17**	−155/−146/−56 (*gg*)	3.08
	−155/−148/58 (*gt*)	3.60
α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-[α-D-NeuAc-(**2→6**)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc, DSL, **18**	−150/−162/47	−7.65

[a] The sialosidic torsion angles can be represented as:

α-D-NeuAc-(2→6) linkage: C_A = C-6, X = C-5
α-D-NeuAc-(2→3) linkage: C_A = C-3, X = H.



φ,ψ = 55,0°) and β-D-Gal-(1→3)-β-D-GalNAc (type 3; φ,ψ = 55,10°), set at values provided by Lemieux and co-workers[16,17]. The effects of sialic acid substitution on these structures were then evaluated by the HSEA effect minimization procedures[17,18]. For all the three disaccharides substituted with the α-D-NeuAc-(2→3)-β-D-Gal linkage, sialic acid had no significant effect on the conformational properties of the remainder of the glycosidic linkages.

The torsion angles defining the conformational properties of the α-(2→3) sialoside linkages are represented by θ and ψ values, while for α-(2→6) sialosides an additional rotational freedom that arises around the flexible C-6 of the galactose or *N*-acetylglucosamine units is included by the ω angle following general conventions[17] (see Fig. 2). The values of glycosidic torsion angles for various sialosides at their global energy minima are shown in Table II. The conformers that are accessible within 10 kcal/mol from the lowest energy one are shown in the isoenergy contour diagrams (Fig. 3). The analyses of the n.m.r. results relevant to the solution conformation of α-(2→6) and α-(2→3) sialosides, and the evaluation of the degree to which the minimum-energy conformers are populated in solution, are discussed for each class separately later.

CONFORMATIONAL ANALYSIS OF α-D-NeuAc-(2→6)-β-D-Gal GLYCOSIDES

For α-D-NeuAc-(2→6)-β-D-Gal linkages, the C-6 arm of the galactose can potentially orient either in "*gt*" or "*tg*" orientation* (shown in Fig. 2). Accordingly, both these orientations were included in the HSEA minimization procedures. The glycosidic torsion angles θ, ψ, and ω for (2→6) sialosides at their global energy minimum in "*gt*" and "*tg*" orientation are (−164, −131, 71°) and (−170, −116, 174°), respectively (Fig. 2)†. The "*gt*" conformer was estimated to be lower in energy than the "*tg*" conformer by ~2.6 kcal/mol. Since this difference in energy between these conformers was small, direct experimental evidence was sought to establish the value of the ω torsion angle. This was done by the selective replacement of one of the C-6 methylene hydrogens, namely C-6(*S*) by deuterium, as seen in sialylgalactoside and sialyllactoside, **5b** and **5c**, respectively. In each case, the stereochemistry of deuteration at the C-6 of the galactose was unambiguous, as **1b** and **1c** were prepared from authentic 6(*S*)-1,6-anhydro-β-D-(6-^{2}H)galactose[40,41] followed by the enzymic transfer of sialic acids to provide **5b** and **5c**, respectively.

For these deuterated sialosides, only H-6(*R*) has the potential for spin coupling with the H-5 of galactose. As seen from the structures shown in Fig. 2, the placement of H-6(*R*) to H-5 of the galactose is antiperiplanar in the "*gt*" orientation, and synclinal in

* The "*gg*" conformation of the C-6 arm of the galactose has not been considered as its contribution to the total rotomer population is known to be insignificant for galactopyranosides[49b]. This is also substantiated by the magnitude of the coupling constants observed for the C-6 hydrogens of galactose in α-D-NeuAc-(2→6)-β-D-Gal derivatives reported in Table III.

† The ideal ω torsion angle in "*gt*" and "*tg*" conformation should be 60 and 180°, respectively. Even though the calculated ω torsion angles of 71 and 174° do not exactly correspond to "*gt*" and "*tg*" values, these have been still used for the purpose of simplification.

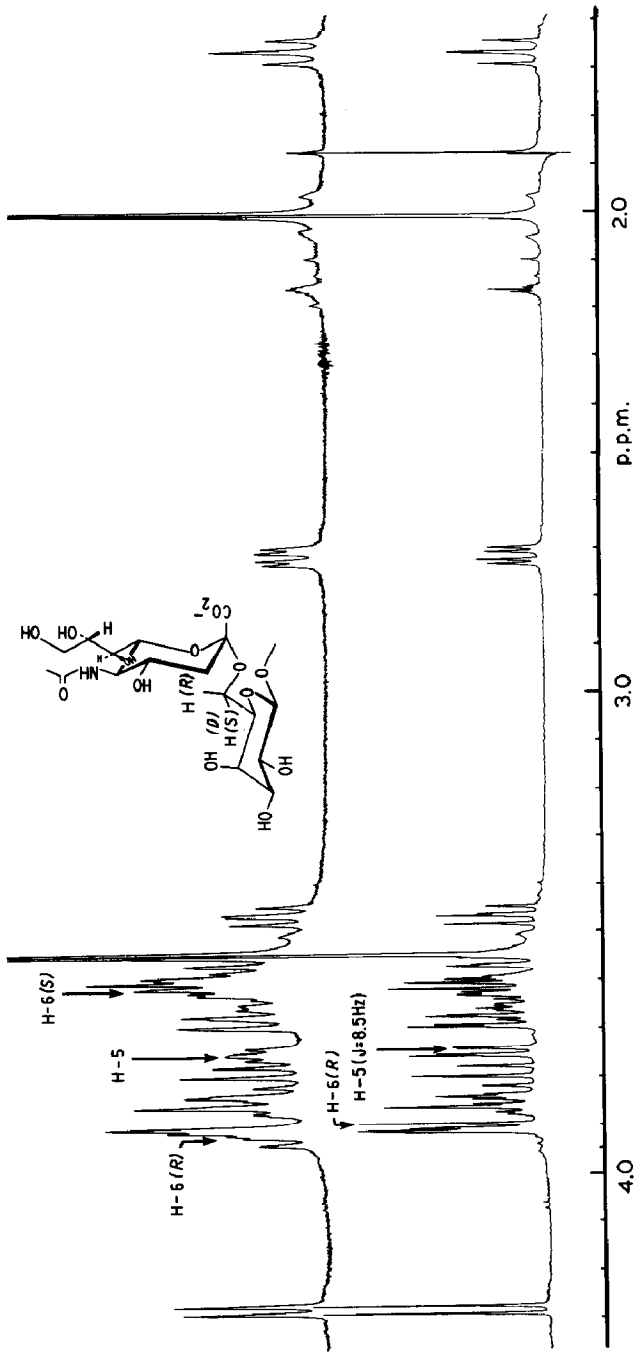


Fig. 1. ^{1}H-N.m.r. spectrum of α-D-NeuAc-(2→6)-β-D-Gal-OMe (top) and α-D-NeuAc-(2→6)-6-(*S*)-β-D-(6-^{2}H)Gal-OMe (bottom). The changes that accompany deuteration for galactose hydrogens (H-6(*R*), H-6(*S*), and H-5) are indicated.

TABLE III

A comparison of ^1H chemical shifts of the natural sialosides **5a**, **5c**, **6b**, and **10a** with the unnatural β anomers **7** and **10b** and the complete assignments of the ^1H chemical shifts for the di- and hexa-saccharide DSL, **16** and **18**, respectively[a]. The ^1H–^1H coupling constants are given in parentheses

Sugar unit	*Hydrogen atom*	**5a**	**5c**	**6b**	**7**	**10a**	**10b**	**16**	**18**
α-D-NeuAc-(2→6)	3*ax*	1.69 (12.1)	1.76 (11.0)	1.72 (12.0)	1.66 (11.4)	—	—	1.73 (12.0)	1.73
	3*eq*	2.73 (4.8)	2.73 (3.0)	2.70 (4.0)	2.40 (5.0)	—	—	2.75 (5.0)	2.77
	4	3.67	3.68 (10.0)	3.63 (10.0)	4.08 (10.0)	—	—	3.72 (10.0)	3.74
	5	3.83	3.88 (10.0)	3.81 (10.0)	3.88 (10.0)	—	—	3.84 (10.0)	3.87
	6	3.71	3.72 (2.7)	3.71 (2.0)	3.81 (0.9)	—	—	3.68 (2.0)	3.62
	7	3.59	3.60	3.58 (9.0)	3.59 (9.0)	—	—	3.60	3.56
	8	3.88	3.91	3.90	3.83	—	—	3.88	3.92
	9a	3.87	3.89 (2.5)	3.88	3.84	—	—	3.89	3.93
	9b	3.64	3.68 (6.0,12.0)	3.66	3.67	—	—	3.66	3.69
	NAc	2.05	2.05	2.06	2.06	—	—	–	–
α-D-NeuAc-(2→3)^6	3*ax*					1.81 (12.0)	1.72 (11.7)	—	1.85
	3*eq*					2.78 (4.7,12.1)	2.49 (4.8,13.1)	—	2.81
	4					3.68 (10.0)	4.20 (9.6)	—	3.70
	5					3.86 (10.0)	3.96 (10.4)	—	3.89
	6					3.66 (2.0)	3.99 (0.9)	—	3.71
	7					3.61 (9.3)	3.54 (9.6)	—	3.64
	8					3.89	3.83	—	3.94
	9a					3.88 (2.5,12.50)	3.85 (3.0)	—	3.89
	9b					3.68 (6.0)	3.69 (5.1,12.0)	—	3.69
β-D-Gal	1	4.31 (8.0)	4.45 (8.0)	4.47 (7.9)	4.48 (7.9)	4.46 (7.9)	4.42 (7.8)		4.59
	2	3.49 (10.0)	3.54 (10.0)	3.56 (10.0)	3.58 (10.0)	3.55 (9.6)	3.63 (10.0)		3.59
	3	3.61 (3.6)	3.65 (3.4)	3.69 (3.6)	3.67 (3.5)	4.09 (3.6)	3.70 (3.3)		4.15
	4	3.93 (0.8)	3.96 (1.0)	3.95 (0.8)	3.95 (0.8)	3.97 (0.8)	4.24 (0.8)		4.00
	5	3.71	3.82 (8.5)	3.83	3.74	3.68	3.64		3.71
	6a	3.94 (8.5)	3.97 (8.5)	4.00 (9.0)	3.85 (3.6)	3.77 (7.5)	3.77 (7.5)		–
	6b	3.63 (4.8,10.6)		3.59 (3.5)	3.67 (9.0)	3.74 (4.5)	3.71 (4.8)		3.83

β-D-GlcNAc	1	—	—	4.57 (8.0)	4.55 (8.2)		4.50 (8.5)	4.77
	2			3.73 (9.5)	3.74 (10.0)		3.70	3.94
	3			3.76 (10.0)	3.79 (9.8)		3.70	3.86
	4			3.67 (10.0)	3.67 (10.0)		3.50	3.67
	5			3.62	3.61		3.52	3.59
	6a			3.99 (2.1)	4.00 (2.4)		3.76 (2.0,11.0)	3.85
	6b			3.84 (5.6)	3.81 (5.2)		3.96 (5.0)	4.01
β-D-Gal	1							4.51
	2							3.65
	3							3.79
	4							4.25
	5							3.77
	6a							3.78
	6b							3.79
β-/α-D-Glc	1		4.43 (8.0)					4.71
	2		3.36 (10.0)					3.39
	3		3.68					3.69
	4		3.68					3.70
	5		3.62					3.65
	6a		3.99 (1.9)					3.87
	6b		3.82 (3.5, 12.0)					—

[a] See Table I for structures.

the "*tg*" orientation (panel B), and thus will be predicted to produce large (~ 10 Hz) and small (~ 3.5 Hz) coupling constants, respectively[50]. Examination of the coupling constant between H-6(*R*) and H-5 should, therefore, establish the relative populations of the "*gt*" and "*tg*" orientations in solution. The results for compounds **5a** and **5b** are shown in Fig. 1.

Fig. 1 (top) shows the ^{1}H-n.m.r. spectrum of α-D-NeuAc-(2→6)-β-D-Gal-OMe (**5a**). In this compound, the signals for the C-6 and C-5 hydrogens of the galactose residue could be identified at 3.94, 3.63, and 3.71 p.p.m., respectively. These assignments were also confirmed by selective decoupling experiments. The replacement of one of the C-6 hydrogens of galactose by deuterium as seen in the deuterated sialoside **5b** (Fig. 1, bottom), caused the complete disappearance of the multiplets at 3.63 p.p.m., originally assigned to H-6 (*S*), and changed the multiplets of the H-5 of the galactose to give a broad doublet ($J_{H6,H5}$ 8.5 Hz). In addition, the signal for the other C-6 hydrogen of the galactose appeared as a doublet at 3.94 p.p.m. with a coupling constant of 8.5 Hz.

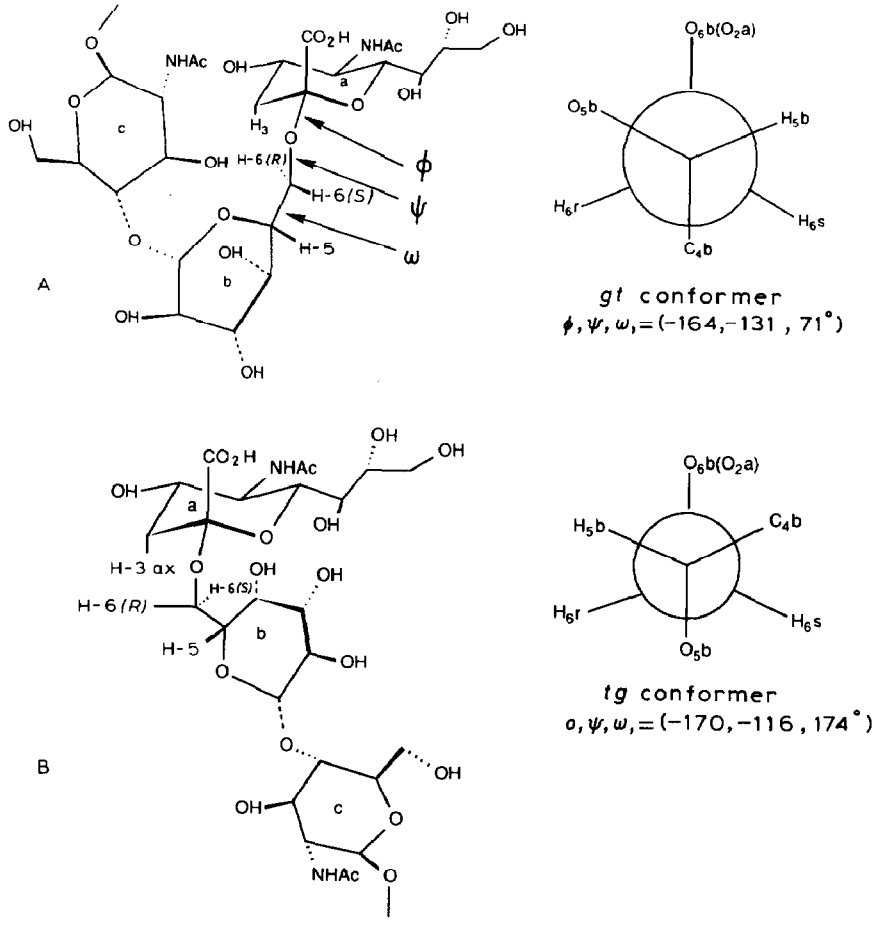


Fig. 2. Lowest energy "*gt*" and "*tg*" conformations for trisaccharide **6a** predicted by HSEA calculations.

This large coupling constant required that H-6(*R*) be antiperiplanar to H-5, confirming the "*gt*" orientation as the predominant one for sialoside **5b** in solution[50,51].

It was equally important to evaluate the trisaccharide **5c**, since the "*gt*" orientation would place the sialic acid closer to a bulky aglycon at the C-1 of galactose, and therefore might invoke interactions between these two units. However, a similar coupling constant of 8.5 Hz was observed for H-5 to H-6 of galactose, requiring these two hydrogens to be antiperiplanar to each other as in disaccharide **5b**. Thus, irrespective of the nature of the aglycon at C-1 of the galactose, the sialylated C-6 arm favors the "*gt*" orientation. This conclusion is also supported from the chemical shifts and the coupling constants for the C-6 hydrogens of the sialylated galactose of other α-D-NeuAc-(2→6)-β-D-Gal derivatives. For example, in the trisaccharide **6b** (Table III), H-6(*R*) of the sialylated galactose appears at 4.00 p.p.m. ($J_{H\text{-}6,H\text{-}5}$ 9.0 Hz), whereas in the pentasaccharide **9**, the corresponding hydrogen appears at 3.987 p.p.m. ($J_{H\text{-}6,H\text{-}5}$ 10.0 Hz) indicating the favored "*gt*" orientation of galactose in these molecules. These observations are in agreement with the conclusions reached recently by Breg *et al.*[52a] and Ohrui *et al.*[52b]

Thus, the establishment of "*gt*" orientation for (2→6) sialosides, simplified the representation of the distribution of conformer populations around the global energy-minimum one (depicted in Fig. 2A) in two-dimensional space. We then calculated for the (2→6) sialosides, all the conformers that were accessible within 10 kcal/mol from the lowest energy one. The results are shown in Fig. 3. As can be seen from the energy contour map for α-D-NeuAc-(2→6)-β-D-Gal-OMe in Fig. 3A, the energy well surrounding the lowest-energy conformation (−163/−132/61°) is very shallow. The same is seen

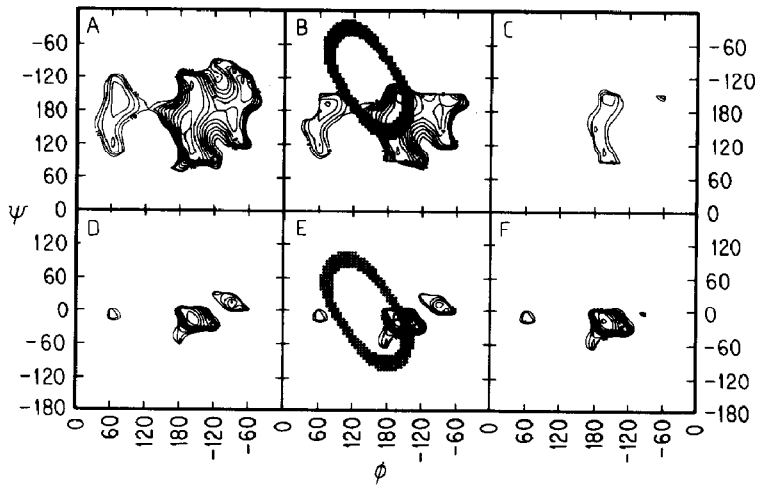


Fig. 3. Energy-contour maps for selected α-(2→6) and α-(2→3) sialoside linkages. Shown are maps for α-D-NeuAc-(2→6)-β-D-Gal-OMe (*A*), α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-OMe (*B* and *C*), α-D-NeuAc-(2→3)-β-D-Gal-OMe (*D*), α-D-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-OMe (*E*), and α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc-OMe (*F*). In each case contour lines represent differences of 1 kcal/mol, and the outside line surrounding each energy well represents a level 10 kcal/mol (except *C*, where the outside line corresponds to 3 kcal/mol) above the minimum-energy conformer. The shaded ovals in *B* and *E* represent conformers expected to provide n.O.e.'s between H-3*ax* (NeuAc) and H-6 (*R*)- and H-3 of galactose of α-(2→6) and α-(2→3) sialosides, respectively.

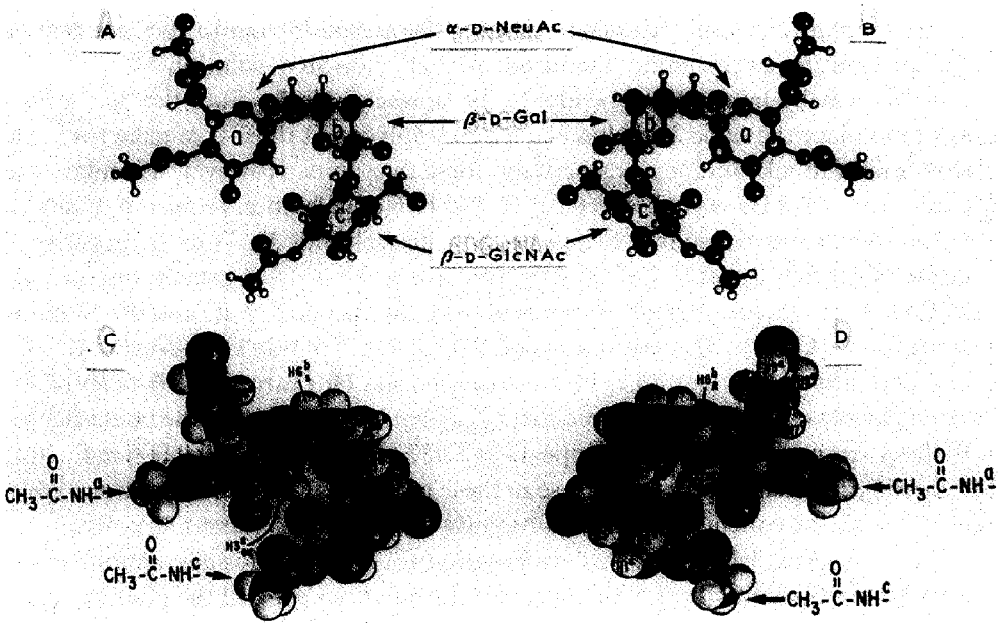


Fig. 4. Computer-drawn ball- and stick- (*A* and *B*) and CPK (*C* and *D*) models for the minimum-energy conformer of α-D-NeuAc^a-(2→6)-β-D-Gal^b-(1→4)-β-D-GlcNAc^c. In *A* and *C* the carboxylate group (see O1) is facing out of plane of the paper and in *B* and *D* it is facing into the plane of the paper.

for the corresponding trisaccharide α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc, although conformers with ψ angles from -90 to $-130°$ are restricted relative to the disaccharide (see Fig. 3B).

Calculation of the population distribution of the conformers over the contour map reveals that >90% of the molecules will be expected to exist in conformations having relative energies within 3 kcal/mol of the lowest energy conformer. Thus, the majority of the molecules will be expected to cover a more restricted range of conformations, as illustrated in Fig. 3C for the trisaccharide **6a**. *Within this boundary, only ~45% of the population is expected to have torsion angles within 15° of −160/−145/60°, including the lowest-energy conformation depicted in Figs. 2 and 4.* It can also be seen that the greatest flexibility is in the ψ domain extending from −130 to 90°, with a full 15% of the population predicted to reside at an extreme within 10° of a conformer defined by −160/100/60°. An additional 4–5% of the molecules are estimated to have the aglycon in the synclinal orientation (−70/−150/60°).

For the analysis of the n.m.r. data, we used the CPK projections of the global minimum-energy conformer (Fig. 4) to extract relevant internuclear distances. It was evident from these models of the global minimum energy conformer, that the aglyconic hydrogen H-6*R* of the galactose and the intraresidual hydrogen H-5 of the NeuAc unit were within 2.5-Å from H-3*ax* of sialic acid, indicating their suitability for detection by n.O.e. experiments. This close proximity between H-6 (*R*) of the galactose and H-3*ax* of

the NeuAc unit however, was seen in less than 35% of the total population of conformers that were estimated to be accessible within 10 kcal/mol from the global minimum one (see the n.O.e. overlaid map, Fig. 3B).

It may be seen from the proton spectrum of the disaccharide α-D-NeuAc-(2→6)-β-D-Gal-OMe, **5a**, (Fig. 1), that the signals for the H-3*ax* of the sialic acid appear well isolated from the signals of the remainder of hydrogen atoms to enable selective saturation. When the n.O.e. experiment was carried out for the disaccharide **5a** by selective saturation of H-3*ax* of the NeuAc unit, only 4.4% n.O.e. was observed for the intraresidual H-5 of NeuAc unit and no n.O.e. was observed for the aglyconic H-6*R*. A full spin-matrix analysis of n.O.e.[53,54] for this disaccharide (assuming isotropic motion[53a,53c] with a rotational correlation-time τ_c of 1×10^{-10} s and dipole–dipole contributions only) estimated that the relative n.O.e. enhancements from the H-3*ax* of sialic acid to the intraresidual H-5 of NeuAc and the interresidue H-6(*R*) of the galactose should be 15 and 8%, respectively. However, if the n.O.e. values were averaged over the whole HSEA energy contour map (Fig. 3A), then the n.O.e.'s calculated for the foregoing two hydrogens were 15.6 and 3%, respectively, indicating the potential difficulty in the experimental detection of the crucial n.O.e. between H-6(*R*) and H-3*ax* of the NeuAc residue. Thus, based on only 4.4% observed n.O.e. for the intraresidual hydrogen H-5 from the H-3*ax* of the NeuAc unit, $< 1\%$ n.O.e. is expected for interresidual hydrogen, namely, the H-6(*R*) of the galactose, even under ideal experimental conditions, and this could have been easily missed. As in the case of **5a**, the n.O.e.'s in the rotating frame[55] on the trisaccharide **6b** also did not give useful results.

In order to improve the chances of n.O.e. between H-6(*R*) (of the β-D-Gal unit) and H-3*ax* of the NeuAc unit, it was decided to replace one of the geminal hydrogens of the C-6 of the galactose by a deuterium. In fact, when the n.O.e. experiment was carried out on the deuterated disaccharide **5b** and trisaccharide **5c**, both H-5 of sialic acid and H-6*R* of the galactose experienced n.O.e.'s (H-6(*R*)Gal/H-5 NeuAc = 0.33, Table V). In this case, the observed n.O.e.'s are within a factor of two of the expected value based on the calculated n.O.e.'s[54]. Given the shallow nature of the potential energy well for (2→6) sialosides, we consider the magnitude of the observed n.O.e. in the deuterated di- and tri-saccharides **5b** and **5c**, respectively, as evidence for the presence of the HSEA estimated lowest energy conformer.

Extension of these results of the conformational analysis obtained for the di- and tri-saccharides to other larger α-(2→6) sialosides was done through comparison of their hydrogen and carbon chemical-shifts (see Tables III and IV). On the basis of the values reported in Tables III and IV, we consider the correspondence among the (2→6) sialosides excellent, indicating the similarity in their conformational properties. Few unusual ^1H and ^{13}C chemical shifts reported in Tables III and IV in fact support the conformation, inwhich the NeuAc unit is placed closer to the aglycon at the C-1 of the sialylated galactose.

TABLE IV

Comparison of ^{13}C chemical shifts of (2→6) sialosides (**5a**, **5c**, **6a**, **7**, **8**, **9**, and **9a**) with their asialo compounds (**2**, **3**, and **4a**)[a]. Data for **2**, **3**, **4**, **5a**, **6a**, and **9** are reproduced from published data[37]

Sugar unit	*Carbon atoms*	**1c**	**2**	**3**	**4**	**5a**	**5c**	**6b**	**7**	**8**	**9**	**9a**
β-D-Glc	1	103.20			96.24		103.28				96.17	96.13
	2	73.06			74.34		73.08				74.25	74.20
	3	74.66			74.88		74.48				74.76	74.76
	4	78.64			78.96		80.08				78.88	78.77
	5	75.04			75.32		74.98				75.23	75.20
	6	60.33			60.69		60.88				60.61	60.51
β-D-Gal	1				103.40						103.36	103.33
	2				70.50						70.43	70.39
	3				82.51						82.41	82.39
	4				68.84						68.89	68.83
	5				75.37						75.32	75.27
	6				61.48						61.40	61.35
β-D-GlcNAc or 3-Deoxy-β-D-GlcNAc	1		102.40	102.94	103.11			101.41	101.60	103.07	102.93	102.94
	2		55.54	49.14	55.75			55.61	55.48	49.16	55.45	55.29
	3		73.08	35.67	72.71			73.11	73.28	35.77	72.67	72.64
	4		79.18	78.74	78.93			81.80	80.68	78.69	80.88	81.02
	5		75.30	74.13	75.08			75.11	75.08	74.86	74.76	74.60
	6		60.71	61.01	60.51			61.11	60.98	61.14	60.67	60.51
	N-C=O		175.30	174.32	175.31			—	—	173.97	175.27	175.22
	Me		22.84	22.40	22.75			23.60	—	22.49	22.76	22.69

β-D-Gal	1	103.34	103.42	104.02	103.40	104.24	103.58	104.01	103.81	104.14	103.83	103.95
	2	71.23	71.54	71.20	71.49	71.02	71.08	71.41	71.01	71.11	71.20	71.02
	3	72.79	73.08	72.99	73.08	73.01	72.78	73.01	73.18	72.99	72.90	73.28
	4	68.81	69.15	68.83	69.08	69.03	68.72	68.81	68.80	68.78	68.89	68.73
	5	75.58	75.89	75.40	75.85	73.81	73.98	74.31	74.28	73.73	74.14	73.87
	6	60.98[b]	61.57	61.16	61.48	63.77	64.38	63.91	62.98	63.40	63.79	63.67
			Me α-D-NeuAc									
α-D-NeuAc	1		173.67			173.74	173.72	—	—	173.84	173.86	170.12
	2		100.99			100.84	100.58	100.80	101.20	100.60	100.61	99.21
	3		40.38			40.57	40.48	40.70	40.18	40.83	40.55	39.10
	4		68.56			68.60	68.78	69.10	67.12	68.66	68.76	67.56
	5		52.21			52.23	52.18	52.50	52.68	52.33	52.36	52.12
	6		72.86			73.01	72.88	73.50	70.98	72.92	72.99	72.84
	7		68.49			68.60	68.78	69.00	69.28	68.50	68.64	68.72
	8		71.94			72.12	72.08	72.30	71.48	72.34	72.16	70.97
	9		62.94			63.02	63.98	63.30	63.98	63.02	63.15	63.58
	C=O		175.39			175.41	175.18	—	—	175.39	173.33	175.39
	Me		22.30			22.35	22.38	22.90	—	22.49	22.50	22.49

[a] See Table I for structures. [b] Triplet centered around 60.98 p.p.m.

TABLE V

The nuclear Overhauser enhancements (n.O.e.'s, shown in parentheses) observed upon saturation of the hydrogens (H-3*ax* and H-3*eq*) of the α-D-NeuAc units in α-D-NeuAc-(2→6)-β-D-Gal-OCH_3 (**5a** and **5b**) and α-D-NeuAc-(2→3)-β-D-Gal sialosides (**10**, LSTa, LSTd, and DSL). The n.O.e.'s observed for the disaccharides **5a**, **5b**, and **10a** were positive, while those for the pentasaccharides LSTa and LSTd, and in the hexasaccharide DSL were negative

Compound	*Hydrogen saturated*	*Observed n.O.e. (%)*
α-D-NeuAc^a-(2→6)-β-D-Gal^b-OMe, **5a**	H-3^a*ax*	H-3^a*eq* (22.0), H-5^a (4.4), H-6(*R*)^b (0.0)
	H-3^a*eq*	H-3^a*ax* (22.0), H-4^a (6.2)
α-D-NeuAc^a-(2→6)-6(*S*)-β-D-(6-^{2}H)Gal^b-OMe, **5b**	H-3^a*ax*	H-3^a*eq* (25.0), H-5^a (6.5), H-6(*R*)^b (2.2)
	H-3^a*eq*	H-3^a*ax* (25.0), H-4^a (5.7)
α-D-NeuAc^a-(2→3)-β-D-Gal^b-O$(CH_2)_5CO_2$Me, **10a**	H-3^a*ax*	H-3^a*eq* (10.0), H-5^a (2.2), H-3^b (1.8)
	H-3^a*eq*	H-3^a*ax* (5.6), H-4^a (0.6)
α-D-NeuAc^a-(2→3)-β-D-Gal^b-(1→4)-β-D-GlcNAc^c-(1→3)-β-D-Gal^d-(1→4)-D-Glc^c, LSTd, **15**	H-3^a*ax*	H-3^a*eq* (13.2), H-5^a (3.0), H-3^b (1.6)
	H-3^a*eq*	H-3^a*ax* (11.0), H-4^a (2.4)
α-D-NeuAc^a-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc^c-(1→3)-β-D-Gal^d-(1→4)-β-D-Glc^e, **14**	H-3^a*ax*	H-3^a*eq*, H-5^a, H-3^b qualitatively obs.
α-D-NeuAc^a-(2→3)-β-D-Gal^b-(1→3)-[(α-D-NeuAc^f-(2→6)]-β-D-GlcNAc^c-(1→3)-β-D-Gal^d-(1→4)-D-Glc^e, DSL, **18**	H-3^a*ax*	H-3^a*eq* (17.5), H-5^a (8.7), H-3^b (1.8)
	H-3^f*eq*	H-3^f*ax* (17.0), H-5^f (9.0)

LONG-RANGE EFFECTS OF SIALYLATION ON THE ^{1}H AND ^{13}C CHEMICAL SHIFTS OF α-(2→6) SIALOSIDES

On the basis of the HSEA calculations and n.m.r. results for the deuterated sialosides, the sialosidic torsion angle, in particular the "ω" in the α-D-NeuAc-(2→6)-β-D-Gal sialosides, could be restricted to values around 60 $\pm 10°$. However, large variations in both φ the ψ domains were indicated (Fig. 3). Yet, in all these conformers that differ especially in the φ and ψ torsion values, the sialic acid residue appeared to reside closer to the aglycon of the sialylated galactose. The ^{1}H and ^{13}C chemical shifts reported in Table III and IV in fact reflected their proximity. As consideration of all individual conformers for meaningful interpretation of the dynamic average of the n.m.r. parameters is impossible, the models shown in Figs. 4 and 5 for the global minimum-energy conformer have been used to explain a number of n.m.r. parameters as described later.

Particularly noteworthy in these models shown in Figs. 4 and 5 for sialoside **6a**, is

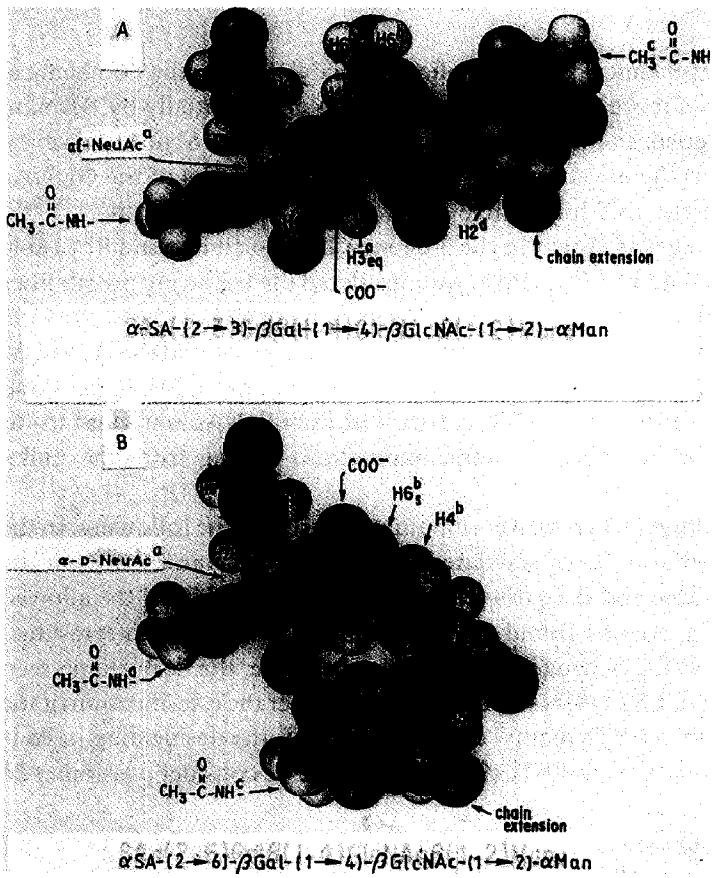


Fig. 5. Computer-drawn CPK models corresponding to the minimum-energy conformers of α-D-NeuAc^{a}-(2→3)-β-D-Gal^{b}-(1→4)-β-D-GlcNAc^{c}-(1→2)-Man^{d} (*A*), and α-D-NeuAc^{a}-(2→6)-β-D-Gal^{b}-(1→4)-β-D-GlcNAc^{c}-(1→2)-Man^{d} (*B*).

that it places the sialic acid in close proximity to the *N*-acetylglucosamine residue. From the inspection of the interatomic distances in trisaccharide **6a** (Fig. 4), it was evident that the 3-hydroxyl group of the GlcNAc unit was within 2.6 Å distance of H-3*ax* of the NeuAc unit. For this reason, a 3-deoxy-GlcNAc analog **8** was prepared to see if the removal of the 3-hydroxyl group would affect the chemical shift of H-3*ax* of the sialic acid. Indeed, this was found to be the case. The hydrogen signals of H-3*ax* of the sialic acid in the deoxysialoside **8** (1.577 p.p.m.), were shielded by 0.128 p.p.m. as compared to **6b** (1.705)[37a], providing additional evidence for the proximity of these atoms. Unexpected hydrogen chemical-shift changes were also observed for H-6(*R*) and H-6(*S*) of the galactose in the α-D-NeuAc-(2→6)-β-D-Gal derivatives. For example, in the sialosides **5a** and **6b**, the chemical shifts for H-6(*R*) of the galactoses are 3.94 and 4.00 p.p.m., respectively (Table III) and these are ~0.2 p.p.m. downfield as compared to their asialo analog. However, the opposite effect is observed for the other C-6 hydrogen, namely H-6(*S*), which is shielded by ~0.15 p.p.m. As shown in the global minimum-energy models (Figs. 4 and 5), the H-6(*R*) is situated ~2.4 Å away from the pyranose ring oxygen of the NeuAc unit. We consider this oxygen atom a likely cause of this deshielding.

Compared to proton chemical shifts, the effects of sialylation on the ^{13}C chemical shifts of aglycons were even more substantial. As has been noted initially by Berman, one of the unique characteristics of the sialic acid substitution at the 6′-position of the *N*-acetyllactosamine core is the unusual deshielding effect seen for C-4 of the GlcNAc unit, although the origin for this long-range effect has not been obvious[35,37,52,56,57]. Surprisingly, this deshielding was absent in the 3-deoxy-GlcNAc compound **8*** (Table IV). To explain this unusual deshielding effect, we considered the following possibilities based on literature precedence[56,58]. (*1*) Anisotropic deshielding of C-4 of GlcNAc by the negatively charged carboxylate unit. (*2*) A conformational change around the glycosidic linkage between the Gal and the GlcNAc residues[58] (*a*) to establish a hydrogen bond between the carboxylate group and the C-3 hydroxyl of the GlcNAc unit; and (*b*) to avoid undesirable repulsive interresidual steric interactions arising from the bulky carboxylate group.

The first two possibilities (*1* and *2a*) were eliminated based on the following. In the "*gt*" orientation of the C-6 arm of the sialylated galactose, the distance between the 3-hydroxyl of GlcNAc residue and the carboxylate unit of sialic acid in all the allowed global-energy minima (Fig. 3) was found to be more than 5 Å. This interresidual distance is too far to establish a hydrogen bond**. Furthermore, the deshielding seen for C-4 of the GlcNAc unit in LSTc (**9**) is still observed even after the esterification of the carboxylate moiety, as seen in LSTc methyl ester (**9a**). In fact, the deshielding in **9a** is greater by 0.14 p.p.m. as compared to LSTc (**9**). On this basis, we consider possibility *2b*

* Here the comparison is made between the deoxy sialoside **8** and its asialo-deoxy derivative **3**.

** To further probe the hydrogen bond interaction between 3-hydroxyl of GlcNAc and the COO^- group of the NeuAc units, enzymic preparation of α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-(3-O-Me)-β-D-GlcNAc-OMe, by reacting CMP-NeuAc and β-D-Gal-(1→4)-(3-O-Me)-β-D-GlcNAc in presence of Galβ 1,4GlcNAc α-(2→6) sialyltransferase was attempted without success.

as the probable one. The bulky carboxylate group can force rotation in the ψ torsion angle involving the C-4 of the GlcNAc unit to minimize its steric interactions with atoms of the GlcNAc residue. The precedence for this can be found in the report by Bock and co-workers[59], who pointed out that the ^{13}C chemical shift of the aglyconic carbons are very sensitive to the variations in ψ angle and rotations of $\sim 20°$ are often sufficient to cause deshieldings of the magnitude seen for the C-4 of GlcNAc in α-(2→6) sialosides **6a**, **9**, and **9a**. These small changes in the ψ torsion angle will not change the overall topography of the molecule, nor would the semiempirical HSEA calculations be expected to reliably detect such small changes.

Based on the HSEA models for the trisaccharide **6a** and the pentasaccharide **9**, the driving force for a change in the ψ angle can arise, as mentioned already, from the steric interactions of the extensively hydrated and bulky carboxylate moiety with the C-4 of the GlcNAc residue. This proposal is in accord with the lack of deshielding of C-4 in the 3-deoxyGlcNAc sialoside **8** (Table IV). Here, deoxygenation at C-3 of the GlcNAc will not only eliminate its direct interaction with H-3*ax* of the sialic acid residue, but also will allow greater freedom of rotation around ψ angle involving the Gal-(1→4)-β-GlcNAc units, to keep the C-4 of the GlcNAc unit away from the carboxylate moiety. In contrast, the esterification of the carboxylate group as seen in **9a**, should make the steric interaction worse causing an even greater deshielding of C-4 of the GlcNAc unit (Table IV).

The unusual deshielding effects observed for the C-4 of the GlcNAc are not restricted to the α-sialosides **6a** and **9**, but can be seen in the β-sialoside **7** as well (Table IV). In **7**, C-6 of the galactose cannot orient antiperiplanar to the carboxylate moiety of the sialic acid, as the C-6 galactose methylene hydrogens would encounter severe nonbonded repulsions with the hydrogens at C-4 and C-6 of the sialic acid units. The only possibility would be to place it synclinal to the carboxylate group and the ring oxygen of the sialic acid, in accordance with the exoanomeric effect. With the hydroxymethylene unit of the galactose in the "*gt*" orientation*, it becomes evident from CPK models that the atoms of the GlcNAc residue (C-4 and C-3 in particular) come in close contact with the carboxylate group. This can be relieved by a similar change in the ψ angle involving β-D-Gal-(1→4)-β-D-GlcNAc units.

Several other long-range effects have also been reported[35,37,57]. For example, Vliegenthart and co-workers have pointed out[35,57] that sialylation of the galactose, as seen in **6a** and **9** (LSTc) causes the *N*-acetyl ^1H signals to shift downfield, as compared to the asialo derivatives **2** and **4**. The CPK models for the lowest-energy conformer indicate that the carbonyl group of the *N*-acetyl group of the GlcNAc unit and the 4-hydroxyl group of the sialic acid are close to each other (the distance is 3.26 Å). Thus, it is possible that a hydrogen bond can exist between these groups either directly or

* While this manuscript was in press, Ohrui *et al.*[52b] reported n.m.r. data of few deuterated sialosides. Particularly noteworthy in the data is the coupling constants for the galactose C-6 hydrogens ($J_{5,6a}$ 6.1 and $J_{5,6b}$ 5.7 Hz), respectively in β-D-NeuAc-(2→6)-β-D-Gal-OMe indicating near equal amounts of "*gt*" and "*tg*" rotomers around the C-5–C-6 bond. However, when this disaccharide was changed to the trisaccharide **7** (Table III), the above C–6 galactose hydrogen coupling constants changed to 9.0 and 3.6 Hz, respectively, indicating the preferential "*gt*" rotomer population.

through the involvement of a water molecule. To better accommodate the intramolecular hydrogen bonds, the orientation of the carbonyl group can deviate from its normal position[60] toward the 4-hydroxyl group of the sialic acid. This may account for the changes in chemical shift of both the *N*-acetyl methyl group and the H-1 of the GlcNAc unit. When these sialosides are present as a part of the large oligosaccharide chain, as in the case of the asparagine-linked carbohydrate groups of glycoproteins, this change in rotation may move the methyl of the *N*-acetyl group closer to the H-1 of the mannose (see Fig. 4B) and, in fact, the chemical shift of H-1 of the mannoses of asparagine-linked oligosaccharides have been shown to be sensitive to sialylation[35,57]. However, these effects are too small to be accounted for precisely, and only a possibility based on the minimum energy conformation of the α-(2→6) sialosides is indicated.

CONFORMATIONAL ANALYSIS OF THE α-D-NeuAc-(2→3)-β-D-Gal GLYCOSIDES

The isoenergy contour maps produced from the HSEA calculations for three α-(2→3) sialosides are shown in Figs. 3D–F. Represented are the simple disaccharide α-D-NeuAc-(2→3)-β-D-Gal-OMe (Fig. 3D), and the trisaccharides α-D-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-OMe (Fig. 3E) and α-D-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc-OMe (fig. 3F). For all the α-(2→3) sialosides, the global minimum-energy conformer exhibited sialosidic torsion angles around $\varphi,\psi = -160, -13°$ (conformer 1). Two other minimum-energy conformers with sialosidic torsion angles $\varphi,\psi = 80, -13°$ (conformer 2), and $\varphi,\psi = -65, -13°$ (conformer 3) were also estimated. Calculation of the conformer population surrounding the global minimum in each potential-energy well indicated that >99% of the total population would have solution structures within the energy well containing the lowest-energy conformer 1*. For α-(2→3) sialosides with the lactosamine core as the aglycon, conformer 2 was estimated to be populated at ~0.5%; and for sialosides with the β-D-Gal-(1→3)-β-D-GlcNAc sequence, population of this conformer would be negligible. Little contribution was estimated from conformer 3 in any of these sialosides.

In conformer 1, predicted to be the most stable, the bulky carboxylate group of the sialic acid and the aglycon, namely, the C-3 of the galactose, are placed farthest away from each other, whereas these are synclinal to each other in conformers 2 and 3. Examination of the interatomic distances shows that in conformer 1, the distance of the H-3*ax* of the sialic acid to H-3 of the galactose is 2.12 Å, whereas in conformer 3, the corresponding internuclear distance is 4.19 Å. In conformer 2, H-4 instead of H-3 of the sialylated galactose is predicted to be closer to both H-3*ax* and H-3*eq* of the NeuAc

* It should be recognized that as the isoenergy contour map indicates that conformer 1 ($\varphi,\psi = -160, -13°$) corresponds to the one at global-energy minimum of potential-energy well 1. All accessible conformers in this well have the aglycon placed between the pyranose ring oxygen and C-3 of the NeuAc unit (this is referred in the text as the conformer in "anti-orientation"). In energy well 2, the lowest energy, conformer 2 has glycoside torsion angles $-80, -13°$ and all the conformers distributed around this have the aglycon placed between C-1 and C-3 of NeuAc unit. Similarly, conformer 3 has the aglycon is placed between C-1 and the pyranose ring oxygen of the NeuAc unit. It should be noted that both conformers 1 and 3 will exhibit the exoanomeric effect.

units. In the proton spectra of all α-(2→3) sialosides listed in Table I, the signals for H-3*ax* of sialic acid and H-3 of the sialylated galactose were well resolved and proved amenable for selective saturation. As summarized in Table V, saturation of H-3*ax* of the sialic acid provided n.O.e.'s for the H-5 of the NeuAc unit, and more importantly for the aglyconic hydrogen, namely the H-3 of the galactose**. No n.O.e. was observed between H-4 of galactose and H-3*ax* or H-3*eq* of the NeuAc unit, and this excluded the appreciable contribution of conformer 2. The magnitude of n.O.e.'s observed (Table V) for H-3 of galactose upon saturation of H-3*ax* of NeuAc unit in α-(2→3) sialosides **10a** and **15** ranges from 81–53% of the theoretical value, based on the calculations of Noggle and Schirmer[53] assuming that 100% of the molecules are in the global minimum-energy conformation 1. Thus, considering the semiempirical nature of the HSEA calculations and within experimental error, we consider the magnitude of n.O.e.'s reported in Table V as a good support for the overwhelming presence of conformer 1 in solution. Based on the n.O.e. overlaid contour map and by considering the broad nature of the potential-energy well around conformer 1, it is to be concluded that the less than expected n.O.e. between H-3*ax* of NeuAc and H-3 of Gal units is an indication of the presence of non-n.O.e. contributing conformers with molecular topography similar to conformer 1 rather than due to the appreciable presence of conformer 3. It is to be mentioned that our n.O.e. results are in good accord with those obtained by Poppe *et al.* for the ganglioside GM4 (α-D-NeuAc-(2→3)-β-D-Gal-ceramide)[61a]. In addition to the n.O.e. between the H-3*ax* of sialic acid and the aglyconic hydrogen, these authors reported additional interresidual n.O.e. between H-8 of the NeuAc unit and H-4 of the sialylated galactose. The HSEA conformer at the global energy-minimum (Fig. 4) in fact placed these two hydrogens within a distance of 2.68 Å.

CONFORMATIONAL ANALYSIS OF THE α-D-NeuAc-(2→6)-β-D-GlcNAc GLYCOSIDES

The glycosidic torsion-angles calculated for the lowest-energy conformers of the α-D-NeuAc-(2→6)-β-D-GlcNAc linkages in sialosides LSTb (**16**) and DSL (**17**) are shown in Table II. As seen for other sialosides, the HSEA calculations predicted the carboxyl group of the sialic acid to orient antiperiplanar to C-6 of the GlcNAc unit. The calculation also showed that the "*gg*" conformation, with C-6–O-6 of GlcNAc unit antiperiplanar to its C-5–H-5 bond was preferred over "*gt*" orientation by about 0.52 kcal/mol. Thus, assuming equilibration between these two orientations, the "*gg*" conformation will be favored over the "*gt*" conformation by ~2.5:1.

The hydrogen coupling constants measured for the C-6 hydrogens of the GlcNAc unit (Table III, $J_{H\text{-}6a,H\text{-}5} = 5.0$, $J_{H\text{-}6b,H\text{-}5} = 2.0$ Hz) of the disaccharide α-D-NeuAc-(2→6)-β-D-GlcNAc-O$(CH_2)_8$ CO_2Me (**16**) suggest that both the "*gg*" and "*gt*" conformers are equally populated with no clear preference of one over the other (These are similar to the results reported by Cumming and Carver for α-D-Man-(1→6)-β-D-Man glycosides[21]).

** Based on the calculated inter nuclear distances, it is to be expected that the saturation of H-3**ax** of NeuAc unit should provide 18% n.O.e. for the intraresidual hydrogen H-5 and ~16% for the aglyconic hydrogen H-3 of the galactose. In other words, the n.O.e. ratio of H-5–H-3 should be ~1. The actual observed ratio in the case of compound **10a** was about 0.8 and in **15a** the ratio was about 0.53.

Compounds **16**, **17**, and **18** exhibited among them excellent correspondence in proton and carbon chemical shifts, suggesting that all these sialosides may have similar conformational properties.

In contrast to the unusual hydrogen and carbon chemical shifts seen in α-D-NeuAc-(2→6)-β-D-Gal sialosides, none was seen for α-D-NeuAc-(2→6)-β-D-GlcNAc sialosides (as seen in **17** and **18**) indicating that the sialic acid does not interact with the aglycon at C-1 of the same GlcNAc unit. This is also evidenced from the construction of the CPK models.

COMPARISON OF THE HSEA GLOBAL MINIMUM-ENERGY CONFORMERS OF α-(2→3) AND α-(2→6) SIALOSIDES

The sialosides examined here constitute common terminal sequences of glycoproteins and glycolipids, and confer specificity in biological interactions with many sialic acid-binding proteins[2,3,6,11,14,15,62]. For comparison, the CPK models of conformers at their global energy-minimum for α-(2→3) and α-(2→6) sialosides with lactosamines as a neutral core, and for the sequences α-D-NeuAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→2)-α-D-Man and α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→2)-α-D-Man that are the terminal branches of asparagine-linked oligosaccharides, are shown in Fig. 5. In these two structures, the sialic acid is held constant to contrast the projections of the remaining oligosaccharide portions. Note that in these structures, the extension of the oligosaccharide and the bulky protein will project into and out of the page for the α-(2→3) and α-(2→6) sialosides, respectively. These qualitative models are presented to highlight the differences in the global conformations of oligosaccharides that are terminated with α-D-NeuAc-(2→3) and (2→6) linkages especially, when these are present as a part of the glycoprotein structure. For sialic acid-binding proteins having the sialic acid bound in a fixed orientation to the combining site, the proper three-dimensional display of the bulky aglycon may be an important factor in determing the binding specificity[11,15].

DISCUSSION

The conformational properties of the glycosidic linkages have been shown to be dependent, among a number of factors, on two key ones, namely the nonbonded interaction of the atoms around the glycosidic linkages and a stereoelectronic term known as the exoanomeric effect[63–65]. The glycosides can exhibit exoanomeric effect in two orientations, where the aglycon is placed between the anomeric substituent and the pyranose oxygen (referred to in the text as synclinal orientation) or the pyranose oxygen and C-2 (carbon adjacent to the anomeric carbon atom, referred to as anti orientation). Between these two, the steric interaction between the anomeric substituent and the aglycon can influence one over the other. When the anomeric substituent is hydrogen, the latter steric effects are minimal and consequently the synclinal orientation of the aglycon is preferentially observed[65]. Sialosides are quite unique in that they carry a

bulky carboxyl group at the anomeric center and this consequently forces the aglycon to adopt the anti orientation. This unusual feature was first reported for a group of sialosides known as gangliosides[16,66]. In this report, we have extended these results to include flexible linear sialosides as well, to indicate that the anti orientation of the aglycon of the sialoside linkage may be a unique feature for all sialyloligosaccharides.

The glycosidic torsion angles shown in Table II for the global energy-minimum conformer of α-D-NeuAc-(2→3)-β-D-Gal linkages in the linear α-(2→3) sialosides are nearly the same as those reported earlier[16] for branched structures of gangliosides GM1 (β-D-Gal-(1→3)-β-D-GalNAc-(1→4)-[α-D-NeuAc-(2→3)]-β-D-Gal-(1→4)-β-D-Glc and GM2 (β-D-GalNAc-(1→4)-[α-D-NeuAc-(2→3)]-β-D-Gal-(1→4)-β-D-Glc]. These two compounds differ from the linear sialosides in that the sialylated galactose is further glycosylated at the 4-position, which renders the sialoside linkages extremely rigid. In addition, a secondary stabilization involving van der Waals attraction of ~3 kcal/mol between the sialic acid and the adjacent GalNAc is established to hold the sialic acids in gangliosides GM1 and GM2 in the rigid conformation[16] as compared to the flexible (2→3) sialosides. These are reflected in their proton and ^{13}C chemical shifts[37] as well.

Recently, other reports have appeared regarding the conformational properties of both (2→3) and (2→6) sialosides[52,61]. The n.O.e. results obtained by Poppe *et al.*[61a] for the disaccharide α-D-NeuAc-(2→3)-β-D-Gal-OH are in good agreement with the results in this paper for the disaccharide **10a** to the hexasaccharide **18**. In addition, a dipolar coupling observed by these authors between the H-8 of the NeuAc unit of and the H-4 the galactose that is sialylated at the 3-position provides clear evidence for the conformational model that we have proposed in this report. However, these authors propose, for the disaccharide just mentioned, the coexistence of conformers with the aglycon placed in both synclinal and anti orientation to the carboxylate group, and they conclude the predominance of syn conformer over the anti. We contend that, in (2→3) sialosides, the barrier to interconversion between the anti and synclinal orientation of the aglycon is high (at least 13 kcal/mol)[46b] and based on the calculated energy-difference between the syn and anti conformers, we propose the contribution of only the anti conformers shown in the isoenergy contour map (Figs. 3D–F) including the lowest-energy one. The evidence for the syn conformer according to Poppe *et al.*[61a], is the observation of a dipolar coupling between H-8 of NeuAc and H-3 of the sialylated galactose. However, the magnitude of the n.O.e. seen is hardly discernable from the noise level, while that for H-4 is very good (favors the anti conformer). As H-3 and H-4 of the galactose are spin coupled to each other, and as the mixing time used in the NOESY experiment was large (500 ms), the conclusion of primary dipolar coupling between H-8 of NeuAc and H-3 galactose is rather inconclusive. Even though the n.O.e. results reported in Table V exclude the exclusive population of the anti conformer having glycosidic torsion-angles $\varphi,\psi = -160,-11°$, it does indicate it as a major conformer. We contend that the less than expected magnitude of the n.O.e.'s (Table V) should be taken as an indication of the other non-n.O.e.-contributing conformers, but which still have the aglycon placed between the sialic acid ring-oxygen and its C-3 atom. These are evident from the n.O.e. overlaid contour map (Fig. 3E), which demonstrates

that rotations in the torsion angles of 10° are sufficient to cause a dramatic change in the calculated n.O.e.'s. For example, in (2→3) sialosides, variations of the glycosidic torsion-angles from $-153, -26°$ to $-165, -10°$ (which are energetically allowed, see Fig. 3E) changes the H-8 (NeuAc)–H-4(Gal) distance from 2.48 to 2.68 Å and these small changes will have dramatic effects in the magnitude of the observed n.O.e.

As noted by Cumming and Carver for (1→6)-linked mannobiosides[21], the problem of estimating the conformational properties for the (2→6) sialosides was more elusive and experimentally difficult, owing to the flexibility in three torsion angles (φ,ψ,ω). In this report, we firmly established the "*gt*" orientation of the C-6 of the galactose through the preparation of sialosides deuterated at C-6 of the galactose. These conclusions have been reinforced in the recent report by Breg *et al.*[52] through 2D COSY and NOESY techniques. The absence of the crucial n.O.e. between the H-3*ax* (NeuAc) and H-6 (Gal) in the undeuterated material have been seen by these authors as well. As described in this report, the deuterated disaccharide **5b** and the trisaccharide **5c** were the only two compounds thus for that provide useful n.O.e. information to establish the bent conformation of the (2→6) sialosides.

The origin of the unusual deshielding upon sialylation of the C-6 of galactose of a lactosamine has been indicated to arise from the steric effect of the carboxyl group of sialic acid rather than its negative charge. The evidence for this has been provided by the observations of similar deshielding effects, even after esterification of the carboxylate moiety. The experimental evidence for the proximity of the GlcNAc unit to sialic acid has also been provided by the preparation of a 3-deoxy-GlcNAc-containing sialoside and establishing that indeed such deoxygenation leads to the shielding of H-3*ax* of the NeuAc unit.

CONCLUSION

In summary, we propose that the sialosides favor having the aglycon oriented remote from the sterically demanding carboxylate moiety. The nature of the potential-energy well representing the sialosidic linkages indicates that the conformer at the global energy-minimum is not a rigid body and the rigidity depends on the steric environment around this glycosidic linkage. On this basis, we can expect that the (2→3) sialosides will be more rigid than the (2→6) sialosides. We consider that these results provide a basis for understanding the conformational properties of these complex molecules.

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