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Fish Egg Polysialoglycoproteins: Circular Dichroism and Proton Nuclear Magnetic Resonance Studies of Novel Oligosaccharide Units Containing One Sialidase-Resistant *N*-Glycolylneuraminic Acid Residue in Each Molecule†

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ABSTRACT: Long-core units having the common sequence GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3GalNAc are one of the major constituents of rainbow trout egg polysialoglycoproteins. The existing ambiguity regarding the anomeric configuration of the sialidase-resistant unsubstituted sialyl group present in this novel type of oligosaccharide chains has been resolved by a circular dichroism difference spectral method. The fact that the negative band originating from the carbohydrate n → π* transition for this sialyl group was observed offers conclusive proof of the α-anomeric configuration. Next particularly interesting is the fact that the chemical shifts of the sialidase-resistant sialyl H-3_{eq} and H-3_{ax} protons were respectively found at relatively higher and lower magnetic field than for the corresponding protons of other sialyl groups. A consideration of molecular models shows that the observed anomalies are all in the directions compatible with expectations on the basis of the magnetic anisotropy effect due to the carboxylate group and steric compression effects by van der Waals interactions between groups that are sterically compressed. In addition to the observed resistance to bacterial sialidases of this sialyl group, it did not behave even as a competitive inhibitor of the sialidase, *Arthrobacter ureafaciens*, indicating that inaccessibility of this unique sialyl group toward the enzyme. Finally, the analysis of the proton nuclear magnetic resonances of sialidase-sensitive mono- and oligosialyl groups present in the long-core units was based on comparisons of diagnostically important regions in the spectra of homologous oligosaccharides of *N*-glycolylneuraminic acid.

We have separated and determined the structures of a series of oligosaccharide alditols of the long-core units with mono- to tetrasialyl groups in the preceding paper (Iwasaki et al., 1984). Long-core units are particularly interesting since a sialidase-resistant *unsubstituted* NeuGc[1] residue is attached to each molecule of these units: sialidases from *Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Streptococcus sp IID6646*, enzymes known to cleave α-linked sialyl residues, exhibited *absolutely no* reactivity against this NeuGc residue. There seemed to be a probability of a β-linked NeuGc residue in this class of carbohydrate chains, so that in the previous papers (Inoue et al., 1981; Iwasaki et al., 1984) the anomeric configuration of the sialidase-resistant NeuGc residue was left unspecified.

The most noteworthy observation on a series of long-core units is that the H-3 protons of the sialidase-resistant NeuGc resonate at 1.86 (H-3_{ax}) and 2.56 ppm (H-3_{eq}) downfield of DSS in the high-resolution ^{1}H NMR spectra in D_2O. Although it is known [e.g., Kamerling et al. (1982)] that H-3 chemical shifts can be correlated with the configuration of the ketosidic linkage, the above data alone are not conclusive in identifying the anomeric configuration of the anomalous NeuGc residue. However, chemical evidence on this point has been secured, and we report in this paper the results of a more extensive investigation on the CD and ^{1}H NMR spectra of the long-core units, which provide fundamental information about certain structural features of a new class of oligosaccharides containing sialidase-resistant sialic acid.

Materials and Methods

In the present work, materials and methods followed closely those described in the preceding paper (Iwasaki et al., 1984) unless otherwise stated.

Preparation of SRO and TlbLP. The following description is for a large-scale preparation procedure that can be used to obtain milligram quantities of SRO. A mixture of oligosaccharide alditols (76 mg in NeuGc) released by the alkali–borohydride treatment of polysialoglycoproteins was subjected to exhaustive digestion in 40 mL of 0.05 M acetate buffer, pH 4.7, at 37 °C for 4 days with *Arthrobacter ureafaciens* sialidase by stepwise addition of 0.5–0.25 unit every several hours (total 2.75 units). The release of free NeuGc was monitored by the thiobarbituric acid method

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; SRO, sialidase-resistant oligosaccharide; GalNAcol, *N*-acetyl-D-galactosaminitol; Galol, D-galactitol; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Cer, *N*-(fatty acyl)sphingosine; G_{M1}, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer; G_{M2}, GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer.

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Table I: Structure and Notation of the Oligosaccharides Prepared and Used in This Work

notation	
long-core units	
T1bL (=SRO)	GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3GalNAcol
asialo-SRO	GalNAcβ1→4GalNAcβ1→3Galβ1→4Galβ1→3GalNAcol
T3L	GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3(NeuGcα2→6)GalNAcol
T6L	GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3(NeuGcα2→8NeuGcα2→6)GalNAcol
T9L	GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3(NeuGcα2→8NeuGcα2→8NeuGcα2→6)GalNAcol
T1bLP	GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galol
short-core units	
T1c	Galβ1→3(NeuGcα2→6)GalNAcol
asialo-T1c	Galβ1→3GalNAcol
Fuc-containing units	
T1bF	Fucα1→3GalNAcβ1→3Galβ1→4Galβ1→3(NeuGcα2→6)GalNAcol
asialo-T1bF	Fucα1→3GalNAcβ1→3Galβ1→4Galβ1→3GalNAcol
sialooligosaccharides	
$(NeuGc)_2$	NeuGcα2→8NeuGc
$(NeuGc)_3$	NeuGcα2→8NeuGcα2→8NeuGc
$(NeuGc)_4$	NeuGcα2→8NeuGcα2→8NeuGcα2→8NeuGc

(Aminoff, 1961) at certain time intervals. After being adjusted to pH 7.6 with NH_4OH and diluted with water (about 60 mL), SRO was isolated from neutral oligosaccharides and free NeuGc by anion-exchange chromatography on a column (1.3 × 50 cm) of DEAE-Sephadex A-25. The yield was 4.4%, on the basis of sialic acid.

Fraction T1b was found to contain a small but sufficient amount for structural analysis of a monosialooligosaccharide having D-galactitol as a reducing terminal residue together with T1bL. This oligosaccharide alditol was presumably formed by a peeling reaction of the parent oligosaccharide of T1bL (=SRO) from the reducing end followed by reduction with sodium borohydride. This oligosaccharide alditol is referred to as T1bLP.

Preparation and Chromatography of Oligosaccharides of NeuGc. Oligosialyl compounds, $(\rightarrow 8NeuGc\alpha 2\rightarrow)_n$, were prepared by limited acid hydrolysis of polysialoglycoproteins, followed by chromatography of the hydrolysate on DEAE-Sephadex A-25. Polysialoglycoproteins (55 mg) were partially hydrolyzed at pH 2.0 for 1 h at 80 °C. The oligosialic acid mixture released from the glycoproteins was separated from the proteins by passing the partial hydrolysate through a Sephadex G-100 column (1.8 × 120 cm). The former mixture was then applied to a column (0.9 × 110 cm) of preequilibrated DEAE-Sephadex A-25 (HCO_3^- form), and the column was eluted with 1.2 L of a 0–0.6 M sodium bicarbonate linear gradient. Fractionation according to chain length yielded nine discrete peaks (data not shown). The material of peak 1 was identified as NeuGc. The materials in peaks 2–5 were isolated and characterized by ^1H NMR spectroscopy.

Steady-State Kinetic Experiments. In order to examine the binding affinity of SRO to sialidase from *A. ureafaciens*, inhibition experiments were made by using SRO as an inhibitor for the sialidase-catalyzed hydrolysis of *N*-acetylneuraminyl α-4-methylumbelliferyl glycoside at pH 5.6 and 37 °C. For this chromophoric substrate, the progress of desialylation was followed by difference spectrometry at 340 nm. The SRO concentration was varied from 3×10^{-2} up to 40×10^{-2} mM while the substrate and the enzyme concentrations were kept constant at 2.22×10^{-2} mM and 3.95×10^{-2} μg/mL. Similar experiments were also conducted for NeuGc and Galβ1→3(NeuGcα2→6)GalNAcol as an inhibitor and a cosubstrate, respectively.

^1H NMR Spectroscopy. The 270-MHz ^1H NMR spectra were recorded on a Bruker WH 270 spectrometer at a probe temperature of 23 or 60 °C. Oligosaccharides were repeatedly treated with D_2O with lyophilization between treatments. Chemical shifts are expressed in ppm relative to DSS indirectly to acetone in D_2O (2.225 ppm at 23 °C or 2.215 ppm at 60 °C) or indirectly to 2-methyl-2-propanol in D_2O (1.237 ppm at 23 °C).

CD Measurements. Salt-free sialo- and asialooligosaccharides were dissolved in water to give about a 0.1–1.6 mg/mL solution, but the exact concentration was determined by the phenol–sulfuric acid method (Dubois et al., 1956). CD measurements were made on a JASCO Model J-40S spectropolarimeter with a 1- or 0.3-cm quartz cell at 25 °C. Blanks were measured before and after the CD measurement of each sample. The CD data are expressed in terms of molar ellipticity, $[\theta]$.

Results and Discussion

Compounds obtained and used in this study are summarized in Table I together with the established structures.

Determination of Anomeric Configuration of the NeuGc Residue of SRO and Its Susceptibility to Enzymatic and Nonenzymatic Hydrolysis. The use of CD data for differentiating C-2 diastereoisomers was suggested in the literature (Ledeen, 1970; Stone & Kolodny, 1971; Dickinson & Bush, 1975; Keilich et al., 1975; Ledeen & Yu, 1976; Melton et al., 1979; Keilich & Brossmer, 1982). The CD curves of α-linked sialic acid derivatives such as NeuAcα2→3Lac and NeuAcα2→6Lac were reported by Dickinson & Bush (1975) and Melton et al. (1979) to exhibit the amide ($-NHCOCH_3$) $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions at about 190–210 nm (strong positive ellipticity) and the carboxylate ($-COO^-$) $n \rightarrow \pi^*$ transition centered at 220–225 nm (weak negative), in contrast to free sialic acid and β-methoxyneuraminic acid, both of which have a weak positive ellipticity band (~220 nm) corresponding to the $n \rightarrow \pi^*$ transition of the carboxylate chromophore. Thus the sign of the ellipticity bands from the carboxylate $n \rightarrow \pi^*$ transition for sialyl groups was found to depend on the configuration at the C-2 atom.

The most promising approach is to compare the CD of SRO with that of the asialo-SRO core. Since the optical activity of the SRO is assumed to be additive at any fixed wavelength, the ellipticity of SRO can be expressed by

$$[\theta]_{SRO} = [\theta]_{core} + [\theta]_{sialyl}$$

The experimental difference CD spectrum, obtained by subtracting the CD spectrum for asialo-SRO from that for SRO, is recorded in Figure 1. To test the reliability of this method, we have made similar measurements for the reference compounds, and the results are also included in Figure 1. The difference spectrum for a pair of short-core units [Galβ1→-

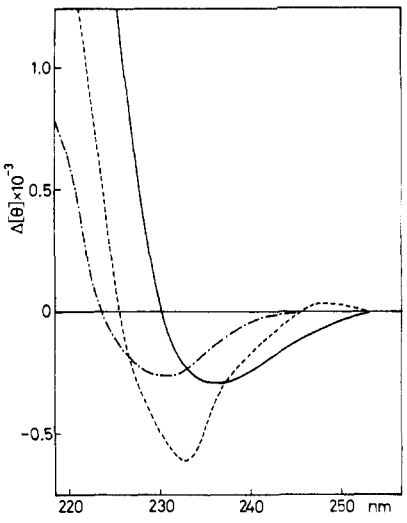


FIGURE 1: Circular dichroic difference spectra for three pairs of oligosaccharide alditols: (—) SRO vs. asialo-SRO; (---) TlbF vs. asialo-TlbF; (-·-) Tlc vs. asialo-Tlc. See Table I for notation of the compounds. The ordinate expresses molar ellipticity in deg cm^2 dmol^{-1}.

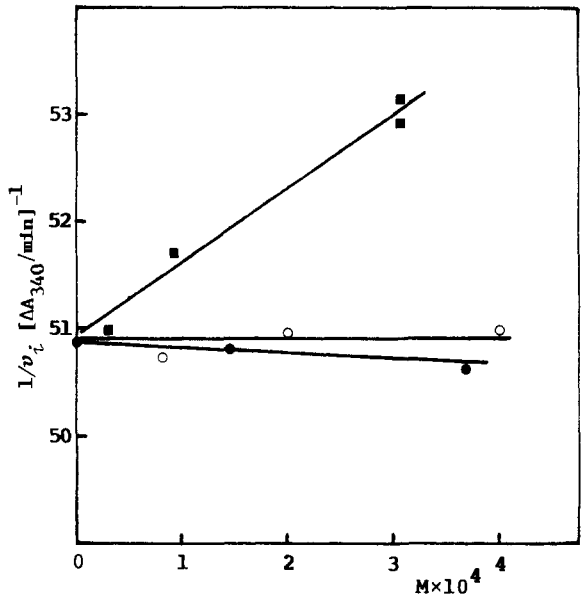


FIGURE 2: Dependence of $1/v_i$ on inhibitor or cosubstrate concentration for the *A. ureafaciens* sialidase-catalyzed hydrolysis of *N*-acetylneuraminyl α-4-methylumbelliferyl glycoside at 37 °C and pH 5.6. (●) SRO; (○) NeuGc; (■) Galβ1→3(NeuGcα2→6)GalNAcol. Enzyme concentration was 3.95 × 10^{-2} μg/mL and substrate concentration was 2.22 × 10^{-2} mM.

3(NeuGcα2→6)GalNAcol vs. Galβ1→3GalNAcol] resembles the CD of NeuAcα2→6Lac (Dickinson & Bush, 1975) in having a negative band at about 230 nm in accord with the established α-configuration for the sialyl group (Nomoto et al., 1982). A pair of the fucose-containing units, Fucα1→3GalNAcβ1→3Galβ1→4Galβ1→3(NeuGcα2→6)GalNAcol vs. Fucα1→3GalNAcβ1→3Galβ1→4Galβ1→3GalNAcol, provides confirmation of the α-D configuration of the NeuGc residue by the method of CD difference spectra in another set. Measurements with SRO vs. asialo-SRO show qualitatively similar difference spectrum, again resembling NeuAcα2→R in having a negative ellipticity, but with lower intensity and with crossover at about 230 nm. The displacement of the negative band associated with the carboxylate n → π* toward somewhat higher wavelength appears to be due to, at least in part, the overlap of an adjacent strongly positive band, originating from the amide n → π* transition, with a negative band due to the carboxylate n → π* transition. Thus, no real significance can at present be attached to the absolute value of the intensity of a band due to the $-COO^-$ group and the apparent position of the band.[2] Nevertheless, the negative sign of the rotational contribution of the $-COO^-$ group in SRO, which in view of the findings (Melton et al., 1979) that the $-COO^-$ group makes a rotatory contribution in terms of the planar rule (Listowsky, 1972), indicates that the $-COO^-$ group is β-oriented. Thus a possibility that the sialidase-resistant NeuGc residue may be linked to the penultimate GalNAc residue by a linkage other than that of the α-ketosidic bond is now precluded.

Efforts to establish the anomeric configuration of the NeuGc in SRO by enzyme cleavage were disappointing: absolutely no cleavage of NeuGc in SRO was observed after prolonged incubation with bacterial sialidases from *A. ureafaciens*, *C. perfringens*, and *S. sp IID6646* irrespective of the presence or absence of 0.1% sodium cholate.[3] It should be noted that, in contrast to the behavior of G_{M1}, the lipid-free carbohydrate moiety of G_{M1} is shown to be completely resistant toward the action of sialidase of *C. perfringens* irrespective of the presence of cholate (Schauer et al., 1979; Gatt et al., 1981). By sharp contrast, in a recent report by Sugano et al. (1978), G_{M1} and its aceramido derivative have been shown to be good substrates for sialidase from *A. ureafaciens*. Attempts were made to remove the terminal GalNAc residue from SRO by treating it with β-*N*-acetylhexosaminidase from *Turbo cornutus* (Muramatsu, 1968) and the enzyme preparation from *Halocynthia roretzi* possessing strong β-*N*-acetylgalactosaminidase activity (Uda & Itoh, 1983). We were unable to observe cleavage of the terminal GalNAc unless the NeuGc residue had been removed by mild acid hydrolysis prior to incubation with these enzyme preparations (S. Inoue et al., unpublished results). The results would indicate that the NeuGc residue in SRO orients itself with these enzymes in a way unfavorable to hexosaminidase action.

We have observed that the SRO does not even act as a competitive inhibitor of the *A. ureafaciens* sialidase when *N*-acetylneuraminyl α-4-methylumbeliferyl glycoside was used as substrate (Figure 2), indicating that the overall conformation prevents it from having some features of the substrate. This is again in contrast to the ability of G_{M2} to act as a competitive inhibitor (Rosenberg & Schengrund, 1976). The resistance of SRO to the action of sialidases is thus attributed to the steric interference of the accessibility of the enzymes caused by the presence of the nonreducing terminal GalNAc attached to the axial 4-O of the penultimate GalNAc residue to which NeuGc is linked. A high degree of crowding of the saccharide chains around the glycosidic bond of the NeuGc residue may be depicted as will be seen in Figure 5A. The resistant character of this NeuGc is also reflected in the behavior of SRO during acid hydrolysis. It was found that, in accordance with their susceptibility to sialidases, an α2→6-linked monosialyl group was hydrolyzed by acid more rapidly than the sialidase-resistant NeuGc in SRO. The apparent first-order rate constants for hydrolysis of the sialyl linkages in 0.1 M trifluoroacetic acid at 80 °C were as follows:

[2] Influence on the magnitude of ellipticity is also exerted by the grouping to which sialic acid is ketosidically linked, as demonstrated by the distinguishable CD spectra observed for the α2→3 and α2→6 isomers of neuraminyllactose (Dickinson & Bush, 1975).

[3] A recent report by Gatt et al. (1981) shows that addition of taurodeoxycholate, below its critical micellar concentration, increased the rate of hydrolysis by sialidase of *Clostridium perfringens* when G_{M3}, glycophorin, orosomucoid, and neuraminyllactose were used as substrates, though these substrates are hydrolyzed even in the absence of the detergent.

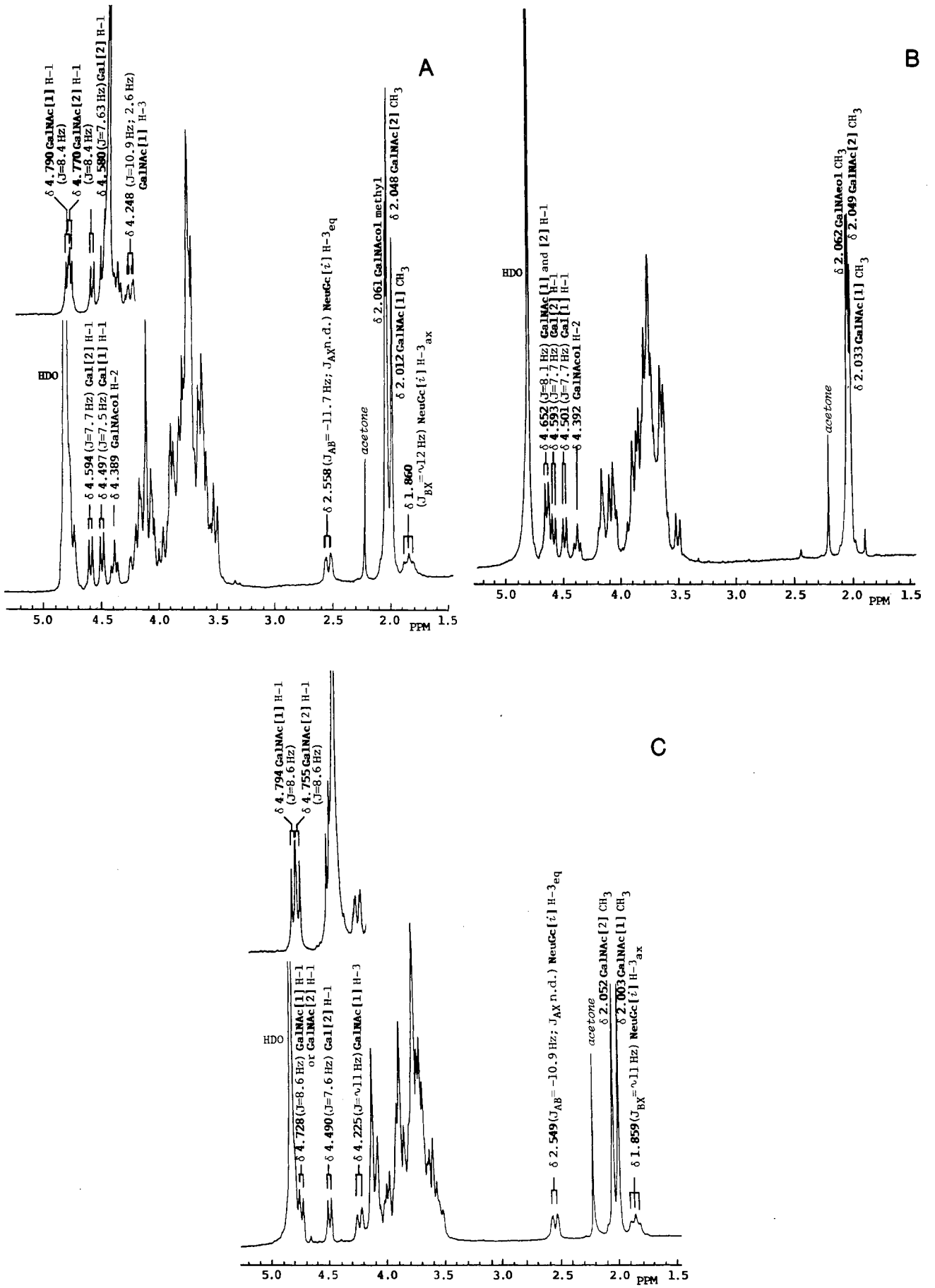


FIGURE 3: ^{1}H NMR spectra at 270 MHz of (A) SRO, (B) asialo-SRO, and (C) TlbLP. Anomeric region of the spectra of SRO and TlbLP at 60 °C is also shown because in the 23 °C spectra the anomeric proton resonances for two GalNAc residues are hidden under the HDO peak.

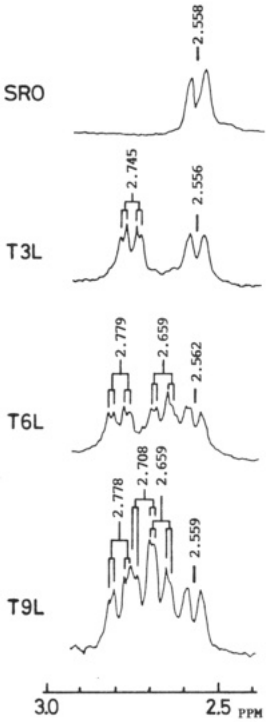


FIGURE 4: NeuGc H-3_{eq} region of the 270-MHz ^{1}H NMR spectra of 23 °C for TlbL (=SRO), T3L, T6L, and T9L.

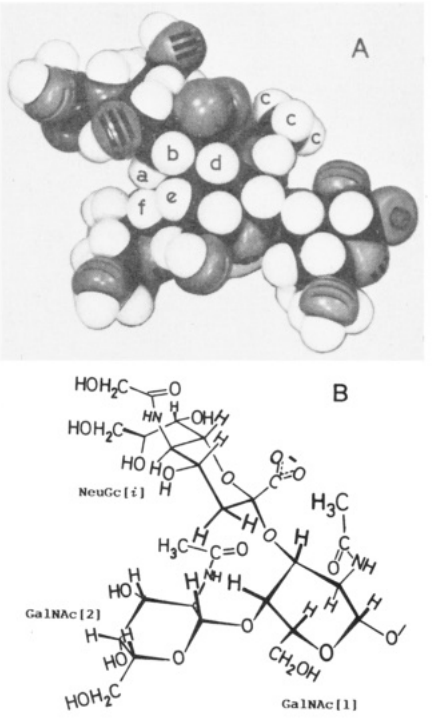


FIGURE 5: (A) A proposed conformational arrangement showing a high degree of crowding of the saccharide chains around the ketosidic linkage of the NeuGc residue in SRO. (a) NeuGc H-3_{ax}; (b) NeuGc H-3_{eq}; (c) penultimate GalNAc acetyl methyl protons; (d) penultimate GalNAc H-3_{ax}; (e) penultimate GalNAc H-4_{eq}; (f) terminal GalNAc H-1_{ax}. (This model is constructed by using space-filling CPK models for SRO by assuming that the relative spatial arrangement of the pyranoid chair structure of NeuGc, the penultimate GalNAc, and the terminal GalNAc residues is analogous to the situation [Figure 3 in the paper by Harris & Thornton (1978)] in NeuAc-1, Gal-1, and GalNAc residues of G_{D1a}, respectively, which has been derived from the ^{13}C NMR shift data for G_{M1} and G_{D1a} gangliosides.) (B) A perspective drawing of the conformational structure for the trisaccharide part, GalNAcβ1→4(NeuGcα2→3)GalNAc, on the basis of construction of Kendrew molecular models. While the picture shown here does not provide unequivocal proof of the correctness of the conformational structure, it is at least self-consistent (see text for details).

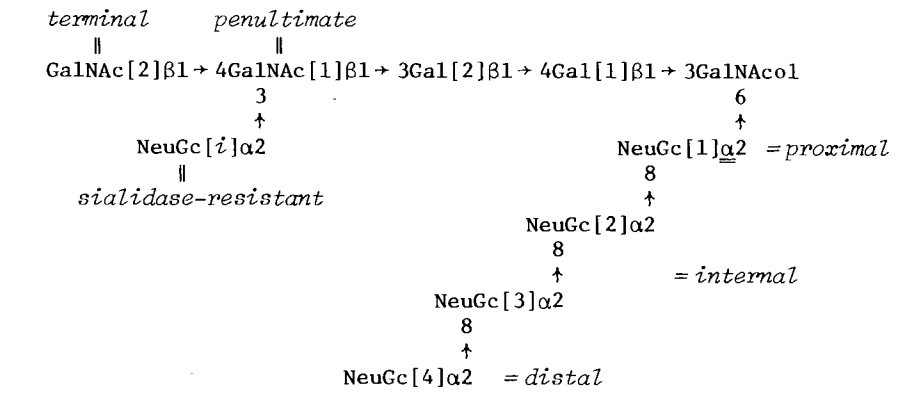
NeuGcα2→6(Galβ1→3)GalNAcol, 0.13 min^{-1}; SRO, 0.016 min^{-1}. This may be coincidental, but it is possible that if sialidase acts by allowing an H_3O^+ catalysis to occur, then the inherently greater resistance to H_3O^+ catalysis may be of some significance in accounting for enzymatic hydrolysis.

^{1}H NMR Studies. The 270-MHz ^{1}H NMR spectra of SRO, asialo-SRO, and TlbLP are given in Figure 3. On close comparison of the spectra of SRO and TlbLP, we see the apparent disappearance of the resonances at 4.595, 4.389, and 2.061 ppm in the spectrum of TlbLP while most resonances in TlbLP are seen in the same positions as in SRO. This suggests that the 4.595 ppm resonance may be assigned to the H-1 proton of Gal[2], and the singlet peak at 2.061 ppm in the spectrum of SRO is attributed to the acetyl methyl protons of the GalNAcol residue, and this assignment is consistent with small downfield shifts (0.012–0.014 ppm) on sialylation at O-6 of GalNAcol (see Table II for T3L, T6L, and T9L). When the sialidase-resistant NeuGc residue is removed from SRO, the most noticeable change in the anomeric proton region is marked upfield shifts of an overlapped pair of doublets at 4.77–4.79 ppm (at 60 °C) to 4.67–4.69 ppm (at 60 °C). It is then reasonable to assign the overlapped signals at 4.77–4.79 ppm (60 °C) in SRO and those at 4.67–4.69 ppm (at 60 °C) (~4.65 ppm at 23 °C) in asialo-SRO to the anomeric protons of the terminal and penultimate GalNAc residues. The H-1 signal at 4.497 ppm for the Gal[1] residue can be assigned by assuming that this peak would be relatively unaffected upon desialylation (SRO vs. asialo-SRO) since this Gal residue is the farthest residue from NeuGc[*i*]. This assignment is also consistent with the presence of a peak at almost the same position (4.48 ppm) in the spectra of Galβ1→3GalNAcol (Vliegenthart et al., 1981) and Galβ1→3(NeuGcα2→8NeuGcα2→6)GalNAcol (Nomoto et al., 1982). In the acetyl methyl proton region, we conclude that the $-NHCOCH_3$ group of the penultimate GalNAc residue must be responsible for the 2.01 ppm resonance because a downfield shift of this peak to 2.033 ppm is observed when the nearby NeuGc residue is removed from SRO. A summary of all of the assignments of the selective protons in the long-core units is presented in Table II.

The resonance (2.558 ppm) due to the H-3_{eq} proton in SRO occurs at much higher field than those for α-anomers. The resonance at 1.860 ppm is, on the other hand, a bit too low downfield to be assigned to a typical α-linked NeuGc H-3_{ax}. Similar peaks are also observed in the spectra of T3L, T6L, and T9L (Figure 4).

Consideration of a space-filling model of SRO shows that the observed anomalies in the chemical shifts of the H-3_{ax} and H-3_{eq} proton resonances are all in the directions compatible with expectations based on effects caused by steric compression and magnetic anisotropy. If one assumes that the conformational environments around GalNAcβ1→4(NeuGcα2→3)GalNAcβ1→ in SRO are analogous to those postulated for gangliosides G_{M1} and G_{D1a} by Harris & Thornton (1978), one such possible conformation would be as illustrated in Figure 5A, in which the overall conformation for this trisaccharide region is semirigid in the SRO molecule and little freedom exists for this unit to move with respect to the other parts of the molecule. In a perspective drawing of this possible structure for the trisaccharide part shown in Figure 5B, it will be noted that the acetamido substituent of the penultimate GalNAc residue lies over a face of the $-COO^-$ group. Consequently, the H-3_{eq} proton must be situated near to the op-

Table II: Summary of Assignments of the Anomeric Proton, Sialic Acid H-3, and *N*-Acetyl Methyl Proton Resonances in Long-Core Units and Sialooligosaccharides[a]



proton	SRO	asialo-SRO	T1bLP	T3L	T6L	T9L	NeuGc	$(NeuGc)_2$	$(NeuGc)_3$	$(NeuGc)_4$
H-1 of										
Gal[1]	4.497 (4.492)	4.501 (4.494)		4.501	4.504	4.508				
Gal[2]	4.594 (4.580)	4.593 (4.579)	4.490 (4.507)	4.598	4.598	4.597				
GalNAc[1]	(4.770)[b]	4.652 (4.672)[c]	4.728 (4.755)[d]							
GalNAc[2]	(4.790)[b]	4.652 (4.686)[c]	(4.794)[d]							
$H\text{-}3_{eq}$ of										
NeuGc[*i*]	2.558		2.549	2.556	2.562	2.559				
NeuGc[1]				2.745	2.659	2.659	2.226	2.235	2.211	2.223
NeuGc[2]					2.779	2.708		2.766	2.701	2.689
NeuGc[3]						2.778			2.763	2.689
NeuGc[4]										2.774
$H\text{-}3_{ax}$ of										
NeuGc[*i*]	1.860		1.859	1.853	1.859	1.855				
NeuGc[1]				1.714	1.666	1.666	1.830	1.752	1.775	1.779
NeuGc[2]					1.741	1.696		1.707	1.659	1.675[e]
NeuGc[3]						1.753			1.729	1.686[e]
NeuGc[4]										1.746
$-NHCOCH_3$ of										
GalNAcol	2.061	2.062		2.075	2.073	2.074				
GalNAc[1]	2.012	2.033	2.003	2.009	2.008	2.009				
GalNAc[2]	2.048	2.049	2.052	2.048	2.038	2.042				

[a] Chemical shifts were measured in D_2O at 23 °C and are given in ppm from DSS. Those in parentheses are the corresponding values measured at 60 °C. The residue number corresponds to the structure shown above. [b–e] Assignments are interchangeable.

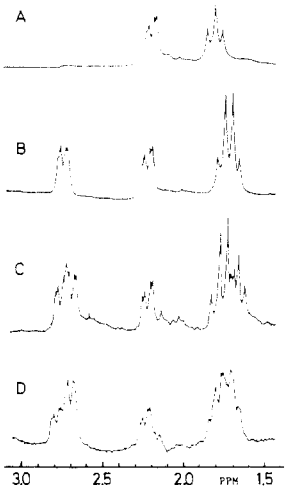


FIGURE 6: 270-MHz ^{1}H NMR spectra of the 1.5–3.0 ppm region for NeuGc and sialooligosaccharides at 23 °C. (A) NeuGc; (B) Neugcα2→8NeuGc; (C) NeuGcα2→8NeuGcα2→8NeuGc; (D) NeuGcα2→8NeuGcα2→8NeuGcα2→8NeuGc.

posite side of the carboxylate group. Such protons should be strongly shielded. Thus the resonance position of H-3_{eq} moves upfield, and likewise, the acetyl methyl protons of the penultimate GalNAc residue resonate at a higher field than other acetamido methyl protons. On the other hand, the H-3_{ax} proton is situated in a hindered portion of the molecule, and a slightly low-field shift of H-3_{ax} (1.86 ppm) is caused at least in part by van der Waals interactions between H-3_{ax} and H-1_{ax} of the terminal 1→4β-linked GalNAc residue and/or H-4_{eq} of the penultimate GalNAc residue, which are sterically compressed (terminal GalNAc H-1_{ax}, 4.77–4.79 ppm at 60 °C; penultimate GalNAc H-4_{eq}, not determined). Pending additional work, the foregoing considerations are also in qualitative accord with the fact that the resonances of H-3_{eq} and H-3_{ax} of the NeuGc[*i*] residue are noticeably broadened in comparison to other corresponding resonances of freely moving NeuGc residues (see Figures 3 and 4). The observed resistance of SRO toward the action of sialidases can now be ascribed to steric hindrance of the sialyl group by the nearby terminal GalNAc residue.

The NMR spectra of T3L, T6L, and T9L (Figure 4) exhibit the usual resonances of the H-3_{eq} protons of NeuGc residues other than NeuGc[*i*]. The C-3 methylene group appeared in T3L as the AB part of an ABX pattern centered at 2.745 ppm for H-3_{eq} and around 1.71 ppm for H-3_{ax}. To facilitate the assignments of the H-3 resonances of oligosialyl groups, the reference compounds, (→8NeuGcα2→)$_n$ with n = 1–5, were prepared, and ^{1}H NMR spectra of these homologous oligosaccharides were measured. The 1.5–3.0 ppm ^{1}H NMR region for the spectra of mono- to tetrasialyl compounds is reproduced in Figure 6. Free sialic acid is known to exist in aqueous solution predominantly in the $^5C_2$ pyranoid form with β-anomeric configuration [e.g., Haverkamp et al. (1978)]. The chemical shift for H-3_{eq} of the reducing terminal sialyl residue is furthest upfield (~2.23 ppm) of the H-3_{eq} in each of (NeuGc)$_n$. The lowest field resonances (2.76–2.77 ppm) in (NeuGc)$_2$, (NeuGc)$_3$, and (NeuGc)$_4$ can readily be assigned to the H-3_{eq} proton of the nonreducing terminal α2→8-linked NeuGc residue simply on the basis of the fact that they should be at almost the same position because they have identical nearest-neighbor environments. The remaining resonance (2.70 ppm) in (NeuGc)$_3$ can be assigned to H-3_{eq} of the internal NeuGc. This was confirmed with (NeuGc)$_4$ in which a resonance at 2.69 ppm, nearly the same position as a 2.70 ppm quartet in (NeuGc)$_3$, is twice the intensity of the other two H-3_{eq} resonances. The assignments of the H-3_{ax} resonances were made on the basis of selective decoupling experiments. In Table II are summarized the NMR spectral data for the long-core units. It is immediately apparent through examination of this table that resonances associated with NeuGc H-3_{eq} could provide useful information about the primary structure of oligosaccharides containing oligosialyl groups.

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Registry No. SRO, 87935-49-7; asialo-SRO, 87862-19-9; T3L, 87935-50-0; T6L, 87862-20-2; T9L, 87862-21-3; Tl6LP, 87862-22-4; Tlc, 83353-80-4; asialo-Tlc, 57123-29-2; Tl6F, 87862-11-1; asialo-Tl6F, 84012-04-4; (NeuGc)$_2$, 68005-95-8; (NeuGc)$_3$, 87862-23-5; (NeuGc)$_4$, 87862-24-6; NeuGc, 1113-83-3; sialidase, 9001-67-6.

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