Comparison of the Conformation of the Epitope of $\alpha(2\rightarrow8)$ Polysialic Acid with Its Reduced and *N*-Acyl Derivatives†

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ABSTRACT: The immunological properties of $\alpha(2\rightarrow8)$ polysialic acid have been rationalized in terms of the presence of an epitope situated on a unique extended helical segment ($n \sim 9$) of the polymer. The critical importance of the carboxylate group to the stability of the extended helical epitope can be ascertained from NMR spectrocopic studies and potential energy calculations on the carboxyl reduced $\alpha(2\rightarrow8)$ polysialic acid. These studies indicate that the extended helix ($n \sim 9$) is not stabilized in the reduced polymer and that the majority of conformers can only have helical parameters with $n = 2$ and 3. This result is also consistent with the fact that the reduced $\alpha(2\rightarrow8)$ polysialic acid, contrary to its acidic counterpart, exhibits conventional immunological properties. Only five to six reduced oligomers are required to inhibit the binding of the reduced polysialic acid to its homologous antiserum. NMR spectroscopic analysis and potential energy calculations on the *N*-propionyl, *N*-butanoyl, *N*-isobutanoyl, *N*-pentanoyl, *N*-hexanoyl, and *N*-glycolyl derivatives of $\alpha(2\rightarrow8)$ polysialic acid indicate that, despite the bulk of some of these substituents, they did not disrupt the extended helical conformer. The presence of the extended helical epitope in some of these *N*-acyl derivatives has also been confirmed from immunological data.

Group B *Neisseria meningitidis* and *Escherichia coli* K1 are major causes of meningitis. The capsular polysaccharide of both organisms consists of $\alpha(2\rightarrow8)$ polysialic acid, which is also an integral component of the neural cell adhesion molecule ([E]N-CAM) (Finne et al., 1983) and human tumors (Troy, 1992). Vaccine development has been hampered by the poor immunogenicity of the group B meningococcal polysaccharide in humans, but by *N*-propionylation of $\alpha(2\rightarrow8)$ polysialic acid, antibodies are produced which are both bactericidal and protective (Jennings, 1989, 1990). Extensive studies have been done on the immunological properties of $\alpha(2\rightarrow8)$ polysialic acid. The presence of a conformational epitope was hypothesized by Jennings et al. (1984) to explain the inability of oligosaccharides of up to five sialic acid residues to inhibit the binding of the group B meningococcal polysaccharide to a homologous horse antiserum. It was later established that an unusually large decasaccharide was required to bind or inhibit group B meningococcal polysaccharide antibodies (Jennings et al., 1985; Finne & Makela, 1985; Hayrinen et al., 1989).

The immunological properties of $\alpha(2\rightarrow8)$ polysialic acid have been rationalized in terms of the presence of an epitope situated on a unique extended helical segment ($n \sim 9$) of the polymer. NMR[1] studies and potential energy calculations indicate that while inherently flexible, the polysialic acid can adopt a wide range of energetically favorable helices, one of which is an extended helix where $n \sim 9$ (Brisson et al., 1992). The presence of identical helices on the $\alpha(2\rightarrow8)$ polysialic acid and poly(A) were also proposed (Brisson et al., 1992) to explain their cross-reactivity with a human monoclonal antibody (IgM^{NOV}) (Kabat et al., 1988).

By means of potential energy calculations and NMR and immunological studies on different chemical modifications of the $\alpha(2\rightarrow8)$ polysialic acid, this paper attempts to identify some of the structural features of the polymer, which contribute to the stability of this unique extended helical epitope.

EXPERIMENTAL PROCEDURES

Materials. Colominic acid, sodium salt (*E. coli*) was obtained from Nacalai Tesque Inc. (Kyoto, Japan) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) was from Aldrich (Milwaukee, WI). Tetanus toxoid was purchased from Institut Armand Frappier (Laval, Quebec) and bovine serum albumin (BSA) was from Sigma Chemical Co. (St. Louis, MO). (Unless otherwise noted, all acyl anhydrides or chlorides were purchased from Aldrich (Milwaukee, WI). NeuAc$\alpha(2\rightarrow8)$NeuAcαOMe was a generous gift from Dr. René Roy (Abbas et al., 1990).

Carboxyl Reduction of Colominic Acid. The carboxylate groups of colominic acid were reduced to primary alcohols according to the method of Taylor and Conrad (1972) via the $NaBH_4$ or $NaBD_4$ reduction of the corresponding EDC activated esters. Two successive treatments were necessary to fully reduce the polymer.

Partial Hydrolysis of the Reduced Colominic Acid. Partial hydrolysis was carried out at 80 °C for 75 min in a 0.2 M sodium acetate buffer (pH 4.5) at a concentration of 10 mg of polysaccharide/mL. The resultant oligosaccharide mixture was fractionated by gel filtration using successive passage through Bio-Gel P-2 and P-4 columns (Bio-Rad Laboratories) with pyridinium acetate (0.02 M, pH 5.4) as eluting buffer. Oligomers ranging from DP1 to DP8 could thus be separated.

Synthesis of N-Acylated Derivatives of Polysialic Acid. The following derivatives were obtained by re-*N*-acylation of de-*N*-acetylated colominic acid (Jennings et al., 1985). Briefly, colominic acid was treated with 2 M NaOH in the presence of $NaBH_4$ at 110 °C for 6 h, followed by treatment of the pure de-*N*-acetylated polymer with the appropriate acyl anhydride,

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[1] Abbreviations: DP, degree of polymerization; NeuAc, *N*-acetylneuraminic acid (sialic acid); NOE, nuclear Overhauser enhancement; NMR, nuclear magnetic resonance; TPPI, time proportional phase increments; *n*, number of residues per turn.

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i.e., propionic, butanoic, isobutanoic, pentanoic, and hexanoic anhydride to afford, respectively, *N*-propionyl-, *N*-butanoyl-, *N*-isobutanoyl-, *N*-pentanoyl-, and *N*-hexanoylpolysialic acid derivatives. The *N*-acryloylated and *N*-glycolylated derivatives were synthesized by treating the de-*N*-acetylated polysaccharide with acryloyl- and acetoxyacetyl chlorides, respectively (Roy & Pon, 1990).

NMR Methods. ^{13}C and ^{1}H NMR spectra were recorded on a Bruker AMX 500 or AMX 600 spectrometer in 5-mm tubes and concentrations of 10–25 mg in 0.5 mL of D_2O at neutral pH. A broadband probe with the ^{1}H coil nearest to the sample was used for experiments in the inverse mode. All experiments were carried out with the standard software provided by Bruker. Proton spin simulation was performed with the program LAOCN5 available with program FTNMR (Hare Research Inc.). The estimated error on the H,H coupling constants is ±0.3 Hz and ±0.005 ppm on the proton chemical shifts. Primary geminal protons were distinguished with a prime for the proton with the largest vicinal coupling constant.

Two-dimensional experiments COSY, relay COSY, and 2D NOE were performed as described before (Michon et al., 1987; Brisson et al., 1992). A mixing time of 150 ms was used for the 2D NOE. ^{1}H detection was used for heteronuclear shift correlation experiments. HMQC (Bax et al., 1983) was recorded in phase-sensitive mode (TPPI) with ^{13}C decoupling in f_1 and 2500 Hz in f_2 and the initial (t_1,t_2) matrix of 512 × 1K data points was zero-filled to give a digital resolution of 29.3 and 2.4 Hz/point, respectively. The long-range heteronuclear shift correlation experiment, HMBC, was done in the absolute value mode according to Bax and Summers (1986). The spectral parameters were the same as described for HMQC except that only 256 data points were collected in f_1. A delay of 80 ms was used for evolution of long-range couplings. Heteronuclear H,C coupling constants were measured with a proton detected experiment proposed by Bermel et al. (1989), which uses a Gaussian pulse for selective excitation of the carbon of interest. The Gaussian shaped pulse (1% truncation) had a duration of 4.2 ms. The sweep width was 625 Hz in f_1 and 510 Hz in f_2, and the original 128 × 2048 data matrix was zero-filled to a final matrix of 512 × 2K data points to give a digital resolution of 1.2 and 0.25 Hz/point, respectively.

Molecular Modeling. The coordinate set for carboxyl reduced α-sialic acid was generated by modification of the crystal structure of β-sialic acid (Flippen, 1973) and energy minimization with MM2(87) (QCPE) (Burket & Allinger, 1982).

The torsional angles φ, ψ, ω_1, ω_7, ω_8, and ω_9 were defined by O6′–C2′–O8–C8, C2′–O8–C8–C7, O6–C2–C1–01, O6–C6–C7–O7, O7–C7–C8–O8, and O8–C8–C9–09, respectively. The bond angle τ, defined by C2′–O8–C8, was set to 117°. The staggered rotamers for ω_7, ω_8, and ω_9 of 60°, –60°, 180° are referred to as g^+, g^-, and t, respectively. The potential energy calculations are described in detail by Brisson et al., (1992).

Synthesis of Conjugates. The conjugates were synthesized as previously described (Jennings et al., 1985) for those of the group B meningococcal polysaccharide. Reduced colominic acid–tetanus toxoid and –BSA conjugates were prepared by direct coupling of the periodate activated polysaccharide with the ε-aminolysine residues of the protein via reductive amination with $NaBH_3CN$ (Jennings & Lugowski, 1981). A high molecular weight fraction (MW ~ 10 000) of the reduced polymer was obtained by sizing through a Bio-Gel A (0.5) column (Bio-Rad Laboratories) using PBS as eluent. The

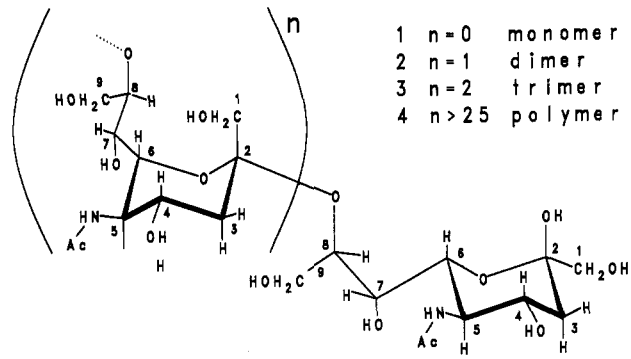


FIGURE 1: Schematic diagram of the reduced α(2→8)-linked sialic acid compounds **1–4**. The linkage conformation is described by the torsional angles φ, ψ, ω_8, and ω_7 defined by O6′–C2′–O8–C8, C2′–O8–C8–C7, O8–C8–C7–O7, and O7–C7–C6–O6, respectively.

above fraction was then treated with sodium metaperiodate to generate terminal aldehyde groups on the polysaccharide fragments. The activated polysaccharide fragments were then coupled to TT monomer (MW ~ 150 000) or BSA using $NaBH_3CN$ as the reducing agent.

Immunization Protocol. Groups of 10 outbred CF1 mice (6–8 weeks old) were immunized ip with 20 μg of conjugate vaccine in a 0.2-mL emulsion of complete Freund's adjuvant (DIFCO Laboratories) and PBS 1:1 (v/v). Immunizations took place at d0, d14, and d28 followed by exsanguinations at d39. The individual bleedings were pooled to yield the reduced α(2→8) polysialic acid antiserum.

Inhibition Studies. Microtiter plates (Corning) were coated with 1 μg/well of the reduced colominic acid–BSA conjugate in PBS buffer at 37 °C for 1 h. The plates were blocked with 1% (v/v) BSA in PBS for 30 min at room temperature and washed (×4) with PBS–Tween 20 (0.05%, v/v). Oligosaccharide inhibitor (100 μL) and 100 μL of a 5×10^{-4} dilution in PBS of mouse antiserum were incubated at room temperature for 90 min. One hundred microliters of the above solutions was then transferred to the coated wells and the plates were incubated for 1 h at room temperature. The plates were then washed (×4) with PBS–Tween 20, and 50 μL of peroxidase labeled goat anti-mouse IgG (1:200) from Kirkegaard & Perry, Gaithersburg, MD, was added to the wells. Following incubation for 30 min at room temperature, the plates were washed (×5) with PBS–Tween 20 and the tetramethylbenzidene (TMB) peroxidase substrate (50 μL) (Kirkegaard & Perry) was added. The reaction was stopped after 15 min by the addition of 50 μL of 1 M H_3PO_4 and the optical densities were read at 450 nm using a microplate reader (Biotek). This data yielded the ELISA inhibitions of each of the reduced α(2→8)NeuAc oligosaccharides of the binding of reduced α(2→8) polysialic acid to its homologous mouse antiserum.

RESULTS AND DISCUSSION

Reduced Compounds. All derivatives in this study are homooligomers or homopolymers of α(2→8)-linked sialic acid. For the sake of simplicity, the reduced compound will mean the carboxyl reduced α(2→8) polysialic acid and the native compound will mean the α(2→8) polysialic acid. Unambiguous assignments of ^{13}C and ^{1}H NMR signals for the reduced compounds **1–4** (Figure 1) were achieved by two-dimensional experiments (Tables I and II). The ^{1}H NMR signals were first assigned with the help of COSY alone and COSY with one- and two-step relay coherence transfer. The relay experiments proved to be more efficient than the TOCSY experiment in this case, due to the very small coupling constant

Table I: ^{13}C NMR Chemical Shifts for Compounds **1–4**[a]

	1	residues of 2[b]		residues of 3[b]			4
carbon	monomer	b	a	c	b	a	polymer
1	69.27	60.85	68.09	60.36	61.00	68.18	60.87
2	97.96	102.18	97.98	102.07	102.69	97.98	102.52
3	38.22	38.80	38.06	39.47	38.13	37.88	38.53
4	68.00	68.75	67.87	68.77	68.39	67.87	68.44
5	53.21	53.06	53.49	53.05	53.44	53.44	53.53
6	70.28	72.76	70.76	72.80	73.24	70.82	73.30
7	68.10	68.05	69.06	68.60	68.66	68.95	69.06
8	71.08	71.79	73.46	70.03	73.79	73.06	73.99
9	64.05	63.89	62.30	63.87	61.76	62.75	62.24
C=O	175.74	175.77[c]	175.89[c]	175.75[c]	175.83[c]	175.88[c]	175.74
CH_3	22.91	23.00[c]	22.92[c]	23.03[c]	23.03[c]	22.93[c]	23.14

[a] Measured at 300 K in D_2O with acetone as internal chemical shift reference (δ 31.07 ppm). [b] Contiguous residues from the reducing residue a. [c] Tentative assignments.

Table II: ^1H NMR Chemical Shifts for Compounds **1–4**[a]

	1	residues of 2[b]		residues of 3[b]			4
proton	monomer	b	a	c	b	a	polymer
H-1	3.525	3.762	3.540	3.814	3.750	3.534	3.80
H-1′	3.542	3.875	3.540	3.914	3.792	3.534	3.80
H-3a	1.710	1.735	1.704	1.701	1.701	1.678	1.69
H-3e	2.126	2.334	2.077	2.314	2.448	2.087	2.42
H-4	4.048	3.905	4.030	3.923	3.861	4.027	3.89
H-5	3.834	3.841	3.830	3.856	3.861	3.843	3.88
H-6	3.985	3.680	3.960	3.692	3.715	3.960	3.76
H-7	3.531	3.560	3.767	3.570	3.747	3.751	3.76
H-8	3.758	3.739	4.162	3.719	4.153	4.132	4.10
H-9	3.840	3.838	3.933	3.844	3.973	3.917	3.96
H-9′	3.612	3.630	3.758	3.643	3.758	3.766	3.74
CH_3	2.052	2.043[c]	2.056[c]	2.054[c]	2.046[c]	2.046[c]	2.06

[a] Measured at 300 K in D_2O with acetone as internal chemical shift reference (δ 2.225 ppm). [b] Contiguous residues from the reducing residue a. [c] Tentative assignments.

Table III: Vicinal and Geminal Coupling Constants (Hz) of Compounds **1–4**

	1	residues of 2[a]		residues of 3[a]			4
(H,H)	monomer	b	a	c	b	a	polymer
1,1′	−11.8	−12	–[b]	−11.8	–[b]	–[b]	
3a,3e	−12.9	−13.2	−13	−13.0	−13.0	−13	−13
3a,4	11.8	11.8	11.8	11.5	11.5	11.5	12
3e,4	5.0	4.8	4.8	4.9	4.9	4.9	5
4,5	10.0	10.5	10.2	10.1	10.1	10.1	10
5,6	10.3	10.5	10.2	10.1	10.1	10.1	10
6,7	0.7	<1.5	<1.5	1.5	0.7	0.7	<3
7,8	9.2	9.6	7.3	9.3	7.5	7.5	7
8,9	2.9	2.6	3.3	2.9	3.0	3.0	–[b]
8,9′	6.6	6.0	4.0	6.0	3.8	3.4	–[b]
9,9′	−11.8	−12.0	−12.5	−11.9	−12.3	−12.3	−12

[a] Contiguous residues from the reducing residue a. [b] Not determined.

between H6 and H7 of each residue (<1 Hz). The cross-peak between H6 and H7 could not be observed in the TOCSY spectrum but was readily observed in relay COSY. Chemical shifts and coupling constants (Table III) of overlapping signals were obtained from a COSY spectrum with high resolution (0.5 Hz/point). The assignments of the ^1H NMR signals were confirmed by spin simulations, and the experimental spectrum together with the simulated spectrum for the reduced trimer (**3**) are shown in Figure 2. The axial H3 proton of the reducing residue in **3** exchanged to deuterium in D_2O solution and the equatorial H3 at the reducing end was observed as a doublet with a coupling to H4 only. The chemical shift and coupling constant for H3a were obtained from a sample that had not been subjected to exchange with D_2O prior to measurements.

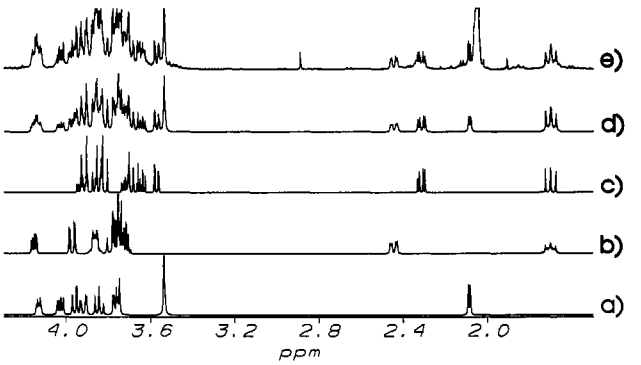


FIGURE 2: Proton NMR spectra for the deuterated reduced α(2→8)-linked sialic acid trimer (**3**): Spin simulated spectra for each residue (a–c), their sum (d), and the experimental spectrum (e).

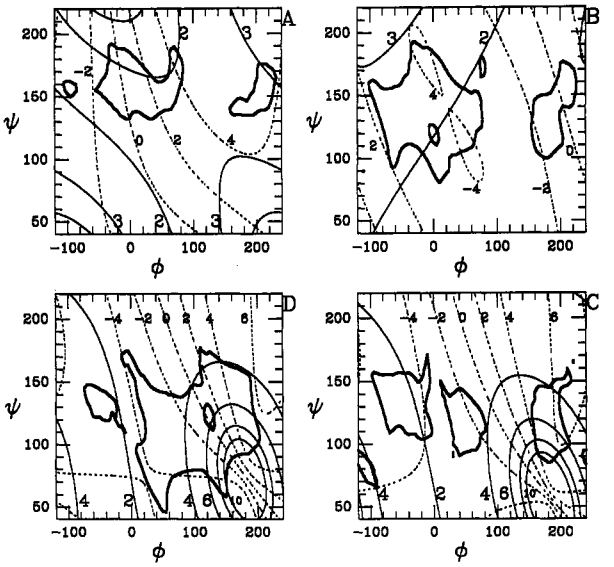


FIGURE 3: Potential energy contour map for the reduced αNeuAc-(2→8)αNeuAc with (ω_7, ω_8) = (g^+, t) (A), (g^+, g^-) (B), and (g^+, g^+) (C). In (D), the map for the isobutanoyl derivative with (ω_7, ω_8) = (g^+, g^+) is shown. With respect to the global minimum of each section, the 15 kcal/mol level is drawn. The helical parameters, *n* (solid lines) and *h* (dotted line) are also displayed.

A proton-detected ^1H–^{13}C correlated experiment then aided in the assignment of the ^{13}C NMR spectrum. The assignment of the quaternary C2 signals and the sequence of the residues in the reduced trimer (**3**) were confirmed by a long-range heteronuclear shift correlation experiment, HMBC. Cross-peaks between C2 and H3 for each residue assigned the anomeric carbon to its respective residue, and a cross-peak between C2 and H8 across the glycosidic linkage confirmed the sequence of the residues. The anomeric configuration at the reducing end is β in all compounds, thus giving rise to a totally different set of chemical shifts for this residue with respect to those for the other α-linked residues.

The conformational differences observed in oligomers of native colominic acid, based on ^{13}C NMR chemical shift differences of carbon signals involved in the glycosidic linkage (Michon et al., 1987), could not be observed in the reduced compounds. The chemical shifts for the linkage carbons in the reduced disaccharide (**2**) and trisaccharide (**3**) differed not more than 0.5 ppm from the corresponding signals in the polymer (Table I). Also, the signals for C7 and C9 in the exocyclic chain of the reduced residue (**2**) have almost identical chemical shifts compared to those in the polymer. This indicates that there is no significant difference in linkage conformation between short oligomers and the polymer in

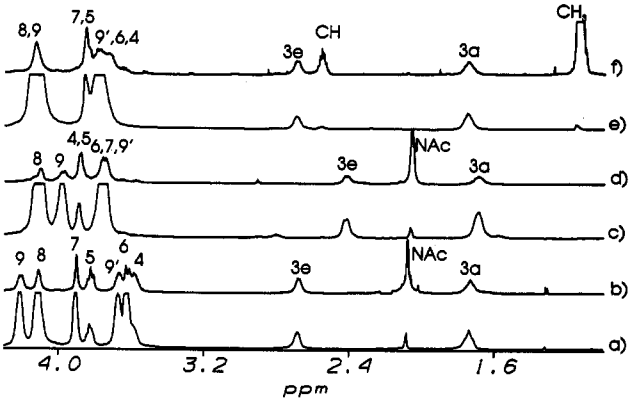


FIGURE 4: Proton NMR spectra of native and derivatives of $\alpha(2\rightarrow8)$ polysialic acid. The 2D NOE trace of the H8 resonance and the normal ^{1}H spectrum for the native compound (a, b), for the deuterated carboxyl reduced compound (c, d), and for the *N*-acyl isobutanoyl derivative (e, f) are shown.

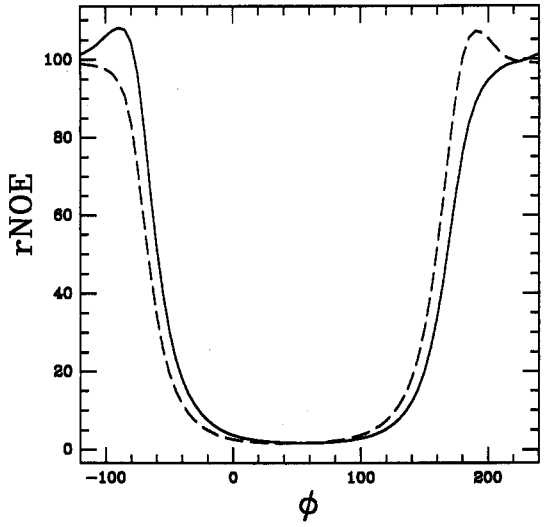


FIGURE 5: Typical 2D NOE variation for $\alpha(2\rightarrow8)$-linked sialic acid of the H3a–H8 NOE (—) and the H3e–H8 NOE (- - -) versus ϕ, with $(\omega_7, \omega_8, \omega_9) = (g^+, t, g^+)$ and $\psi = 150°$. A mixing time of 150 ms and a correlation time of 6 ns were used for the calculation. The NOE's are relative to the H3a–H3e NOE which is set to 100.

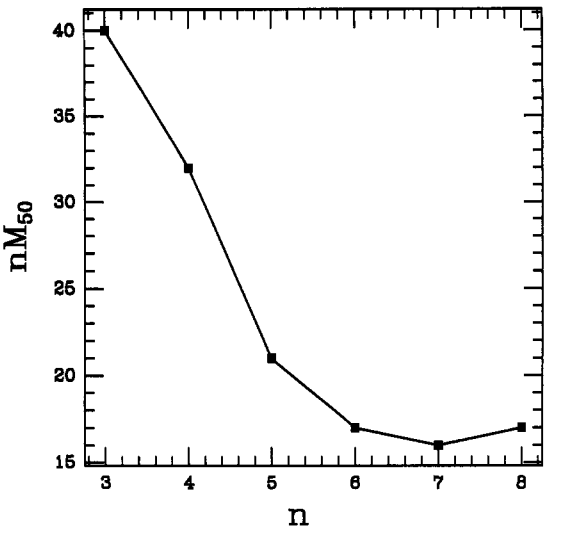
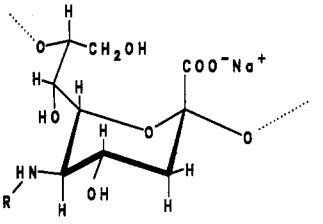


FIGURE 6: Quantity (nanomoles) of reduced $\alpha(2\rightarrow8)$-linked sialic acid oligosaccharides required to cause 50% inhibition (nM_{50}) of the reaction between reduced $\alpha(2\rightarrow8)$ polysialic acid and its homologous antiserum. Data were obtained from an ELISA inhibition experiment using the individual oligosaccharides.



R	
Acetyl	$COCH_3$
Propinoyl	$COCH_2CH_3$
Butanoyl	$CO(CH_2)_2CH_3$
Isobutanoyl	$COCH(CH_3)_2$
Pentanoyl	$CO(CH_2)_3CH_3$
Hexanoyl	$CO(CH_2)_4CH_3$
Acryloyl	$COCH{=}CH_2$
Glycolyl	$COCH_2OH$

FIGURE 7: *N*-Acyl derivatives of $\alpha(2\rightarrow8)$ polysialic acid.

contrast to the conformational heterogeneity observed for the native compounds.

Further evidence for this can be obtained by observation of the ^{1}H NMR chemical shifts and coupling constants for compounds **2**–**4** (Tables II and III). The lack of an anomeric proton in these compounds emphasizes the importance of protons in the exocyclic chain of the substituted residues (H7, H8, H9, and H9'). Only the chemical shifts of the exocyclic protons involved in glycosidic linkages are to be compared with the corresponding shifts in the polymer. There are only minor differences observed (<0.06 ppm) between the ^{1}H NMR chemical shifts of internal side chain protons of residue a in the dimer and of residues a and b in the trimer with respect to those in the polymer.

The linkage region between two residues is composed of four bonds (Figure 1) which can all adopt numerous conformations. Two of the bonds are located in the exocyclic chain (C6–C7 and C7–C8) and an estimate of their rotamer distribution can be made with help of the vicinal coupling constants and their relation to the dihedral angle (Haasnoot et al., 1980). The small coupling constant between H6 and H7 (~1 Hz) (Table III) is consistent with ω_7 being near 60°. This is also observed for sialic acid in various derivatives (Christian et al., 1987; Michon et al., 1987). The coupling constant between H7 and H8 is large (~9 Hz) in the monomer (**1**) and in the nonreducing end residues of **2** and **3**, indicating that these two protons are trans to each other. When the exocyclic chain is involved in a glycosidic linkage, as in **2** and **3**, the H7–H8 coupling constant is slightly decreased to ~7.5 Hz. This suggests that the trans rotamer is still predominant but that there is a small amount of one or both of the gauche rotamers (assuming only staggered rotamers).

The H7–H8 coupling constants in the reduced trimer (**3**) were compared to those in the native trimer (Michon et al., 1987). In the first linkage between residues a and b, no difference in the coupling constant can be observed, thus the rotamer distribution must essentially be the same. In the second linkage, however, there is a large difference observed between the two compounds. No change from the first linkage is observed in the reduced trimer (**3**), but in the native trimer the coupling constant has decreased to 2.3 Hz, indicating that these protons are now in a gauche orientation. The only difference between these two compounds is that for **3** the negatively charged carboxyl group has been reduced to a neutral hydroxymethyl group, thus strongly suggesting that the negative charge in the carboxyl group is the cause for the heterogeneity observed in the linkage conformations. The

Table IV: ^{13}C NMR Chemical Shifts[a] for *N*-Acyl Derivatives of Colominic Acid

	N-acyl derivative							
carbon	acetyl	propionyl	butanoyl	isobutanoyl	pentanoyl	hexanoyl	acryloyl	glycolyl
1	174.00	173.97	174.03	173.71	174.09	174.09	174.17	174.16
2	101.78	101.83	101.98	101.91	102.05	102.05	102.19	101.50
3	40.76	40.86	40.90	41.24	40.88	40.88	39.93	41.09
4	69.13	69.13	69.15	69.01	69.14	69.08	69.48	68.50
5	53.23	53.10	53.12	52.94	53.12	53.10	53.29	53.14
6	74.01	74.00	74.09	74.03	74.13	74.17	74.07	73.77
7	69.86	69.95	70.13	69.90	70.19	70.23	70.40	69.70
8	78.72	78.53	78.44	78.09	78.43	78.39	78.48	79.05
9	62.06	62.11	62.16	62.09	62.18	62.17	62.24	62.03
C=O	175.76	179.66	178.87	182.84	179.07	179.07	170.13	176.99
CH	–	–	–	36.10	–	–	–	–
CH_2	–	–	–	–	–	36.90	–	–
CH_2	–	–	–	–	36.70	31.38	–	–
CH_2	–	–	38.87	–	28.25	25.75	–	–
CH_2	–	30.10	19.70	–	22.50	22.53	–	–
CH_3	23.30	10.17	13.84	19.75	13.98	14.11	–	–
CH=	–	–	–	–	–	–	130.84	–
CH_2=	–	–	–	–	–	–	128.91	–
CH_2OH	–	–	–	–	–	–	–	62.03

[a] Measured at 300 K (±0.05 ppm) in D_2O with acetone as internal chemical shift reference (δ 31.07 ppm).

Table V: ^1H NMR Chemical Shifts[a] for *N*-Acyl Derivatives of Colominic Acid

	N-acyl derivative						
proton	acetyl	propionyl	butanoyl	isobutanoyl	pentanoyl	hexanoyl	glycolyl
3a	1.74	1.73	1.74	1.75	1.75	1.75	1.72
3e	2.67	2.68	2.68	2.69	2.68	2.68	2.71
4	3.60	3.59	3.59	3.72	3.60	3.59	3.68
5	3.82	3.84	3.84	3.84	3.84	3.83	3.87
6	3.63	3.63	3.63	3.76	3.63	3.64	3.71
7	3.90	3.88	3.88	3.85	3.88	3.88	3.86
8	4.10	4.10	4.11	4.13	4.11	4.11	4.12
9	4.19	4.18	4.17	4.13	4.16	4.15	4.19
9′[b]	3.66	3.67	3.67	3.79	3.67	3.66	3.70
CH	–	–	–	2.56	–	–	–
CH_2	–	–	–		–	2.33	–
CH_2	–	–	–		2.34	1.61	–
CH_2	–	–	2.30		1.59	1.61	–
CH_2	–	2.36	1.63		1.34	1.31	–
CH_3	2.07	1.14	0.93	1.15	0.90	0.88	–
CH_2OH	–	–	–	–	–	–	4.10, 4.21

[a] First-order chemical shifts (±0.03 ppm) obtained at 300 K in D_2O with acetone as internal chemical shift reference (δ 2.225 ppm).

large difference for *J*(H7–H8) between the first and second linkage of the native trimer is probably due to the β-configuration of the reducing end for which the carboxyl groups of residues a and b are much further apart relative to the second linkage c–b. To confirm this, an investigation of NeuAcα-(2→8)NeuAcαOMe (Abbas et al., 1990) was undertaken. The proton coupling constants in the interresidue side chain of the dimer were identical to those of the second linkage in the native trimer (data not shown), providing further evidence regarding the presence and orientation of the charge in the conformational behavior of α(2→8)-linked sialic acid oligomers.

Potential energy calculations were performed on the reduced dimer as a model for the reduced α(2→8) polysialic acid. The methods and potentials used were the same as reported earlier for the native compound by Brisson et al. (1992). The value for ω_7 was set to the g^+ rotamer as deduced from the coupling constant. Figure 3 shows the conformational energy contour plots for each of the three rotamers (t, g^-, g^+) of the C7–C8 bond. The maps show essentially the same minima as the maps for the native compound. The native compound showed in general more flexibility around the glycosidic linkage than the reduced compound, probably due to the small but significant difference in size and geometry between the carboxyl group and the hydroxymethyl group.

The large *J*(H7–H8) coupling constant of 7.5 Hz in the reduced compound indicated that the ω_8 rotamer of 180° was predominant. As can be seen in the potential energy map for this rotamer (Figure 3A), the C2–O2 bond (i.e., the ϕ angle) is very flexible whereas the C2–O8 bond is less flexible with ψ in the range 130°–190°. In contrast, the g^+ rotamer for the C7–C8 bond in the native compound was found to predominate. This rotamer had a much larger flexibility around the glycosidic linkage, and furthermore, the O2–C8 bond was not found to attain values between 60° and 160° (Brisson et al., 1992). In an attempt to verify the differences in the ψ angle distribution between the native and reduced compound by NMR, the ^3J(C2,H8) coupling constant was measured for both the reduced and native trisaccharides. Values of 4.5 Hz and 3.0 Hz were found for the native and reduced compounds, respectively. Tvaroska et al. (1989) have shown the angular dependence of the ^3J(CH) coupling constant for various glycosides behaves in a Karplus-type manner. The experimental values obtained for the trisaccharides suggest a good agreement with the difference in ψ observed in the potential energy calculations. However, the difference in electronegativity between a carboxyl group and a hydroxymethyl group may also affect the magnitude of the coupling constant, and due to the lack of model compounds, these data should be interpreted with care.

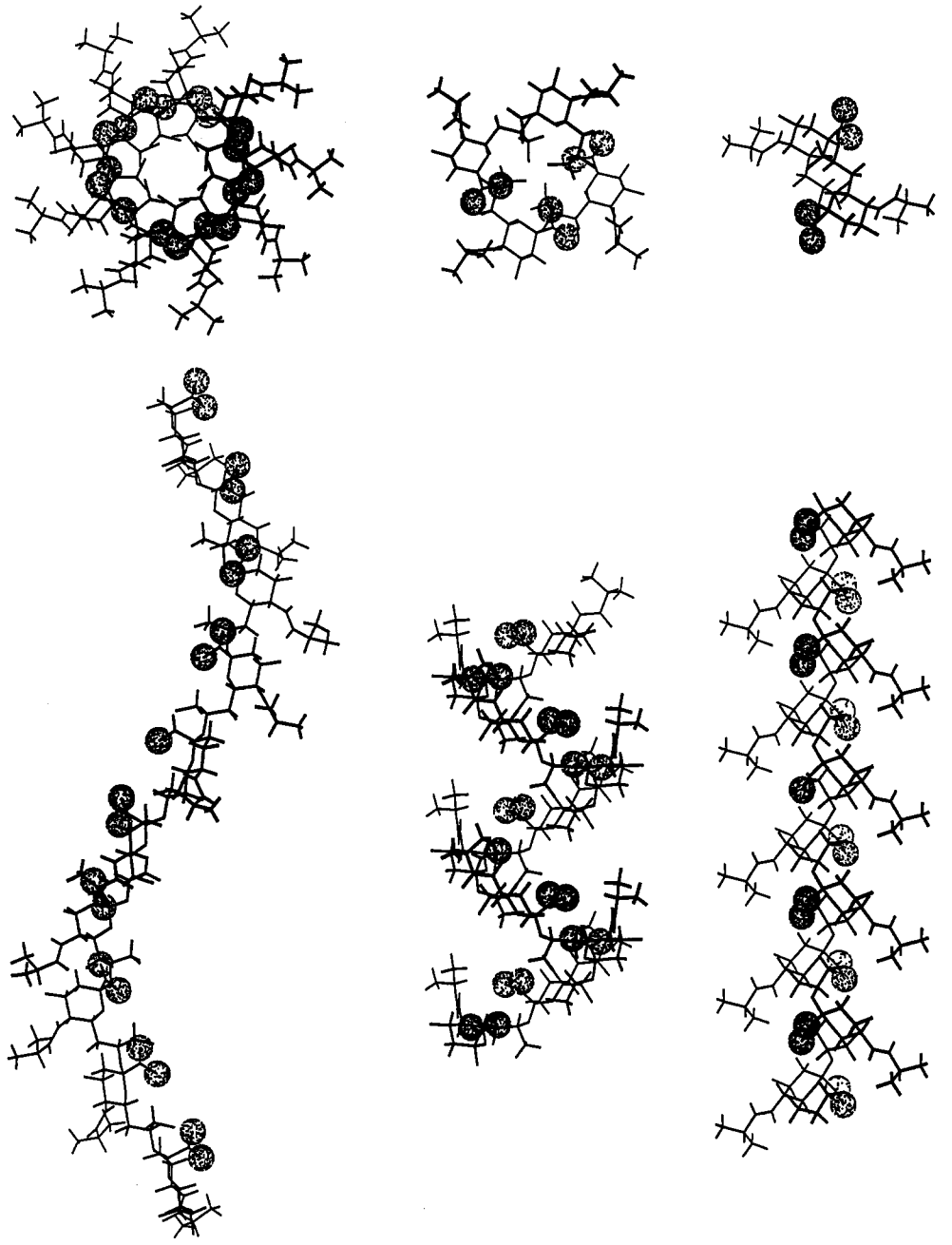


FIGURE 8: Side and top views of various helices of the *N*-acyl isobutanoyl derivative of $\alpha(2\rightarrow8)NeuAc_{10}$ where n = 2, 4, and 9 with (ω_7, ω_8) = (g^+, g^+) (see Figure 3d). The carboxyl oxygens are displayed as spheres.

NOE's were also performed on reduced $\alpha(2\rightarrow8)$ polysialic acid and compared to those of its native counterpart in order to assess the (ϕ,ψ) distribution (Figure 4). Due to overlap of resonances, only two NOE's H3a–H8 and H3e–H8 could be used for the conformational analysis. As shown before, these NOE's are not highly sensitive to ω_7, ω_8, ω_9, and ψ and they depend mostly on the value of ϕ. Also due to the wide range of ϕ angles energetically possible, motional averaging of the NOE's must be considered (Brisson et al., 1992). The variation of the H3a–H8 and H3e–H8 NOE with ϕ is shown in Figure 5. In the range ϕ = (–120,–20), the H3a–H8 NOE is bigger than the H3e–H8 NOE, while in the range ϕ = (120,180) the reverse is true. In the range ϕ = (–20,120), both sets of NOE's are very small. For the potential energy maps given in Figure 3, the majority of conformers will contribute very little to the observed NOE. Since experimentally the H3a–H8 NOE is bigger than the H3e–H8 NOE (17 and 11 relative to H3a–H3e set at 100), conformers in the range ϕ = (–20,120) are contributing more to the observed NOE than conformers in the range ϕ = (120,180). Hence, as expected from the similarity in the energetically allowable ϕ range between the reduced and native compounds, the H3a–H8 and H3e–H8 NOE's are expected to show the same conformational dependence.

In contrast with the unusually large $\alpha(2\rightarrow8)$-linked sialic acid oligomer (decasaccharide) required to inhibit $\alpha(2\rightarrow8)$ polysialic acid specific antibodies (Jennings et al., 1985), the carboxyl reduced polymer contains a more conventional determinant (Figure 6). Optimum inhibition of the binding of reduced polymer with homologous antibodies could be accomplished with an $\alpha(2\rightarrow8)$-linked carboxyl reduced hexasaccharide, consistent with the number of residues required to define a conventional epitope (Kabat, 1960). This result would indicate that the negative charge is important in stabilizing the conformationally defined native epitope.

The results of these inhibition studies can be correlated with the helical parameters of the polymers. As explained previously for native $\alpha(2\rightarrow8)$ polysialic acid, an extended

helical epitope ($n \sim 9$) is postulated to be necessary for recognition by an antibody (Brisson et al., 1992). Whereas from the data above, the majority of conformers for the reduced α(2→8) polysialic acid will have helical parameters with n between 2 and 3 (Figure 3A), and the topographical properties of short oligomers would be expected to be the same as those present in the polymer. Hence, if the recognition site in the reduced α(2→8) polysialic acid corresponds to helices of short pitch, the dependence of inhibition on chain length is not expected to be important.

N-Acyl Derivatives. In order to investigate the importance of the *N*-acyl substituent in terms of conformational behavior and antibody binding, a number of *N*-acyl derivatives of α-(2→8) polysialic acid were synthesized (Figure 7). To measure any conformational changes resulting from such chemical modifications, a comparative study of the ^{13}C and ^{1}H NMR chemical shifts for each derivative was undertaken. The ^{13}C and ^{1}H NMR chemical shifts of the *N*-acyl derivatives of colominic acid are given in Tables IV and V, respectively. Initial assignments were based on those derived from native colominic acid and then confirmed by two dimensional COSY and H,C correlated HMQC experiments. It was shown by Michon et al. (1987) that the ^{13}C NMR chemical shifts of the linkage carbons (C2 and C8) are very sensitive to conformational changes. An examination of these chemical shifts in the *N*-acyl derivatives (Table IV) shows that they are very similar; the largest difference relative to colominic acid (*N*-acetyl) is observed for the C8 signal of the *N*-isobutanoyl derivative (–0.6 ppm). Large differences are observed in the chemical shifts of the *N*-acyl carbonyl signal which are not unexpected due to the variety of the acyl groups. The chemical shift values for the signals of the other carbons in the ring and in the exocyclic side chain of the sialic acid moiety are very similar, and minor differences in the values may be due to direct electronic effects from the *N*-acyl substituent.

The ^{1}H NMR chemical shifts (Table V) show the same similarities between the different *N*-acyl derivatives as observed for the ^{13}C NMR chemical shifts. Although there are some changes in the chemical shifts for the H4, H6, and H9′ signals in the *N*-isobutanoyl derivative (~0.13 ppm), they cannot be attributed to changes in the linkage conformation. A possible explanation is that the bulky isobutanoyl group has a steric interaction with the carboxyl group at C1 and/or the hydroxymethyl group at C9. A change in rotamer distribution for one or both of these groups could well induce the chemical shift differences observed for the above-mentioned protons. Supporting this hypothesis is the observed cross-peak in the 2D NOESY spectrum between the CH_3 of the isobutanoyl group and H9 (Figure 4e). The H3a–H8 NOE is also bigger than the H3e–H8 NOE (Figure 4e) and similar to the ones observed with the native compound (Figure 4a), indicating a similar linkage conformation for both the native and *N*-acyl derivatives.

Potential energy calculations for the *N*-isobutanoyl derivative were undertaken to explain the similarity in the observed linkage conformation between the native and *N*-acyl derivatives. Only the map for the major ω_8 conformer is shown in Figure 3D, the other maps being similar to the native one also. The isobutanoyl group together with the carboxyl group at C1 and the hydroxymethyl group at C9 were all allowed to adopt preferred rotamers during the calculations. This shows that the bulky isobutanoyl group has no significant steric effect on the conformational behavior of the polymer as can be observed for some of the helices, ranging from $n = 2$ to $n = 9$, which the polymer can adopt (Figure 8). Hence, the conformational behavior of α(2→8) polysialic is mostly determined by the presence of its negative charge.

In summary, from an analysis of the NMR spectroscopic data and potential energy calculations it can be inferred that chemical modifications of α(2→8) polysialic acid involving replacement of its *N*-acetyl group by the *N*-acyl groups cited (Figure 7) do not result in major changes of the conformation of the polymer. Thus, the increasing size of the *N*-acyl substituent does not disrupt the formation of the extended helix. This is consistent with the models of the helix on the basis of the above data, which show that in fact the bulky *N*-acyl groups protrude outward from the helix (Figure 8). Additional immunological evidence for the preservation of the extended helix in these chemically modified derivatives was also derived from the fact that the *N*-acetyl, *N*-propionyl, *N*-butanoyl, *N*-pentanoyl, and *N*-hexanoyl derivatives of the α(2→8) polysialic acid precipitate the same amounts of a human monoclonal antibody (IgM^{NOV}) (Lifely & Esdaile, 1991). The latter antibody has been demonstrated to be specific for the extended helical epitope of α(2→8) polysialic acid (Kabat et al., 1988).

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