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Efficient Synthesis of α(2-8)-Linked *N*-Acetyl and *N*-Glycolylneuraminic Acid Disaccharides from Colominic Acid

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Controlled acid hydrolysis of poly-α(2,8)-linked homopolymers of *N*-acetylneuraminic acid (colominic acid) and its homologous poly-*N*-glycolylneuraminic acid afforded high yields of the corresponding disaccharides useful in block synthesis of disialylated gangliosides. The poly-*N*-glycolyl analog was derived from de-*N*-acetylated colominic acid by two different reaction sequences. The first one involved reaction with acetoxyacetyl chloride followed by de-*O*-acetylation. The second and most interesting one required *N*-acryloylation and reductive ozonolysis.

Sialic acids in glycolipids and glycoproteins have been recognized to play essential roles in a number of biological functions [1-3] with *N*-acetylneuraminic acid (Neu5Ac, **6**) representing the most ubiquitous form of the sialic acids. In spite of the fact that Neu5Ac (**6**) and its α(2-8)-linked disaccharide (**8**) constitute the immunodominant epitope of many tumor associated antigens [4] and human erythrocyte autoantigens [5], little is known concerning the analogous *N*-glycolylneuraminic acid (Neu5Gc, **7**) and its corresponding α(2-8)-linked disaccharide (**9**). Neu5Gc (**7**) has been found in some GM3-gangliosides and is known as the Hanganutziu-Deicher antigen [6] which may also be expressed on human tumor cells [7].

In a research program directed toward the synthesis and the study of structure-function relationship amongst sialyloligosaccharides, chemically modified α(2-8)-linked polysialic acids and their corresponding oligomers were required. Several sources of polysialic acids comprising this particular sequence were identified. The capsular polysaccharides of *Escherichia coli* (K1 antigen) [8, 9] and *Neisseria meningitidis* group B [10] are well studied cases. They also represent the main carbohydrate constituents of glycoproteins of neural cell adhesion molecules (*N*-CAM) [11] while the homologous poly-α(2-8)Neu5Gc has been isolated from the glycoproteins of the cortical alveoli of Salmonidiae fish eggs [12].

We describe herein, efficient syntheses of Neu5Gc (**7**), α(2-8)-linked Neu5Ac (**8**) and Neu5Gc (**9**) disaccharides from *E. coli* K1 antigen.

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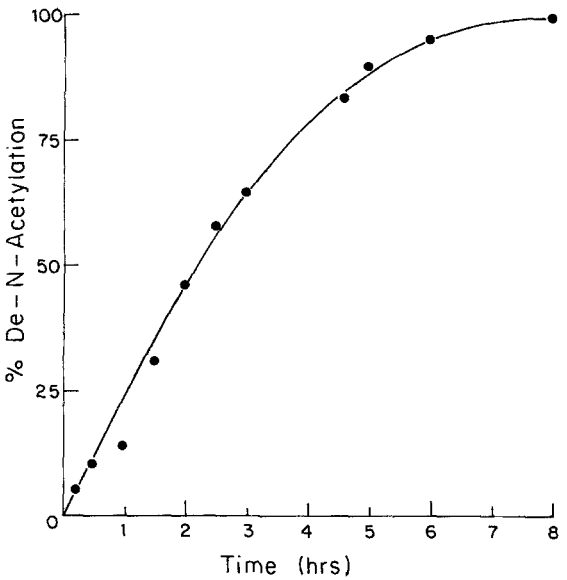


Figure 1. Rate of de-*N*-acetylation of colominic acid (**1**) in 2 M NaOH at 110°C (sealed tube).

Results and Discussion

De-N-acetylation of Colominic Acid (1)

The capsular polysaccharide colominic acid (**1**) is an α(2-8)-linked homopolymer of *N*-acetylneuraminic acid. It is also known as the *Escherichia coli* K1 antigen [8] and was shown to present structural and serological similarities to the bacterial polysaccharide of *Neisseria meningitidis* group B [10]. The two polysaccharides differ only in their molecular weight and by the absence in **1** of 1,2-diacyl-glycerophosphate residues linked to the reducing end of the polysaccharide [13]. Due to its commercial availability, colominic acid (**1**) constitutes an appropriate starting material for the large scale preparations of sialic acid analogs. In order to obtain substantial quantities of Neu5Gc (**7**) and its homologous α(2-8)-linked disaccharide (**9**), colominic acid (**1**) was first transformed into poly-α(2-8)Neu5Gc (**5**).

Low molecular weight colominic acid (**1**) (10 kDa, ≈30 residues) was first treated with 2 M NaOH containing $NaBH_4$ at 110°C for 7 h in a sealed tube. The pure de-*N*-acetylated derivative (**2**) was obtained in 82% yield after dialysis and freeze-drying. The extent of de-*N*-acetylation was followed as a function of time and the rate of hydrolysis was found to be similar to that observed for the group B polysaccharide of *N. meningitidis* (Fig. 1) [14]. The residual level of *N*-acetyl groups was established by ^1H and ^{13}C-NMR spectroscopy. The disappearance in the ^1H-NMR spectrum of the *N*-acetamido proton signal at 2.08 ppm was measured relative to that of the H-3e and H-3a signals at 2.64 and 1.76 ppm respectively. Furthermore, a new peak (dd) at 2.98 ppm attributed to H-5 of **2** slowly increased as de-*N*-acetylation proceeded, corresponding to an upfield shift of 0.84 ppm. The ^{13}C-NMR

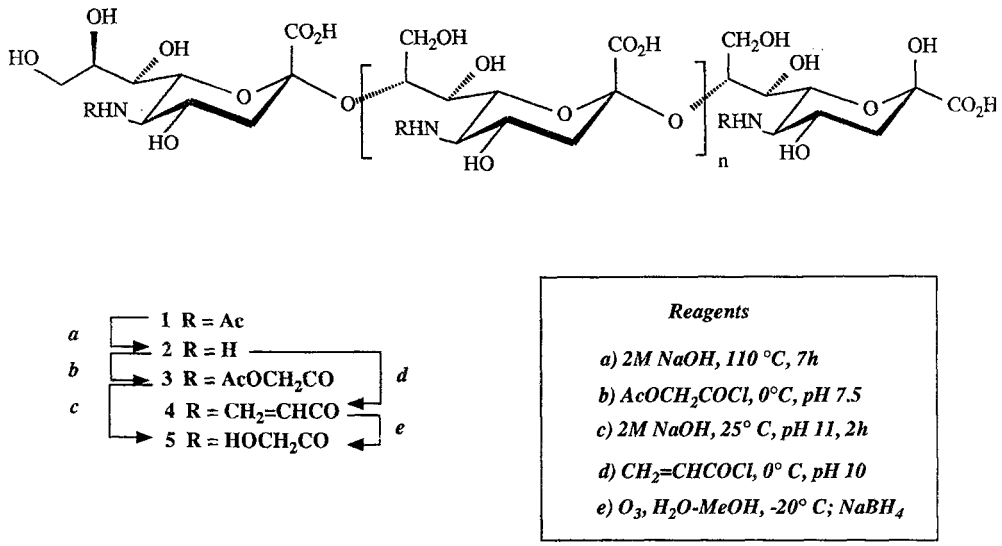


Figure 2. *N*-Glycolylation of **2**.

spectrum of **2** was also indicative of a complete de-*N*-acetylation (7 h) since both *N*-acetamido carbon signals in **1** had disappeared (Table 1). Interestingly, the expected downfield shift (1.0 ppm) of the C-5 signal was accompanied with the C-4 resonance being shifted upfield by 1.5 ppm. Since this effect has not been observed in the case of the vicinal C-6 signal, it can not be solely attributed to a substituent effect. It has been previously shown that a hydrogen bond between the C-5 acetamido carbonyl and the C-4 hydroxyl group exists in the monomeric unit [15], therefore the above observations may indicate a similar situation within the polysaccharide.

*Synthesis of Poly-α(2-8)-N-glycolylneuraminic Acid (**5**)*

Although the preparation of *N*-glycolylneuraminic acid (**7**) from Neu5Ac (**6**) has already been described [1], the previous synthesis required preliminary blocking of the anomeric position through glycoside formation. In addition, unstable and non-commercially available glycolylating reagents such as 1,3-dioxolan-2,4-dione are required. The strategy involved with using the naturally occurring polysaccharide (**1**) as a precursor was to take advantage of the α(2-8)-linkage in the role of a blocked glycoside. Thus, *N*-glycolylation of **2** was performed under two different sets of conditions: (1) using acetoxyacetyl chloride followed by de-*O*-acetylation and (2) reductive ozonolysis of a preformed *N*-acryloyl polysaccharide (**4**) (Fig. 2). The latter procedure was found to be more versatile in that it allows more flexibility in the design of other *N*-acyl analogs. Thus in the first case, de-*N*-acetylated colominic acid (**2**) was treated with an excess of the commercially available acetoxyacetyl chloride (H_2O, 0°C, pH 7.5). The pH was kept constant with 2 M NaOH. After the complete disappearance of ninhydrin positive material, the solution was divided into two equal fractions in an attempt to isolate completely *O*-acetylated *N*-glycolyl polysaccharide (**3**). The title polysaccharide (**3**) was purified on a fast desalting column (Bio-Gel P-6DG)

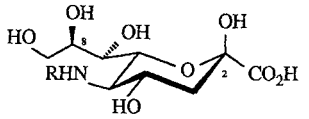
Table 1. 75.41 MHz ^{13}C-NMR chemical shifts of colominic acid derivatives ($^{2}H_2O$,25°C).

Compound	C-1	C-2	C-3	C.4	C-5	C-6	C-7	C-8	C-9	C=O	R
1[a,c]	173.9	101.8	40.7	69.2	53.3	74.0	70.0	78.7	62.1	175.7	23.4
2[a,d]	174.1	102.0	40.8	67.7	54.3	73.7	70.2	78.0	62.1	-	-
4[b,c]	174.0	102.0	40.3	69.3	53.3	74.1	70.5	78.0	62.2	170.0	130.6 129.0
5[b,c]	174.1	101.6	40.9	68.5	53.2	73.7	70.0	78.5	62.1	176.7	62.1
7[c,e]	177.5	97.2	40.2	67.8	52.8	70.8	69.3	71.2	64.1	176.3	61.8
8[d,e]	173.6 (177.1)[g]	102.9 (97.3)	41.9 (40.1)	69.3 (67.8)	52.7 (53.3)	73.8 (71.2)	68.9 (68.4)	73.0 (76.0)	63.6 (61.9)	175.8[f] (175.5)[f]	23.1 (23.1)
9[d,e]	173.3 (177.5)[g]	102.6 (97.4)	41.9 (40.1)	71.0 (67.8)	52.2 (52.9)	73.3 (70.9)	68.7 (68.0)	72.9 (76.2)	63.3 (61.8)	176.3[f] (176.2)[f]	61.8 (61.8)

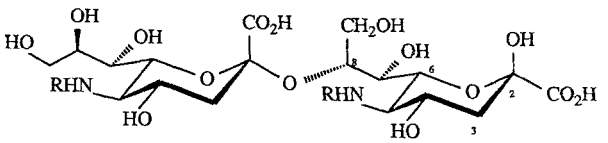
[a] In ppm from internal dioxane (67.4ppm).
[b] In ppm from external dioxane.
[c] Na^+ salt.
[d] NH_4^+ salt.
[e] In ppm from internal acetone (31.1 ppm).
[f] Tentative assignments.
[g] Numbers in parentheses refer to reducing ends.

where the high molecular weight material was isolated and freeze-dried to yield **3** in 70% yield based on the half scale used. Although the ^{1}H- and ^{13}C-NMR spectra of **3** showed the characteristic signals of *O*-acetyl residues at 2.0 ppm in the ^{1}H-NMR and at 21.0 ppm in the ^{13}C-NMR spectra, the presence of heterogeneity was evident. This was attributed to the partial hydrolysis of some of the acetoxy groups which had occurred during the mildly basic conditions used for the incorporation of the *N*-glycolyl residues. Partial hydrolysis was confirmed based on the results obtained from treating the remaining fraction of the reaction mixture with base. Indeed, when the pH of the remaining reaction mixture was raised to pH 12 with 2 M NaOH and the reaction allowed to proceed for a further 2 h at room temperature, the fully de-*O*-acetylated *N*-glycolylated polysaccharide (**5**) was obtained in 89% yield after dialysis and lyophilization. The ^{13}C-NMR spectrum is in full agreement with the structure proposed (Table 1). For scale-up purposes, the de-*N*-acetylation/*N*-acylation sequence was performed in a one pot reaction without adverse effects and generally in higher overall yields.

An alternative approach was also considered since it appeared of interest to offer a procedure which could be of broader utility (e.g. radiolabeling). Consequently, *N*-acryloylation of **2** was achieved with a slight excess of a dioxane solution of acryloyl chloride added slowly to an aqueous solution of the amine derivative (**2**) (NaOH, pH 10-11, 0°C). The reaction was deemed complete based on a negative ninhydrin test. The reaction was allowed to stand for an additional hour at pH 11 to ensure complete hydrolysis of esters which might be present. The yield of **4** obtained as a white powder after dialysis and



6 R = CH_3CO *N-Acetylneuraminic acid*
7 R = $HOCH_2CO$ *N-Glycolylneuraminic acid*



8 R = CH_3CO
9 R = $HOCH_2CO$

lyophilization was 79%. The ^{13}C-NMR spectrum of the poly-α(2-8)-*N*-acryloylneuraminic acid (**4**) showed the characteristic signals of *N*-acryloyl residues. The carbonyl carbon appeared at 170.0 ppm corresponding to an upfield shift of 5.7 ppm (Table 1) consistent with an α,β-unsaturated system; the olefinic methylene and methine carbons appeared at 130.6 and 129.0 ppm respectively. The C-5 carbon signal of the unsubstituted amine at 54.3 ppm was completely absent and now appeared at 53.3 ppm. The ^{1}H-NMR spectrum of **4** in ^2H_2O showed a broad multiplet (2 H) and a broad doublet (1 H) centered at 6.08 and 5.62 ppm respectively. The integration of these olefinic proton signals relative to those of H-3 were in complete agreement with all the amine residues being substituted.

Reductive ozonolysis of the *N*-acryloylated analogue (**4**) was used for the preparation of the poly-*N*-glycolyl derivative (**5**). The ozonolysis of **4** was first accomplished in a water/methanol mixture, 2/1 by vol (-20°C, 20 min). After the removal of excess ozone with nitrogen, sodium borohydride was directly added to the reaction mixture to ensure reduction of α-hydroxyperoxides. After 2 h at room temperature and following dialysis and lyophilization, the reaction mixture afforded a 78% yield of a white fluffy powder which had all the characteristic ^{1}H- and ^{13}C-NMR signals consistent with pure poly-α(2-8)-linked homopolymer (**5**) of Neu5Gc. All the NMR features were identical to those of **5** obtained by the first procedure (Table 1). It is noteworthy to mention that in the present case both the C-9 and the *N*-glycolyl methylene signals appeared at 62.1 ppm, while the latter was shifted to 63.8 ppm in the *O*-acetoxylated derivative (**3**). Additional proof for **5** resides in its degradation to the oligomeric units **7** and **9**. Contrary to colominic acid (**1**) and the homologous *N*-propionylated polysaccharide [14], the poly-*N*-glycolyl analog (**5**) showed no precipitin bands with equine (H.46, IgM) anti group B *N. meningitidis* polysaccharide in double immunodiffusion experiments (R. Roy and R.A. Pon, unpublished results). These results are analogous to previous observations obtained with an α(2-8)-linked poly-Neu5Gc containing polysialoglycoprotein isolated from fish eggs [12].

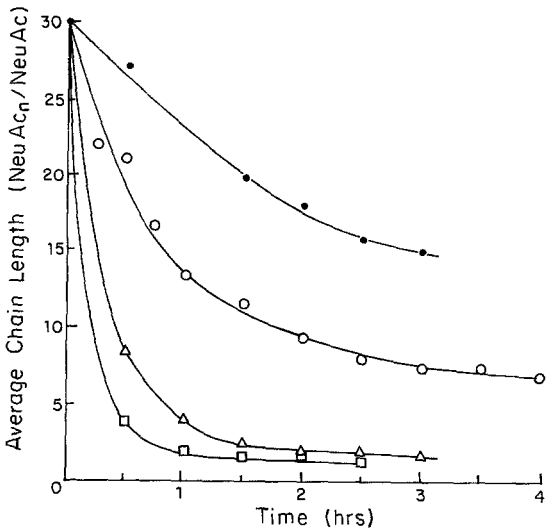


Figure 3. Rate of acid hydrolysis of colominic acid (**1**) at: pH 7.0, 100°C (●); pH 3.0, 50°C (○); pH 3.0, 70°C (△); and pH 2.0, 85°C (□).

*Acid Hydrolysis of **1** and **5***

It was previously shown that controlled acid hydrolysis of colominic acid (**1**) could provide access to discrete oligomers of α(2-8)-linked Neu5Ac (up to 18) [16] while more stringent conditions (0.1 M H_2SO_4, 80°C, 1 h) or enzymatic hydrolysis afforded monomeric Neu5Ac (**6**) [1]. In this work reasonable quantities of material were desired which could be used for synthetic purposes and for inhibition studies. It therefore appeared that concurrent synthesis of the corresponding dimers (**8** and **9**) should be considered a priority over the monomers (**6** and **7**) which are obtained as side products in high yields, because exclusive formation of **6** and **7** is possible by alternative approaches [1]. Moreover, while it was previously shown that chemical synthesis of an α(2-8)-linked neuraminyl disaccharide was met with only limited success [17, 18], it was recently demonstrated that the disaccharide (**8**), prepared by the procedure described below, could be efficiently transformed into a useful glycosyl donor (Abbas SZ, Sugiyama S, Diakur J, Pon RA, Roy R; unpublished results). Hence, simple access to disaccharides **8** and **9** should be considered an appreciable improvement toward the synthesis of complex gangliosides and neoglycoproteins.

Detailed evaluation of conditions were required to optimize the yield of the disaccharides using colominic acid (**1**) as model compound. As shown in Fig. 3, the extent of hydrolysis was determined under various conditions of temperature and pH. The average chain length of the residual oligomer mixture was based on the ratio of the total number of sialic acid residues (resorcinol method [19]) to the number of free reducing ends (Park-Johnson method

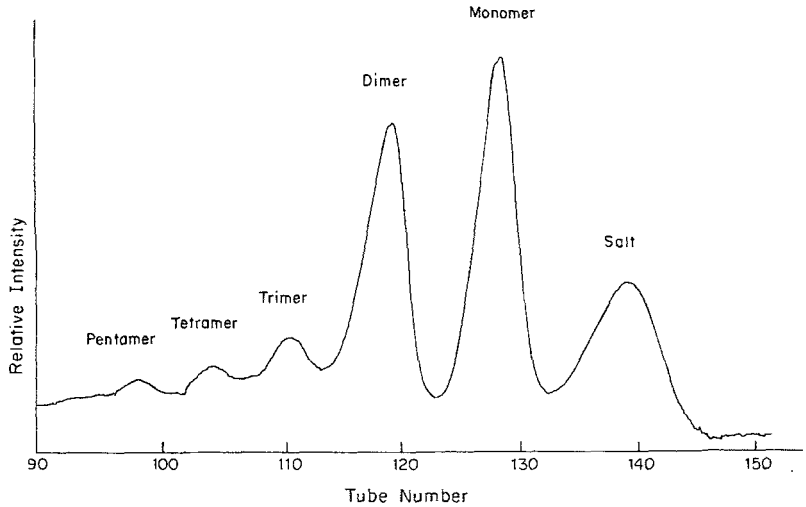


Figure 4. Fractionation of acid hydrolysate (pH 3.0, 70°C, 3 h) of poly-α(2-8)-*N*-glycolylneuraminic acid (**5**) by size exclusion chromatography on Bio-Gel P-10 in 0.03 M NH_4HCO_3 (3.5 ml fractions).

[20]) and was followed as a function of time for various sets of conditions. For practical purposes and because the chromatographic separation gave the expected chromatogram, the hydrolytic conditions using a pH of 3.0 and a temperature of 70°C for 3 h were adopted. The monomer (**6**) was obtained in 29% yield while the dimer (**8**) was obtained in 28% yield based on the recovered material (84% yield) after fractionation on a Bio-Gel P-10 column using 0.03 M NH_4HCO_3 as eluant. Incidentally, this method of purification avoids the previously described [16] tedious separation using a DEAE-Sephadex A-25 column (chloride) requiring a salt gradient and consequently, a Sephadex G-10 desalting column for each oligomer.

The above hydrolytic conditions were then transposed to the *N*-glycolyl analog (**5**). The results were similarly successful (Fig. 4). The material recovered from the Bio-Gel P-10 column accounted for a 76% yield comprising 38% of the monomer (**7**) and 32% of the dimer (**9**). The physical data for Neu5Gc (**7**) were in complete agreement with a purified authentic sample from Sigma, St. Louis, MO, USA (TLC, FAB-MS, $[\alpha]_D$, ^{1}H- and ^{13}C-NMR). The ^{13}C-NMR spectrum of the corresponding disaccharide (**9**) is shown in Fig. 5 and illustrates the purity of the product (see Table 1 for details).

In conclusion, the above procedures describe a striking improvement over the existing methodology [17, 18] for the synthesis of the α(2-8)Neu5Ac disaccharide (**8**) found in numerous disialyl gangliosides such as GD2, GD3, GT1b and GQ1b. Moreover, access to large quantities of the immunodominant epitope of the Hanganutziu-Deicher antigen Neu5Gc (**7**) and its homologous α(2-8)Neu5Gc disaccharide (**9**) is clearly demonstrated. It is also of interest to mention that the methodology described herein represents an entry into the synthesis of the mixed disialyl Neu5Ac(Gc) α(2-8)Neu5Gc(Ac) disaccharides [21].

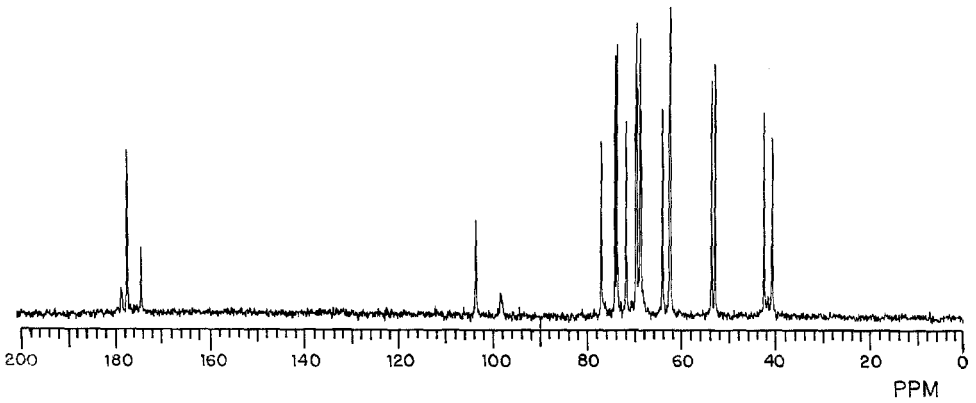


Figure 5. 75.41 MHz ^{13}C-NMR spectrum of Neu5Gcα2-8Neu5Gc disaccharide (**9**) (ammonium salt) in $^{2}H_2O$ at 25°C.

Furthermore, the possibility also exists for the synthesis of α(2-8)-linked oligomers derived from a number of other *N*-acyl analogs of colominic acid (Roy R, Pon RA; unpublished results). Polyacrylamides (Roy R, Pon RA; unpublished results: [22-24]) and protein conjugates (Roy R, Pon RA; unpublished results) of some of the above derivatives have been prepared and the results will be described in due course.

Experimental

General Methods

Distilled water and reagent grade solvents were used. Colominic acid (Kyoto Research Laboratories, Japan) was used without prior treatment. Commercial sodium borohydride (BDH, Toronto, Canada), acetoxyacetyl chloride and acryloyl chloride (Aldrich, Milwaukee, WI, USA) were used as received. Extrusion gel chromatography was performed on a Bio-Gel P-6DG column in water and on a Bio-Gel P-10 column (Bio-Rad, Richmond, CA, USA) in 0.03 M ammonium bicarbonate. Peaks were detected using a Waters Associates (Millford, MA, USA) differential refractometer. NMR Spectra were recorded on a Varian XL-300 MHz NMR at 25°C in $^{2}H_2O$ (>99.9%, MSD, Montreal, Canada). Acetone was used as an internal reference for both ^{1}H-NMR (δ=2.225) and ^{13}C-NMR (δ=31.07). Only selected NMR data are reported. Optical rotations were recorded at 25°C with a Perkin-Elmer 241 polarimeter. FAB mass spectra were recorded on a VG 7070-E spectrometer.

*De-N-acetylation of Colominic Acid (**1**)*

Colominic acid (100 mg) in 4 ml 2 M NaOH containing 10 mg $NaBH_4$ was treated at 110°C in a sealed tube for 7 h. The slightly yellow solution was exhaustively dialyzed against 0.01 M NH_4HCO_3 (pH 7.5) and lyophilized to give 70.5 mg (82%) of white fluffy product (**2**). ^{13}C-NMR: see Table 1.

*N-Acryloylation of De-N-acetylated Colominic Acid (**2**)*

The following is an example of a one pot, preparative scale reaction: Colominic acid (100 mg) was de-*N*-acetylated as above followed by direct *N*-acryloylation in the following manner. The solution was diluted ten-fold and the pH lowered to 10 with 1 M HCl. A 1:1 solution of acryloyl chloride (500 µl) in dioxane was added in increments to the ice cold solution until complete amine derivatization occurred based on a negative ninhydrin test. The pH was maintained between 9 and 11 with periodic additions of 2 M NaOH. The reaction was allowed to stand for an additional hour at pH 11 to ensure the complete hydrolysis of possible esters formed. The solution after dialysis (running water) was lyophilized to a white powder (**4**) (82.0 mg, 79%). ^{1}H-NMR: $CHCH_2$ (δ=5.62, d); $CHCH_2$ (δ=6.08, m). ^{13}C-NMR: see Table 1.

*Poly-α(2-8)-N-glycolylneuraminic Acid (**5**)*

*a) From **1** by acylation with acetoxyacetyl chloride:* To an ice cold solution of 100 mg de-*N*-acetylated colominic acid (**2**) in 10 ml water at pH 7.5, was added single drop aliquots (≈50 µl) of a dioxane solution of acetoxyacetyl chloride (500 µl). The pH was maintained below pH 8 at all times with 2 M NaOH. The acid chloride was added until the complete disappearance of amino groups as determined by a negative ninhydrin test. One half of the solution was applied to a Bio-Gel P-6DG column in water where the higher molecular weight fraction was collected and lyophilized to yield 45.6 mg (70%) of white powder. ^{1}H-NMR: disappearance of H-5 (δ=2.98). ^{13}C-NMR: see Table 1. De-*O*-acetylation: To the remaining half, the pH of the solution was raised to 12-13 with 2 M NaOH and was maintained in this range for a period of 2 h at room temperature. The solution was neutralized with 1 M HCl, dialyzed against running water, and then lyophilized to yield 55.0 mg (89%) of **5** as a fluffy white material.

*b) Reductive ozonolysis of **4**:* 100 mg of **4** was dissolved in a 2:1 water-methanol mixture and was cooled to -20°C. Ozone was bubbled through the solution for 25 min, residual ozone was removed with nitrogen, and an excess of sodium borohydride (≈20 mg) was added. The solution was allowed to stand at room temperature for 2 h, dialyzed against running water, and lyophilized to give **5** (95.6 mg, 94%).

*Acid Hydrolysis of **1** and **5***

The pH of 100 mg of **1** or **5** in 10 ml water was adjusted to 3.0 with 1 M HCl and heated at 70°C for 180 min. The solution was immediately cooled to room temperature and the pH was raised to 7.6 with 1 M NaOH and the solution was lyophilized. The material was chromatographed on a Bio-Gel P-10 (200-400 mesh) column in 0.03 M NH_4HCO_3 (3.5 ml fractions). The individual peaks were lyophilized into a white fluffy powder with a total recovered amount of 84 mg for **1** and 76 mg for **5**.

N-Acetyl monomer (**6**): 24.2 mg (29%), negative FAB mass spectrum: M-1 = 308. Dimer (**8**): 23.3 mg (28%), $[\alpha]_D$ = -11.2° (H_2O, c=5 mg/ml), M-1 = 599 (negative FAB-MS).

N-Glycolyl monomer (**7**): 28.7 mg (38%), $[\alpha]_D$ = -21.8° (H_2O, c=5 mg/ml), M-1 = 324 (negative FAB-MS). Dimer (**9**): 23.7 mg (32%), $[\alpha]_D$ = -14.2° (H_2O, c=5 mg/ml), M-1 = 631 (negative FAB-MS). See Table 1 for ^{13}C-NMR data.

Acknowledgments

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