Synthesis of protein conjugates and analogues of *N*-acetylneuraminic acid

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N-Acetylneuraminic acid (**1**) was prepared from the salivary gland mucins of the Chinese swiftlet by an improved procedure using acidic resin hydrolysis. Compound **1** was transformed into the known acetochloroneuraminic acid (**3**) by a new two-step procedure. Koenigs–Knorr glycosylation of **3** followed by subsequent reductive ozonolysis of the 2-propenyl α-glycoside afforded the key aldehyde precursors **7** and **8**, which were coupled to bovine serum albumin or tetanus toxoid by reductive amination. The factors influencing the extent of incorporation were investigated. A series of *N*-acetylneuraminic acid analogues modified at strategic functionalities were also synthesized.

Key words: sialic acid, *N*-acetylneuraminic acid, neoglycoproteins, bovine serum albumin, tetanus toxoid.

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L'acide *N*-acétylneuraminique (**1**) a été préparé selon une procédure améliorée à partir de la mucine des glandes salivaires d'hirondelles asiatiques. La méthode fait intervenir une hydrolyse par de la résin cationique acide. Le composé **1** a été transformé en dérivé acide acétochloroneuraminique connu (**3**) par une nouvelle méthode en deux étapes. Une glycosylation de **3** par la méthode de Koenigs–Knorr, suivie d'une ozonolyse réductive du α-glycoside de propèn-2-yle conduit aux précurseurs aldéhydes clés **7** et **8** qui sont ensuite couplés à l'albumine bovin et à l'anatoxine tétanique par amination réductrice. Les facteurs influençant le niveau d'incorporation ont été étudiés. Une série d'analogues de l'acide *N*-acétylneuraminique modifiés aux fonctionnalités stratégiques a aussi été synthétisée.

Mots clés : acide sialique, acide *N*-acétylneuraminique, néoglycoproteines, albumine bovin, anatoxine tétanique.

Introduction

The sialic acids represent a family of more than 30 naturally occurring derivatives of neuraminic acid (1). The most ubiquitous member of the sialic acids is *N*-acetylneuraminic acid (Neu5Ac, **1**). It is present in the capsular polysaccharide fractions of a number of pathogenic bacteria (2, 3). It was also identified in the glycoprotein mucins of higher animals, the most abundant source being the *Collocalia* mucoid, the nest-cementing glycoprotein substance of the Chinese swiftlet (4) from whose nests it can be isolated in large quantity (5% w/w) (5). *N*-Acetylneuraminic acid (**1**) is also an important constituent of the cell wall glycolipids (gangliosides) (1). More recently, the N-linked oligosaccharide sequences of the envelope glycoproteins gp120 of the human immunodeficiency viruses (HIV) were characterized and were shown to contain high levels of Neu5Ac (**1**) (6).

In the glycoproteins and glycolipids, the sialic acids, which are exclusively α-linked, occupy the penultimate nonreducing end of the oligosaccharide chains. Therefore, it is not surprising to find that they play forefront roles in a number of biochemical and immunochemical events. For instance, Neu5Ac alone or Neu5Ac terminated oligosaccharides were shown to be responsible for influenza virus adhesion onto erythrocytes (7) and human nasal mucins. Bacterial adherence and colonization of intestinal tracts by *Escherichia coli* is another event caused by the binding interactions between the bacterial pili and the sialic acids of the epithelial cell walls (8). Bacterial toxins (tetanus, diphtheria) (9), animal and plant lectins (10), interferon (11), and mycoplasma pneumoniae (12) are other representative examples of such interactions, which use Neu5Ac as cell surface receptors. Moreover, the sialic acids in the glycoprotein hormone of human chorionic gonadotropin (hCG) can modulate its hormonal activity (13). On the other hand, the absence of sialic acids on glycoproteins may have beneficial effects. Thus, the uptake or sequestration of senescent cells by macrophages (14) and liver hepatocyte receptors (15) can trigger the catabolism of useless cells. Perhaps the most interesting features of the sialic acids are their roles as differentiation (16) and oncodevelopmental (17) antigens. As such, they constitute key functional entities in the design of specific oncogenic markers.

It was therefore anticipated that the synthesis of well-defined artificial neoglycoproteins, containing sialic acids as the sole carbohydrate antigen, would constitute excellent probes for studying some of the interactions mentioned above. Such tools may have useful applications in the study of lectins and antibody–carbohydrate interactions at the molecular level (10). These neoglycoproteins may also be useful for the detection and screening of endogenous lectins by immunocytochemical staining (18), which might permit cell targeted drug delivery. We have also previously mentioned that neoglycoproteins and copolymers containing Neu5Ac represented good inhibitors of the adherence of influenza A virus to chicken erythrocytes (19). This phenomenon could constitute the basis of a therapy against the common cold.

As mentioned previously (20, 21), our initial goals were to produce specific anti-sialic acid antibodies (potentially monoclonals) that might be useful for the quantitation of sialyloligosaccharides present in large quantities in the serum of cancer patients (17). Our first attempts were directed to terminal anti-Neu5Ac antibodies, irrespective of the type of linkages between the Neu5Ac and the next carbohydrate residues, usually α-(2,6) or α-(2,3)-galactose. Therefore, the present work describes the synthesis of antigenic Neu5Ac residues α-linked to two different carrier proteins, namely bovine serum albumin (BSA) and tetanus toxoid (TT). These two proteins are known for their large difference in immunogenicities in model animals. The synthesis of some analogues modified at strategic functionalities is also depicted. These sialic acid derivatives are intended to be used as inhibitors in the study of sialic acid interactions with lectins and antibodies.

Results and discussion

Preparation of the aldehyde precursors 7 and 8

For the present study, sialic acid as an α-linked glycoside of *N*-acetylneuraminic acid (Neu5Ac, **1**) was required in a form

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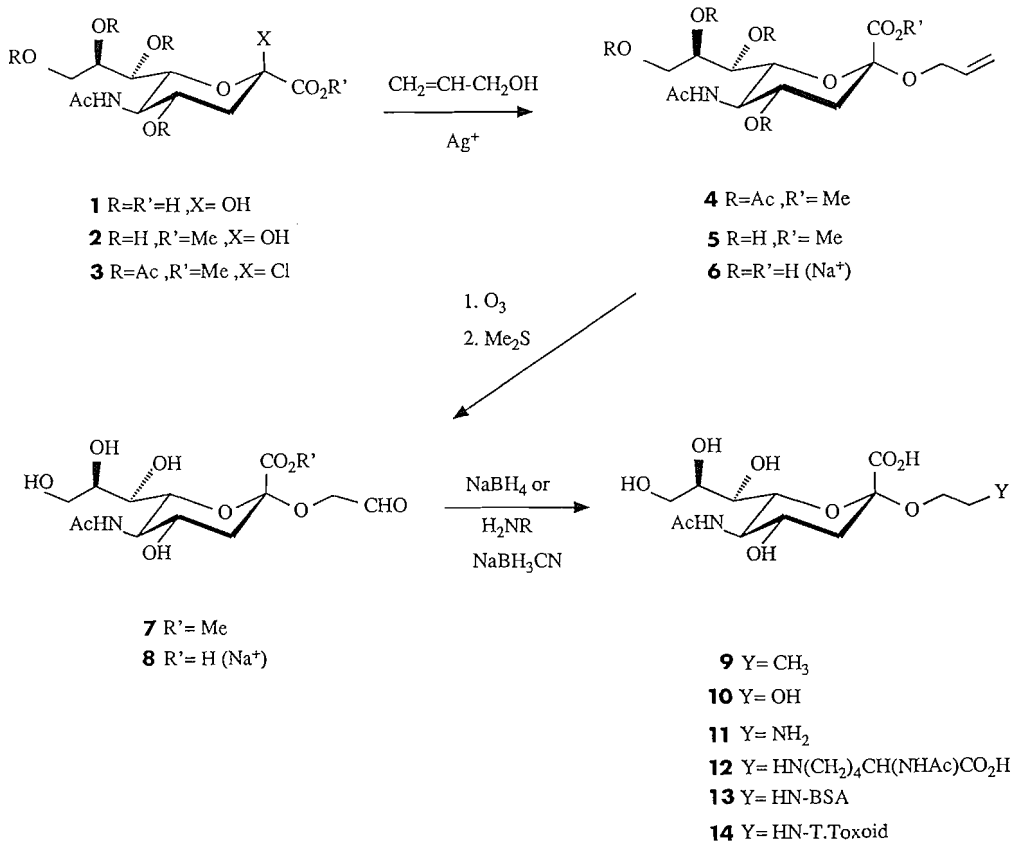


SCHEME 1

suitable for covalent attachment to the ϵ-amino groups of the lysine residues of the carrier proteins. Previous studies by us (22) and by others (23) demonstrated the usefulness of reductive amination (24) for the direct reduction of a Schiff base formed between aldehyde groups and the amine functionalities present on the proteins. Hence, 2-oxoethyl α-glycoside of Neu5Ac (**8**) was sought as an appropriate precursor for the conjugation. Large scale preparation of the starting Neu5Ac (**1**) could have been achieved as described previously using 0.025 M H_2SO_4 hydrolysis of the *Collocalia* mucins present in the bird's nest substances (5). The method suffers, however, from being tedious in the neutralization step because of the large volume of solution required. We anticipated that hydrolysis by a cationic resin (H^+) should be amenable to a straightforward isolation sequence since, after filtration, the reaction mixture could be directly loaded onto the formate anion column as originally suggested (5). After optimization of the reaction conditions, a temperature of 75°C and a hydrolysis time of 6 h with a 2:1 weight ratio of commercial bird's nest and resin Rexyn 101 (H^+) were used. The yield of pure Neu5Ac (**1**) varied from 2.5 to 5% by weight depending on the source of the bird's nest.

Having secured the supply of Neu5Ac (**1**), it was then transformed into the known (25) acetochloroneuraminic acid (**3**) via a new two-step sequence (21) (Scheme 1). The methyl ester (**2**) was prepared in methanol containing H^+ resin according to Kuhn *et al.* (25) in an almost quantitative yield. The chloride (**3**) was directly prepared by treatment of the methyl ester (**2**) with acetyl chloride in acetic acid whereby the intermediate peracetylated ester was further tranformed into the chloride (**3**) because the required HCl was generated *in situ* during the reaction conditions. This is in contrast to a recent publication (26) in which acetic acid was omitted and which failed, in our hands, to give good yield of the desired chloride (**3**). The unstable **3** thus obtained was of >95% purity (^{1}H NMR) and was usually suitable for the following transformations. The α-allyl glycoside (**4**) (21, 27, 28) was prepared using silver salicylate (29) as catalyst rather than the polymeric one originally employed (27). The Koenigs–Knorr glycosylation occurred with complete stereocontrol giving rise to the α-allyl glycoside **4** in 90% yield. The anomeric configuration of the sialic acid, for which there is no anomeric proton, was determined indirectly by empirical rules (30), which predict the proton chemical shifts of H-4 and H-7 of Neu5Ac α-glycosides to occur at 4.83 and 5.30 ppm respectively. It was also directly determined (*vide infra*) by the three-bond heteronuclear gated decoupling spectrum (31) between C-1 and H_{3ax} ($^3J_{C\text{-}1,H\text{-}3ax}$, ∠180°) which showed a coupling of 5.7 Hz in the free acid **6**. Deacetylation of **4** with sodium methoxide in methanol provided crystalline ester **5** in 93% yield. Treatment of the methyl ester **5** with 0.1 N aqueous NaOH gave the free acid **6** also in 93% yield. Reductive ozonolysis of the allyl group in **6** afforded the key aldehyde **8** in quantitative yield after lyophilization. The ozonolysis was also performed on the allyl ester **5** to provide the aldehyde ester **7** in 95% isolated yield. Evidence that the aldehyde group exists in the hydrated form in aqueous solution was seen by the ^{1}H NMR spectra of **7** and **8**, which showed the aldehydic proton at 5.12 ppm (Table 1), and by the ^{13}C NMR spectrum, which showed the carbonyl at 89.6 ppm (acetal) (Table 2).

*Synthesis of the protein conjugates **13**, **14** by reductive amination*

Direct covalent attachment of the aldehydes **7** or **8** onto bovine serum albumin (BSA) and tetanus toxoid (TT) could be

TABLE 1. ^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) of the NeuAc derivatives in D_2O at 25°C

Compound no.	H3$_{ax}$ (J_{a-e}, J_{3-4})	H3$_{eq}$ (J_{e-a}, J_{3-4})	H5 (J_{4-5}, J_{5-6})	H6 (J_{5-6}, J_{6-7})	H7 (J_{6-7}, J_{7-8})	H9 (J_{8-9}, $J_{9-9'}$)	H9′ (J_{8-9}, $J_{9-9'}$)	Methyl ester (s)	N*Ac* (s)	Aglycon: allyl, others				
										H_a	H_b	H_c	H_d	H_e
5	1.83 (12.2, 12.2)	2.72 (12.2, 4.6)	[a]	[a]	3.56 (1.7, 8.9)	[a]	3.65 (6.0, 11.9)	3.87	2.03	5.25	5.32	5.91	4.31	4.07
6	1.66 (11.8, 12.1)	2.75 (12.4, 4.5)	3.82 (9.8, 10.2)	3.71 (10.2, 1.9)	3.59 (1.7, 8.9)	3.87 (—, 9.6)	3.64 (6.3, 12.2)	—	2.03	5.23	5.32	5.94	4.24	4.01
7	1.83 (11.9, 12.6)	2.73 (12.8, 4.5)	[a]	[a]	3.55 (—, 8.9)	[a]	3.65 (6.7, 12.5)	3.88	2.03	—	—	5.12[b] (4.8)	3.47 (10.2, 4.9)	3.76 (10.1, 4.7)
8	1.70 (11.9, 12.6)	2.74 (12.8, 4.5)	3.83 (9.8, 10.3)	3.70 (9.5, 1.7)	3.58 (1.6, 8.8)	3.87 (—, 9.8)	3.63 (6.6, 12.5)	—	2.03	—	—	5.12[b] (4.9)	3.43 (10.2, 4.8)	3.68 (11.4, —)
9	1.64 (11.7, 12.3)	2.74 (12.3, 4.5)	3.82 (9.7, 9.1)	[a]	3.58 (1.6, 8.9)	[a]	[a]	—	2.03	0.88 (7.4)		1.56 (7.0)	[a]	3.37 (9.3, 6.9)
10	1.73 (11.9, 12.3)	2.72 (12.3, 4.6)	[a]	3.73 (10.5, 1.9)	3.57 (1.6, 8.9)	3.83 (1.8, 9.7)	3.63 (6.6, 12.5)	—	2.03	—	—	[a]	[a]	[a]
11	1.73 (11.9, 12.2)	2.72 (12.3, 4.5)	3.83 (9.7, 10.3)	3.70 (9.7, 2.0)	3.59 (1.8, 8.7)	3.86 (2.7, 9.8)	3.65 (5.7, —)	—	2.03	—	—	3.19 (4.8, 5.4)	[a]	3.97 (10.6, 4.9)
12	[a]	2.72 (12.3, 4.6)	[a]	3.70 (10.2, 1.7)	3.40 (1.5, 8.8)	[a]	3.65 (5.6, 12.1)	—	2.03			[c]		
15	1.63 (11.6, 13.5)	2.44 (13.5, 5.4)	[a]	[a]	[a]	[a]	[a]	—	2.03	—	—	[a]	[a]	[a]
16	1.74 (11.0, 11.2)	2.30 (13.0, 4.7)	3.85 (9.9, 10.5)	3.68 (10.5, 1.4)	3.52 (1.2, 9.3)	[a]	3.61 (6.0, 11.5)	—	2.03	5.22	5.34	5.96	4.23	
17	1.77 (11.2, 12.8)	2.75 (12.9, 3.8)	[a]	[a]	3.63 (—, 9.7)	[a]	[a]	—	2.02	5.23	5.33	5.94	4.26	4.09
18	1.70 (12.4, 12.5)	2.80 (12.5, 4.6)	3.24 (9.7, 10.3)	4.05 (10.3, 2.1)	3.77 (2.2, 11.0)	3.90 (2.5, 12.0)	3.72 (5.7, 12.0)	—	—	5.23	5.33	5.93	4.24	[a]
19	1.68 (12.2, 12.5)	2.76 (12.5, 4.5)	[a]	[a]	3.61 (—, 9.0)	3.66 (2.3, 7.0)	3.70 (6.0, 11.9)	—	3.93[d]	5.23	5.33	5.94	4.27	4.02
20	1.68	2.75	[a]	[a]	3.57	[a]	3.64	—	4.65[d] 2.18 (OAc)	[a]	[a]	[a]	[a]	[a]
21	1.68 (11.8, 13.1)	2.77 (12.4, 4.6)	[a]	[a]	3.58 (1.7, 8.9)	3.87 (1.1, 9.8)	3.64 (6.5, 12.3)	—	4.12[d]	5.22	5.33	5.94	4.25	4.01
22	1.84 (11.4, 12.5)	2.71 (13.5, 3.5)	[a]	[a]	[a]	—	—	3.87	2.03	[a]	[a]	5.92	4.33	4.08
23	1.63 (11.7, 12.4)	2.69 (12.4, 4.0)	3.69 (9.3, 9.6)	[a]	3.62 (5.9, 12.9)	3.76 (1.9, 12.9)	—	—	—	2.03	5.23	5.34	5.94	4.27	3.97

[a]H4, H8 and others not assigned due to the complexity of the spectrum.
[b]Aldehyde proton as a hydrate.
[c]See experimental section.
[d]Methylene of *N*-acyl group.

TABLE 2. ^{13}C NMR chemical shifts (ppm) of the sialic acid derivatives (H_2O, 25°C)

Compound no.	(C-1, NHAc)	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CH_3 (NHAc)	CH_3 (Me ester)	Aglycon
5	171.2, 176.1	100.0	40.6	68.3	53.0	74.1	69.5	71.8	64.3	23.3	54.6	67.0, 119.7, 134.3
6	174.6, 176.3	101.8	41.7	69.4	53.1	73.9	69.4	72.9	63.8	23.3	—	67.1, 119.4, 135.0
7	171.2, 176.1	100.5	40.8	69.9	53.4	74.5	68.8	72.2	64.8	23.8	55.1	68.4, 89.6
8	174.6, 176.3	101.7	41.3	69.4	53.1	73.8	69.4	72.9	63.9	23.3	—	68.2, 89.6
9	175.0, 176.3	101.7	41.4	69.3[a]	52.8	73.5	69.1[a]	72.7	63.5	23.3	—	10.6, 22.9, 67.7
10	173.2, 175.7	100.6	40.6	69.1	52.7	73.5	68.7	72.1	63.7	23.0	—	61.4, 66.4
11	174.1, 175.9	101.3	40.7	69.0	52.8	73.6	69.0	72.5	63.6	23.0	—	40.3, 61.1
12	174.1, 175.8	101.3	40.5	69.0	52.8	73.6	69.0	72.5	63.5	23.0	—	[b]
16	61.3, 176.4	101.7	37.4	69.0[a]	53.4	71.7	69.5[a]	72.8	64.1	23.2	—	64.2, 119.0, 135.7
17	172.3, 176.3	101.1	40.1	68.8[a]	52.8	74.6	68.3[a]	72.2	63.8	23.3	—	66.6, 119.7, 134.7
18	173.5, —	101.3	41.3	67.4	53.9	71.9	68.6	72.3	63.0	—	—	66.9, 119.0, 134.3
19	170.8, 177.2	100.7	40.7	69.5[a]	53.4	73.7	68.6[a]	72.4	63.9	29.0	—	67.0, 119.8, 134.6
20	[c]	[c]	40.9	68.7[a]	52.2	72.9	68.4[a]	72.3	63.4	63.2 (20.4)[d]	—	66.5, 119.0, 134.3
21	[c]	101.7	41.5	69.2	52.6	73.4	69.0	72.7	63.6	61.9	—	67.1, 119.4, 135.0
22	171.3, 176.3	100.1	40.1	68.0	54.4	76.6	62.2	—	—	23.1	53.0	66.8, 120.0, 134.3
23	174.6, 176.0	101.9	41.4	68.6	53.0	75.6	62.0	—	—	22.8	—	67.7, 119.4, 134.6

[a]Tentative assignments.
[b]See experimental.
[c]Signals lost into the noise.
[d]Acetate signal.

TABLE 3. Factors influencing the reductive amination of **7** or **8** onto BSA

Entry	Molar ratio[a] $CHO/NH_2/H^-$	Temperature, °C (±3°)	Conditions[b]	NeuAc/protein[c] 3 days (6 days)	Yield, %[d] 3 days (6 days)
On **8**					
1	1:1:10	37°C	P	15	25
2	5:1:25	37°C	P	22	37
3	5:1:25	50°C	P	23	39
4	10:1:25	50°C	P	21	36
5	5:1:25	37°C	P + 20% DMSO	20	34
6	10:1:100	37°C	P	18(18)	31
7	10:1:100	50°C	P	17(22)	29
8	10:1:100	37°C	P + 20% DMSO	19(21)	32
9	5:1:25	37°C	B	6	10
On **7**					
10	7:1:33	37°C	P	32(35)	54(59)
11	7:1:33	37°C	P + 25% MeOH	36(42)	61(71)
12	7:1:33	37°C	P + 25% DMSO	36(41)	61(69)
13	8:1:33	37°C	P, pH 6	33	56
14	8:1:33	37°C	P, pH 7	36(45)	61(76)
15	8:1:33	37°C	P, pH 8	38(42)	64(71)
16	8:1:33	37°C	P, pH 9	36(38)	61(64)

[a]Reaction mixtures. H^- refers to $NaBH_3CN$.
[b]P = 0.2 M phosphate buffer, pH 7 unless stated otherwise. B = 0.2 M borate buffer, pH 7.
[c]In the conjugate as determined by colorimetric methods.
[d]Based on 59 lysine residues.

achieved using sodium cyanoborohydride because this reducing agent is known to be selective for the imine functionality while being inert toward the carbonyl functions. This procedure, which was pioneered by Gray (24) on reducing sugars, was further improved by Roy *et al.* (22) using optimized conditions of buffers, pH, and temperature. To obtain protein conjugates covering the entire range of Neu5Ac incorporation, optimization of the reaction conditions was again set forth (Table 3). Using borate buffer (0.2 M, pH 9.0) as suggested by Roy *et al.* (22) on reducing sugars and BSA as model protein, the level of incorporation of **8** onto the lysine group was only marginal even after 6 days at 37°C (entry 9), as opposed to neutral sugars (21–24). Thus, it became apparent that the free aldehyde in the acid **8** did not behave like neutral free reducing sugars (22–24). Therefore, the original procedures (23, 24) using 0.2 M phosphate buffer were reinvestigated. As observed from Table 3, there was no improvement in coupling efficiency when only the molar ratio of the aldehyde to the amine was increased (entries 3 and 4) at 50°C in phosphate buffer pH 7.0. There was a slight improvement when both the aldehyde and the hydride

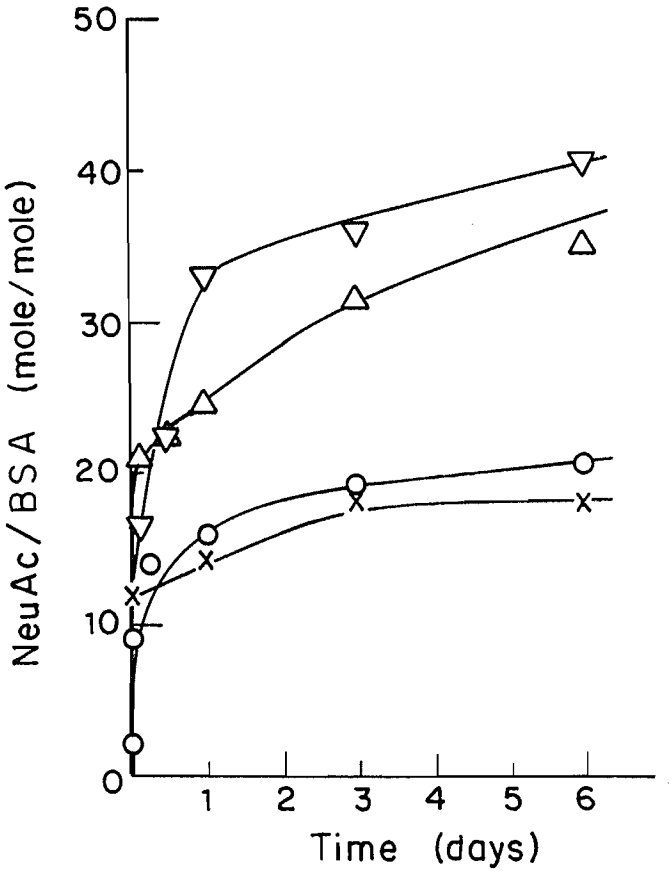


FIG. 1. Time course of reductive amination of **7** (▽,△) or **8** (○,×) into BSA. (×) entry **6** (Table 3); (○) entry **8**; (△) entry **10**; (▽) entry **12**.

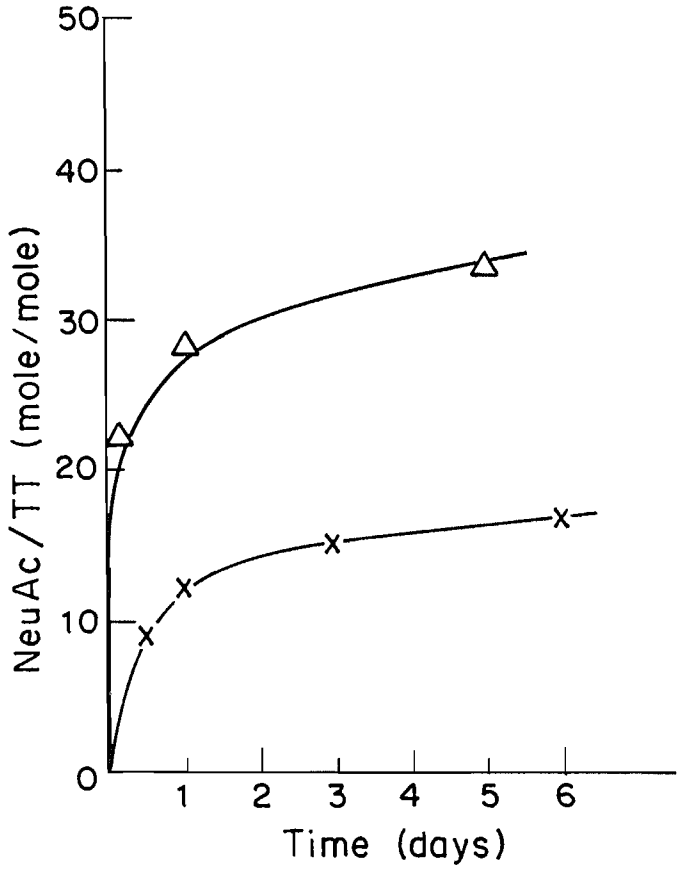


FIG. 2. Time course of reductive amination of **7** (△) or **8** (×) into TT. (×) 0.2 M phosphate buffer pH 7.0, 37°C, **8**: NH_2: $NaBH_3CN$ (10:1:30); (△) like (×) with **7** and 25% DMSO.

ratios were increased relative to that of the amine (entries 1 and 2). There was also no temperature effect observed between 37°C and 50°C in the same buffer (entries 2,3 and 6,7). A detrimental effect could be seen when the amount of hydride reagent used was too high (entries 4 and 7) at 50°C. No solvent effect could be noticed (entries 2,5 and 6,8). Thus, a maximum of approximately 23 Neu5Ac residues out of 59 lysines (32) were effectively coupled onto BSA, while 16 Neu5Ac residues out of ~66 lysines could be coupled to TT (33). The level of incorporation of Neu5Ac in all the above procedures was conveniently measured by a specific colorimetric method using resorcinol (34), while the amount of protein within the sample was determined using the Lowry method (35). Previous workers (22–24) using amino acid analysis showed the level of incorporation of the sugar content to agree within ±1–2 residues compared to the colorimetric tests.

We postulated that the restricted incorporation of the acid **8** in comparison to neutral sugars (22–24), where complete substitution was possible, could be due to the anionic nature of both the BSA protein (pI 4.9) and the aldehyde **8**, which would cause repulsive electrostatic interactions. Therefore, conjugations with the aldehyde ester **7** were initiated in the hope of minimizing these repulsive interactions (Fig. 1). As shown from Table 3 (entry 10 vs. entry 2), there was a noticeable improvement in the level of reductive amination. This was further increased (entries 11, 12 vs. entry 10) when methanol or dimethyl sulfoxide was used as cosolvent. There was almost no pH effect (between 6 and 9) in the 0.2 M phosphate buffer at 37°C, a pH of 6 being only slightly less efficient than the other three higher pH's. Under the optimum conditions (see Experimental), a conjugate having 53 Neu5Ac residues per BSA molecule was achieved with the methyl ester **7**. A similar set of experiments was effected on the tetanus toxoid; the results are illustrated in Fig. 2.

Interestingly, at the end of the reaction time (usually 3–6 days) the Neu5Ac–protein conjugates **13** or **14** obtained from the reductive amination of the ester **7** did not have any of the remaining ester functionality. This was ascertained by ^1H NMR spectroscopy, by the colorimetric hydroxamic acid test (36) for esters, by immunoelectrophoresis, and by *Vibrio cholerae* neuraminidase treatment (1) of the protein conjugates (not shown). The above enzyme was known to be inactive toward the esters of sialic acids (1) but in the present study it hydrolysed 96% of the Neu5Ac **1** in the conjugates, thus confirming the absence of the ester function within the conjugates.

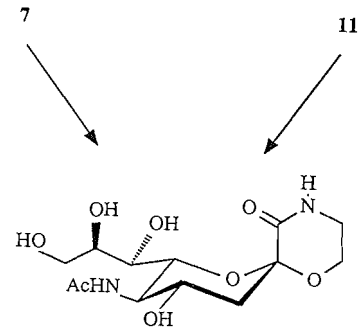
As control experiments, it was shown that the reagent $NaBH_3CN$ or BSA alone was not capable of inducing the ester hydrolysis of **5** in the phosphate buffer. It is suggested that the hydrolysis could have been catalyzed by the neighboring amine group, which was reductively alkylated in the aglycon portion, or by the intramolecular assistance of a suitably positioned hydroxyl group. The basis for this assumption was supported by the set of transformations leading to the sialic acid analogues described below. These specifically modified sialic acid derivatives were prepared in order to be utilized as potential inhibitors for the interaction studies between sialic acid and specific lectins such as wheat germ agglutinin (10) or antibodies (19) prepared against the protein conjugates **13** and **14**.

Synthesis of sialic acid analogues

The 2-propenyl glycoside ester **5** was reduced to the corresponding 2-propyl glycoside ester using hydrogen on 5% palladium–charcoal in 95% yield. The ester was then hydrolyzed to the free acid **9** by 0.1 N aqueous NaOH in 92% yield. The aldehyde **8** was converted to the 2-hydroxyethyl glycoside **10** in 67% yield using sodium borohydride. The corresponding 2-aminoethyl glycoside **11** was obtained directly from the aldehyde **8** by reductive amination in the presence of ammonium actate (51%). The $[\alpha]_D$ of **11** (−18.5°) agreed well with the one previously obtained ($[\alpha]_D$ −17°) by Holmquist and Brossmer (37), who synthesized it by glycosylation of **3** with 2-(benzyloxycarbonylamino)ethanol in the presence of silver carbonate in 14% overall yield after deprotection. When the above reductive amination was effected on the methyl ester **7** rather than the free acid **8**, a side product tentatively assigned the lactam structure **15** was produced together with **11** (Scheme 2). This is of particular interest since the reductive amination of **7** with the two proteins might have given a similar cyclic lactam during the conjugation. To establish whether or not this was the case, the amino acid **11** was treated with a carbodiimide (EDC), and the same product (**15**) resulting from the reductive amination of the ester **7** with ammonium acetate was obtained. When the same sequence of reductive amination of **7** was performed with N^{α}-acetyl-(L)-lysine, the free acid **12** was obtained as a single product; no methyl ester was present. The acid **12** proved to be the same as the one obtained from the acid **8**. Thus, the above results indicated that the formation of the lactam (**15**) is selective for the primary amine **11** and that ester hydrolysis also occurred upon reductive amination of **7** with either N^{α}-acetyl-(L)-lysine alone or with the proteins. These results confirmed the observations made above, although the mechanism by which the ester self-hydrolyzed during the reductive amination was not fully understood. As mentioned above, it is possible that the hydrolysis occurred by an intramolecular catalyzed reaction due to the proximity of the ε-amino groups. We have also mentioned previously that most of the Neu5Ac residues (>96%) could be hydrolyzed from the protein conjugates **13** and **14** by *Vibrio cholerae* neuraminidase. This could not have been the case with a cyclic amide such as **15**.

The acid group was the next functionality to be modified. This was done by reduction of the C-1 carbomethoxy group of **5** by lithium borohydride in a MeOH–THF mixture. The resulting hydroxymethyl derivative **16** (~quant.) showed the disappearance of the carbonyl band of **5** at 1725 cm^{-1} together with the C-1 ^{13}C NMR signal at 171.2 ppm. The new methylene signal was shifted to 61.3 ppm. The amide analogue **17** was obtained by ammonolysis of the ester **5** in 84% yield. These two derivatives constitute important modifications because they deprive the Neu5Ac residue of its anionic character, which is essential to most of its biochemical and immunochemical functions (1).

The other functional group manipulations were then centered on the acetamido function (Scheme 3). The amide **5** was hydrolyzed in refluxing tetramethylammonium hydroxide to afford the free amine **18** in 80% yield after purification by size exclusion chromatography. The product was ninhydrin positive on tlc. Furthermore its ^1H NMR showed the disappearance of the *N*-acetamido signal at 2.03 ppm with concomitant upfield shift of its H-5 signal at 3.24 ppm. Similarly, the ^{13}C NMR spectrum of **18** showed the displacement of the C-5 signal from 53.1 ppm in **5** or **6** to 53.9 ppm (0.8 ppm downfield). The signals for C-4 and C-6 were also affected since they shifted



15

SCHEME 2

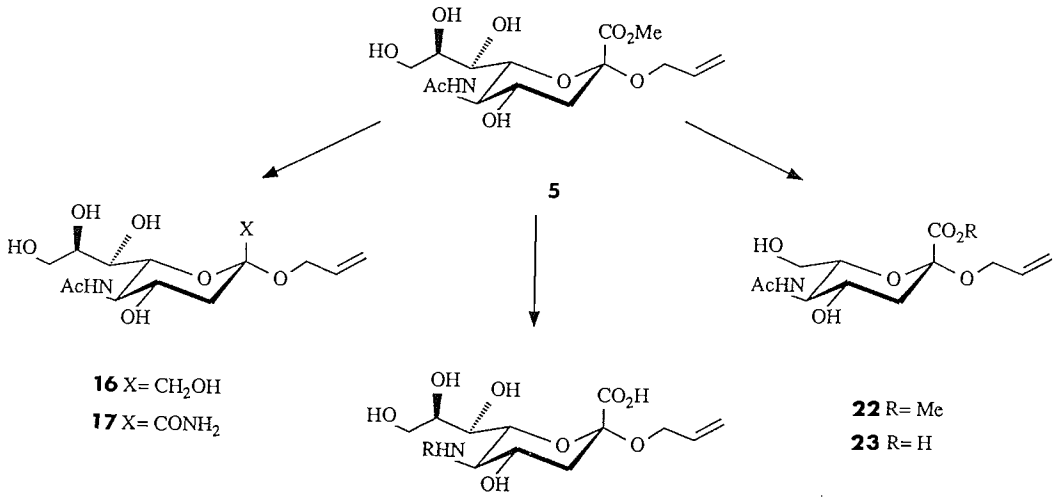
upfield by 2.0 ppm (67.4 and 71.9 ppm respectively) relative to that in **6**. These observations were previously made on a polymer of Neu5Ac (38). The new amide derivatives **19** and **21** were obtained in yields of 80 and 86% respectively upon treatment of **18** with the corresponding bromoacetyl and acetoxyacetyl chloride. The intermediate acetoxy derivative **20** needed to be hydrolyzed at the ester functionality during the isolation of **21**. It is of interest to mention that **21** corresponds to an α-allyl glycoside of the important *N*-glycolylneuraminic acid (Neu5Gc). It is also worth mentioning that the above entry to the Neu5Gc series was more convenient (38) than the previous one (1) using the unstable 1,3-dioxolan-2,4-dione as glycolylating reagent.

Finally, the last derivative of Neu5Ac synthesized was the corresponding C-7 analogue of the α-allyl glycoside. Hence, the vicinal diol array in the side chain was simultaneously cleaved and reduced using periodate and borohydride anions supported on anionic resins (39). The isolation of **22** thus appeared straightforward since only a filtration of the resin was required. The low yield obtained (59%) was attributed to the partial hydrolysis of the methyl ester under the reaction conditions that generated the acid **23** *in situ*, which then remained on the resin. In fact, further treatment of the recuperated resin with acetic acid showed the release of some **23** (tlc).

In conclusion, antigenic and immunogenic (19–21) forms of *N*-acetylneuraminic acid were synthesized having BSA or TT as protein carriers. The series of conjugates having different levels of incorporation will be used to evaluate the role of the avidity phenomena encountered in lectin– and antibody–Neu5Ac interactions. The key functional group manipulations should allow the systematic evaluation of the exact binding epitopes with rabbit anti-sialic antibodies already obtained (19).

Experimental

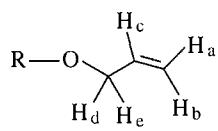
Melting points were determined on a Gallenkamp apparatus and are uncorrected. The ^1H and ^{13}C NMR spectra were recorded on a Varian XL-300 or a Varian Gemini 200 at 300 and 200 MHz respectively for protons and at 75.4 and 50.3 MHz respectively for carbons. The proton chemical shifts (δ) are given relative to internal Me_4Si (δ = 0) for $CDCl_3$ solutions and relative to acetone (δ = 2.225) for D_2O solutions. The carbon chemical shifts are given relative to dioxane (δ = 67.4) for D_2O solutions. The analyses were done as a first-order approximation. Optical rotations were measured on a Perkin Elmer 241 polarimeter and were run at room temperature (~25°C). IR spectra were taken as KBr pellets using a Perkin Elmer 783 spectrophotometer. Mass spectra were recorded on a VG 7070-E spectrometer for CI, EI, and FAB-MS. Combustion analyses were performed by Guelph Chemical Laboratories Ltd. (Ont.). Thin-layer chromatography (tlc) was performed



SCHEME 3

using silica gel 60 F-254 plates and column chromatography on silica gel 60 (230–400 mesh). The developed plates were sprayed or dipped with a solution of ceric sulfate (1%) and ammonium molybdate (2.5%) in 10% aqueous sulfuric acid and heated at ~150°C. The following solvent combinations (v/v) were used for tlc on silica gel: *n*-propanol–water, 7:3 (A) and 8:2 (B); ethyl acetate (C); chloroform–methanol 7:3 (D); acetonitrile – 10% acetic acid 8:2 (E); ethyl acetate – acetic acid – water 3:2:1 (F). Water soluble products were purified or desalted by size exclusion chromatography on Sephadex G-10. The products were monitored with a Waters R403 refractive index monitor. The *Collocalia* mucoids (edible bird's nest) were obtained from local Chinese groceries. Ozone was generated from a Welsbach instrument.

Protons of the allyl group present in some of the compounds are designated as H_a, H_b, H_c, H_d, and H_e as defined below and as previously described by Tahir and Hindsgaul (40).



They were, however, described using a lower resolution instrument and thus some coupling constants (ex. H_a–$H_{d,e}$) were not observed. These protons generally showed the same multiplicity pattern throughout and only their chemical shifts varied: H_a, dd, $J_{a,b}$ = 1.5 Hz, $J_{a,c}$ = 10.5 Hz; H_b, dd, $J_{a,b}$ = 1.5 Hz, $J_{b,c}$ = 17.0 Hz; H_c, m; H_d and H_e ddd, $J_{d,e}$ = 12.5 Hz, $J_{c,d}$ = $J_{c,e}$ = 5.5 Hz.

*5-Acetamido-3,5-dideoxy-D-*glycero-*D-*galacto-*2-nonulopyranosonic acid* **1** *(Neu5Ac)*

Water (1.6 L) was added to edible bird's nest (200 g) and the resulting suspension was blended for ~5 min in a Waring blender. Rexyn 101 cation exchange resin (100 g) in the acid form was then added to the mixture. The reaction vessel was immersed in a thermostated water bath at 75°C and the content was mechanically stirred. After 6 h, the mixture was cooled to room temperature and filtered over gauze bandage in a large Büchner funnel. The resulting solution was directly loaded onto a Dowex 1X8 (20–50 mesh) column (3.5 × 40 cm) in the formate form. The column was washed with 2 L water followed by a linear gradient of formic acid (0–2 M). Fractions of 10 mL were collected and aliquots (100 μL) testing positive for sialic acid by the resorcinol method (34) were pooled and freeze-dried. The yields of pure **1** varied from 5 to 10 g (2.5–5% w/w) depending on the brand of bird's nest used. The solid obtained had mp 177–183°C (dec.); $[\alpha]_D$ −30.8° (*c* 1, H_2O) (lit. (5) mp 184–186°C; $[\alpha]_D$ −32.1°); R_f 0.47 (A). Negative FAB-MS for $C_{11}H_{18}NO_9$: 308 (M − 1). Both ^1H and ^{13}C NMR spectral data are in complete agreement with literature values (1, 5).

*Methyl 5-acetamido-3,5-dideoxy-D-*glycero-*D-*galacto-*2-nonulopyranosonate* **2** *(Neu5Ac methyl ester)*

The methyl ester **2** was prepared following the procedure of Kuhn *et al.* (25). Briefly, a solution of **1** (2.99 g, 9.7 mmol) in 50 mL methanol containing Amberlite IR-120 (H^+) (2.0 g) was stirred overnight at room temperature. The clear solution was filtered on a filter paper and evaporated to a volume of ~5 mL. Ether was added to turbidity and **2** crystallized on standing. It was recuperated by filtration (3.18 g, 9.7 mmol, ~quant.); mp 193.5–194.7°C; $[\alpha]_D$ −28° (*c* 3.5, H_2O) (lit. (25) mp 179–180°C; $[\alpha]_D$ −28°); R_f 0.55 (A). ^1H and ^{13}C NMR spectra were identical to those published (1).

*Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero-*β-D-*galacto-*2-nonulopyranosylchloride)onate* **3** *(acetochloro-neuraminic acid)*

Compound **2** (0.47 g, 1.5 mmol) was added to freshly distilled acetyl chloride (10 mL, some preparation required preactivation with 1.5 mL MeOH) containing acetic acid (5 mL) and cooled to 0°C. The flask was sealed and kept 24–48 h at room temperature. The extent of the reaction was followed by tlc in ethyl acetate. When the transformation was deemed complete, the reaction mixture was evaporated to dryness and coevaporated a few times with dry toluene. The chloride **2** was obtained as a foam (0.75 g, 1.5 mmol, ~quant.); $[\alpha]_D$ −61° (*c* 1, $CHCl_3$) (lit. (25) $[\alpha]_D$ −63°); R_f 0.32 (C). Its ^1H NMR showed it to be >95% pure in both $CDCl_3$ (41) and C_6D_6 (42), and it was used directly for the next step.

*Methyl (allyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosid)onate* **4**

The chloride **3** (0.75 g, 1.4 mmol) was dissolved in dry (4A molecular sieves) allyl alcohol (5 mL) containing freshly prepared silver salicylate (0.35 g) and 4A molecular sieves (0.5 g). The reaction was stirred for 2 h at room temperature in the dark. The slurry was filtered over Celite and washed with CH_2Cl_2. The resulting clear solution was evaporated to dryness and the residue was dissolved in CH_2Cl_2 (25 mL). The organic solution was successively washed with cold 5% aqueous sodium bicarbonate, 5% aqueous sodium thiosulfate, and finally with water. The organic layer was dried over sodium sulfate

and evaporated to a clear oil that was homogeneous by tlc (90% yield). An analytical sample was obtained by crystallization from methanol–ether (0.59 g, 1.1 mmol, 73%). The glycoside **4** has mp 153.6–157.7°C; $[\alpha]_D$ −14.2° (*c* 1, $CHCl_3$) (lit. (27) mp 154–156°C, $[\alpha]_D$ −13° (MeOH)); R_f 0.30 (C); ^1H NMR ($CDCl_3$) δ: 5.83 (4 × d, 1H, $J_{b,c}$ = 17.0, $J_{a,c}$ = 10.5, $J_{c,d} \approx J_{c,e} \approx$ 5 Hz, H_c), 5.35 (m, 1H, H8), 5.30 (dd, 1H, $J_{7,8}$ = 8.5, $J_{6,7}$ = 1.8 Hz, H7), 5.26 (dd, 1H, $J_{b,c}$ = 17.0, $J_{a,b} \approx J_{b,d} \approx J_{b,e} \approx$ 1.5 Hz, H_b), 5.14 (d, 1H, $J_{5,NH} \approx$ 10 Hz, NH), 5.13 (dd, 1H, $J_{a,c}$ = 10.5, $J_{a,b} \approx J_{a,d} \approx J_{a,e} \approx$ 1.5 Hz, H_a), 4.83 (m, 1H, H4), 4.27 (dd, 1H, $J_{9,9'}$ = 12.5, $J_{8,9}$ = 2.7 Hz, H9), 4.27 (m, 1H, H5 or H6), 4.03–4.11 (m, 3H, H_d, H_e, H5 or H6), 3.84 (dd, 1H, $J_{9,9'}$ = 12.5, $J_{8,9'}$ = 5.8 Hz, H9′), 3.76 (s, 3H, Me-ester), 2.59 (dd, 1H, $J_{3ax,3eq}$ = 12.0, $J_{3eq,4}$ = 4.8 Hz, H_{3eq}), 1.86, 2.00, 2.02, 2.11, 2.13 (5 × s, 15H, N, O-Ac), 1.96 (dd, 1H, $J_{3ax,3eq} \approx J_{3ax,4} \approx$ 12 Hz, H_{3ax}); ^{13}C NMR ($CDCl_3$) δ: 171.2, 170.9, 170.4, 170.3 (N, O-Ac), 168.5 (CO_2Me), 133.6, 117.4 (allyl), 98.5 (C-2), 72.5 (C-6), 69.0 (C-8), 68.5 (C-7), 67.3 (CH_2-allyl), 66.0 (C-4), 62.3 (C-9), 49.3 (C-5), 38.0 (C-3), 23.1 (N-Ac), 21.0, 20.7 (2×), 20.61 (O-Ac). Anal. calcd. for $C_{23}H_{33}NO_{13}$: C 51.97, H 6.26, N 2.64; found: C 52.22, H 6.46, N 2.82.

*Methyl (allyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosid)onate* ***5***

Compound **4** (1.00 g, 1.9 mmol) was de-*O*-acetylated in dry methanol (20 mL) containing a few drops of 1 M NaOMe. The reaction was complete after 2 h at room temperature. The solution was neutralized with Amberlite 120 (H^+) resin, filtered, and evaporated to ~5 mL. Compound **5** crystallized upon addition of ether to afford 0.64 g of pure material (93%) having mp 143–144°C; $[\alpha]_D$ −10.1° (*c* 1, MeOH); R_f 0.49 (D); ν_{max}: 3380, 2944, 1750, 1650, 1570, 1440 cm^{-1}; ^1H and ^{13}C NMR, see Tables; pos. FAB-MS for $C_{15}H_{25}NO_9$: 364 (M + 1). Anal. calcd. for $C_{15}H_{25}NO_9$: C 49.58, H 6.94, N 3.86; found: C 49.47, H 7.10, N 3.98.

*Allyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosidonic acid* ***6*** *(Neu5Ac allyl glycoside)*

The ester **5** (0.28 g, 0.77 mmol) was treated for <1 h at room temperature with 0.1 M aqueous NaOH (5 mL). The reaction mixture was cooled to 0°C and neutralized with Rexyn 101 (H^+) resin (pH reached 2). After the solution was filtered, the pH was quickly readjusted to 7 with 0.1 M NaOH and then the solution was lyophilized to yield **6** as a white powder (0.25 g, 0.72 mmol, 93%); mp 245–250°C (dec.); $[\alpha]_D$ −9.1° (*c* 0.66, H_2O); R_f 0.57 (B); neg. FAB-MS for $C_{14}H_{23}NO_9$: 348 (M − 1).

*Methyl (2-oxoethyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosid)onate* ***7***

The allyl ester **5** (68 mg, 0.19 mmol) was dissolved in methanol (15 mL). The solution was cooled to −78°C, purged with oxygen for 5 min, and treated with ozone by bubbling till the solution remained blue. After 10 min, the ozone was evacuated with nitrogen for 15 min and, while at −78°C, dimethyl sulfide (0.5 mL) was added. The resulting solution was allowed to warm to room temperature over a 1 h period. The clear solution was then evaporated to give **7** as a homogeneous oil (66 mg, 0.18 mmol, 95%). The product was freed of its DMSO by-product by desalting on a G-10 column using water as eluent; $[\alpha]_D$ −10.3° (*c* 1, H_2O); R_f 0.35 (E), 0.61 (B); pos. FAB-MS for $C_{14}H_{23}NO_{10}$: 366 (M + 1). Anal. calcd.: C 46.07, H 6.35, N 3.83; found: C 45.65, H 6.39, N 3.79.

*2-Oxoethyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosidonic acid* ***8***

The above procedure was repeated on the sodium salt of **6** (1.0 g, 2.70 mmol) in methanol (50 mL). The yield of **8** was 1.0 g (2.70 mmol, quant.); $[\alpha]_D$ −1.8° (*c* 1, H_2O); R_f 0.27 (E); ν_{max}: 3390, 2940, 1620, 1560, 1060 cm^{-1}; neg. FAB-MS for $C_{13}H_{20}NO_{10}$: 350 (M − 1).

*Propyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosidonic acid* ***9***

The allyl ester **5** (26 mg, 0.072 mmol) was dissolved in methanol (10 mL) containing 5% Pd on charcoal (25 mg). Hydrogen gas was bubbled through the solution for 1 h. The reaction mixture was centrifuged to remove the catalyst and evaporated to dryness. Crystals of the methyl ester of **9** formed upon standing in the cold (25 mg, 0.068 mmol, 95%); mp 145.5°C; $[\alpha]_D$ −8.9° (*c* 0.7, H_2O); R_f 0.52 (E); ν_{max}: 3390, 1725, 1650, 1560, 1440, 1040 cm^{-1}. Anal. calcd. for $C_{15}H_{27}NO_9 \cdot H_2O$: C 46.99, H 7.10; found: C 46.56, H 6.93.

The above ester (10 mg) was dissolved in 0.1 N NaOH (3 mL) and the hydrolysis was allowed to proceed for 30 min at room temperature. The reaction mixture was cooled to 0°C, neutralized with H^+ resin, and filtered. The pH of the filtrate was readjusted to 7.0 with 0.1 N NaOH and the solution was lyophilized to give **9** as a homogeneous white powder (9 mg, 92%); $[\alpha]_D$ −4.1° (*c* 0.37, H_2O); pos. FAB-MS for $C_{14}H_{25}NO_9$: 352 (M + 1), 374 (M + Na).

*2-Hydroxyethyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosidonic acid* ***10***

The aldehyde **8** (27 mg, 0.077 mmol) was dissolved in methanol (2 mL). Sodium borohydride (10 mg) was then added. After 2 h, when tlc indicated the complete disappearance of **8**, a few drops of acetic acid were added to neutralize the excess borohydride. The reaction mixture was evaporated to dryness. The residue was dissolved in water and desalted on a column of Sephadex G-10 using water as eluant. The combined fractions in the void volume were pooled and lyophilized to give **10** as a white powder (18 mg, 0.051 mmol, 67%); $[\alpha]_D$ −7.6° (*c* 0.3, H_2O); R_f 0.39 (B); neg. FAB-MS for $C_{13}H_{23}NO_{10}$: 352 (M − 1).

*2-Aminoethyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosidonic acid* ***11***

Compound **8** (18 mg, 0.051 mmol) was reductively aminated in methanol (2 mL) saturated with ammonium acetate. Sodium cyanoborohydride (10 mg) was added and the solution was refluxed. After 24 h, the cooled reaction mixture was evaporated several times with methanol and finally dissolved in water (1 mL). The aqueous solution was desalted on a Sephadex G-10 column using water as eluant. The combined required fractions were freeze-dried to yield **11** (9.2 mg, 0.026 mmol, 51%); $[\alpha]_D$ −18.5° (*c* 1, H_2O) (lit. (37) $[\alpha]_D$ −17°); R_f 0.17 (B); neg. FAB-MS for $C_{13}H_{25}N_2O_9$: 352 (M − 1).

*2-[N$^\epsilon$-(N$^\alpha$-Acetyl-(L)-lysyl)]ethyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosidonic acid* ***12***

The aldehyde **8** (34 mg, 0.097 mmol) was dissolved in 0.2 M phosphate buffer pH 7.0 (1 mL) containing N^α-acetyl-(L)-lysine (30 mg, 0.15 mmol, Sigma). Sodium cyanoborohydride (13 mg, 0.21 mmol) was added to the reaction mixture, which was stirred at room temperature for 48 h. Acetic acid (0.25 mL) was added, and after 3 h the solution was directly loaded on a Sephadex G-10 desalting column. The desired product **12** was eluted with 30 mM aqueous ammonium bicarbonate to yield 40 mg (0.076 mmol, 78%) of a white powder after lyophilization; $[\alpha]_D$ −6.5° (*c* 0.9, H_2O); R_f 0.31 (F); ^1H NMR (D_2O) δ (aglycon): 4.16 (dd, 1H, $J_{\alpha,\beta}$ = 8.2 and 4.9 Hz, H_α), 3.87 (m, 1H, OCH_2CH_2N), 3.25 (t, 2H, *J* = 4.7 Hz, OCH_2-CH_2N), 3.08 (t, 2H, *J* = 7.8 Hz, H_ϵ), 2.04 (s, 3H, N^α-Ac), 1.69–1.82 (m, 5H, H_{3ax} + $4H_{\beta,\gamma}$), 1.42 (q, 2H, H_γ); ^{13}C NMR (D_2O) δ (aglycon): 22.8, 23.2, 26.0, 31.8, 47.9, 48.1, 55.5, 60.0, 174.4, 179.2; FAB-MS for $C_{21}H_{37}N_3O_{13}$: 522 (M − 1). Anal. calcd. for $C_{21}H_{37}N_3O_{13} \cdot NH_4HCO_3$: C 43.91, H 7.03, N 9.30; found: C 43.30, H 7.53, N 9.07.

Conjugation of ***7*** *or* ***8*** *onto BSA and tetanus toxoid* ***13***, ***14***

The aldehydo acid **8** or ester **7** added in various proportions (see Table 3) was dissolved in 0.2 M phosphate buffer of the pH indicated in the table but generally being at pH 7.0 (2 mL). The protein bovine serum albumin or tetanus toxoid (10 mg) was added to the buffer solution and stirred for 1 h at 37°C. Cyanoborohydride was then added. Aliquots were taken at appropriate time intervals and dialyzed exhaustively against running water for 24 h, then with distilled water for 48 h. The sample was then diluted to a fixed volume and the sialic acid content was measured by the resorcinol method (34). The protein content was measured by the Lowry procedure (35). The samples were lyophilized and weighed. The conjugates were characterized

by SDS-PAGE and by serological assays (double immunodiffusion, ELISA, quantitative precipitation, and immunoelectrophoresis) (to be published).

Typical procedure for a conjugate Neu5Ac$_{53}$/BSA

Bovine serum albumin (BSA, Pentex, Miles Laboratories) (11 mg, 0.16 μmol) was dissolved in 0.2 M phosphate buffer pH 7.0 (2 mL). The ester **7** (22 mg, 60 μmol) dissolved in the same buffer (0.3 mL) was added to the protein solution, followed by methanol (1 mL). The solution was stirred for 20 min at 37°C. Then $NaBH_3CN$ (20 mg, 317 μmol) was added to the reaction mixture, which was stirred for 4 days at 37°C. The solution of the conjugate was transferred to a dialysis bag (10 kDa MW cutoff) and dialyzed for 3 days against distilled water. The solution was diluted to 10 mL and three aliquots of 250 μL each were tested for sialic acid by the resorcinol method as above. The solution was lyophilized, giving a white powder (14 mg) containing an average of 53 Neu5Ac residues per mole of BSA.

*Bicyclic lactam **15***

Method A

The 2-aminoethyl glycoside **11** (7 mg, 0.020 mmol) was dissolved in MeOH (3 mL) containing EDC (3 mg). After 24 h at room temperature, an additional portion of EDC was added (3 mg) and the solvent evaporated to dryness after 24 h. The lactam **15** was purified by silica gel chromatography using a gradient of ethanol and methanol as eluant (2 mg).

Method B

The aldehydo ester **7** (4 mg) was dissolved in MeOH (1 mL) containing ammonium acetate (20 mg). After 1 h at room temperature, sodium cyanoborohydride was added (2 mg). The reaction mixture was evaporated to dryness after 4 h. The residue was dissolved in 0.03 M ammonium bicarbonate (~1 mL) and desalted on a Sephadex G-10 column using the same solvent as eluant. The pooled fractions containing **15** were freeze-dried to yield 3 mg of a mixture of **11** and **15** as white powder. Compound **15** isolated from method A had ν_{max}: 3420, 1650, 1560, 1350, 1040 cm^{-1}; ^{1}H NMR (D_2O) δ: 1.63 (dd, 1H, $J_{3ax,3eq} \approx J_{3ax,4} \approx 12$ Hz, H-3ax), 2.03 (s, 3H, NAc), 2.44 (dd, 1H, $J_{3ax,3eq} = 12$, $J_{3eq,4} = 5$ Hz, H-3eq), 3.32–4.50 (m, 11H, remaining); ^{13}C NMR (D_2O) δ: 97.5, 74.2, 71.5, 69.6, 69.0, 64.7, 56.1, 53.4, and 23.3 (signals lacking because signal-to-noise ratio was too high for 3 mg sample); pos. FAB-MS for $C_{13}H_{22}N_2O_8$: 335 (M + 1).

*Allyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranoside **16***

The ester **5** (21 mg, 0.058 mmol) was dissolved in methanol (0.5 mL) to which was added THF (5 mL). $LiBH_4$ (1 mg) was added to the solution and the reduction was allowed to proceed for 1 h at room temperature. The reaction mixture was neutralized with resin (H^+) and evaporated after filtration. The remaining boric acid was eliminated as trimethyl borate after repeated coevaporation of the residue from methanol. The reduced product **16**, which resisted crystallization, was obtained in quantitative yield (19 mg, 0.058 mmol); $[\alpha]_D$ −15.4° (*c* 1.8, H_2O); R_f 0.50 (E); ν_{max}: 3400, 1640, 1560, 1050 cm^{-1}; pos. FAB-MS for $C_{14}H_{25}NO_8$: 336 (M + 1).

*Allyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonamide **17***

The ester **5** (40 mg, 0.11 mmol) was dissolved in methanol (5 mL). Methanol (5 mL) presaturated with NH_3 gas was then added to the ester solution. The reaction was allowed to proceed for 1 h at room temperature. The solution was concentrated on the Büchi evaporator and **17** crystallized upon standing. The crystals were recuperated by filtration to give **17** in 84% yield (32 mg, 0.092 mmol); mp 82–87°C; $[\alpha]_D$ −3.6° (*c* 2.0, H_2O); R_f 0.63 (E); ν_{max}: 3400, 1690, 1600, 1385, 1040 cm^{-1}; pos. FAB-MS for $C_{14}H_{24}N_2O_8$: 349 (M + 1).

*Allyl 5-amino-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **18***

The acetamido ester **5** (95 mg, 0.26 mmol) was dissolved in water (1 mL). To the solution was added 20% tetramethylammonium hydroxide (1 mL) (20% w/w in MeOH, Aldrich). The reaction was refluxed and monitored by tlc, which indicated the transformation to be complete after 5 h. The reaction mixture was neutralized with acetic acid and evaporated to dryness. The remaining residue was dissolved in water (1 mL) and desalted on a Sephadex G-10 column (3.0 × 60 cm) by elution with water. The positive fractions (resorcinol and ninhydrin) were pooled and lyophilized to afford **18** (64 mg, 0.21 mmol) as a white powder in 80% yield; $[\alpha]_D$ −18.0° (*c* 0.9, H_2O); R_f 0.26 (E); ν_{max}: 3400, 1610, 1400, 1130 cm^{-1}; FAB-MS for $C_{12}H_{21}NO_8$: 308 (M + 1) and 306 (M − 1).

*Allyl 5-bromoacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **19***

The amine derivative **18** (67 mg, 0.22 mmol) was dissolved in methanol (2 mL) containing triethylamine (0.5 mL). The solution was cooled to −70°C and a few drops of bromoacetyl chloride were added. After disappearance of starting material by tlc (molybdate and ninhydrin), the solvents were evaporated to dryness. The residue was dissolved in water and desalted on a Sephadex G-10 column as before. The pooled fractions of **19** were freeze-dried to give a white powder (75 mg, 0.18 mmol, 80%); $[\alpha]_D$ −8.0° (*c* 0.1, H_2O); R_f 0.53 (E); neg. FAB-MS for $C_{14}H_{22}BrNO_9$: 426 and 428 (M − 1).

*Allyl 3,5-dideoxy-5-hydroxyacetamido-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **21***

Compound **18** (34 mg, 0.11 mmol) was dissolved in methanol (2 mL) containing triethylamine (0.5 mL) and the solution was cooled to −70°C. Acetoxyacetyl chloride (50 μL, Aldrich) was added dropwise. The reaction was judged instantaneous by tlc, whereby **20** could be isolated or could be allowed to proceed for an additional hour to ensure complete de-*O*-acetylation of the intermediate **20** (0.1 N NaOH may be added to accelerate the hydrolysis). The reaction mixture was evaporated to dryness and treated as above for **19**. The yield of **21** obtained as a white powder after lyophilization was 86% (39 mg, 0.095 mmol); mp 245°C (dec.); $[\alpha]_D$ −3.8° (*c* 0.4, H_2O); R_f 0.48 (d); ν_{max}: 3430, 1600, 1410, 1080 cm^{-1}; FAB-MS for $C_{14}H_{23}NO_{10}$: 366 (M + 1) and 364 (M − 1) respectively.

*Allyl 5-acetamido-3,5-dideoxy-α-D-*galacto*-2-heptulopyranosidonic acid **23***

Sodium borohydride (5 g) was dissolved in water (20 mL) and mixed with Amberlite IRA 400 anion exchange resin (10 g) (Cl^- form). After 1 h, the resin was decanted and fresh sodium borohydride in water was again added. After 10 min, the resin was filtered and rinsed with water and allowed to dry at room temperature. Similarly, a fresh portion of the same resin (5 g, Cl^- form) was treated with three 30-mL portions of 0.5 M aqueous sodium metaperiodate over several hours. The resin was finally rinsed with water and allowed to dry at room temperature.

The methyl ester **5** (20 mg, 0.055 mmol) was dissolved in dry methanol (2 mL) to which was added the above resin (0.5 g each, of the IO_4^- and BH_4^- forms). After stirring for 3 h at room temperature, the solution was filtered and evaporated to give **22** (9.8 mg, 0.032 mmol, 59%). A sample of pure **22** was obtained after freeze-drying; mp 67.5–72.4°C; $[\alpha]_D$ +2.0° (*c* 0.5, H_2O); R_f 0.72 (D); ν_{max}: 3390, 1725, 1650, 1560, 1440, 1040 cm^{-1}; pos. FAB-MS for $C_{13}H_{21}NO_7$: 304 (M + 1).

A sample of the ester **22** was treated with 0.1 N NaOH as previously for **5** to afford a quantitative yield of **23** stored in the sodium form; $[\alpha]_D$ −21.3° (*c* 0.5, H_2O); R_f 0.31 (E).

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