Synthesis and Enzymatic and NMR Studies of Novel Sialoside Probes: Unprecedented, Selective Neuraminidase Hydrolysis of and Inhibition by C-6-(Methyl)-Gal Sialosides

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Abstract: We report here the synthesis of sialoside analogs, namely, αDNeuAc(2-6)(6-Me,*R*)βDGal-OR^1 (*R* isomer, tg rotamer analog) and αDNeuAc(2-6)(6-Me,*S*)βDGal-OR^1 (*S* isomer, gt rotamer analog, R^1 = $CH_2CH_2SiMe_3$ or H) and the corresponding sulfur linked thiosialosides useful for the determination of carbohydrate structural requirements in neuraminidase hydrolysis and for the design of neuraminidase inhibitors. The purpose of methyl substitution at C-6 of the galactose in these analogs is (a) to render the rotation around C6–C5 bond of the galactose more rigid, (b) to maintain the C-6–O-6 arm of the galactose predominantly in "tg" or "gt" rotamer orientation, and (c) to evaluate the importance of these two rotamer orientations in neuraminidase catalyzed hydrolysis. Compared to the natural disaccharide αDNeuAc(2-6)βDGal-OR, the gt rotamer analogs are very poorly hydrolyzed by neuraminidases from the influenza A virus, *Arthrobacter ureafaciens* (A.U.), *Vibrio cholerae* (V.C.), and *Clostridium perfringens* (C.P.). In contrast, the tg rotamer analogs are hydrolyzed by all four neuraminidases at comparable rates relative to the natural disaccharide. Detailed enzyme kinetic analysis indicates that the gt rotamer analogs bind less efficiently to the neuraminidases and have 4- to 18-fold smaller V_{max}, as compared to the tg rotamer analogs. Evaluation of the sulfur analogs as neuraminidase inhibitors indicates that only a "tg rotamer" thiosialoside analog is a good competitive inhibitor of the four neuraminidases. The inhibition constant K_i ranges from 0.3 to 1 mM. Neither the natural thiosialoside analog nor the gt thiosialoside analogs are effective inhibitors ($K_i > 5$ mM). Detailed NMR investigations of these sialosides show that in tg rotamer analogs there is a preferential anti orientation of the sialoside aglycon as compared to the natural or the "gt sialosides". Computer assisted docking of these analogs into the binding pocket of the influenza A neuraminidase–sialic acid crystal structure shows that the tg rotamer analog fits favorably into the neuraminidase binding pocket, whereas the natural isomer in the gt rotamer orientation or the gt rotamer analog encounters severe respulsive interactions with the arginine residues at the catalytic site. The perturbation of these important arginine residues appear to be responsible for the lack of neuraminidase catalyzed hydrolysis or inhibition by the gt rotamer analogs. These findings may have important implications in the rational design of neuraminidase inhibitors.

Introduction

Viral and bacterial neuraminidases play key roles in the infectious processes and the design of inhibitors for these is of therapeutic interest.[1,2] Many 2,3-dehydrosialic acid derivatives,[2-4] thiosialosides,[5,6] C-linked sialosides,[7,8] and 6-amino-6-deoxysialic acids[9] have been made either as inhibitors or as a nonhydrolyzable substrates of neuraminidases. Of these, the *N*-acetyl-4-guanidino-2,3-dehydro-D-neuraminic acid derivative described by von Itzstein *et al.*[2] is noteworthy, as it inhibits the influenza neuraminidase activity at the low nanomolar range. These dehydrosialic acid derivatives in spite of being potent neuraminidase inhibitors have very little effect on the influenza virus hemagglutinin activity,[3,10] the key protein involved in the attachment of influenza virus to target cells. This is due to the strict requirement of an α-ketosidically linked sialoside ligand in a chair conformation[11-13] for the hemagglutinin binding. Our interest concerns the design of α-linked sialoside-based common inhibitors for both hemagglutinin and neuraminidase of the influenza virus where the sialosides are stable to neuraminidase hydrolysis and, if possible, also inhibit its action. The hemagglutinin and the neuraminidase of the influenza virus are two envelope glycoproteins present on the outer membranes of the virus.[14] These two proteins bind to the αDNeuAc(2-6/3)βDGal structures of the glycoproteins or glycolipids present on the host cell membranes. Hemagglutinin–sialic acid interaction facilitates the virus to establish a foothold on the surface of host cells,[15] whereas at high pathogen concentration, neuraminidase-sialic acid interaction facilitates the virus to elute from the host cell

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and enables its movement across the mucous layers.[16] The hemagglutinin–carbohydrate recognition is a very complex event involving multisite attachment between the protein and the sialoside.[17,18] The design of multivalent sialoside inhibitors for this protein has been described in several recent reports.[19-23]

Recently we disclosed[21,24] that synthetic molecules bearing two sialic acid resides bound to the hemagglutinin more efficiently than monovalent sialosides, and this suggested their potential use as viral inhibitors. Since these molecules are subject to the hydrolytic action of neuraminidase present on the viral envelope, we sought to selectively modify the aglycon attached to the sialic acid in such a way that it would not only be resistant to the neuraminidase but also would inhibit its action. Many thiosialoside analogs known to date are poor inhibitors of influenza neuraminidases. Therefore, we sought to determine the key carbohydrate structural features needed for neuraminidase inhibition. Since the sialosides of interest in the influenza inhibition studies were αDNeuAc(2-6)βDGal derivatives, we wanted to assess the importance of gt and tg rotamers of the aglycon galactose in neuraminidase recognition and to evaluate whether a conformationally biased sialoside analog may be bound better by the enzyme. Lemieux and co-workers have earlier shown[25-27] that such a conformational bias toward the gt or tg rotamers around the C-5–C-6 of a galactose could be accomplished by a methyl substitution at the C-6 of the hexopyranose. Such models were subsequently refined by Hindsgaul and co-workers when examining the *N*-acetylglucosaminetransferase specificity toward mannoligosaccharides.[28,29] In this report, we describe the preparation of novel C-6 C-methylated αDNeuAc(2-6)βDGal sialosides and their sulfur analogs and the evaluation of their hydrolytic susceptibility or inhibitory potencies toward the influenza A neuraminidase. The purpose of C-6 methylation at the galactose is to maintain the C-5–O-6 arm of the galactose in tg and gt rotamer orientation and to investigate its significance in the neuraminidase hydrolysis or inhibition. We have extended these investigations to include three bacterial neuraminidases in an effort to elucidate the common structural features required for the neuraminidase hydrolysis of, or inhibition by αDNeuAc-(2-6)βDGal sialoside analogs. Finally, we have carried out combined molecular modeling and NMR studies to establish the three dimensional structures of these analogs in solution and to fit these structures into the influenza A neuraminidase binding site.[30,31]

Results

Sialosides such as αDNeuAc(2-6)βDGal-OR exist in solution as an equilibrium mixture of predominantly gt and minor tg and "gg" rotamers as indicated in Figure 1 (gg rotamer is not shown). As representatives of the gt and tg rotameric populations [At this time, we could not prepare an analog that would be a representative of the gg rotamer population. Lemieux and co-workers have shown[27] that the C5–C6 bond in 6,6-C-dimethylgalactopyanose derivatives exists predominantly in the gg rotamer orientation, even though the extent of such rotamer population could not be assessed with certainty. Therefore, sialylation of such derivatives may provide a model sialoside representing the gg rotamer population of αDNeuAc(2-6)βDGal structures. However, the C-6 hydroxyl groups in such galactopyranosides have been shown to be very unreactive even toward acylations, and the preparations of sialosides of such poorly reactive hydroxyls may be a very difficult synthetic challenge. We hope to address this in the future.] and for detailed enzymatic evaluations, we set as synthetic targets the oxygen analogs **16a–d**, **19a,b**, and the thio analogs **22a** and **22b**. The natural sialoside **23a**, **23b**, and its thio analog (**23c**) were also prepared for comparison of their enzymatic behavior relative to these analogs.

Synthesis of *O*-Sialoside Analogs. The list of intermediates involved in the preparation of sialosides **16a**, **16b**, **16c**, **19a**, and **19b** are indicated in Schemes 1–3 starting from 1,2:3,4-di-*O*-isopropylidene-1,6-dialdohexopyranose (**1**).[32] As reported by Lemieux and co-workers,[25,26] reaction of **1** with methyl magnesium iodide gave a 1:3 mixture of **2a** and **2b**. The stereoselectivity of **2a** to **2b** was about 1:1 with methyl magnesium bromide and 2.5:1 with methyl magnesium chloride. These two alcohols, which had identical mobility on silica gel, were separated after derivatization to their *O*-allyl ethers **3a** and **3b** or *O*-acetates **3c** and **3d**. To establish the structural identity, the pure O-acetylated products were de-O-acetylated to get authentic samples of **2a** and **2b** and compared to literature data.[26]

The mixture of **2a** and **2b** was converted to the bromide **7a** and **7b** via O-allylation with allyl bromide–NaH followed by the separation of the two diastereomers to give **3a** and **3b**, de-O-isopropylidenation with 90% aqueous trifluoroacetic acid, acetylation with acetic anhydride–pyridine to give **6a** and **6b**, followed by the selective removal of the anomeric acetates with hydrazine–acetate[33] and then reaction with Vilsmeier bromide.[34] The bromides **7a** and **7b** were then reacted with 2-(trimethylsilyl)-ethanol in the presence of silver triflate–collidine complex to give the β-glycosides **8a** and **8b**. The 2-(trimethylsilyl)ethyl group was chosen for temporary protection[35] at the anomeric center and for subsequent removal in order to carry out larger oligosaccharide synthesis. Fortunately, it also turned out to be useful in the separation of anomeric mixture of sialosides (see below), which could not be accomplished with other anomeric protecting groups such as the corresponding methyl glycosides.

The conversion of **8a** and **8b** to **11a** and **11b** was achieved under well-established conditions, namely de-O-acetylation followed by alkylation with benzyl bromide and sodium hydride to give **10a** and **10b**. Removal of the O-allyl group with iridium reagent[36] afforded the alcohol **11a** and **11b**.

The sialosides **14a** and **14b** were prepared by glycosylation of **11a** and **11b** with the thioglycoside **12b**. The thioglycoside glycosyl donor **12b** was prepared in one step from the chlorosugar[37] (**12a**)

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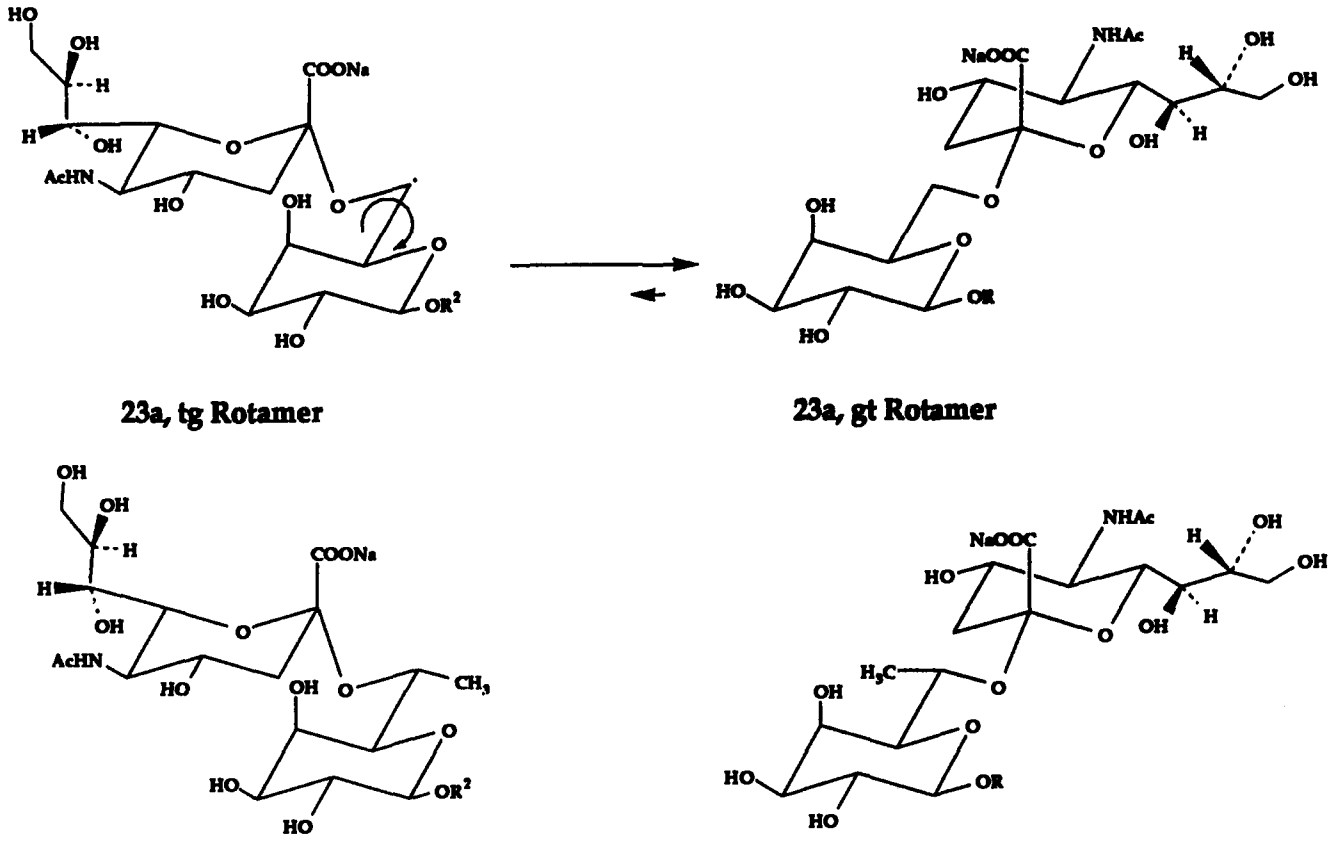

Figure 1. The equilibration between the tg and gt rotamer orientations for the sialoside αDNeuAc(2-6)βDGal-OR (**23a**) and the structures of the corresponding analogs methylated at C-6 of the galactose.

by reaction with sodium thiomethylate in acetonitrile. The yield was over 86%. Due to satisfactory purity of the crude product (>90%, the remainder of the material was the glycal **13**), chromatographic purification was not required at this point. This one step procedure for α-thiosialoside is superior in terms of purity and yield compared to the multistep literature method.[38]

The condensation of the alcohol **11a** or **11b** with thioglycoside **12b** in presence of methylsulfenyl triflate gave greater than 60% yield of the desired α-sialosides **14a** or **14b**. The minor β-sialoside was separated by chromatography. The structures of the α- and β-sialosides were established by NMR using the chemical shifts of H-4 of NeuAc unit (4.80 ppm for α-sialoside and 5.3 ppm for β-sialoside). Our use of the methylsulfenyl triflate for condensation of **11a** or **11b** and **12b** was prompted by the recent report of Lönn and Stenvall.[39] These authors prepared α-sialosides in high yield by condensation of secondary alcohols with the *N*-acetylneuraminyl xanthate derivative **12c**. We found that the thioglycoside **12b** also gave yields of the α-sialoside which were comparable to those provided by the 2-xanthate glycosyl donor **12c**.[39]

Dimethyl(methylthio)sulfonium triflate mediated condensation[40] of **11a** or **11b** with **12b** was not very successful in our hands. In the case of **11a**, even though the α-sialoside **14b** was the major product, the yield was low, and more than 70% of the starting material remained unreacted. In the case of **11b**, the β-sialoside was the major product with most of the starting alcohol remaining unreacted.

The benzyl protecting groups were removed by hydrogenation. Subsequent treatment with sodium methoxide gave the methyl ester of the sialosides (**15a** and **15b**). These methyl esters were converted to the sodium salts **16a** and **16b**, respectively, by treatment with Chelex-resin (sodium form). The 600-MHz proton spectra of **16a** and **16b** are shown in Figure 2, and the complete proton and carbon chemical shift assignments are presented in Tables 1 and 2. The α-sialosidic configuration in sialosides **16a** and **16b** was confirmed by the observation of larger coupling constants between C-1′ and H-3′ax (5.6 Hz) of the NeuAc unit,[41,42] as compared to the coupling constant of 2–3 Hz for the β-sialosides (supplementary material, Table 2).

The two di-*O*-isopropylidene sialosides **19a** and **19b** were synthesized via the protected sialosides **17a** and **17b**, which in turn were prepared by the condensation of alcohol **2a** and **2b** with the thioglycoside **12b** or **12c** under the conditions described for **14a**. In contrast to **14a** or **14b**, the yield of the disaccharides **17a** and **17b** was low. We did not optimize the reactions, since enough material could be obtained for biochemical evaluations. Even though the sialosides **17a** and **17b** were contaminated with the byproduct glycal **13** (due to identical mobility on silica gel), the impurities could be removed by gel permeation chromatography of the de-O-acetylated derivatives **18a** and **18b**. The methyl esters in **18a** and **18b** were hydrolyzed to the sodium salts **19a** and **19b** by treatment with Chelex resin as described above. Finally, the reducing sugar **16c** was prepared from **19a** by the removal of the isopropylidene groups with aqueous trifluoroacetic acid.

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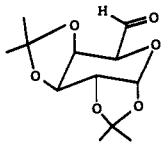
Scheme 1. The Monosaccharide Intermediates Involved in the Synthesis of Sialosides **16a**, **16b**, **16c**, **19a**, **19b**, **22a** and **22b**



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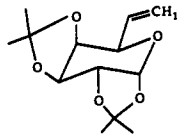

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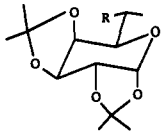
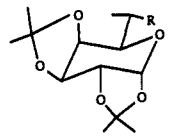
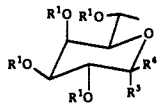
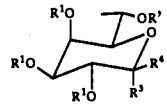




2a	R= OH	2b
3a	R = $OCH_2CH=CH_2$	3b
3c	R = OAc	3d
4a	R = $SCOCH_3$	4b
5a	R = SH	5b





6a,	R^1 = allyl, R^2 = OAc, R^3 = OAc and R^4 = H or R^3= H and R^4 = OAc;	6b
7a,	R^1 = allyl, R^2 = OAc, R^3 = Br, R^4 = H;	7b
8a,	R^1 = allyl, R^2 = OAc, R^3 = H, R^4 = $O(CH_2)_2SiMe_3$	8b
9a,	R^1 = allyl, R^2 = R^3 = H, R^4 = $O(CH_2)_2SiMe_3$;	9b
10a,	R^1 = allyl, R^2 = OBn, R^3 = H, R^4 = $O(CH_2)_2SiMe_3$	10b
10c,	R^1 = allyl, R^2 = OBn, R^3 = OBn, R^4 = H	
11a,	R^1 = R^3 = H, R^2 = OBn, R^4 = $O(CH_2)_2SiMe_3$;	11b
11c,	R^1 = R^3 = H, R^2 = R^4= OBn,	
24a,	R^1 = R^2 = R^3 = H, R^4 = $O(CH_2)_2SiMe_3$;	24b

The natural sialoside derivative of αDNeuAc(2-6)βDGalOCH$_2$-CH$_2$Si(CH$_3$)$_3$ (**23a**) was prepared by reacting (2-trimethylsilyl)-ethyl 3-*O*-benzoyl-β-D-galactopyranoside[43] with **12b** according to the procedure described for **14a**, followed by the removal of the protecting groups. Comparison of NMR data with the literature report[44a] confirmed its structure. Similarly, the sialoside **23b** was prepared by reacting 1,2;3,4-di-*O*-isopropylidene-α-D-galactopyranose with **12b** followed by removal of the protecting groups.

Synthesis of *S*-Sialoside Analogs. To prepare the thiosialoside analogs **22a** and **22b**, the sodium thiolate derivatives of **5a** and **5b** were required for condensation with the chlorosugar **12a**. These were prepared from **2a** and **2b** by converting them to their 6-*O*-trifluoromethanesulfonates (triflates) followed by displacement with potassium thioacetate. The displacement of the 6-*O*-triflate of **2a** with potassium thioacetate was slow. The desired product **4b** was obtained (46% from **2a**) together with an equal amount of the eliminated product **4c** (about 49%). In contrast, the formation of **4a** from the 6-*O*-triflate of **2b** was more facile, and the product **4a** was obtained from **2b** in 83% yield. The *S*-acetyl groups from **4a** and **4b** were removed by treatment with ammonium hydroxide in the presence of 1,4-dithiothreitol[44b] to give **5a** and **5b**. Condensation of the sodium salt of **5a** or **5b** with the chlorosugar **12a** gave the thiosialosides **20a** or **20b** in greater than 60% yield. The products were contaminated with about 10% of the glycal **13** (compounds **20a**, **20b**, and **13** exhibited identical mobility on silica gel). This latter impurity was removed by gel permeation chromatography of the de-O-acetylated derivatives **21a** and **21b**. Analytical samples of **20a** and **20b** were prepared by O-acetylation of pure **21a** and **21b**. The isopropylidene groups in **21a** and **21b** were removed by treatment with 50% aqueous trifluoroacetic acid, and the methyl ester was hydrolyzed with Chelex resin to give **22a** and **22b**.

The thiosialoside **23c** was prepared similarly from 1,2;3,4-di-*O*-isopropylidene-α-D-galactopyranose via the sodium salt of 6-deoxy-1,2:3,4-di-*O*-isopropylidene-6-thio-α-D-galactopyranose, which was then reacted with **12a** as described above for **22a** or **22b**.

It should be noted that many thiosialosides have been prepared previously in a circuitous way by the alkylation of the *S*-sodium salt of 2-thio-*N*-acetylneuraminic acid derivatives with the halides of the desired aglycon.[45] Since secondary halides are less reactive, we decided to retain the thio group in the aglycon and then condense it with the reactive pyranosyl chloride **12a**. Using this method, even sterically hindered sialosides could be prepared in high yields.

Synthesis of ^{14}C-Labeled Sialosides. ^{14}C-Labeled αDNeuAc-(2-6)βDGal(1-4)βDGlcNAc (**25**) was needed for evaluation of the inhibitory potencies of the thiosialosides. It was prepared enzymatically from ^{14}C-*N*-acetyllactosamine using Galβ1,-4GlcNAcα2,6 sialyltransferase as described in the published procedure.[46,47] The ^{14}C-labeled *N*-acetyllactosamine was made enzymatically from the commercially available ^{14}C-GlcNAc, using a modification of a published method[46,48] (see Experimental Section).

Neuraminidase Assays. Neuraminidase from influenza A virus/WSN/H1N1 (virus suspension) and three bacterial neuraminidases from *Arthrobacter ureafaciens* (A.U.), *Clostridium perfringens* (C.P.), and *Vibrio cholerae* (V.C.) were used in the neuraminidase assays according to published methods.[49] Figure 3 compares the rate of hydrolysis of 0.75 mM solutions of **16a**, **16b**, and the parent sialoside **23a** by the A. U. (panel A), C. P. (panel B), V. C. (panel C), and influenza A (panel D) neuraminidases. A. U. Neuraminidase efficiently hydrolyzed the natural (**23a**) and the tg sialoside (**16a**) equally well, whereas there was little hydrolysis of the gt sialoside (**16b**) under the same experimental conditions. The C. P. neuraminidase hydrolyzed **23a** faster than the tg sialoside **16a**. Here again, the gt sialoside **16b** was hydrolyzed very slowly. In the case of V. C. neuraminidase, the natural sialoside **23a** was the fastest hydrolyzed substrate of the three sialosides. Comparison between the tg **16a** and gt sialoside **16b** indicated that **16a** was preferentially hydrolyzed. Influenza A neuraminidase hydrolyzed both the natural sialoside **23a** and the tg sialoside **16a** (about 2-fold slower than **23a**), whereas the gt sialoside was not hydrolyzed at all. Thus, all four neuraminidases hydrolyze the tg sialoside **16a** preferentially as compared to **16b**, even though the selectivity between **16a** and **16b** appeared to be dependent on the source of the neuraminidase.

The hydrolysis of sialosides **16a**, **16b**, and **23a** was carried out at various substrate concentrations in order to determine detailed enzyme kinetic parameters. Control reactions were carried out at all substrate concentrations without the enzyme, and a

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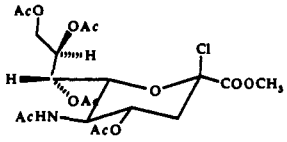
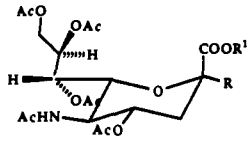
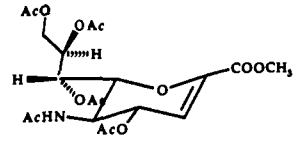
Scheme 2. The Disaccharide Intermediates Involved in the Synthesis of Sialosides **16a**, **16b**, **19a**, **19b**, **22a** and **22b**



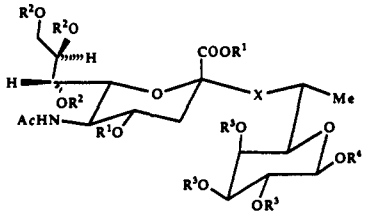
12a



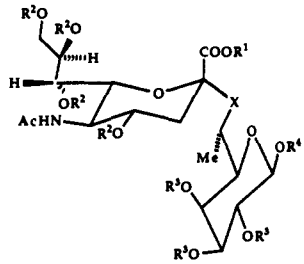
12b, R = SMe, R^1 = Me
12c, R = SCSOEt



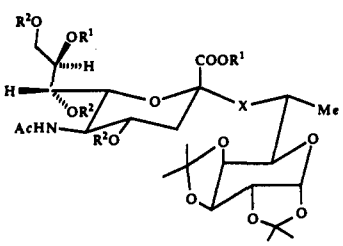
13

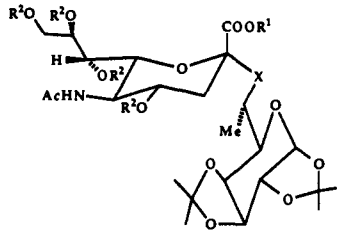


14a, X = O, R^1 = Me, R^2 = OAc, R^3 = Bn, R^4 = $(CH_2)_2SiMe_3$,
15a X = O, R^1 = Me, R^2 = R^3 = H, R^4 = $(CH_2)_2SiMe_3$,
16a X = O, R^1 = Na, R^2 = R^3 = H, R^4 = $(CH_2)_2SiMe_3$,
16c X = O, R^1 = Na, R^2 = R^3 = H, R^4 = H
22a X = S, R^1 = Na, R^2 = R^3 = R^4 = H



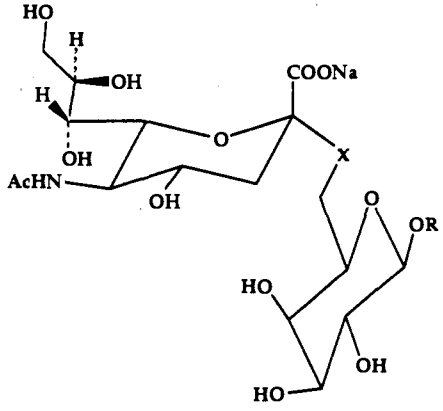
14b X = O, R^1 = Me, R^2 = OAc, R^3 = Bn, R^4 = $(CH_2)_2SiMe_3$,
15b X = O, R^1 = Me, R^2 = R^3 = H, R^4 = $(CH_2)_2SiMe_3$,
16b X = O, R^1 = Na, R^2 = R^3 = H, R^4 = $(CH_2)_2SiMe_3$,
16d X = O, R^1 = Na, R^2 = R^3 = H, R^4 = CH_3
22b X = S, R^1 = Na, R^2 = R^3 = R^4 = H

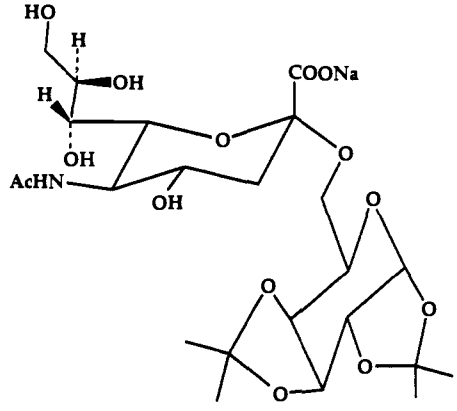




17a,	X = O, R^1 = Me, R^2 = OAc,	17b
18a	X = O, R^1 = Me, R^2 = H,	18b
19a	X = O, R^1 = Na, R^2 = H,	19b
20a,	X = S, R^1 = Me, R^2 = OAc,	20b
21a	X = S, R^1 = Me, R^2 = H,	21b

Scheme 3. Structures of the Sialoside **25**, **23b**, and **23a** and Its Thio Derivative **23c**





X = O, R = $CH_2CH_2SiMe_3$; 23a
X = S, R = H ; 23c
X = O, R = DGlcNAc ; 25

23b

correction for the nonenzymic hydrolysis was done. The hydrolysis profile in Figure 4 shows the difference in enzymatic hydrolysis behavior of the two analogs **16a** and **16b**. The Michaelis constant K_m and the maximum rate of enzyme hydrolysis V_{max} for the three substrates **16a**, **16b**, and **23a** were calculated from the Lineweaver–Burk plots, and the results are presented in Table 4.

As can be seen from Figure 4, the neuraminidase cleavage of sialic acid was facile for the tg sialoside **16a** and the natural sialoside **23a** (Figure 1, supplementary material) but was very slow for **16b**. Since no significant amount of hydrolysis took place for gt sialoside **16b** under the conditions used for **16a** or **23a**, a 10–20-fold higher concentration of the enzymes (A. U., C. P., and V. C., see Experimental Section) relative to that used

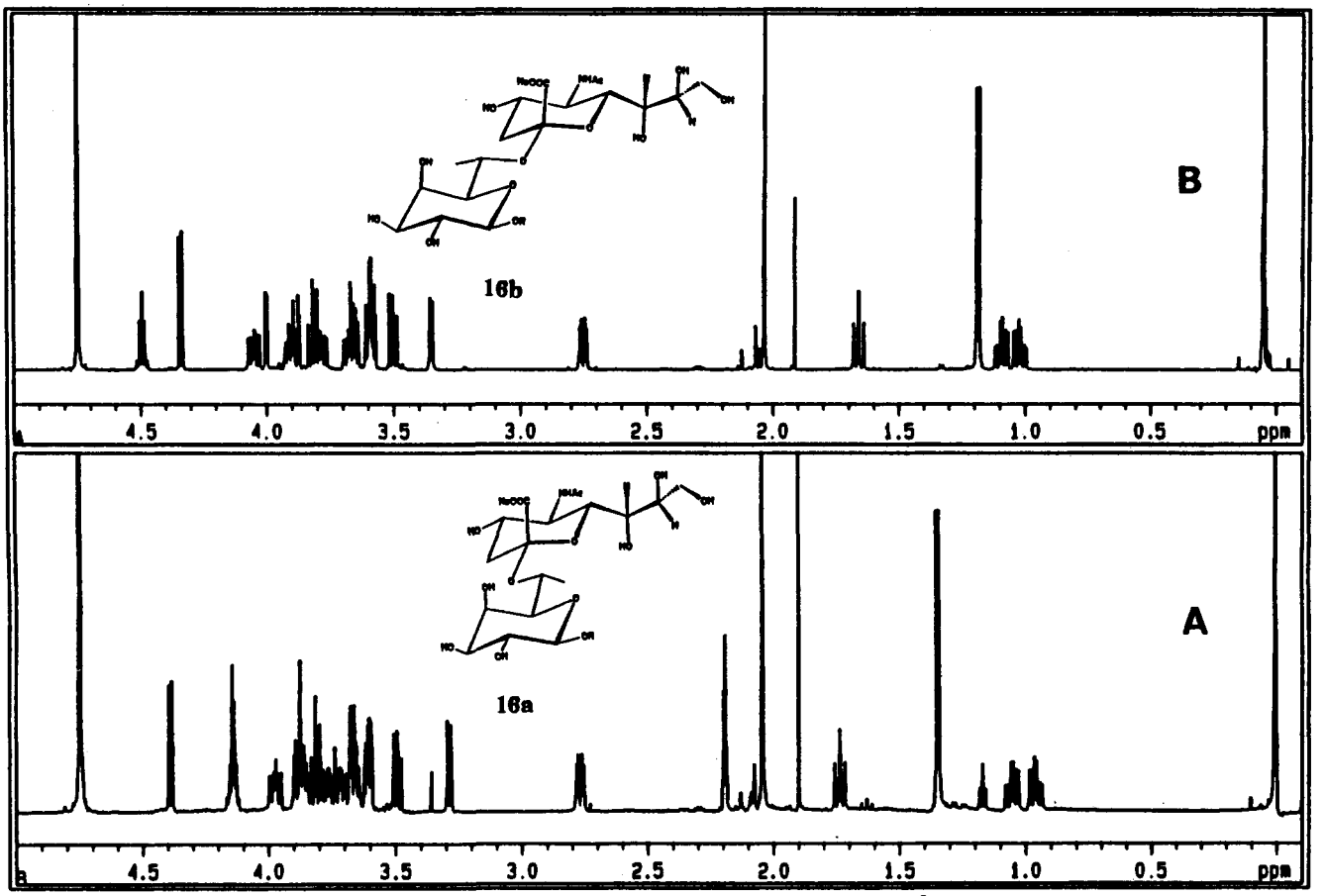


Figure 2. 600 MHz proton NMR spectra in deuterium oxide of the tg rotamer analog **16a** (panel A) and gt rotamer analog **16b** (panel B).

for **16a** was used to determine the K_m and V_{max}. Due to the limited solubility of the influenza virus, a higher concentration of viral suspension could not be prepared to effect measurable hydrolysis of gt sialoside **16b**; therefore, the K_m and V_{max} could not be determined.

Table 4 shows that with influenza virus and C. P. and V. C. neuraminidases, the K_m for **16a** was 2–3-fold higher than for the natural sialoside **23a**, whereas the V_{max} was nearly the same. However, with the A. U. neuraminidase, these two substrates behaved nearly identically. On the other hand, the K_m for the gt sialoside **16b** was generally higher than that of either **16a** or **23a**. Most notably, the V_{max} was substantially smaller (4–18-fold lower than **16a** or **23a**).

In order to elucidate whether the galactose hydroxyl groups or the sialoside aglycon itself has any role in the enzymatic reactions, the hydrolysis of the isopropylidene analogs of the natural sialoside **23a** and the two analogs **16a** and **16b** were carried out (compounds **23b**, **19a**, and **19b**, respectively). It is to be noted that the isopropylidenation in these derivatives not only masks the galactose hydroxyls but also changes the pyranose ring chair conformation into a boat structure.[26] As shown in Figure 5 (panels A–D), the isopropylidene derivative of the natural sialoside **23b** was in fact more efficiently hydrolyzed by all four neuraminidases (Figure 1, supplementary material). The K_m for **23b** was lower compared to **23a** with A. U. neuraminidase (Table 4) and higher with C. P. and V. C. neuraminidases. The lower affinity of **23b** for these latter two enzymes was compensated by higher V_{max} as compared to **23a** (Figure 1, supplementary material). The isopropylidenation of the tg sialoside as seen in **19a** had no effect on the hydrolysis with A. U. and V. C. neuraminidase (Figure 5, panels A and C), as compared to **16a**, but had a detrimental effect with the C. P. and influenza A neuraminidases (Figure 5, panels B and D). The isopropylidene derivative **19b** of the gt sialoside was inactive with all the four enzymes and, in fact, was more resistant to enzymatic hydrolysis as compared to **16b** (compare Figures 4 and 5). Based on this, we can conclude that the substitution at galactose hydroxyls has a marginal effect only on the enzymatic hydrolysis of sialic acid.

Inhibition of Neuraminidases by Thiosialosides. Based on the results with the oxygen analogs **16a**, **16b**, and **23a**, the corresponding thioanalogs **22a**, **22b**, and **23c** were tested as inhibitors of the neuraminidases. This was done by monitoring the neuraminidase catalyzed liberation of ^{14}C-*N*-acetyllactosamine (LacNAc) from αDNeuAc(2-6)βDGal(1-4)DGlcNAc (**25** labeled in the LacNAc residue) in the absence and presence of the inhibitor. In Figure 6, panel A shows the influenza virus neuraminidase catalyzed hydrolysis of **25** in the absence ($I = 0$) and in the presence of **22a** ($I = 0.6$ mM), panel B for **22b** ($I =$ 0 and 5 mM), and panel C for **23c** ($I = 0$ and 8 mM). It is evident from these results that only the tg thiosialoside analog **22a** was a good inhibitor ($K_i = 0.3$ mM) of influenza neuraminidase. Similar experiments with A. U., C. P., and V. C. neuraminidases showed that only **22a** was a good inhibitor (K_i for **22a** with A. U., C. P., and V. C. are 0.3, 1.0, and 0.35 mM, respectively; see Experimental Section and Figure 2, supplementary material for details). The gt analog **22b** showed very little inhibition at 5 mM or higher concentrations (Figure 3, supplementary material). Similarly, **23c** exhibited only weak inhibition when examined with A. U. neuraminidase. Thus, there was a parallel in the behavior of oxygen and the sulfur analogs of tg (**16a** and **22a**) and gt (**16b** and **22b**) sialosides which was not the case with the natural sialoside **23a** and its thio analog **23c**.

Molecular Modeling and NMR. To correlate the differential enzymatic behavior of the sialosides **16a** and **16b**, and **22a** and **22b** with their solution structure, we carried out conformational analysis of these compounds using molecular modeling studies and detailed NMR investigations. Molecular modeling was done using Monte Carlo (MC) simulations at 600 K according to the

Table 1. ^1H Chemical Shifts (500.13 or 600.13 MHz) for the "tg" and "gt" Sialosides (**15a**, **15b**, **16a**, **16b**, **16d**, **22a**, and **22b**) in D_2O at 300 K[a]

							22a		22b	
units	H atom	15a	15b	16a	16b	16d	α-OH	β-OH	α-OH	β-OH
αDNeuAc	H3'ax	1.94 (11.7, 13.0)	1.91 (12.9, 12.2)	1.74 (12.5, 12.0)	1.64 (12.4, 12.2)	1.67 (12.2)	1.77 (12.5, 11.2)		1.73 (12.0)	1.75 (12.0)
	H3'eq	2.73 (4.6)	2.63 (4.5)	2.77 (4.7)	2.73 (4.5)	2.73 (4.6)	2.80 (4.7)	2.79 (4.7)	2.79 (4.5)	2.80 (4.5)
	H4'	3.78 (10)	3.73 (11)	3.70 (10.0)	3.65 (10.0)	3.65 (10.0)	3.68 (10.0)		3.67	
	H5'	3.87 (10.4)	3.86 (10.2)	3.81 (10.3)	3.78 (10.0)	3.79 (10.0)	3.79 (10.2)		3.80 (10.4)	
	H6'	3.71 (1.1)	3.69 (0.6)	3.66 (1.8)	3.56	3.58	3.57		3.59 (1.7)	
	H7'	3.56 (9.2)	3.54 (9.0)	3.59 (9.0)	3.57 (10)	3.58	3.57 (10)		3.56 (9.6)	
	H8'	3.80	3.85	3.84	3.89	3.88	3.83		3.87	
	H9a'	3.86 (12.0, 2.5)	3.84 (11.9)	3.88 (12.5, 2.5)	3.86 (11.4, 2)	3.87	3.84 (12.0, 2.4)		3.86 (12.0, 2.5)	
	H9b'	3.67 (6.0)	3.65 (6.0)	3.65 (6.0)	3.63 (6.1)	3.63	3.62 (6.1)		3.62 (6.5)	
	NAc	2.05	2.01	2.05	2.01	2.01	2.02		2.02	
	$COOCH_3$	3.87	3.85							
βDGal	H1	4.39 (8.0)	4.29 (7.9)	4.38 (7.9)	4.32 (7.9)	4.23 (8.0)	5.20 (3.4)	4.50 (7.9)	5.22 (2.3)	4.52 (7.8)
	H2	3.48 (9.8)	3.46 (9.8)	3.48 (9.9)	3.48 (9.8)	3.50 (9.9)	3.76	3.44 (9.9)	3.79	3.48 (10.0)
	H3	3.58 (3.5)	3.58 (3.4)	3.60 (3.5)	3.58 (3.3)	3.59 (3.5)	3.76	3.56 (3.5)	3.79	3.56 (3.3)
	H4	4.09 (<1)	3.92 (<1)	4.13 (<1)	3.98 (<1)	4.00 (<1)	4.22	4.17 (<1)	4.11	4.10 (<1)
	H5	3.28 (8.6)	3.34 (8.5)	3.28 (8.6)	3.33 (6.5)	3.36 (6.0)	3.77 (8.4)	3.38	3.80 (8.8)	3.46 (7.9)
	H6	4.30 (6.2)	4.45 (6.4)	4.13 (6.2)	4.48 (6.3)	4.49 (6.4)	3.35 (6.9)	3.39 (6.6)	3.40 (7.2)	3.42 (7.1)
	H7	1.34	1.18	1.36	1.16	1.18	1.42	1.44	1.31	1.33
aglycon	OMe					3.57				
	OCHa	3.97	4.00 (10.1, 13.0, 5.2)	3.96 (10.0, 12.5, 5.3)	4.03 (10.3, 13.0, 5.4)					
	OCHb	3.76	3.75 (5.3, 13.0)	3.75 (5.5, 12.5)	3.77 (5.2, 13.0)					
	SiCHa	1.07	1.08 (13.0)	1.06 (13.2)	1.07 (13.0)					
	SiCHb	0.97	0.99	0.97	1.00					
	$SiCH_3$	0.03	0.04	0.02	0.03					

[a] The chemical shifts are expressed relative to internal acetone (2.23 ppm) which sets the HDO signal at 4.75 ppm. The proton–proton couplings constants in Hz are given in parentheses.

Table 2. ^{13}C Chemical Shifts (125.76 MHz) for the "tg" and "gt" Sialosides (**15a**, **15b**, **16a**, **16b**, **16d**, **22a**, and **22b**) in D_2O at 300 K[a]

							22a		22b	
unit	C atom	15a	15b	16a	16b	16d	α-OH	β-OH	α-OH	β-OH
αDNeu-Ac	C1'	169.8	170.7	173.9 [5.6] (2.8)	174.4 [5.6] (2.9)	174.3 [5.6]	174.75		174.7	174.6
	C2'	100.3	99.4	102.3 (3.7)	99.9 (5.0)	100.2	87.3	87.4	85.7	86.2
	C3'	38.5	38.9	40.4 (0.21)	41.0 (0.17)	40.8	41.61	41.56	41.48	41.54
	C4'	67.6	67.6	67.6 (0.41)	68.55 (0.35)	68.7	68.8		68.8	
	C5'	51.9	52.1	52.1 (0.33)	52.2 (0.35)	52.2	52.0		52.0	
	C6'	73.1	73.3	73.2 (0.35)	73.0 (0.34)	73.0	75.3		75.1	75.2
	C7'	68.4	68.6	68.44	68.58 (0.33)	68.6	68.4		68.6	
	C8'	71.0	71.1	72.3 (0.34)	72.1 (0.36)	72.2	72.4		72.2	72.4
	C9'	63.4	63.4	63.0 (0.26)	62.9 (0.24)	62.9	62.9		63.0	
	$NHCOCH_3$	22.4	22.4	22.3 (1.0)	22.3 (1.4)	22.4	22.3		22.3	
	NHCO	175.2	175.3	175.4 (2.2)	175.4 (2.2)	175.4	175.3		175.3	
	$COOCH_3$	53.7	53.8							
βDGal	C1	102.6	102.1	102.5 (0.42)	102.5 (0.37)	104.3	92.7	97.1	92.6	97.0
	C2	71.1	71.0	71.2 (0.35)	71.2 (0.37)	71.1	68.7	72.2	68.7	72.2
	C3	73.5	73.5	73.2 (0.35)	73.5 (0.36)	73.4	69.9	73.3	70.1	73.6
	C4	67.7	68.9	68.0 (0.37)	69.7 (0.34)	69.8	69.5	69.1	69.6	69.1
	C5	77.2	77.5	77.7 (0.36)	77.2 (0.35)	77.1	72.7	77.5	72.0	76.6
	C6	68.9	70.6	69.8 (0.29)	70.3 (0.33)	70.4	39.6	39.4	39.7	40.2
	C7	19.3	17.8	19.4 (0.98)	17.2 (0.55)	17.5	20.7	20.5	18.9	18.6
aglycon	OMe					57.4				
	OCH_2	69.3	68.4	68.50	68.47 (0.43)					
	$SiCH_2$	17.8	17.8	17.9 (0.78)	17.8 (0.69)					
	$SiCH_3$	−2.2	−2.2	−2.2 (4.3)	−2.1 (4.3)					

[a] The chemical shifts are expressed relative to 1,4-dioxane (66.9 ppm) using the deuterium lock of the spectrometer. The T1's (s) are given in parentheses. The values in brackets are selected ^1H–^{13}C coupling constants in Hz.

published procedures.[24,50] For the tg and gt rotamer analogs (**16a** and **16b**, respectively), the "ω angles" [51] in these calculations were fixed at 180° (tg rotamer orientation) and 60° (gt rotamer orientation), respectively, while varying the torsion angles around the other exocyclic bonds. The local mimina obtained in the MC simulations were energy minimized in a second step to obtain the lowest global minimum energy conformers. From these MC simulations, a 2:1 population of "anti" (around ϕ, ψ, ω = 179°, 132°, 180°, Figure 7a) versus "syn" (around ϕ, ψ, ω = −70°, 134°, 180°, Figure 7b) for the sialoside **16a** and a near equal population of anti (around ϕ, ψ, ω = 172°, −126°, 60°, Figure 8a) versus syn conformer (around ϕ, ψ, ω = −62°, −119°, 60°, Figure 8b) for the sialoside **16b** were estimated (see Figure 4, supplementary material). From these models, it became evident that the H-6 of the galactose was close to the H-3'ax of the sialic acid in **anti** orientation in both **16a** and **16b**, (Figures 7a and 8a), whereas the H-7 of galactose and H-8 of NeuAc unit in **16a** (Figure 7b) and the H-6 of galactose and H-8 of NeuAc unit in **16b** (Figure 8b) were close in the **syn** conformation. To assess the validity of the models and to experimentally evaluate the population of the two conformers, we tried to establish relevant internuclear distances by NMR methods.

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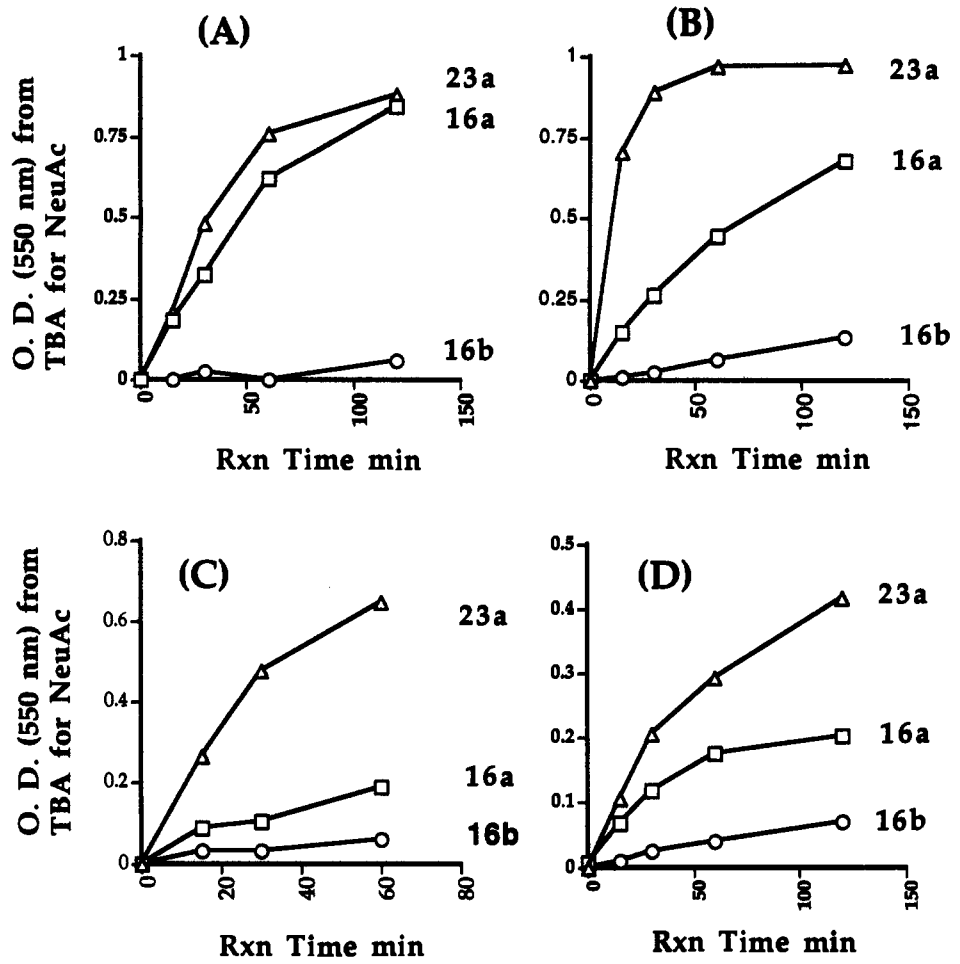

Figure 3. The time course of hydrolysis of sialosides **23a**, **16a**, and **16b** by neuraminidases from *Arthrobacter ureafaciens* (A), *Clostridium perfringens* (B), *Vibrio cholerae* (C), and influenza A neuraminidase (D).

The proton NMR spectra of **16a** and **16b** were well resolved at 600 MHz (Figure 2), the chemical shifts and hydrogen–hydrogen coupling constants of all hydrogen could be readily established using 1- and 2-D NMR spectroscopy (Table 1). The ^{13}C chemical shifts were established by heteronuclear ^{1}H–^{13}C 2-D correlation experiments (Table 2). These NMR parameters confirmed the structural identity and chair conformation of the pyranoside rings in both of the disaccharides **16a** and **16b**. Also, the ^{13}C spin–lattice relaxation times (T_1's, Table 2) showed that the exocyclic carbon C-6 in both **16a** and **16b** were nearly as rigid as the pyranose ring carbon. Therefore, the conformational properties of these two disaccharides **16a** and **16b** can be defined on the basis of the sialosidic torsion angles ϕ and ψ and by the ω torsion angle around the C-5–C-6 bond of the galactose (Figure 1).

Determination of Rotamer Orientation (ω Torsion Angle) in Sialosides 16a and 16b. The extent of rotamer populations defined by the ω torsion angle could be determined from the coupling constants between H-5 and H-6 of galactose combined with an NOE experiment. The coupling constant between H-5 and H-6 of galactose in **16a** was 8.6 Hz requiring them to be almost antiperiplanar.[52] This orientation was further confirmed by the NOE experiment where the saturation of methyl signals (H-7) at 1.36 ppm resulted in strong NOEs to H-5 and H-6 but none to the H-4 of the galactose (Figure 9, panel B, for NOE values, see Table 3). In the case of **16b**, the observed coupling constant between H-5 and H-6 of the gal unit was only 6.5 Hz (it was 8.0 Hz for the asialo derivative **24b** and 8.5 Hz in the methyl ester **15b**), thus suggesting the presence of other rotamers in addition to the major gt rotamer population. This was also evident from the NOE experiment where a weak NOE from the H-6 of galactose to its H-4 was observed (Figure 10, panel B), suggesting [The MC simulation for **16b** suggested a weak NOE between H-6 and H-4 of the galactose, even when the gg rotamers were not included. Thus, on the basis of NOE alone, the presence of gg rotamers cannot be suggested. Our proposal is based on these NOE results taken together with the smaller than expected coupling constant between H-5 and H-6 galactose in **16b** as compared to its methyl ester **15b** and the asialo-derivative **24b**.] the presence of unexpected gg rotamer particularly in the sialoside **16b**. However, the highly populated form appears to be the gt rotamer, as is evident from the strong NOE between the H-7 and H-4 of the galactose (Table 3). The fact that the gg rotamer may be present only in the sialoside derivative **16b** and not in the methyl ester **15b** (based on coupling constants between H-5 and H-6 of the galactose) suggests that the carboxyl group may be providing the stabilizing force for the presence of the unexpected gg rotamer by establishing a hydrogen bond with O-4 of the galactose.

Determination of Sialosidic Torsion Angles. To establish the sialosidic torsion angle ϕ and ψ, NOE experiments at 300 and 500 MHz (Table 3) were carried out to obtain relevant interring hydrogen distances. Compounds **16c** and **16d** containing modified aglycon at C-1 of galactose were investigated together with **16a** and **16b** due to the unfavorable rotational correlational time of the latter two sialosides for NOE experiments at 500 MHz. On the basis of the minimum energy models, we infer that the anti conformer for **16a** (Figure 7A) would place the H-3′ax of the NeuAc to within 2.2 Å of H-6 of gal unit, whereas in the syn conformer (Figure 7B), the H-8′ of NeuAc unit would be within 2.7 Å distance of H-7 of the gal unit (H-6 to H-3′$_{ax}$ distance in syn conformer would be 4.38 Å). Consequently, interring NOEs between these hydrogens would be expected depending on their relative abundance. In fact, as shown in Table 3, for the sialosides **16a** (Figure 9A) and **16c**, the saturation of H-3′$_{ax}$ of the NeuAc

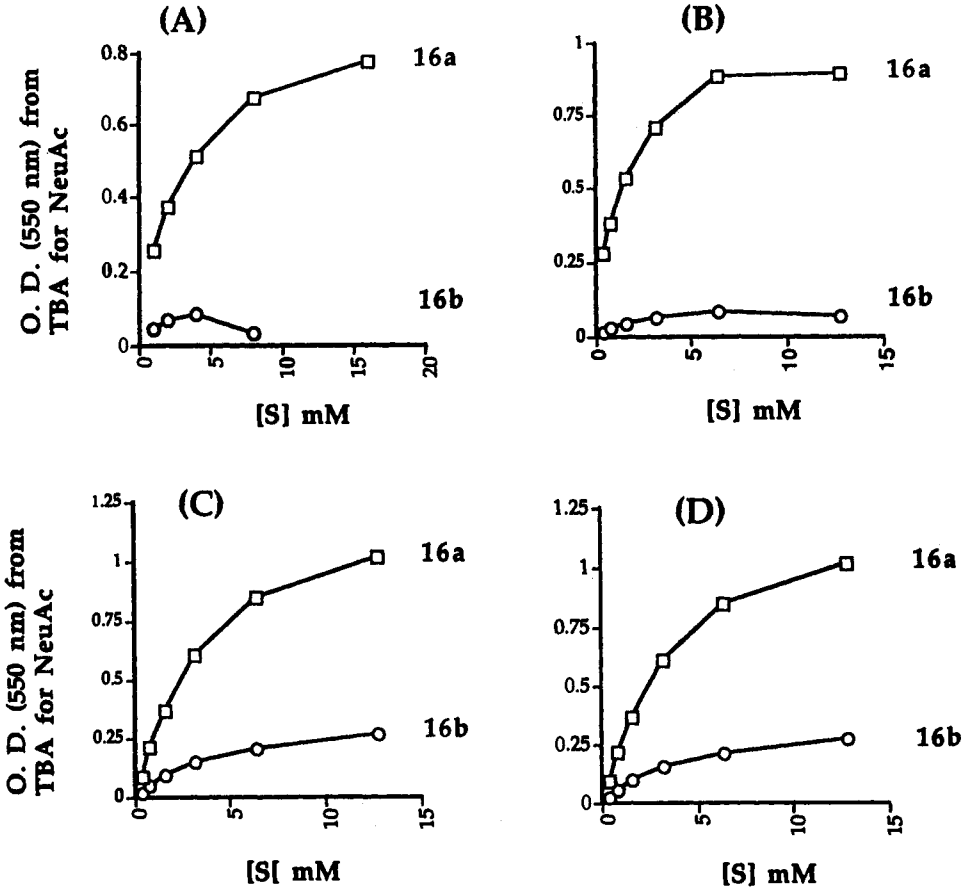

Figure 4. Hydrolysis of sialoside analogs **16a** and **16b** at various concentrations by neuraminidases from influenza A (A), *Arthrobacter ureafaciens* (B), *Clostridium perfringens* (C), and *Vibrio cholerae* (D).

Table 3. The NOEs (at 500 or 300 MHz) Measured and Calculated (at 500 MHz) for tg and gt Sialosides (**16a**, **16b**, **16c**, **16d**, **22a**, and **22b**)[d]

	observed NOE							calculated NOE			calculated NOE		
	tg ω = 180°				gt ω = 60°			tg ω = 180°			gt ω = 60°		
	16a NOE	**16a** ROE	**16c** NOE (NOE)[c]	**22a** NOE	**16d** NOE	**16b** ROE (NOE)[c]	**22b** NOE	anti min.	syn min.	MC 600 K	anti min.	syn min.	MC 600 K
ϕ/ψ								179/132	−70/134		172/−126	−62/−119	
energy								2.1	4.5		3.3	2.4	
anti/syn										65/35			45/55
{H3′$_{ax}$}-H3′$_{eq}$	2.7 (15.3)[c]		10.0 (18.5)[c]	11.4	11.1	20.2 (13.6)[c]	14.0	11.6	12.4	11.8	10.9	12.2	11.6
{H3′$_{ax}$}-H5′	*a* (4.4)[c]		2.4 (5.8)[c]	3.2	3.5	3.2 (5.4)[c]	4.9	4.8	5.4	4.0	4.5	4.6	3.8
{H3′$_{ax}$}-H6	0.51 (5.3)[c]		2.6 (5.1)[c]	1.0	2.3	4.8 (2.3)[c]	0.49	7.4	0.17	5.1	7.3	0.2	4.4
H3′$_{ax}$-H6/H3′$_{ax}$-H3′$_{eq}$	0.19 (0.35)[c]		0.26 (0.32)[c]	0.09	0.21	0.24	0.04	0.64	0.01	0.43	0.67	0.02	0.38
H3′$_{ax}$-H6/H3′$_{ax}$-H5′	*a* (1.2)[c]		**1.08 (0.9)**[c]	**0.31**	**0.66**	1.5	0.10	1.52	0.03	**1.26**	1.62	0.04	**1.16**
{H7}-H8′	0.53 (0.91)[c]	1.2	0.91 (1.3)[c]	1.9	<0.4	0	0	0	3.0	0.63	2.0	0	0.36
{H7}-H4			<0.1	0.42	2.9	7.8 (2.3)[c]	2.7	0.1	0.08	0.08	5.3	5.3	5.9
{H7}-H6	3.8[b] (2.4)[c]	5.6[b]	6.9 (4.0)[c]	7.3	6.9	10.5 (3.4)[c]	2.4	4.3	8.2	5.4	4.1	7.7	5.3
H7-H8′/H7-H6	0.11	0.21	**0.13 (0.3)**	**0.26**	**<0.06**	0	0	0	0.36	**0.12**	0.49	0	**0.07**
H7-H4/H7-H6	*a*	*a*	0	0.06	0.42	0.74	1.13	0.02	0.01	0.01	1.28	0.68	1.12
{H-4}-H7					0.6	7.8	0.68				1.6	1.6	1.9
{H4}-H5					3.0	9.4	2.1				2.7	2.7	2.7
H4-H7/H4-H5					0.20	0.83	0.32				0.59	0.59	0.70
{H6}-H3′A					0.8	4.8					3.4	0	1.8
{H6}-H8′					0.7	2 (1.97)[c]					0.3	1.5	0.7
{H6}-H7					1.5	10.5 (7.0)[c]					1.4	1.4	1.4
{H6}-H4					0.4	3.8 (1.79)[c]					0.1	0.7	0.6

[a] weak NOE; b = overlapping signals from H-4; c = measured at 300 MHz. [d] The NOE values are expressed as a percent relative to the intensity of the saturated hydrogen signal. The calculated minima by the GEGOP methods and the conformer populations estimated from Monte-Carlo simulations are included (see Experimental Section). { } indicates the hydrogen saturated.

unit around 1.74 ppm resulted in large NOEs to two intraring hydrogens (H-5′ and H-3′$_{eq}$) and to the interring hydrogen H-6. In addition, when the H-7 of the galactose was saturated (Figure 9B), a weak but definite NOE to H-8′ of the NeuAc unit and strong NOEs to H-5 and H-6 of the galactose unit were seen (Table 3). Since this interring NOE between H-7 and H-8′ can arise only from the syn conformer, we suggest that in **16a** and **16c** both the anti and syn conformers coexist in solution. On the basis of the relative magnitude of the NOEs (Table 3) and the NOEs derived from MC simulations, contribution from the anti conformer appears to be more than from the syn conformer.

For the sialoside analog **16b** and **16d**, we can expect on the basis of the minimum energy models that the H-3′ax of NeuAc unit will be within 2.5 Å distance of the H-6 of the gal unit in

Table 4. The Michaelis Constant (K_m in mM) and V_{max} (O.D./mU) of αDNeuAc(2-6)βDGal Derivative **23a** and Its Isopropylidenated Derivative **23b** and the tg (**16a**) and gt (**16b**) Rotamer Analogs with Neuraminidases from Influenza A Virus, *Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Vibrio cholerae*

	influenza A		*A. urefaciens*		*C. perfringens*		*V. cholerae*	
sialoside	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
23a	1.0	1.0	1.1	0.7	0.65	0.88	2.0	2.2
23b			0.50	0.73	1.44	1.70	3.18	4.5
16a	2.3	0.84	0.94	0.91	2.15	0.90	4.50	1.42
16b			3.0	0.05	3.2	0.22	4.8	0.37

anti conformation (Figure 8A), whereas the H-8′ of the NeuAc unit will be within 3.1 Å of the H-6 of the gal unit in the syn conformer (Figure 8B, also see Table 3 of supplementary material). Indeed, as seen in Table 3, the saturation of signals H-3′$_{ax}$ in sialosides **16b** (Figure 10A) and **16d** resulted in NOEs to H-5′ and H-3′$_{eq}$ and to the interring hydrogen H-6. As expected for the syn conformer, the saturation of H-6 resulted in NOEs to H-4, H-5, H-7, and to two inter-ring hydrogens H-3′$_{ax}$ and H-8′. On the basis of these interring NOEs and the MC simulations (Table 3), we propose that **16b** and **16d** coexist in solution in near equal amounts in both the anti and syn conformers.

Similar NOE results at 300 MHz were also observed for the isopropylidene sialosides **19a** and **19b**.

For the thiosialosides **22a** and **22b**, the C-5–O-6 arm of the galactose appears to orient predominantly in the tg (ω = 180°) and gt (ω = 60°) rotamer orientation, respectively which was evident from the large coupling constants between the H-5 and H-6 of galactose ($J_{H5-H6} \geq 8$ Hz, Table 1). These results are similar to those observed in sialosides **16a** and **16b**. Detailed NOE data at 500 MHz are presented in Table 3 (Figure 6, supplementary material). Comparison of these data with those obtained for the sialosides **16a** and **16b** clearly shows that in the case of thioanalogs, one family of conformers (syn) is populated predominantly.

In summary, in the case of **16a** and its sulfur analog, the NOE results show that the C-5–O-6 of the galactose orients predominantly in the tg rotamer orientation. Whereas, the carboxyl group of the sialic acid maintains anti and syn orientations with respect to the C-6 of galactose in the oxygen analog **16a** in a relative ratio of 2:1. On the other hand, the syn conformer appears to be populated to a high degree in the sulfur analog **22a**. In the case of **16b** and its sulfur analog **22b**, the gt rotamer orientation for the C-5–O-6 of the galactose appears to be most populated, whereas the conformational properties around the sialoside linkage appears to be similar to those observed for **16a** and **22a**.

Discussion

The design of common inhibitors for both the hemagglutinin and neuraminidase of the influenza virus may provide a novel way to increase the efficacy of carbohydrate based drugs. A wealth of information is available surrounding the synthesis and carbohydrate structural requirements of hemagglutinin inhibitors. Such inhibitors are oxygen sialoside substrates in most cases, which are subject to the hydrolytic action of the neuraminidases. Recently, carbon-linked sialoside analogs have been prepared[7,8] that have been shown to be resistant to neuraminidase hydrolysis. However, these compounds do not bind to neuraminidase and as such are not neuraminidase inhibitors. Detailed crystal structures of the sialic acid bound influenza A and B neuraminidase have been published recently.[2,30,31] Recently the crystal structure of a bacterial sialidase has also been reported.[53] However, very little information is known regarding the natural sialoside conformational requirements for neuraminidase recognition[4] and the mechanism of enzymatic hydrolysis.[54,55] The enzymatic hydrolysis and inhibition behavior of the sialoside analogs of the αDNeuAc(2-6)βDGal sequences described in this report reveal for the first time a conformational requirement of sialosides that may be important for neuraminidase recognition. These results, together with the recent information[2] on the crystal structure of the influenza neuraminidase are expected to aid in the design of tight binding inhibitors of the influenza virus.

The four neuraminidases that we have examined show a clear preference for the sialoside analog **16a** over **16b**. Of these, the neuraminidases from influenza A and *Arthrobacter ureafaciens* show a similarity in their enzymatic behavior (Table 4) toward **16a**. The fact that **16a** is hydrolyzed and bound nearly as efficiently as the natural disaccharide **23a** shows that **16a** behaves more like a 2,6 gal linked sialoside and that both **16a** and **23a** may be bound to the enzyme in a similar conformation. This is unexpected since it is known[56–58] that in the unbound state, sialoside **23a** exhibits a different rotamer (gt) preference about the C-5–O6 bond of the galactose than does **16a** (tg rotamer preference). On the other hand, compound **16b**, which resembles **23a** closely in terms of rotamer preference, is practically resistant to enzymatic hydrolysis. Thus, methylation not only affects the binding of **16b** to the enzyme as evident from the K_m values (assuming that the methyl group does not contribute to the binding) but also dramatically lowers the V_{max}. In contrast to the A. U. neuraminidase (which preferentially cleaves αDNeuAc-(2-6)Gal linkages), the C. P. and V. C. neuraminidases (which shows a preference for a 2,3-sialoside linkages)[49,59,60] hydrolyze **16a** less efficiently as compared to the natural disaccharide **23a**, probably due to less efficient enzyme binding of **16a** (Table 4). However, the other analog **16b** was even a worse substrate, where the most notable difference appears to be in the V_{max} values. In all of the neuraminidase hydrolysis, neither the galactose hydroxyl groups nor the conformation of the galactose pyranose ring appear to play a significant role consistent with the computer assisted docking studies described below. Thus, compound **23b** is hydrolyzed as efficiently as **23a** by all four neuraminidases. The same results are seen for the isopropylidene derivatives of **16a**, especially with A. U. neuraminidase. The isopropylidenation of **16b** rendered the substrate (**19b**) even more resistant to neuraminidase hydrolysis. On this basis, we conclude that only the substitution of the carbon immediately connected to the O-2 of the NeuAc unit may have a significant effect on neuraminidase binding and hydrolysis.

To understand the origin of the preferential hydrolysis of **16a** versus **16b** and the selective neuraminidase inhibition by **22a** and not by **22b**, we tried to fit the two sialoside analogs **16a** and **16b** in the conformation described above into the crystal binding pocket of the influenze A neuraminidase.[30] The published structure[30] is a complex with α-sialic acid. The α-sialic acid in this complex is distorted so as to accommodate the carboxyl group in the equatorial orientation; thereby establishing salt bridges with three arginines residues at 118, 292, and 371. This arginine triad at the neuraminidase active site appears to be conserved in all known

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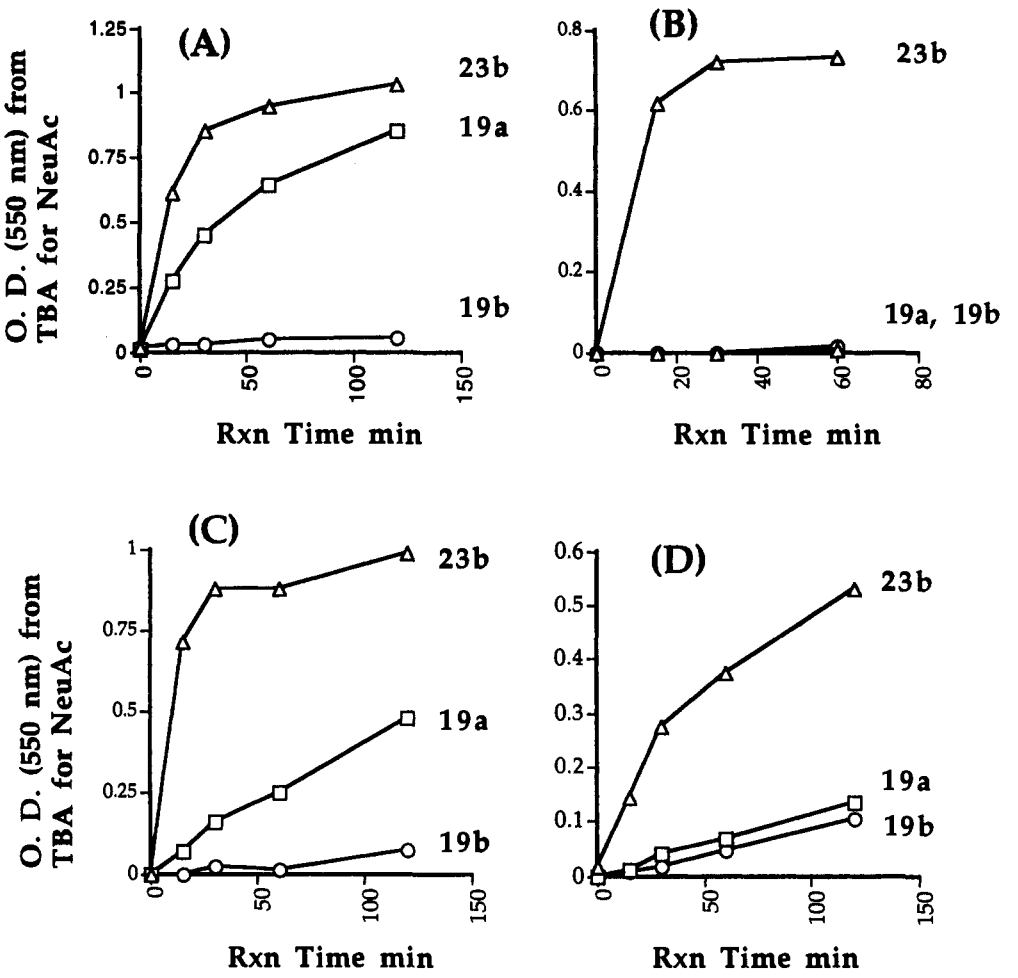

Figure 5. The time course of hydrolysis of the isopropylidentated sialosides **23b**, **19a**, and **19b** by neuraminidases from *Arthrobacter ureafaciens* (A), *Clostridium perfringens* (B), *Vibrio cholerae* (C), and influenza A neuraminidase (D).

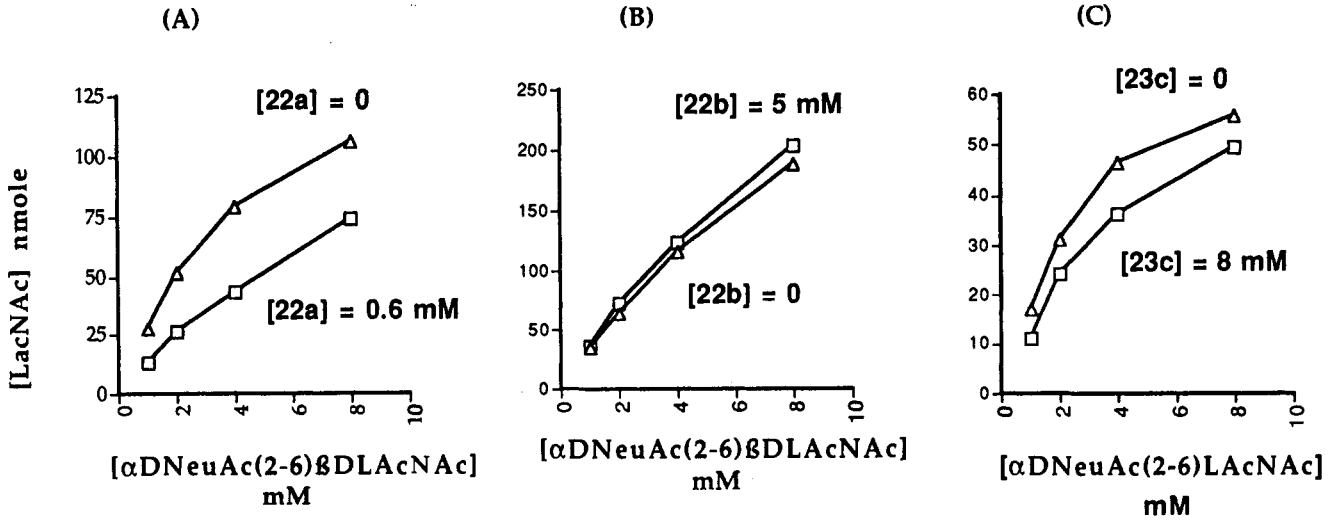

Figure 6. The hydrolysis of αDNeuAc(2-6)βDLAcNAc (**25**) by influenza A neuraminidase in the absence (△) and in the presence (□) of thiosialosides inhibitors **23c**, **22a**, and **22b**.

influenza strains and in a bacterial neuraminidase described very recently.[53] On this basis and active site mutation experiments,[61] these arginines residues have been implicated as being essential for the catalytic function of the influenza neuraminidase. Recently, Chong *et al.* has proposed that the protonated[55] arginine-371 may be the amino acid involved in the protonation of the sialoside glycosidic oxygen.

When we superimposed the minimum energy model obtained for the sialoside analog **16a** on the sialic acid–neuraminidase complex by aligning the glycerol side chain, we could see that in the tg anti conformation, the NeuAc unit, even when maintained in the chair conformation, could fit (except for the interaction of the NeuAc carboxyl with tyr-410) into the protein binding pocket (Figure 11) in such a way that the aglycon galactose projected out of the binding pocket and into the solvent. Similar facile fit into the binding site was seen when **16a** was maintained

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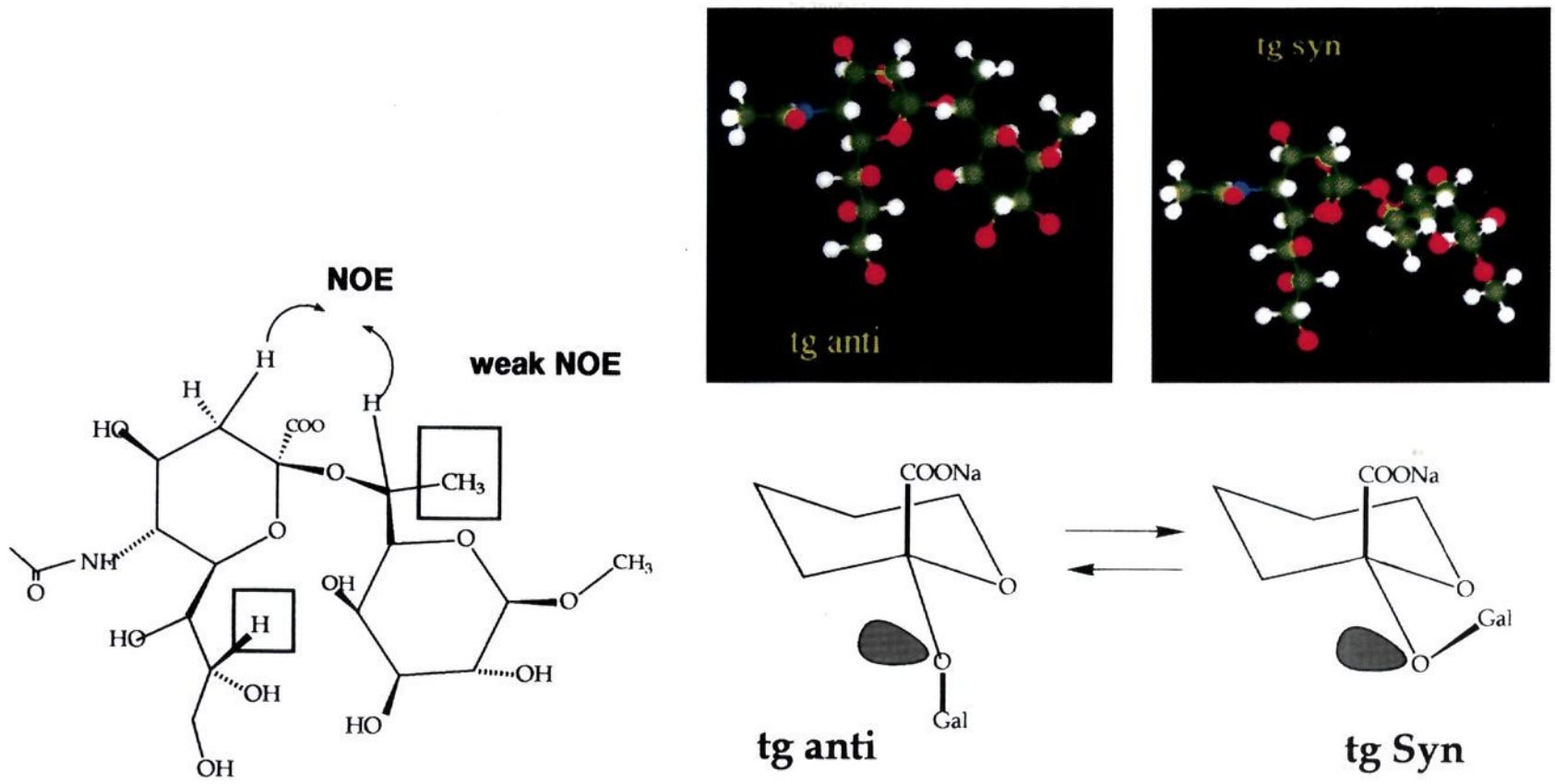

Figure 7. The calculated minimum energy structures for the sialoside analog **16a** in the anti (A) and syn (B) orientation. In the tg anti orientation, the H-3′ax of NeuAc unit and H-6 of galactose (indicated by arrows) are within 2.2 Å, whereas in the tg syn orientation the H-8′ of NeuAc and the H-7 of the galactose (indicated by boxes) are within 2.7 Å distance.

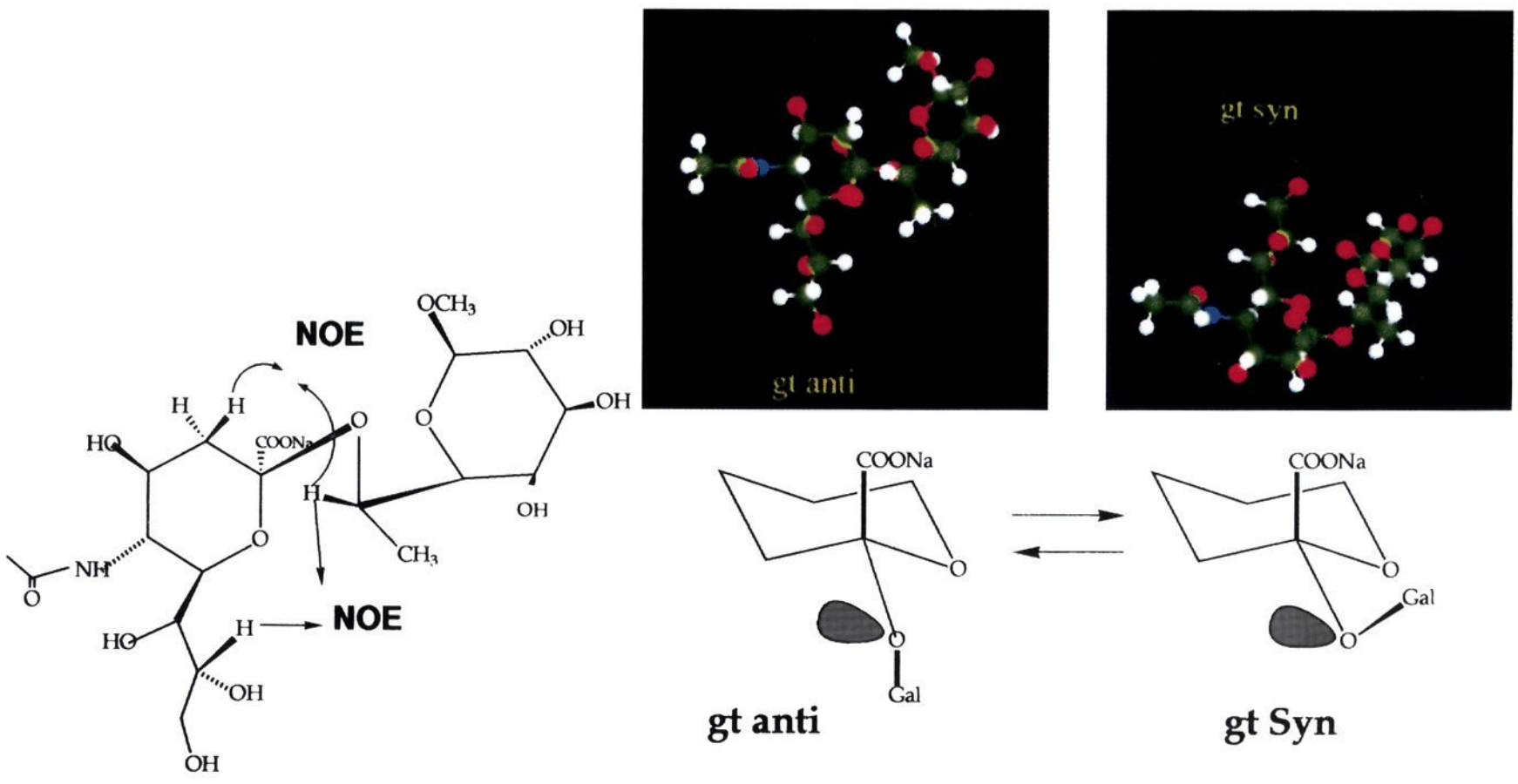

Figure 8. The calculated minimum energy structures for the sialoside analog **16b** in the anti (A) and syn (B) orientation. In the gt anti orientation, the H-3′ax of NeuAc unit and H-6 of galactose (indicated by bent arrows) are within 2.2 Å, whereas in the gt syn orientation, the H-8′ of NeuAc and the H-6 of the galactose (indicated by straight line arrows) are within 3.1 Å distance.

in the tg syn conformation (stereoview provided in the supplementary material, Figure 7). Further twisting of the NeuAc unit of **16a** into a boat conformation along the position occupied by free α-sialic acid placed the carboxyl carbon away from the tyrosine but close to the arginine triad for establishing salt bridges. This could be accomplished without encountering any unfavorable interaction between the galactose and the protein atoms. In contrast, the placement of **16b** in the binding pocket of influenza neuraminidase and in the gt syn conformation caused severe steric repulsions between the atoms in the galactose unit, especially the O-3, O-4, and the C-7 atoms and the two arginines (arg-292 and -371) in the catalytic site (Figure 12). For example, the O-4 gal to arg-292 distance was 1.4 Å, O-3 gal to arg-292 was 0.87 Å, and C-7 to arg-371 was 1.9 Å in the complex. Similarly in the gt anti conformation, the galactose unit encountered severe steric repulsions with arginines 371 and 118 (stereoview provided in supplementary material, Figure 8)). This unfavorable interaction with the catalytic arginines may interfere with the formation of salt bridges between the NeuAc carboxyl group of **16b** and the arginine triad and may seriously affect the transport of protons to the glycosidic oxygen; the low V_{max} for the **16b** in neuraminidase hydrolysis may be a reflection of this feature.

The steric environment around the NeuAc carboxyl unit may be equally important since it has been proposed that the NeuAc carboxylate may participate in the enzyme hydrolysis by stabilizing the neuraminyl cation.[62,63] The stabilization of the glycosyl

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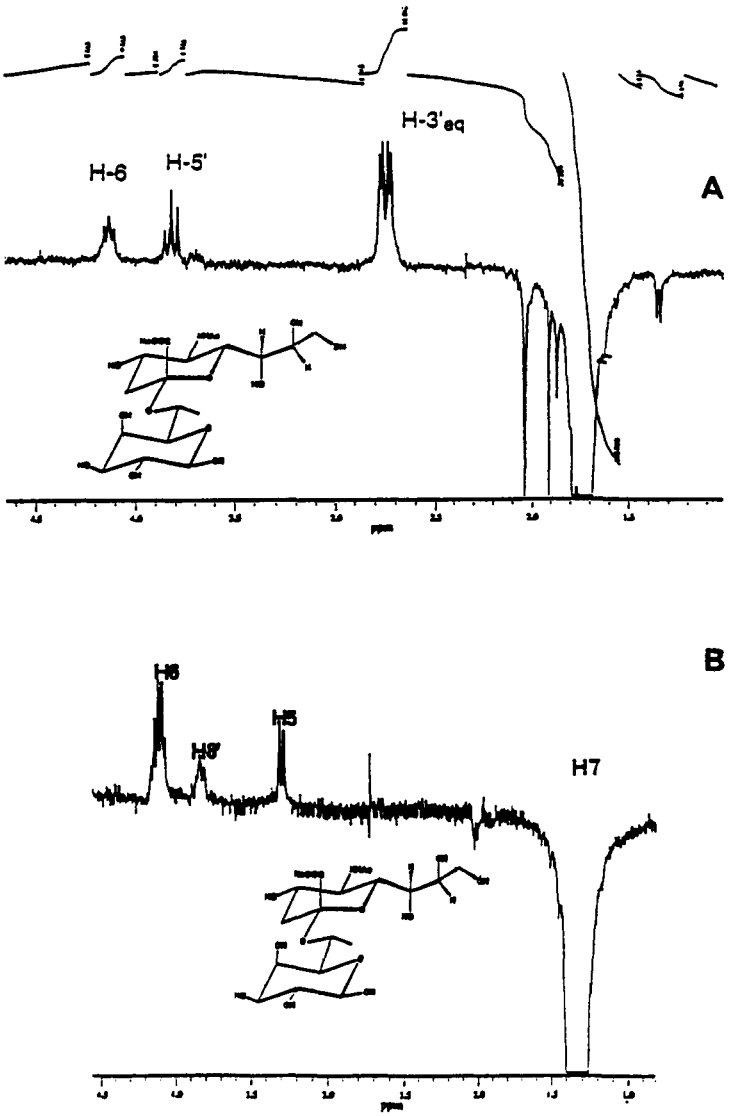

Figure 9. The NOE difference spectra at 300 MHz for the sialoside **16a**. Panel A shows the saturation of signals for H-3′ax of NeuAc unit and the NOEs obtained to the intraring hydrogens H-3′eq and H-5′ and to the interring hydrogen H-6. Panel B shows saturation of signals for H-7 of the Gal unit and the observation of NOEs for intraring hydrogens H-6 and H-5 and to the interring hydrogen H-8′ of the NeuAc unit.

cation by the carboxylate group present in the substrate instead of the enzyme appears to be a unique feature of sialosides. This contrasts with the conventional enzymatic glycoside hydrolysis where a protein carboxylate anion in the active site has been implicated in the stabilization of the glycosyl cations.[64] Even though there is an aspartic acid in the active site of the neuraminidase, its direct participation in sialoside hydrolysis is unlikely, as it is placed on the same side as the glycosidic oxygen and which will lead to the formation of β-sialic acid. The NMR evidence has clearly established the formation of the α-sialic acid as the initial neuraminidase hydrolysis product.[55] Therefore, the retention of configuration must arise as result of the participation of the NeuAc carboxylate group.[62] To enable the facile participation of the carboxyl group, the placement of the bulky aglycon in a sterically favorable orientation, namely in the anti conformer as seen in Figure 7A or 8A, may be desired.

On the basis of the arguments presented above, it is likely that the natural 2,6-sialosides, such as **23a** or the NeuAc units linked to the 6-position of galactose of glycoproteins, may fit better in the enzyme active site with the C-5–O-6 arm of the galactose maintained in the tg rotamer orientation rather than the gt rotamer found in the unbound state.[58] Other than this indirect role, the galactose (except for the C-6 atoms) residue does not appear to have a direct role either in the binding or in enzyme catalysis, as borne out by the facile hydrolysis of the isopropylidene derivatives **19a**, and **23b**.

Based on the analogy with **16a** and **16b**, only the sulfur analog **22a** and not **22b** should fit well into the enzyme active site. This is borne out by the fact that **22a** was the only good inhibitor of influenza A and other bacterial neuraminidases. [Recently Crennel *et al.* reported[53] the crystal structure of a bacterial neuraminidase from *Salmonella typhimurium* LT2. Even though this bacterial enzyme shows only 15% sequence similarity to the influenza A neuraminidase, it contains remarkably similar amino acids at the enzyme active site as the influenza neuraminidase, especially around the C-1 and C-2 of the 2,3-dehydro-neuraminic acid unit. Thus, a similar type of enzymatic mechanism may be involved in the cleavage of the neuraminic acids from the glycoconjugates by the viral enzymes investigated in the present work and this bacterial enzyme.] The lack of inhibition by the sulfur analog **23c** was indeed surprising

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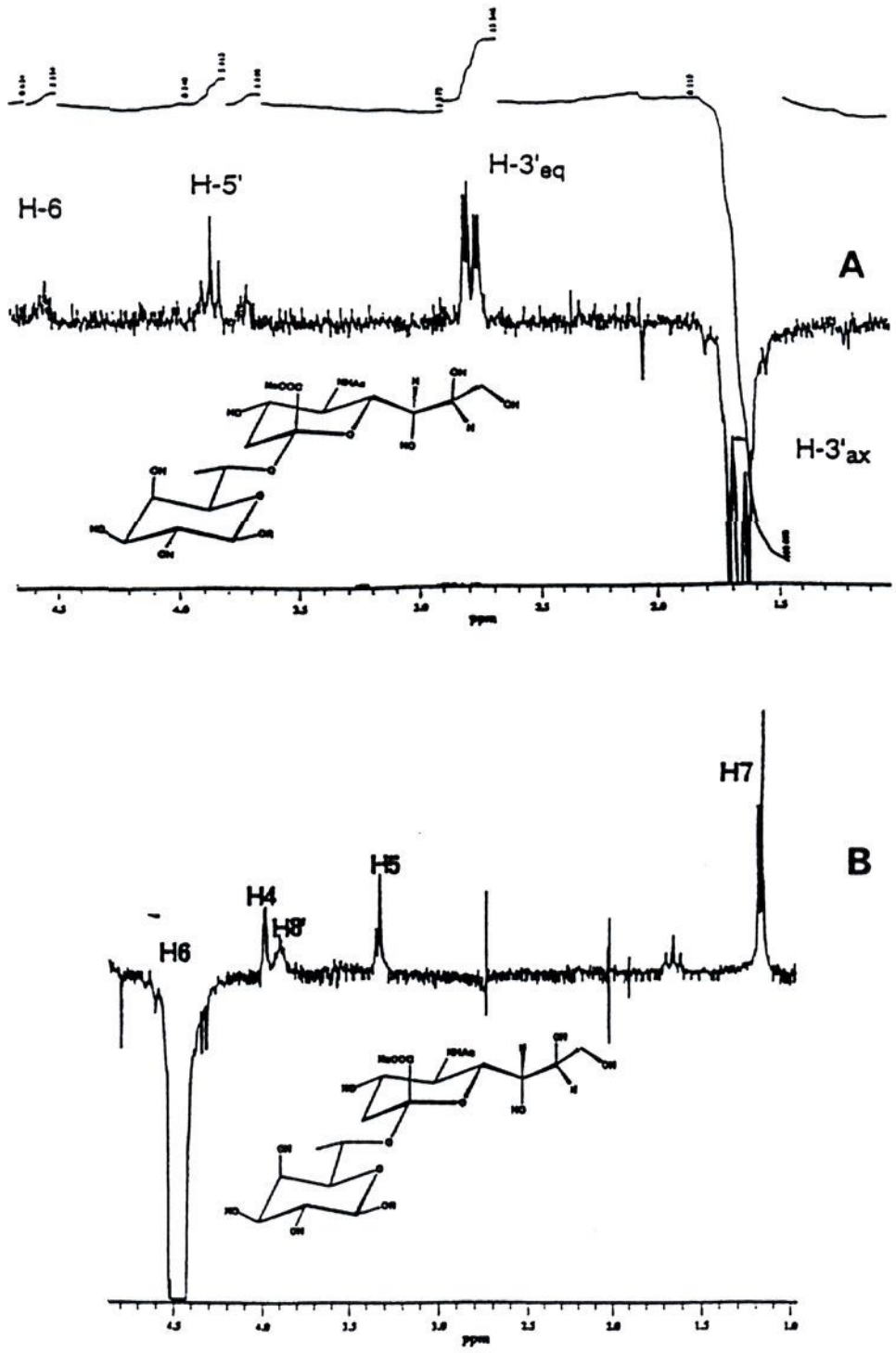

Figure 10. The NOE difference spectra at 300 MHz for the sialoside **16b**. Panel A shows the saturation of signals for H-3′ax of NeuAc unit and the NOEs obtained to the intraring hydrogens H-3′eq and H-5′ and to the interring hydrogen H-6. Panel B shows saturation of signals for H-6 of the Gal unit and the observation of NOEs for intraring hydrogens, H-7, H-5, and H-4 and to the interring hydrogen H-8′ of the NeuAc unit.

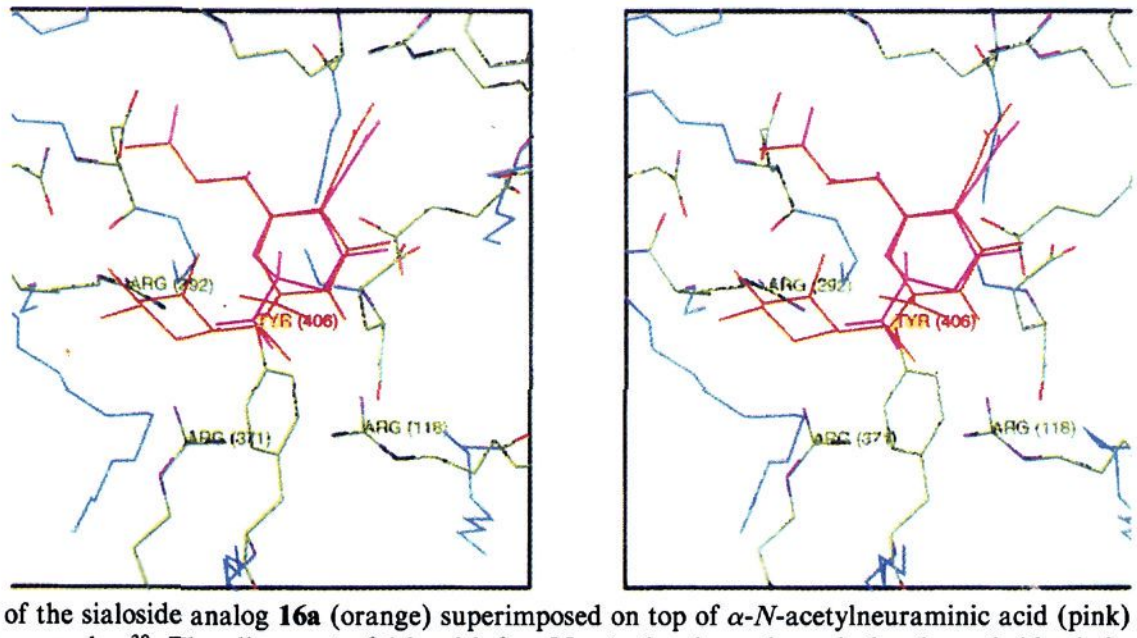

Figure 11. The stereoview of the sialoside analog **16a** (orange) superimposed on top of α-*N*-acetylneuraminic acid (pink) in the published structure of sialic acid–neuraminidase complex.[30] The alignment of **16a** with free NeuAc has been through the glycerol side chain C7–C-9. It is to be noted that the NeuAc unit of **16a** is in chair conformation as opposed to a boat structure found for free sialic acid. Twisting the chair of NeuAc unit of **16a** to a boat will bring the carboxyl group away from the tyr-406 and closer to the arg-371 and -118 for establishment of salt bridges and this can be accomplished without involving steric repulsion between the galactose and active site amino acids.

since the parent compound **23a** was bound and hydrolyzed well by all four neuraminidases. Also, an analog of **23c** carrying a ceramide at C-1 of galactose had been shown by Suzuki *et al.*[6] to be a good inhibitor of influenza neuraminidase (K_i = 0.1 mM; no inhibition data has been reported for bacterial neuraminidases). These compounds, including an analog of **23c**, show preferential potent inhibition of membrane-bound neuraminidases such as influenze virus (as opposed to poor inhibition of membrane free

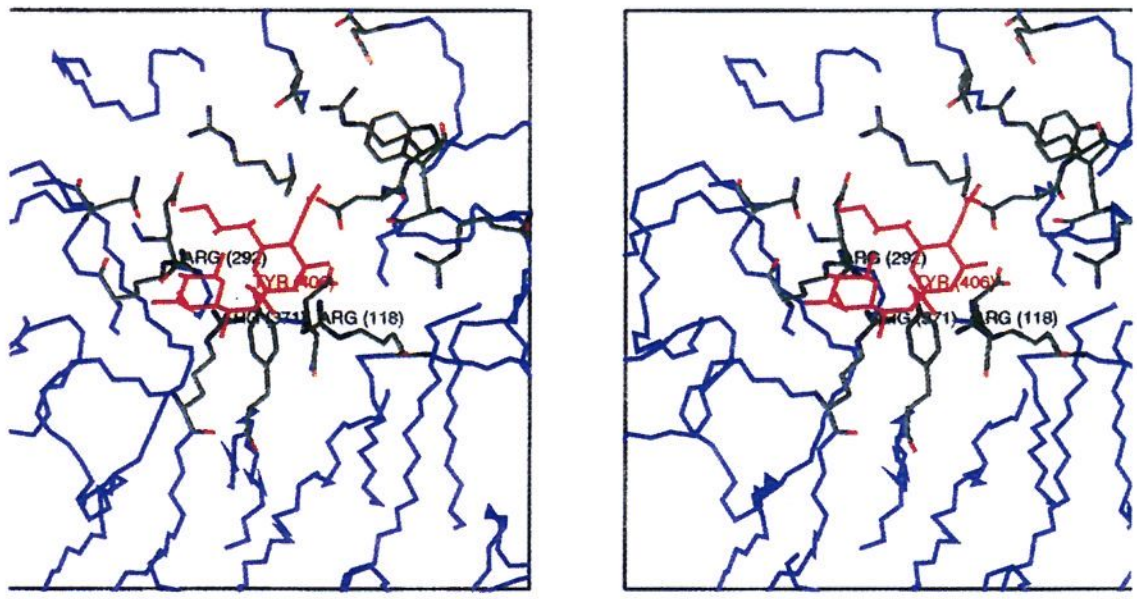

Figure 12. The stereoview of sialoside analog **16b** (red) at the active site of the neuraminidase.[30] The alignment of **16b** with free NeuAc (not shown) has been through the glycerol side chain C7–C-9. It is to be noted that the galactose unit of **16b** in gt syn conformation encounters severe nonbonded repulsions with Arg-292 and -371 that are required for the catalytic function of the enzyme.

bacterial neuraminidases). However, it is not clear whether the ceramide facilitates the anchoring of these compounds into viral membranes, or whether the ceramide changes the conformational properties around the sialoside linkages. We plan to explore the role of ceramide in influenza neuraminidase inhibition by incorporating it into the already good inhibitor **22a**.

Summary. We have reported here that unexpected conformational properties of certain sialoside structures may play an important role in the recognition by neuraminidases and in this regard our efforts to design common inhibitors for influenza hemagglutinin and neuraminidase based on this new information. Preliminary enzyme active site docking studies show that **16a**, **16b**, **22a**, and **22b** fit into the published hemagglutinin binding pockets.[11] Thus, an analog of **16a** and **22a** or their derivatives may become the first common inhibitors on influenza HA and neuraminidases. Further refinement in structures such as the 4-amino or guanidino sialic acid derivatives reported recently by von Itzstein *et al.*[2] may be required for developing tighter binding and truly useful anti influenza drugs.

Experimental Section

All the reagents were purchased from Aldrich Chemical Co. (St. Louis, MO). Thin-layer chromatography was performed on precoated plates of silica gel 60 F_{254} (EM Science), and the spots were visualized with a spray containing 5% sulfuric acid in ethanol, followed by heating. Column chromatography was done on silica gel 60 (230–400 mesh, EM Science). ^{1}H NMR spectra were recorded at 300, 500, or 600 MHz (GE Omega-300, GE Omega 500, or Bruker AM-500, AMX-600) and the ^{13}C NMR spectra were recorded with the above instruments operating at 75.48 or 125.74 MHz (300 and 500 MHz, respectively for proton). The hydrogen and carbon chemical shifts [The hydrogen and carbon atoms are numbered from the reducing end units. Thus, the galactose unit atoms in **16a** or **16b** are denoted by 1 to 7 and the NeuAc unit atoms are denoted by 1′ to 9′.] in organic solvents are expressed relative to tetramethylsilane (TMS). For solutions of compounds in deuterium oxide or deuterated methanol, the hydrogen chemical shift values are expressed relative to the HOD signal (4.75 ppm at 296 K, internal acetone 2.23 ppm), and the carbon chemical shifts are expressed relative to external TMS using the deuterium lock of the spectrometer, which set the chemical shifts of 1,4-dioxane at 66.9 ppm. Carbon-13 spin–lattice relaxation time (T_1) measurements, 1-D NOE difference spectroscopy, and DQFCOSY experiments were performed as described earlier.[51] Monte Carlo simulations and calculations of minimum energy conformations were carried out using the GEGOP program[50] as described earlier.[24] The GEGOP program uses a modified HSEA force field and a Metropolis algorithm for the Monte Carlo simulations. The details of the docking of the minimum energy sialoside structures into the neuraminidase binding pocket will be described elsewhere.

6-*O*-Allyl-7-deoxy-1,2;3,4-di-*O*-isopropylidene-α-D-*glycero*-D-*galacto*-heptopyranose (3a) and 6-*O*-allyl-7-deoxy-1,2;3,4-di-*O*-isopropylidene-β-L-*glycero*-D-*galacto*-heptopyranose (3b). 1,2;3,4-Di-*O*-isopropylidene-1,6-dialdohexapyranose[32] (71.5 g) was reacted with methyl magnesium iodide according to Lemieux *et al.*[26] to get a syrup (71.6 g). This was dissolved in anhydrous dimethylformamide (DMF, 800 mL). Sodium hydride (6.3 g) followed by allyl bromide (23 mL) was added, and the reaction mixture was stirred at room temperature for 24 h. Another portion of sodium hydride (3.5 g) and allyl bromide (10 mL) was added, and the reaction was continued for 3 more days. The reaction mixture was quenched with methanol (5 mL), and the solvents were evaporated. The residue was dissolved in dichloromethane and washed with water, ice cold hydrochloric acid, and saturated sodium bicarbonate solution. The solvent was evaporated, and the two major products were separated by chromatography on a column of silica gel using ethyl acetate–hexane (1:15 in the beginning and 3:8 at the end), as column eluant. The less polar component was identified as 6-*O*-allyl-7-deoxy-1,2;3,4-di-*O*-isopropylidene-α-D-*glycero*-D-*galacto*-heptopyranose (**3a**, TLC, ethyl acetate–hexane = 3:8; R_f 0.56, 15.2 g), whereas the more polar product R_f 0.50) was established as 6-*O*-allyl-7-deoxy-1,2;3,4-di-*O*-isopropylidene-β-L-*glycero*-D-*galacto*-heptopyranose (**3b**) (28.1 g).

3a: $[\alpha]^{25}_D$ −40 ± 2° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) ∂ 5.94 (m, –C*H*=C), 5.51 (d, *J* = 4.9 Hz, H-1), 5.31–5.11 (m, CH_2=C), 4.57 (dd, *J* = 2.4, 8.2 Hz, H-3), 4.45 (dd, *J* = 1.8, *J* = 8.2 Hz, H-4), 4.26 (dd, *J* = 2.4, 4.9 Hz, H-2), 4.15–3.99 (m, O-CH_2-C=), 3.64 (d × quartet, *J* = 6.1, 9.2 Hz, H-6), 3.53 (dd, *J* = 1.5, 9.2 Hz, H-5), 1.51, 1.44, 1.34 and 1.31 (isopropylidene methyls), 1.24 (d, *J* = 6.1 Hz, H-7); ^{13}C NMR ($CDCl_3$) ∂ 135.4, 116.5, 108.7, 108.4, 96.43, 72.8, 70.9, 70.7, 70.6, 70.4, 70.3, 26.04, 25.97, 24.9, 24.3, 17.2. Anal. Calcd for $C_{16}H_{26}O_6$: C, 61.15; H, 8.28. Found: C, 61.10; H, 8.32.

3b: $[\alpha]^{25}_D$ −73.5 ± 2° (*c* 1.03, $CHCl_3$); ^{1}H NMR ($CDCl_3$) ∂ 5.94 (m, C*H*=C), 5.57 (d, *J* = 4.9 Hz, H-1), 5.31–5.11 (m, CH_2=C), 4.56 (dd, *J* = 2.4, 8.2 Hz, H-3), 4.28 (dd, *J* = 2.4, 5.2 Hz, H-2), 4.22 (dd, H-4), 4.20–4.05 (m, OCH_2-C=), 3.68 (m, H-5 and H-6), 1.53, 1.44 and 1.32 and 1.31 (isopropylidene methyls), 1.24 (d, *J* = 6.1 Hz, H-7); ^{13}C NMR ($CDCl_3$) ∂ 135.8, 116.1, 109.1, 108.5, 96.4, 74.5, 72.2, 71.4, 70.9, 70.8, 70.4, 26.0, 25.9, 24.9, 24.4, 16.6. Anal. Calcd for $C_{16}H_{26}O_6$: C, 61.15, H, 8.28; Found: C, 61.0; H, 8.27.

2-(Trimethylsilyl)ethyl 2,3,4-Tri-*O*-benzyl-7-deoxy-β-D-*glycero*-D-*galacto*-heptopyranoside (11a). To an ice cold solution of **3a** (11.2 g) in dichloromethane (150 mL), 90% aqueous trifluoroacetic acid (75 mL) was added. After 60 min, the solvents were evaporated, and the residue was dissolved in acetic anhydride (75 mL) and pyridine (100 mL) containing 4-(*N*,*N*-dimethylamino)pyridine (10 mg). After 16 h, the reaction mixture was poured over ice, and the product was extracted with dichloromethane. The dichloromethane layer was washed with ice cold hydrochloric acid (1 M) and saturated sodium bicarbonate solution. The solution was dried over anhydrous magnesium sulfate, and the solvent was evaporated to get a syrup (compound **6a**, 11 g). A portion of the syrup (5 g) was dissolved in DMF (100 mL) containing acetic acid (0.9 g) and hydrazine (0.5 g) and heated to 50 °C for 2 h under dry nitrogen. The solvent was then evaporated, and the residue was dissolved in dichloromethane. The dichlormethane layer was washed with ice cold hydrochloric acid (0.5 M) and saturated sodium bicarbonate solution. The solution was dried over anhydrous magnesium sulfate, and the solvent was evaporated to get a syrup (3.8 g).

The above syrup (3.8 g) was dissolved in dichloromethane (100 mL). 2,4,6-Trimethylpyridine (s-collidine, 1.3 g) and Vilsmeier bromide (4.8 g) were added and stirred for 2 h. The reaction mixture was then washed with water, ice cold hydrochloric acid (0.5 M), and saturated sodium bicarbonate solution. The solution was dried over anhydrous magnesium sulfate, and the solvent was evaporated. Purification by chromatography on a column of silica gel using ethyl acetate–hexane (1:5) gave pure 6-*O*-allyl-2,3,4-tri-*O*-acetyl-7-deoxy-α-D-*glycero*-D-*galacto*-heptopyranosyl bromide (**7a**, 2.9 g): ^{1}H NMR ($CDCl_3$) ∂ 6.71 (d, H-1), 5.79 (m, –C*H*=C), 5.74 (d, H-4), 5.38 (dd, *H*-3), 5.26–5.13 (m, CH_2=C), 5.02 (dd, H-2), 4.03 and 3.76 (m, O-CH_2-C=), 4.00 (dd, H-5), 3.55 (m, H-6), 2.14, 2.11 and 2.00 (3 s, CH_3COO), 1.20 (d, H-7).

To a cold solution (–28 °C) of 2-(trimethylsilyl)ethanol (9.1 g) in nitromethane (100 mL) containing silver trifluoromethanesulfonate (2.0 g) and s-collidine (0.65 g), a solution of **7a** (2.9 g) in dichloromethane (30 mL) was added in drops. After 1 h at –28 °C, the reaction mixture was diluted with dichloromethane and filtered. The filtrate was washed with water, 5% sodium thiosulfate solution, ice cold hydrochloric acid (0.5 M), and saturated sodium bicarbonate solution. The solvent was removed under reduced pressure to give a homogeneous syrup (3.3 g), which was identified by NMR as 2-(trimethylsilyl)ethyl 2,3,4-tri-*O*-acetyl-7-deoxy-β-D-*glycero*-D-*galacto*-heptopyranoside (**8a**): ^{1}H NMR ($CDCl_3$) ∂ 5.78 (m, 1H, –C*H*=C–), 5.61 (dd, *J* = 1.0, 3.4 Hz, H-4), 5.25–5.10 (m, 3H, CH_2=C, H-2), 5.00 (dd, *J* = 3.4, 10.4 Hz, H-3), 4.44 (d *J* = 7.9 Hz, H-1), 4.1–3.5 (m, 5H, O-CH_2 of aglycon, O-CH_2-C=, H-6), 3.34 (dd, *J* = 1.1, 8.8 Hz, H-5), 2.13, 2.03, and 1.97 (3 s, CH_3COO), 1.25 (d, *J* = 6.0 Hz, H-7). Compound **8a** was dissolved in dry methanol (50 mL) containing sodium methoxide solution (0.5 M, 0.8 mL) and stirred for 16 h. The reaction mixture was then neutralized with H^+ resin, filtered, and evaporated to get a solid (2.6 g). This was dissolved in dry DMF (50 mL) containing sodium hydride (1.2 g) followed by the addition of benzyl bromide (8.5 g). After 2 h, methanol (3 mL) was added, and the solvent was evaporated. The residue was extracted with dichloromethane and washed with water, ice cold hydrochloric acid (0.5 M), and saturated sodium bicarbonate solution. Purification by chromatography on a column of silica gel using ethyl acetate–hexane (1:12) gave pure 2-(trimethylsilyl)ethyl 6-*O*-allyl-2,3,4-tri-*O*-benzyl-7-deoxy-β-D-*glycero*-D-*galacto*-heptopyranoside (**10a**, 3.1 g): ^{1}H NMR ($CDCl_3$) ∂ 7.4–7.2 (m, aromatic hydrogens), 5.86 (m, –C*H*=C), 5.26–5.10 (4 m, 2H, CH_2=C), 5.04–4.61 (benzylic hydrogens), 4.34 (d, *J* = 7.7 Hz, H-1), 4.14 (d, *J* = 2.3 Hz, H-4), 4.1–3.5 (O-CH_2 of aglycon, O-CH_2C=, H-3, H-2 and H-6), 3.07 (dd, *J* = 0.7, 8.9 Hz, H-5), 1.24 (d, *J* = 6.0 Hz, H-7), 1.04 (dd, CH_2Si).

A solution of **10a** (8.45 g) in dry tetrahydrofuran (THF, 100 mL) containing 1,5-cyclooctadienebis(diphenylmethylphosphine)iridium hexafluorophosphate (300 mg) was gently evacuated and equilibrated under nitrogen pressure twice followed by brief exposure to hydrogen for 10 min. The reaction mixture was stirred under nitrogen atmosphere for 16 h. The solvent was evaporated, and the residue was dissolved in 90% aqueous acetonitrile (220 mL) containing lithium bromide (7.2 g) and acetonitrile washed H^+ resin (7.2 g). After 15 min, the solution was filtered, neutralized with triethylamine, the solvent was evaporated, and the residue was dissolved in dichloromethane. The dichloromethane was washed with water, ice cold hydrochloric acid (0.5 M), and saturated sodium bicarbonate solution, dried over anhydrous magnesium sulfate, and evaporated. The product was purified on a column of silica gel using ethyl acetate–hexane (1:6) as eluant to get pure **11a** (4.5 g): $[\alpha]^{25}_D$ –23.3 ± 2° (*c* 0.97, $CHCl_3$); ^{1}H NMR ($CDCl_3$) ∂ 7.40–7.28 (m, aromatic hydrogens), 5.01–4.72 (six benzylic hydrogens, 4.33 (d, *J* = 7.7 Hz, H-1), 4.01–3.5 (6 H, O-CH_2- of aglycon, H-2, H-4, H-6 and H-3), 2.87 (dd, *J* = 0.7, 8.3 Hz, H-5), 1.18 (d, *J* = 6.3 Hz, H-7), 1.03 (m, CH_2-Si); ^{13}C NMR ($CDCl_3$) ∂ 138.9, 138.5, 128.8, 128.5, 128.4, 128.2, 128.1, 127.6, 127.5, 103.5, 82.5, 79.7, 78.3, 75.1, 73.8, 73.3, 71.3, 67.0, 65.4, 20.3, 18.3, –1.5. Anal. Calcd for $C_{33}H_{44}O_6Si$: C, 70.28, H, 7.80; found: C, 69.97; H, 7.92.

2-(Trimethylsilyl)ethyl 2,3,4-Tri-*O*-benzyl-7-deoxy-α-L-*glycero*-D-*galacto*-heptopyranoside (11b). Compound **3b** (34.5 g) was converted to **7b** via **6b** (yield of **6b** from **3b** was 20.5 g; 13.8 g of this was converted to **7b**) according to the procedure described earlier for **7a**. Purification of the crude product by chromatography on a column of silica gel using ethyl acetate–hexane (1:5) gave pure 6-*O*-allyl-2,3,4-tri-*O*-acetyl-7-deoxy-β-L-*glycero*-D-*galacto*-heptopyranosyl bromide (**7b**, 11.4 g): ^{1}H NMR ($CDCl_3$) ∂ 6.76 (d, *J* = 4.2 Hz, H-1), 5.87 (m, –C*H*=C–), 5.52 (br d, *J* = 2.2 Hz, H-4), 5.36 (dd, *J* = 3.2, 10.7 Hz), H-3), 5.28–5.14 (m, CH_2=C), 5.04 (dd, *J* = 3.9, 10.5 Hz, H-2), 4.14 (d, *J* = 7.3 Hz, H-5), 4.03 (m, O-CH_2-C=), 3.64 (m, H-6), 2.13, 2.11 and 2.03 s, CH_3COO), 1.11 (d, *J* = 6.6 Hz, H-7).

Compound **7b** (11.4 g) was converted to **8b** according to the procedure described for **8a**. Purification on a column of silica gel using ethyl acetate–hexane (1:4) as eluant, gave pure 2-(trimethylsilyl)ethyl 6-*O*-allyl-2,3,4-tri-*O*-acetyl-7-deoxy-α-L-*glycero*-D-*galacto*-heptopyranoside (**8b**, 9.8 g): ^{1}H NMR ($CDCl_3$) ∂ 5.89 (m, C*H*=C) 5.36 (dd, *J* = 0.8, 3.4 Hz, H-4), 5.3–5.1 (m, CH_2=C), 5.18 (dd, *J* = 8.0, 10.4 Hz, H-2), 4.97 (dd, *J* = 3.4, 10.4 Hz, H-3), 4.45 (d, *J* = 8.0 Hz, H-1), 4.15 (m, *O*-CH_2)-C=), 4.0–3.6 (m, O-CH_2 of aglycon, H-6), 3.50 (dd, *J* = 0.9, 7.9 Hz, H-5), 2.14, 2.04 and 1.96 (3 s, CH_3COO), 1.08 (d, *J* = 6.3, H-7).

Compound **8b** (9.8 g) was converted to **11b** as described earlier for **11a**. The product was purified on a column of silica gel using ethyl acetate–hexane (1:4) as eluant to get pure **11b** (4.5 g): $[\alpha]^{25}_D$ –5.0 ± 2° (c 1.04, $CHCl_3$). ^{1}H NMR ($CDCl_3$) ∂ 7.4–7.2 (m, aromatic hydrogens), 5.00–4.63 (benzylic hydrogens), 4.37 (d, *J* = 7.8 Hz, H-1), 4.0 and 3.58 (m, O-CH_2- of aglycon and H-6), 3.83 (dd, *J* = 7.8, 9.8 Hz, H-2), 3.77 (d, *J* = 2.2 Hz, H-4), 3.51 (dd, *J* = 2.9, 9.8 Hz, H-3), 2.97 (d, *J* = 7.6 Hz, H-5), 1.04 (m, CH_2-Si), 0.87 (d, *J* = 6.4 Hz, H-7); ^{13}C NMR ($CDCl_3$) ∂ 138.7, 138.4, 138.1, 128.5, 128.4, 128.3, 128.2, 128.1, 127.8, 127.6, 127.52, 127.49, 103.6, 82.9, 79.5, 78.8, 75.1, 74.1, 73.54, 73.48, 67.5, 66.7, 18.5, 17.7, –1.5. Anal. Calcd for $C_{33}H_{44}O_6Si$: C, 70.28; Found: C, 69.97; H, 7.92.

Methyl 5-Acetamido-3,5-dideoxy-4,7,8,9-tetra-*O*-acetyl-2-thiomethyl-α-D-*glycero*-D-*galacto*-nonulopyranosidonate (12b). *N*-Acetylneuraminic acid (20 g) was converted to methyl 5-acetamido-3,5-dideoxy-2,4,7,8,9-penta-*O*-acetyl-β-D-*glycero*-D-*galacto*-nonulopyranosylate (31.3 g, crude) according to the literature methods.[65,66] A portion of this (5.0 g) was dissolved in glacial acetic acid (50 mL) containing acetic anhydride (2 mL), and anhydrous HCl gas was gently bubbled (45 min) into the solution. After 16 h, the reaction mixture was worked up, and the crude product (4.8 g) was dissolved in acetonitrile (50 mL) containing sodium thiomethylate (0.82 g). After 2 h, the solvent was evaporated, and the residue was dissolved in CH_2Cl_2 and washed with water, ice cold 1 M HCl and saturated sodium bicarbonate solution. The crude product **12b** (4.3 g) obtained by this short procedure was greater than 90% pure as evidence from the ^{1}H NMR spectrum. The NMR parameters were identical to those published by Hasegawa *et al.*[38] This crude product was employed in subsequent glycosylation reactions.

Sodium Salt of 5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonate(2–6)-7-deoxy-β-D-*glycero*-D-*galacto*-heptopyranosyl-OCH_2CH_2Si(CH_3)$_3$ (16a). Methylsulfenyl bromide solution in 1,2-dichloromethane (1 M, 2.4 mL) was added in drops to a cold (–50 °C) solution of **11a** (601 mg), **12b** (1.12 g), and silver trifluoromethanesulfonate (590 mg) in 5:3 mixture of acetonitrile–propionitrile (40 mL) containing powdered 3Å molecular sieves (2.1 g). After 18 h at –50 °C, the reaction mixture was warmed to room temperature followed by the addition of saturated sodium bicarbonate solution (3 mL). The reaction mixture was filtered through a pad of Celite, and the residue was washed with dichloromethane. The filtrate was washed with water, ice cold hydrochloric acid (0.5 M), and saturated sodium bicarbonate solution. The solvent was then evaporated, and the products were isolated by chromatography using ethyl acetate–hexane–ethanol (10:15:1) as eluant. About 260 mg of **11a** was recovered, and the products were eluted in the following order: β-Anomer of **14a** (60 mg), β + **14a** mixture (190 mg), **14a** (320 mg), and finally compound **13** (490 mg). This procedure was repeated to obtain a larger amount of **14a**. The structure of **14a** was confirmed by NMR: $[\alpha]^{25}_D$ +1.6 ± 2° (*c* 1.02, $CHCl_3$); ^{1}H NMR ($CDCl_3$) ∂ 7.37–7.25 (aromatic hydrogens), 5.30 (m, H-7′ and H-8′), 5.11 (d, *J* = 9.9 Hz, NH), 5.10–4.72 (6 d, benzylic hydrogens), 4.81 (m, H-4′), 4.31 (d, *J* = 7.7 Hz, H-1), 3.49 (dd, *J* = 2.6, 9.5 Hz, H-3), 3.42 (s, $COOCH_3$), 3.11 (d, *J* = 7.7 Hz, H-5), 2.64 (dd, *J* = 4.7, 12.8 Hz, H-3$_{eq}$), 2.14–1.88 (5 × s, 4 OAc and 1 NHAc), 1.97 (t, *J* = 12.5 Hz, H-3$_{ax}$), 1.33 (d, *J* = 6.2 Hz, H-7), 1.02 (dd, 2 H, –CH_2Si); ^{13}C NMR ($CDCl_3$) ∂ 170.9, 170.6, 170.2, 170.11, 170.08, 167.4, 139.3, 138.8, 138.6, 128.3, 128.2, 128.1, 128.0, 127.4, 127.0, 126.9, 103.7, 100.2, 82.5, 79.5, 77.2, 75.0, 73.9, 73.4, 73.0, 72.7, 72.1, 69.5, 69.0, 67.5, 67.3, 62.1, 52.8, 49.4, 39.1, 23.2, 21.0, 20.9, 20.8, 20.7, 18.9, 18.4, –1.5. Anal. Calcd for $C_{53}H_{71}O_{19}$-NSi: C, 60.4; H, 6.7, N, 1.3. Found: C, 60.46; H, 6.78; N, 1.41.

Compound **14a** (439 mg) was dissolved in 90% aqueous ethanol (80 mL) containing 20% palladium hydroxide on carbon (300 mg) and gently evacuated and equilibrated under hydrogen atmosphere. After 4 h, the reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated to a dry residue. This was dissolved in dry methanol (30 mL) and followed by the addition of sodium methoxide solution (0.5 M, 0.25 mL). After 4 h, the solution was neutralized with H^+ resin and

filtered; the filtrate was then evaporated to a dry residue. This was then redissolved in water and lyophilized to get a colorless material (254 mg). A portion of this material (244 mg) was dissolved in deionized water (10 mL) containing Chelex resin (sodium form, 100–400 mesh, 1.02 g) and stirred at room temperature for 24 h. Additional resin (1.1 g) and water (5 mL) were added, and the reaction was continued for additional 24 h. The resin was then filtered through a pad of celite, and the filtrate was lyophilized to get a residue (240 mg) which was homogeneous on TLC (ethyl acetate–ethanol–water = 4:2:1). This was dissolved in water and applied to a column of Bio Gel P-2 (200–400 mesh, 1400 mL), equilibrated, and eluted with deionized water. The fractions (7.5 mL) containing the product (as evidenced by U.V. absorption at 220 nm) were pooled and lyophilized to get a colorless product **16a** (224 mg): $[\alpha]^{25}_D$ −22.5 ± 2°, (*c* 0.99, H_2O); ^1H NMR (see Figure 2A, Table 1); ^{13}C NMR (see Table 2). Anal. Calcd for $C_{23}H_{42}O_{14}NSiNa\cdot 2H_2O$: C, 42.92; H, 7.15; N, 2.18. Found: C, 42.21; H, 7.21; N, 2.37.

Sodium Salt of 5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonate(2–6)-7-deoxy-α-L-*glycero*-D-*galacto*-heptopyranosyl-$OCH_2CH_2Si(CH_3)_3$ (16b). Methylsulfenyl bromide solution in 1,2-dichloroethane (1 M, 3.5 mL) was added in drops to a cold (−50 °C) solution of **11b** (895 mg), **12b** (1.68 g), and silver trifluoromethanesulfonate (885 mg) in 5:3 mixture of acetonitrile–propionitrile (40 mL) containing powdered 3Å molecular sieves (2.1 g). Workup and purification as described above for **14a** gave compound **14b** (530 mg), a mixture of **14b** and its β-anomer (190 mg), and compound **13** (870 mg). This procedure was repeated to obtain more **14b**. Its structure was confirmed by NMR: $[\alpha]^{25}_D$ −9.1 ± 2° (*c* 1.04, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.38–7.26 (aromatic hydrogens), 5.30 (m, 2 H, H-7′ and H-8′), 5.10 (d, *J* = 9.9 Hz, 1 H, NH-), 5.04–4.64 (6 × d, benzylic hydrogens), 4.89 (m, H-4′), 4.35 (d, *J* = 7.7 Hz, H-1), 4.22 (m, 1H, H-6), 3.77 (s, 3H, $COOCH_3$), 3.51 (dd, *J* = 2.6, 9.5 Hz, H-3), 3.25 (d, *J* = 7.7 Hz, H-5), 2.49 (dd, *J* = 4.8, 13.2 Hz, H-3′ eq), 2.13 (t, *J* = 12.5 Hz, $H\text{-}3_{ax}$), 2.12–1.88 (5 × s, 4 × OAc and NHAc), 1.03 (d, *J* = 6.2 Hz, H-7), 0.00 {$(CH_3)_3Si$}; ^{13}C NMR ($CDCl_3$) δ 170.9, 170.6, 170.2, 170.0, 169.8, 168.4, 138.9, 138.6, 138.5, 128.4, 128.3, 128.24, 128.18, 128.15, 129.1, 127.5, 127.50, 127.4, 103.3, 99.5, 83.4, 79.6, 76.2, 75.0, 73.9, 73.7, 73.5, 72.6, 70.7, 69.2, 68.8, 67.5, 67.0, 62.3, 52.8, 49.4, 36.7, 23.1, 21.0, 20.8, 18.3, 17.9, −1.5. Anal. Calcd for $C_{53}H_{71}O_{19}NSi$: C, 60.4; H, 6.7, N, 1.38. Found: C, 60.33; H, 6.80; N, 1.38.

Compound **14b** (955 mg) was converted to **16b** (514 mg) using the procedure described for **16a**. An analytical sample was prepared by gel filtration of a portion of the residue (118 mg) on a column of Bio Gel P-2 (200–400 mesh, 1400 mL), equilibrated, and eluted with deionized water. The fractions (7.5 mL) containing the product (as evidenced by UV absorption at 220 nm) were pooled and lyophilized to get a colorless product **16b** (101 mg): $[\alpha]^{25}_D$ −28.6 ± 2°, (*c* 1.0, H_2O); ^1H NMR (see Figure 2B, Table 1); ^{13}C NMR (see Table 2). Anal. Calcd for $C_{23}H_{42}O_{14}NSiNa\cdot 2H_2O$: C, 42.92; H, 7.15; N, 2.18. Found: C, 42.46; H, 7.38; N, 2.17.

Sodium *Salt* of 5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonate(2–6)-7-deoxy-α,β-D-*glycero*-D-*galacto*-heptopyranose (16c). To a solution of **8a** (crude, obtained from 8.9 g of **7a**) in methanol (200 mL) was added sodium methoxide solution (0.5 M, 6 mL), and the solution was stirred at room temperature for 2 h. In addition to the *O*-acetate groups, the 2-(trimethylsilyl)ethyl group was partially removed. The reaction mixture was worked up and benzylated as described for **10a**. Purification by chromatography using ethyl acetate–hexane (1:10) gave **10a** (8.5 g) and **10c** (3.7 g): ^1H NMR **10c** ($CDCl_3$) δ 7.35 (m, aromatic hydrogens), 5.86 (m, CH═C), 5.30-5.15 (m, C═CH_2), 4.95 (d, H-1), 3.66 (dd, H-2), 3.53 (m, H-6), 1.25 (d, H-7).

The *O*-allyl group of **10c** (3.7 g) was removed, and the product **11c** (2.7 g) was purified as described for **11a**: $[\alpha]^{25}_D$ +41.8 ± 2°, (*c* 1.1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.42–7.25 (aromatic hydrogens), 4.98 (d, *J* = 4.0 Hz, H-1), 4.82–4.38 (benzylic hydrogens, H-4), 4.40 (m 2 H, H-5 and H-3), 3.91 (m, H-6), 3.47 (dd, *J* = 4.0 (dd, H-2), 1.22 (d, H-7).

Compound **11c** (554 mg, 1 mmol) was condensed with **12b** (1.04 g, 2 mmol) and purified as described for **14a**. In addition to the starting material **11c** (60 mg), this gave the pure α-sialoside **14c** (610 mg): $[\alpha]^{25}_D$ 20.4 ± 2° (*c* 0.9, $CHCl_3$); ^1H NMR ($CHCl_3$) δ 7.42–7.22 (aromatic hydrogens), 5.40 (m, H-8′), 5.32 (dd, *J* = 1.8, 8.8 Hz, H-7′), 5.13 (d, *J* = 9.4 Hz, N*H*), 4.90 (d, *J* = 3.8 Hz, H-1), 4.88 (m, H-4′), 4.87–4.22 (m, 8 H, benzylic hydrogens), 3.80 (s, $COOCH_3$), 2.59 (dd, H-3′eq), 2.10, 2.07, 2.02, 1.96 and 1.87 (5 × s, CH_3COO), 1.40 (d, H-7). Compound **14c** was converted to **16c** (270 mg) as described for **16a**. The complete proton and carbon chemical shift assignments are provided in Tables 1 and 2 of the supplementary material.

Sodium Salt of 5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonic acid(2–6)-1,2;3,4-di-*O*-isopropylidene-7-deoxy-α-D-*glycero*-D-*galacto*-heptopyranose (19a). Methylsulfenyl bromide solution (1 M in 1,2-dichloroethane, 3 mL) was added to a suspension of **2a** (548 mg, 2 mmol), glycosyl xanthate **12c**[39] (1.487 g, 2.5 mmol), silver triflate (642 mg), and 3Å molecular sieves (3.0 g) in 1:1 acetonitrile–propionitrile mixture (50 mL) at −50 °C. After 18 h at −50 °C, the reaction mixture was worked up as described for **14a**, and the product was purified by chromatography using ethyl acetate–ethanol–water (10:15:1) as eluant. The products were eluted in the following order: the starting alcohol **2a** (230 mg), the β-anomer of **17a** (50 mg), a mixture of β- and α-sialoside (400 mg), a 2:3 mixture of α-sialoside (**17a**) and glycal **13** {700 mg, the ratio of **17a** and **13** was determined by the integrations of NMR signals at 2.68 (H-3′eq) ppm for **17a** and 6.1 ppm (H-3′) for **13**, the α-anomeric configuration of **17a** was confirmed by identification of H-4′ signal at 4.83 ppm} and pure **13** (140 mg). The mixture of **17a** and **13** (700 mg) was de-O-acetylated with NaOMe (0.5 M, 0.2 mL) and methanol (20 mL, 36 h), and the crude product was applied to a column of Bio Gel P-2. The disaccharide **18a** which eluted first (218 mg) was converted to the sodium salt **19a** (219 mg) by Chelex resin as described earlier: $[\alpha]^{25}_D$ −26.9 ± 2°, *c* 1.0, H_2O); ^1H NMR (D_2O) δ 5.56 (d, *J* = 5.0 Hz, H-1), 4.65 (dd, *J* = 2.2, 7.9 Hz, H-3), 4.44 (dd, *J* = 1.5, 7.9 Hz, H-4), 4.40 (dd, *J* = 2.0, 5.0 Hz, H-2), 4.23 (m, H-6), 3.82–3.76 (m, 2H, H-8′, H-9′), 3.73 (t, *J* = 10.3 Hz, H-5′), 3.69 (dd, *J* = 1.3, 5.9 Hz, H-7′), 3.58 (m, H-4′), 3.56 (dd, H = 6.8, 12.6 Hz, H-9′), 3.48–3.52 (m, 2H, H-6′ and H-5), 2.69 (dd, *J* = 4.6 Hz, 12.5 Hz, H-3′eq), 1.96 (s, N*Ac*), 1.61 (t, *J* = 12.3 Hz, H-3′ax), 1.52, 1.39, 1.32 and 1.31 (4 × s, isopropylidene methyls), 1.23 (d, *J* = 6.4 Hz, H-7); ^{13}C NMR (D_2O) δ 174.9, 172.6, 109.61, 109.59, 101.3, 95.8, 72.4, 71.8, 70.6, 70.2, 70.1, 70.0, 69.8, 68.3, 67.9, 62.4, 51.6, 40.2, 24.9, 24.6, 23.9, 22.9, 21.9, 18.1. Anal. Calcd for $C_{24}H_{38}O_{14}NNa\cdot 3H_2O$: C, 44.92, 6.86; N, 2.18. Found: C, 44.00; H, 6.77; N, 2.13.

Sodium Salt of 5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonic acid(2–6)-1,2;3,4-di-*O*-isopropylidene-7-deoxy-α-D-*glycero*-D-*galacto*-heptopyranose (19b). To a suspension of **2b** (600 mg, 2.2 mmol), thioglycoside **12b** (1.14 g), and 3Å molecular sieves (1.5 g) in 2:1 acetonitrile–propionitrile mixture (24 mL) at −50 °C was added dimethyl(methylthio)sulfonium triflate (1.50 g) solution in CH_3CN (10 mL) over a period of 10 min. After 3 days at −35 °C, the reaction mixture was worked up as described for **14a**, and the product was purified by chromatography using CH_2Cl_2–MeOH (30:1) as eluant. After the starting material (420 mg), a homogeneous product (1.20 g) was eluted. ^1H NMR showed that the product was a mixture of the sialoside **17b** and the glycal **13** (**17b**:**13** = 15:4) along with minor β-sialosides. This mixture was treated with sodium methoxide (0.5 mL) in methanol (20 mL) and worked up after 18 h (yield: 670 mg). A portion (115 mg) of this was dissolved in deionized water (5 mL) and applied to a column of Bio Gel P-2, and fractions (7.5 mL) were collected. The disaccharide appeared in fractions 84–95. These fractions were pooled and lyophilized to obtain **18b** (34 mg), contaminated with minor amounts (<20%) of the β-anomer. This was then converted to the sodium salt **19b** as described earlier: $[\alpha]^{25}_D$ −35.1 ± 2°, (*c* 1.0, H_2O); ^1H NMR (D_2O) δ 5.80 (d, *J* = 5.1 Hz, H-1), 4.70 (dd, *J* = 2.4, 7.9 Hz, H-3), 4.47 (dd, *J* = 2.6, 5.1 Hz, H-2), 4.41 (dd, *J* = 1.8, 7.9 Hz, H-4), 4.35 (m, H-6), 3.82 (m, H-8′, H-9′), 3.78 (t, *J* = 9.7 Hz, H-5′), 3.62–3.50 (m, H-4′, H-6′, H-7′, H-9′ and H-5), 2.64 (dd, *J* = 4.6, 12.3 Hz, H-3′eq), 1.95 (s, N*Ac*), 1.63 (t, *J* = 12.1 Hz, H-3′ax), 1.51, 1.42 and 1.32 (3 × s, isopropylidene methyls), 1.12 (d, *J* = 6.2 Hz, H-7); ^{13}C NMR (D_2O) δ 175.3, 174.5, 110.3, 110.2, 110.14, 110.09, 99.6, 96.5, 72.9, 72.2, 71.2 (2 × C), 70.6, 70.0, 69.6, 68.8, 68.5, 62.7, 52.1, 40.8, 25.5, 25.2, 24.3, 23.8, 22.4, 16.7

Sodium Salt of 5-Acetamido-3,5-dideoxy-2-thio-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-*S*-6)-7-deoxy-D-*glycero*-D-*galacto*-heptopyranose (22a). To a solution of **2b** (7.8 g) in CH_2Cl_2 (150 mL) at 0 °C were added pyridine (10 mL) and trifluoromethanesulfonic anhydride (10.2 g), and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with dichloromethane and washed with water, ice cold 1 M hydrochloric acid, and saturated sodium bicarbonate solution. The solvent was evaporated, and the crude produce was dissolved in DMF (150 mL) containing potassium thioacetate (5.5 g, Janssen Chimica, New Brunswick, NJ) and stirred at room temperature for 18 h. The solvent was evporated, and the residue was dissolved in dichloromethane and washed with water, ice cold 1 M hydrochloric acid, and saturated sodium bicarbonate solution. Purification by chromatography on silica gel using ethyl acetate–hexane (1:12) afforded 6,7-dideoxy-1,2;3,4-di-*O*-isopropylidene-6-(acetylthio)-α-D-*glycero*-D-*galacto*-heptopyranose (**4a**, 5.6 g): ^1H NMR ($CDCl_3$) δ 5.58 (d, H-1), 4.58

(dd, *H*-3), 4.37 (dd, H-4), 4.29 (dd, H-2), 3.88 (dd, H-5), 3.74 (m, H-6), 2.31 (s, S-Ac), 1.52, 1.45, 1.33 and 1.32 (isopropylidene methyls), 1.43 (d, H-7).

A portion of the above residue **4a** (4.6 g) was dissolved in dry methanol (40 mL) at 0 °C containing 30% ammonium hydroxide (4.6 mL) and dithiothreitol (2.8 g). After 16 h at 0 °C, the solvent was evaporated, and the residue was dissolved in CH_2CL_2, dried with anhydrous $MgSO_4$, and concentrated. Purification of the product by chromatography on a column of silica gel (ethyl acetate–hexane = 1:20) afforded product **5a** (3.3 g): $[\alpha]^{25}_D$ −59.8 ± 2°, (*c* 1.02, $CHCl_3$); ^1H NMR ($CDCl_2$) ∂: 5.54 (d, H-1), 4.65–4.59 (m, 2H), 4.30 (dd, 1 H), 3.45 (dd, 1 H), 3.12 (m, H-6), 1.66 (d, SH), 1.4 (d, H-7), 1.53, 1.44, 1.35 & 1.33 (isopropylidene methyls); ^{13}C NMR ($CDCl_3$) ∂ 109.1, 108.6, 96.8, 73.6, 71.1, 70.97, 70.4, 33.63, 26.0, 25.9, 24.9, 24.4, 21.7. Anal. Calcd for $C_{13}H_{22}O_5S$: C, 53.79; H, 7.58. Found: C, 53.90; H, 7.71.

To a solution of **5a** (3.0 g) in DMF (60 mL) at −20 °C was added sodium hydride (223 mg). After 10 min, a solution of **12a** (4.74 g) in DMF (5 mL) was added, and the solution was stirred at −20 °C for 16 h. The solvent was evaporated, and the residue was dissolved in CH_2Cl_2, washed with water, ice cold HCl (1 M), and saturated sodium bicarbonate solution. It was then dried over anhydrous $MgSO_4$ and concentrated. Purification by chromatography on a column of silica gel (ethyl acetate–hexane–ethanol 10:10:1) gave a homogeneous product (5.5 g). ^1H NMR showed it to be the desired product **20a** contaminated with about 10% of **13**. Pure **20a** was made from this mixture by removal of the acetate groups with sodium methoxide in methanol, followed by gel permeation chromatography (see below) and then reacetylation of the product with pyridine–acetic anhydride: $[\alpha]^{25}_D$ −8.1 ± 2°, (*c* 0.98, $CHCl_3$); ^1H NMR ($CDCl_3$) ∂ 5.50 (d, J = 5.1 Hz, H-1), 5.30 (m, 2H, H-7′ and H-8′), 5.12 (d, J = 10.1 Hz, NH), 4.85 (m, H-4′), 4.50 (dd, J = 2.4, 7.9 Hz, H-3), 4.36 (dd, J = 1.7, 7.9 Hz, H-4), 4.29 (dd, J = 2.2, 12.5 Hz, H-9′a), 4.26 (dd, J = 2.6, 5.1 Hz, H-2), 4.12 (dd, J = 4.6, 12.5 Hz, H-9′b), 4.03 (t, J = 10.5 Hz, H-5′), 3.85 (dd, J = 10.8 Hz, H-6′), 3.79 (s, $COOCH_3$), 3.50 (dd, J = 1.5, 8.1 Hz, H-5), 3.30 (m, H-6), 2.74 (dd, J = 4.8, 12.7 Hz, H-3′eq), 2.16, 2.13, 2.03, 2.02, and 1.87 (5 × CH_3CO), 1.99 (H-3′ax), 1.46 (d, J = 7.0 Hz, H-7), 1.49, 1.44, 1.32, and 1.31 (4 s, isopropylidene methyls; ^{13}C NMR ($CDCl_2$) ∂ 171.0, 170.6, 170.1, 169.9, 168.2, 109.4, 108.6, 96.7, 84.5, 74.4, 71.4, 71.1, 70.0, 69.0, 67.4, 62.1, 52.9, 49.4, 39.4, 38.4, 29.7, 26.0, 24.9, 24.8, 23.2, 21.3, 20.9, 20.8. Anal. Calcd for $C_{33}H_{49}O_{17}SN$: C, 51.90; H, 6.42: Found: C, 49.23; H, 6.18.

This mixture of **20a** and **13** (500 mg) was dissolved in methanol (20 mL) to which sodium methoxide solution (0.5 M, 0.2 mL) was added. After 4 h, the reaction mixture was neutralized with H^+ resin and filtered, and the solvent evaporated. The residue was dissolved in 10% aqueous ethanol (5 mL) and applied to a column of Bio Gel P-2 (200–400 mesh, 1400 mL), equilibrated, and eluted with water. The eluant was monitored by UV absorption at 220 nm. Fractions (7.5 mL) 77–84 were pooled and lyophilized to give methyl 5-acetamido-3,5-dideoxy-2-thio-α-D-*glycero*-D-*galacto*-nonulopyranosylonate(2-*S*-6)-6,7-dideoxy-1,2;3,4-di-*O*-isopropylidene-6-thio-α-D-*glycero*-D-*galacto*-heptopyranose (**21a**) (280 mg): $[\alpha]^{25}_D$ 2.0 ± 2°, (*c* 1.03, H_2O); ^1H NMR (D_2O) ∂ 5.56 (d, H-1), 4.66 (dd, H-3), 4.48 (dd, H-4), 4.44 (dd, H-2), 3.80 (s, $COOCH_3$), 3.62 (H-4′), 3.47 (H-5), 3.26 (m, H-5), 2.74 ($d3)_{eq}$), 1.95 (s, NAc), 1.78 (dd, $H\text{-}3_{ax}$), 1.47, 1.44, 1.32, 1.31 (isopropylidene methyls), 1.39 (d, H-7).

A solution of **21a** (200 mg) in 50% aqueous trifluoroacetic acid (10 mL) was stirred at room temperature for 4 h, and the solvent was evaporated. The residue was dissolved in water (5 mL) and applied to a column of Bio Gel P-2 (200–400 mesh, 1400 mL) equilibrated, and eluted with water. The eluant was monitored by UV absorption at 220 nm. Fractions (7.5 mL) 108–121 were pooled and lyophilized to get the methyl ester of **22a** (156 mg). $[\alpha]^{25}_D$ 50.9 ± 2°, (*c* 1.05, H_2O). This was dissolved in water (10 mL) containing Chelex resin (sodium form, 200–400 mesh, 2 g) and stirred for 6 days. The resin was filtered, and the solution was lyophilized to get obtain **22a** as a colorless solid (87 mg). $[\alpha]^{25}_D$ +49.5 ± 2°, (*c* 0.94, H_2O). ^1H and ^{13}C NMR data—see Tables 1 and 2. Anal. Calcd for $C_{18}H_{30}O_{13}SNa{\cdot}2H_2O$: C, 38.64; H, 6.08; N, 2.50. Found: C, 38.83; H, 6.16; N, 2.57.

Sodium Salt of 5-Acetamido-3,5-dideoxy-2-thio-α-D-*glycero*-D-*galacto*-nonulopyranosylonic acid(2-*S*-6)-7-deoxy-L-*glycero*-D-*galacto*-heptopyranose (22b). To a solution of **2a** (7.2 g) in CH_2Cl_2 (150 mL) at 0 °C were added pyridine (10 mL) and trifluoromethanesulfonic anhydride (9.4 g), and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with dichloromethane and washed with water, ice cold 1 M hydrochloric acid, and saturated sodium bicarbonate solution. The solvent was evaporated, and the crude product was dissolved in DMF (100 mL) containing potassium thioacetate (5.1 g) and stirred at 0 °C for 18 h. The product was worked up as described above for **4a**. Purification by chromatography on silica gel using ethyl acetate–hexane (1:10) afforded 6,7-deoxy-1,2;3,4-di-*O*-isopropylidene-6-(acetylthio)-β-L-*glycero*-D-*galacto*-heptopyranose (**4b**, 3.3 g) and 1,2;3,4-di-*O*-isopropylidene-α-D-*galacto*-hept-6-eneopyranose (**4c**, 3.2 g). Data for **4b**: $[\alpha]^{25}_D$ −52.7 ± 2° (*c* 1.05, $CHCl_3$); ^1H NMR of **4b** ($CDCl_3$) ∂ 5.54 (d, J = 4.9 Hz, H-1), 4.61 (dd, J = 5.4, 7.8 Hz, H-3), 4.32–4.29 (m, H-2, H-4), 3.77 (m, H-6), 3.68 (dd, J = 1.7, 10.0 Hz, H-5), 2.30 (s, S-Ac), 1.52, 1.46, 1.34 and 1.32 (CH_3 of isopropylidene groups), 1.41 (d, J = 6.8 Hz, H-7); ^{13}C NMR ($CDCl_3$) ∂: 195.6, 109.5, 108.7, 96.7, 71.0, 71.0, 70.4, 68.8, 40.8, 30.9, 25.9, 24.9, 24.5, 17.7.

A portion of the above residue **4b** (2.3 g) was dissolved in dry methanol (40 mL) at 0 °C containing 30% ammonium hydroxide (2.3 mL) and dithiothreitol (1.4 g). After 16 h at 0 °C, the solvent was evaporated, and the residue was dissolved in CH_2Cl_2, dried with anhydrous $MgSO_4$, and concentrated. Purification of the product by chromatography on a column of silica gel (ethyl acetate–hexane = 1:20) afforded **5b** (1.8 g): $[\alpha]^{25}_D$ −58.2 ± 2°, (*c* 1.02, $CHCl_3$); ^1H NMR ($CDCl_3$) ∂ 5.56 (d, J = 5.1 Hz, H-1), 4.60 (dd, J = 2.6, 8.1 Hz, H-3), 4.32 (dd, J = 1.8, 7.3 Hz, H-4), 4.31 (dd, J = 2.6, 5.1 Hz, H-2), 3.50 (dd, J = 1.5, 9.2 Hz, H-5), 3.27 (m, H-6), 2.24–2.26 (m, SH), 1.56, 1.44, 1.34, and 1.33 (H-7 and isopropylidene methyls); ^{13}C NMR ($CDCl_3$) ∂ 109.3, 108.8, 96.6, 74.1, 70.9, 70.7, 70.53, 70.52, 35.1, 26.0, 25.9, 25.0, 24.4, 18.7.

To a solution of **5b** (1.8 g) in DMF (50 mL) at −20 °C was added sodium hydride (134 mg). After 10 min, a solution of **12a** (2.8 g) in DMF (5 mL) was added, and the solution was stirred at −20 °C for 16 h. The solvent was evaporated, and the residue was dissolved in CH_2Cl_2, and washed with water, ice cold HCl (1 M), and saturated sodium bicarbonate solution. It was then dried over anhydrous $MgSO_4$ and evaporated. Purification by chromatography on a column of silica gel (ethyl acetate–hexane–ethanol, 10:10:1) gave a homogeneous product (3.5 g). ^1H NMR showed it to be the desired product **20b** contaminated with about 10% of **13**. Pure **20b** was made from this mixture by removal of the acetate groups with sodium methoxide in methanol, followed by gel permeation chromatography (see below) followed by reacetylation of the product with pyridine–acetic anhydride: $[\alpha]^{25}_D$ −38.3 ± 2°, (*c* 0.98, $CHCl_3$); ^1H NMR ($CDCl_3$) ∂ 5.56 (d, J = 5.1, H-1), 5.37 (m, H-8′), 5.31 (dd, J = 2.1, 7.3 Hz, H-7′), 5.12 (d, J = 10.0 Hz, NH), 4.85 (m, H-4′), 4.59 (dd, J = 2.4, 7.9, H-3), 4.35–4.28 (m, H-4, H-2) and H-9′a), 4.19 (dd, J = 7.2, 12.6 Hz, H-9′b), 4.02 (m, H-5′), 3.91 (dd, J = 2.0, 10.7 Hz, H-6′), 3.79 (s, $COOCH_3$), 3.54 (dd, J = 1.7, 8.6 Hz, H-5), 3.50 (m, H-7), 2.72 (dd, J = 4.6, 13.1 Hz, H-3′eq), 2.21 (dd, J = 12.0, 12.9 Hz, H-3′ax), 2.14, 2.11, 2.04, 2.02 and 1.87 (5 × CH_3CO), 1.37 (d, J = 6.8 Hz, H-7), 1.51, 1.48, 1.33, and 1.31 (isopropylidene methyls); ^{13}C NMR ($CDCl_3$) ∂ 171.0, 170.7, 170.1, 170.0, 169.97, 169.3, 109.4, 108.5, 96.7, 83.7, 74.3, 71.4, 71.1, 70.4, 69.9, 69.6, 69.4, 67.7, 62.0, 53.0, 49.5, 39.6, 38.4, 26.0, 25.98, 24.9, 24.5, 23.2, 21.1., 20.9, 20.8, 20.7, 19.7.

A portion of **20b** (1.0 g) was de-O-acetylated with 0.5 M sodium methoxide (0.3 mL) in methanol (20 mL, 16 h), and the product from this was purified by chromatography on Bio Gel P2 (200–400 mesh) to obtain pure **21b** (590 mg). The isopropylidene group was removed with 50% aqueous trifluoroacetic acid (30 mL, room temperature for 4 h), followed by filtration on a column of Bio gel P2. Finally, the methyl ester was converted to the sodium salt **22b** with Chelex resin (Na form, 3.0 g suspended in 20 mL of water, 6 days); this was followed by purification on a column of Bio gel P2 eluted and equilibrated with deionized water. The product fractions (as evidenced by UV absorption at 214 nm) were pooled and lyophilized to give a colorless material (400 mg). The structure was confirmed on the basis of its ^1H and ^{13}C NMR data (Tables 1 and 2). Anal. Calcd for $C_{18}H_{30}O_{13}SNa{\cdot}2H_2O$: C, 38.64; H, 6.08; N, 2.50. Found: C, 39.23; H, 6.19; N, 2.70.

Preparation of ^{14}C-Labeled αDNeuAc(2–6)βDGlcNAc (25). The compound was made according to a modified literature method.[46] ^{14}C-Labeled *N*-acetyl-D-glucosamine (^{14}C-D-GlcNAc, 50 uCi, NEN, MA) was mixed with D-GlcNAc (13.5 mg, 61.1 μmol) and UDP-galactose (45.3 mg, 80 μmol), dissolved in a buffer (1.7 mL, pH 7.4) containing $MnCl_2$ (10 μmol), sodium cacodylate (50 μmol), and galactosyl transferase (5 U, E.C. 2.4.1.22, Sigma Chemical Company, St. Louis, MO), and incubated at 37 °C for 24 h. The reaction mixture was passed through a Dowex phosphate resin column (200–400 mesh) containing Chelex resin (500 mg) packed on the top. The column was eluted with deionized water (30 mL), and the eluant was concentrated to a dry residue, which was dissolved in 100 mM sodium cacodylate buffer (2.5 mL, pH 6.5) containing CMP-NeuAc (50 mg, Sigma Chemical Co.), bovine alkaline phosphatase (6 U), bovine serum albumin (5 mg), and Galβ1,4GlcNAc α2,6 sialyl transferase (500 mU, E.C. 2.4.99.5), and incubated at 37 °C

for 24 h. The reaction mixture was diluted with water to 12 mL, applied to a column of Dowex-phosphate resin (200–400 mesh), and eluted with water (75 mL). The elution buffer was then changed to 5 mM sodium phosphate buffer (pH 6.8), and fractions (7.5 mL) were collected. A sample of the fraction (10 μL) was diluted with scintillation liquid (3 mL, Formula 989, NEN, MA), and the radioactivity was measured. The products appeared in fractions 17–30. These were pooled, evaporated to a dry residue, redissolved in water, and applied on a column of Sephadex G-15 (75 mL), equilibrated, and eluted with deionized water. The fractions (2 mL) containing the radioactivity were pooled and lyophilized to obtain a colorless material (38 mg).

Neuraminidase Assays. Stock solutions (10 or 20 mM) of **16a**, **16b**, **19a**, **19b**, **22a**, **22b**, **23a**, **23b**, **23c**, and **25** were made, and the molarity was determined based on the sialic acid content estimated by the periodate–resorcinol method.[67] The *Arthrobacter ureafacien* neuraminidase (E.C. 3.2.1.18, specific activity 81.6 U/mg), *Clostridium perfringens* neuraminidase (E.C. 3.2.1.18), and *Vibrio cholerae* neuraminidase (EC 3.2.1.18) were purchased from Calbiochem (La Jolla, CA). Influenza A virus (WSN H1N1) was provided by Dr. E. Cheng of DuPont-Merck Pharmaceutical, Wilmington, DE. The virus (5.3 mg total protein content) was suspended in 50 mM sodium phosphate buffer (1 mL, pH 7.0), and the suspension was thoroughly mixed to obtain a uniform suspension and stored at −80 °C as 100-μl aliquots. Prior to use, the suspension was warmed up to 37 °C and thoroughly mixed. The following buffers were used in the enzymatic sialoside hydrolysis: For *Arthrobacter ureafaciens* neuraminidase, the buffer was 0.075 M sodium acetate–0.013 M calcium chloride at pH 5.0; for *Clostridium perfringens* neuraminidase, the buffer was 50 mM sodium acetate at pH 4.5; for *Vibrio cholerae* neuraminidase, the buffer was 50 mM sodium acetate–10 mM calcium chloride–50 mM sodium chloride at pH 5.5; and for influenza neuraminidase, the buffer was 100 mM sodium acetate at pH 5.5. The time course of neuraminidase hydrolysis (Figures 3 and 4) was done by incubating a 0.75 mM solution of the sialoside (in 500 μL total reaction volume, except for influenza neuraminidase reaction, where the substrate concentration was about 1 mM and the total reaction volume was 165 μL) with the neuraminidase (A. U. neuraminidase 10 mU; C.P. and V. C. neuraminidase 100 mU; influenza neuraminidase 30 μL of virus suspension) at 37 °C. Samples (50 μL, except in the case of influenza neuraminidase assay, where 25 μL of sample was used) were withdrawn at 0, 15, 30, 60, and 120 min, and the free sialic acid liberated was estimated by the thiobarbituric acid method[68] as follows: the sample was diluted to 100 μL with deionized water, followed by the addition of 0.2 M sodium periodate in 9 M phosphoric acid solution (50 μL). After 20 min at room temperature, the excess periodate was destroyed with 10% sodium arsenite in 0.5 M sodium sulfate–0.1 N sulfuric acid solution (250 μL), followed by the addition of 0.6% thiobarbituric acid in 0.5 M sodium sulfate solution (1.5 mL). This mixture was kept in boiling water bath for 15 min. The samples were then cooled to room temperature, the color was extracted with cyclohexanone (2 mL), and the optical density was measured at 550 nm. To determine the Michaelis constant (K_m) and V_{max} (Table 4), the solutions of the sialoside substrates at six different concentrations (0.1–12.8 mM) were incubated at 37 °C for 20 min with neuraminidase in a total volume of 100 μL of the buffers (60 μL for influenza neuraminidase reaction) for 20 min. The amounts of the neuraminidase used for various sialosides are as follows: For sialoside **16a**, 2 mU of A. U. neuraminidase, or 25 mU of C. P. neuraminidase, or 50 mU of V. C. neuraminidase, or 12 μL of the influenza virus suspension were used, for **16b**, 50 mU of A. U. neuraminidase, or 165 mU of C. P. neuraminidase, or 50 mU of V. C. neuraminidase were used, and for **23a** and **23b**, 2 mU of A. U. neuraminidase, or 12 mU of C. P. neuraminidase, or 50 mU of V. C. neuraminidase, or 12 μL of the influenza virus suspension were used. Following incubation, a portion of the reaction mixture (50 μL) was assayed for free sialic acid content by the thiobarbituric acid method described above. As controls, the reaction was also carried out at these substrate concentrations without the neuraminidase and a correction was made for the sialic acid liberated nonenzymatically. The K_m (expressed in mM) and V_{max} (O. D. unit/mU of enzyme/20 min) values were determined from Lineweaver–Burk double reciprocal plots using linear fits of the data points.

The inhibition constants (K_i) for **22a** and **22b** were determined by incubating (37 °C) a solution of **25** at four different concentrations (approximately at 0.5, 1, 2, and 4 times the K_m of **25** for the enzyme) with the neuraminidase, in the presence (at three inhibitor concentrations) or absence of the inhibitors, for 20 or 30 min. This was followed by estimating the amount of free LacNAc liberated. The buffers used in these neuraminidase reactions were the same as described above. The four concentrations of **25** used in these neuraminidase assays were as follows: A. U. neuraminidase, 0.5, 1.0, 2.0, and 4.0 mM; influenza A neuraminidase, 1.0, 2.0, 4.0, and 8.0 mM; C. P. neuraminidase, 0.5, 1.0, 2.0, and 4.0 mM; V. C. neuraminidase 4.0, 6.0, 8.0, and 10 mM. The inhibitor concentrations were as follows: **22a**, 0.5, 1.0, and 2.0 mM for A. U. and C. P. neuraminidases, 0.6, 1.2, and 2.4 mM for influenza A neuraminidase, 1.0, 2.0, and 2.9 mM for V. C. neuraminidase; **22b**, 2.5, 5.0, and 10 mM for influenza A neuraminidase, 0.95, 1.90, and 3.8 mM for C. P. neuraminidase and 1.9, 3.8, and 5.5 mM for A. U. and V. C. neuraminidases. The neuraminidase concentrations were as follows: 2 mU of A. U. neuraminidase in 100 μL of total reaction mixture, 53 μg of influenza A virus in 50 μL of total reaction volume, 12.5 mU of C. P. neuraminidase in 50 μL total reaction volume, and 12.5 mU of V. C. neuraminidase in 25 μL of total reaction volume. After the reaction, the reaction mixture was diluted with deionized water (1 mL) and passed through a column of Dowex resin (phosphate form, 200–400 mesh, 2 mL of a 1 g/mL suspension of the resin in deionized water). The column was then further eluted with deionized water (2 mL). Under these conditions, only the free LacNAc eluted. The eluant was diluted with scintillation fluid (10 mL, Formula 989, NEN, MA) and the radioactivity was measured for 5 min with a Liquid Scintillation Counter. From the Lineweaver–Burk plots for the hydrolysis of **25**, the slopes at various concentrations of the inhibitors were obtained, and these were plotted against the inhibitor concentration. From the slope of the second plot, the inhibition constant K_i was calculated.[69]

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Supplementary Material Available: ^1H and ^{13}C chemical shifts of sialoside **16c** and the β-anomers of sialosides **16a** and **16d** and **15a** (Tables 1 and 2); selected intra- and interring hydrogen distances estimated by the GEGOP program for sialosides **16a** and **16b** (Table 3); plots of V_o versus [S] of the Michaelis–Menten equation for the neuraminidase hydrolysis (Influenza, A. U., C. P., and V. C.) of sialosides **23a** and **23b** (Figure 1); Dixon plots for the inhibition of neuraminidase (Influenza, A. U., C. P., and V. C.) hydrolysis of αDNeuAc(2-6)βDGal(1-4)DGlcNAc (**25**) by **22a** (Figure 2); the neuraminidase (Influenza, A. U., C. P., and V. C.) hydrolysis of αDNeuAc(2-6)βDGal(1-4)DGlcNAc (**25**) in the presence of thiosialoside **22b** (Figure 3); the population contour plots for the sialoside linkages of **16a** and **16b** (Figure 4); the 500-MHz 1D NOE difference spectra of **16d**, where the hydrogen signals of H-6 and H-3′ax are saturated (Figure 5); the 300-MHz 1D NOE difference spectra of the thiosialoside **22a**, where the hydrogen signals of H-7 and H-3′ax are saturated (Figure 6); the stereoview of the sialoside **16a** in "tg syn" conformation at the active site of influenza N2 neuraminidase (Figure 7) and the stereoview of the sialoside **16b** in "gt anti" conformation at the active site of influenza N2 neuraminidase (Figure 8) (11 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.