Cluster Sialoside Inhibitors for Influenza Virus: Synthesis, NMR, and Biological Studies

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Abstract: Two αDNeuAc(2→6)βDGal(1→4)βDGlcNAc units which are the receptor determinants for the influenza virus hemagglutinin have been anchored on a galactose in order to design structures capable of bimodal viral binding. To determine the optimum αDNeuAc distance for the best intramolecular binding to the hemagglutinin trimer, the attachment sites of the two αDNeuAc(2→6)βDGal(1→4)βDGlcNAc units have been systematically varied by proper choice of the galactose glycosylation sites. Thus, we have chemoenzymatically prepared five heptasaccharides of the general formula αDNeuAc(2→6)βDGal-(1→4)βDGlcNAc(1→x)[αDNeuAc(2→6)βDGal(1→4)βDGlcNAc(1→y)]βDGalOR, where x and y are 2 and 3, 2 and 4, 2 and 6, 3 and 6, and 4 and 6, respectively. The structural identity and the complete proton and carbon chemical shift assignments of the tri-, penta-, and heptasaccharides have been established by a combination of 1D TOCSY, 1D and 2D NOESY, and $^{1}H-^{13}C$ correlation techniques. One-dimensional and two-dimensional NOESY experiments have been used to assess the conformational properties of these molecules. These, together with Monte Carlo simulations, suggested that the end C-2 to C-2 atoms of the sialic acid residues in these bivalent receptor structures are separated by ~19 Å in 2,4-heptasaccharides to ~9 Å in 3,6- and 4,6-heptasaccharides, with the others falling between these two distances. The five heptasaccharides, when evaluated as inhibitors of the influenza virus adsorption to resialylated human erythrocytes, showed that relative to the methyl α-sialoside the 3,6- and 4,6-disialosides with a 9-Å end distance exhibited 10- and 8.4-fold better inhibitory activity, respectively, whereas the 2,4-disialoside with a ~19-Å sialic acid end distance had no increased inhibitory activity. Conjugation of the 3,6-heptasaccharide to bovine serum albumin increased the inhibitory potency even more, suggesting the therapeutic potential of a macromolecule containing the 3,6-heptasaccharides for influenza viral inhibition.

Introduction

Host cell surface oligosaccharides are involved as receptor ligands for protein molecules such as enzymes,[1] antibody,[2] and lectins,[3,4] and they initiate many critical biological reactions. Unfortunately, these receptor ligands also initiate many harmful biological reactions by providing attachment sites to viruses,[5] toxins,[1] and bacteria,[6] etc. Understanding the mechanism of such a carbohydrate–protein interaction may provide us with a clue to promote or prevent a biological reaction of interest and to aid in the eventual design of pharmaceuticals. A case in point is the influenza infection process. The virus binds via its hemagglutinins to a trisaccharide αDNeuAc(2→6)βDGal(1→4)βDGlcNAc residue present on the host cell, and this has been recognized as a key event in the infection process.[7]

It appears that the binding of influenza virus hemagglutinins (HAs) to the host cell surface carbohydrates enables a pH-mediated conformational change of the HAs and the fusion of the virus to the target cell.[8] This protein–carbohydrate-specific interaction appears to be only transient in the cascade of events that follows the initial viral attachment. As it is known that the synthetic trisaccharide exhibits only weak affinity to the hemagglutinin molecules,[9,10] it is important to understand how such weak carbohydrate–protein interactions are eventually amplified in the successful virus–target cell fusions, but with extreme carbohydrate specificity. Over the last 10 years, Lee et al. have elegantly demonstrated[11] for the galactose–hepatocyte binding systems that a cluster or multipoint carbohydrate–protein interaction may be involved in the successful binding phenomena. Since there are hundreds of copies of HA trimers on the viral envelope,[5] it is reasonable to expect that these engage in both intra- and intermolecular target cell–carbohydrate binding (Figure 1), leading to the overall stability of the complex. To understand and design inhibitors for this biological process, it will be desirable to mimic such a process by the design of suitable synthetic molecules. In recent years, Gamian et al.[12,13] and Spaltenstein et al.[14] designed such macromolecules and showed that they possess markedly increased binding properties to the hemagglutinin molecules as compared to monovalent sialosides.

Since the HAs have the potential to bind both intra- and intermolecularly to the cell surface carbohydrates (Figure 1), it is important to elucidate the key carbohydrate structural elements required for such a process. Glick et al. recently have reported[15] that, when two sialic acids are held apart by 55 Å, they bind to the adjacent HAs and this results in stronger binding as compared to the monovalent sialosides. On the basis of the three-dimensional structure of the protein, they proposed that these bivalent sialosides bind intermolecularly. Recently, we showed[16] that the bivalent

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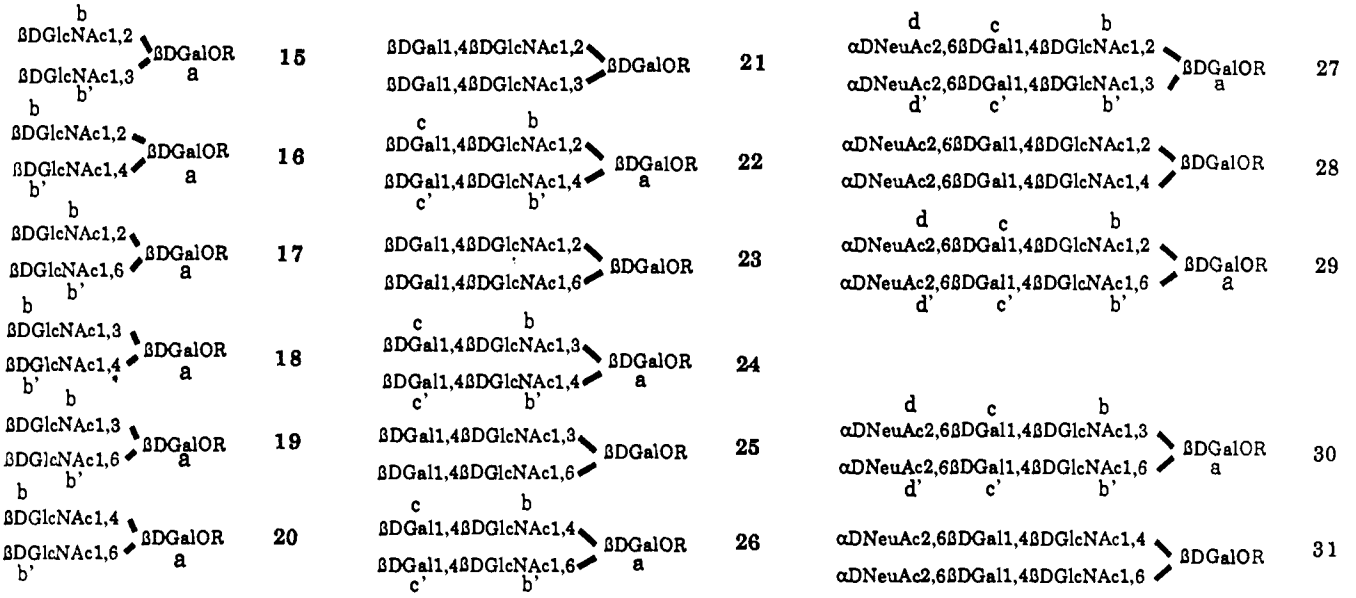
Table I. List of Oligosaccharides Prepared and Studied in this Report (The Pyranose Units Are Designated by a–d′ Starting from the Reducing End; R = $(CH_2)_5COOCH_3$)



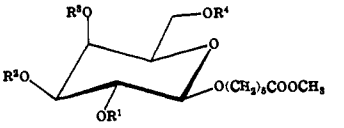
sialosides attached to lactosamine cores on a single carbohydrate moiety exhibited increased binding potencies. Since the compound which bound the best appeared to have the sialic acids placed ~9 Å apart, it suggested to us that these may be binding intramolecularly to the hemagglutinin molecule. We wish to disclose in this report our systematic design and synthesis of a number of bivalent heptasaccharides (Figure 2) for evaluation as inhibitors of the influenza virus of human isolates. These compounds were designed with the expectation that (1) these heptasaccharides would be more rigid than the simple bivalent sialosides reported in the literature[15] and consequently should have favorable entropic factors for binding to proteins; (2) these compounds could be investigated in detail by NMR in order to understand their conformational properties and result in the eventual understanding of the structure–activity relationship; (3) these molecules could be coupled to proteins for biological evaluation; (4) the tether could be used to eventually polymerize these materials to generate macromolecules of therapeutical interest; and (5) finally, these compounds could be coupled to hydrophobic molecules such as (1-naphthylmethyl)amine[17] or fatty acids in order to make liposomes.

Results

Synthesis. As shown in Figure 2, our objective was to anchor two trisaccharides, namely, αDNeuAc(2→6)βDGal(1→4)-βDGlcNAc residues in six possible combinations to a galactose residue carrying a tether at the anomeric center. The 5-(methoxycarbonyl)pentyl tether can be modified at the end of the synthesis to carry polymerizable groups[14] or to derivatives suitable for attachment to carrier proteins.[18] The end sialic acids and the penultimate galactose residues in the heptasaccharides were, hopefully, to be added enzymatically to the trisaccharides **15–20** (Table I), as the corresponding enzymes and the sugar nucleotide cofactors are available commercially or through purification processes. Thus, our first task was to synthesize chemically the six trisaccharides **15–20** (Table I) via intermediates **9–14** (Chart I).

Preparation of Trisaccharides 9–20. In order to prepare the trisaccharides **9–20**, the monosaccharide diols **3–8** were needed. These were made from 5-(methoxycarbonyl)pentyl β-D-galactopyranoside[19] following well-established literature methods (see the supplementary material). The diols **3–8** were then converted to the protected trisaccharides **9–14** by reaction with 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranosyl bromide in the presence of AgOTf and collidine.[20] The reaction was facile in the case of diols **5** and **8**, which have a primary and a secondary hydroxyl group. The choice of the protecting groups in the diol was very important as well. For example, when 5-(methoxycarbonyl)pentyl 3,6-di-*O*-benzoyl-β-D-galactopyranoside or the corresponding 3,6-bis-*O*-(*tert*-butyldimethylsilyl) derivative was reacted with the phthalimido bromide, the glycosylation of only the 2-hydroxyl group could be accomplished. However, when the protecting groups were changed to 3-*O*-allyl-6-tBDMS groups, as seen in **4**, the diol reacted to afford the trisaccharide **10**. It is to be noted that even though the introduction of the 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl group causes severe steric crowding at the adjacent positions, the vicinally substituted 2,3-trans (compound **9**) or 3,4-cis trisaccharides (compound **12**) could still be made. Even though the diols **3**, **4**, and **7** reacted slowly and incompletely, we did not use more than 3 equiv of the bromide, as the protected trisaccharide products comigrated with the hydrolyzed product, namely, the 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranose (arising from the hydrolysis of excess bromide). The structural identities of the products **9–14** were firmly established on the basis of proton and carbon chemical shifts (provided in the supplementary material) and by comparison with the literature data.[20–22]

Chart I. Structures of the Synthetic Intermediates



1; $R^1 = R^2 = R^3 = R^4 = H$
2; R^2 = allyl, $R^1 = R^3 = R^4 = H$
3; $R^1 = R^2 = H$, $R^3 = R^4$ = benzylidene
4; R^2 = allyl, R^4 = t-butyldimethylsilyl (tBDMS), $R^1 = R^3 = H$
5; $R^2 = R^3$ = isopropylidene, $R^1 = R^4 = H$
6; $R^1 = R^4$ = allyl, $R^2 = R^3 = H$
7; $R^1 = R^3$ = benzoyl, $R^2 = R^4 = H$
8; $R^1 = R^2$ = allyl, $R^3 = R^4 = H$
9; $R^1 = R^2$ = 2-deoxy-3,4,6-tri-O-acetyl-2-phthalimido-ß-D-glucopyranosyl, $R^3 = R^4$ = benzylidene
10; R^2 = allyl, R^4 = tBDMS, $R^1 = R^3$ = 2-deoxy-3,4,6-tri-O-acetyl-2-phthalimido-ß-D-glucopyranosyl
11; $R^2 = R^3$ = isopropylidene, $R^1 = R^4$ = 2-deoxy-3,4,6-tri-O-acetyl-2-phthalimido-ß-D-glucopyranosyl
12; $R^1 = R^4$ = allyl, $R^2 = R^3$ = 2-deoxy-3,4,6-tri-O-acetyl-2-phthalimido-ß-D-glucopyranosyl
13; $R^1 = R^3$ = benzoyl, $R^2 = R^4$ = 2-deoxy-3,4,6-tri-O-acetyl-2-phthalimido-ß-D-glucopyranosyl
14; $R^1 = R^2$ = allyl, $R^3 = R^4$ = 2-deoxy-3,4,6-tri-O-acetyl-2-phthalimido-ß-D-glucopyranosyl

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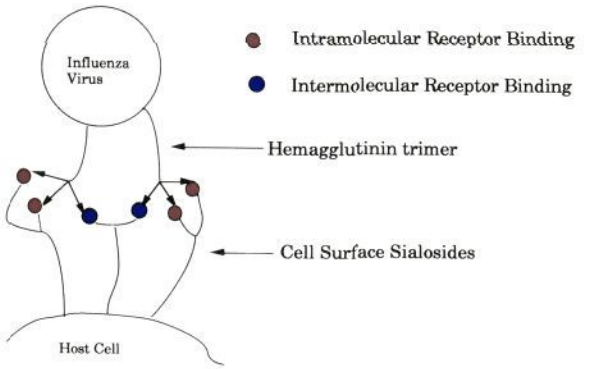

Figure 1. Hemagglutinin recognition of sialosides. The inter- and intramolecular binding of multivalent cell surface sialosides to the influenza virus hemagglutinin trimers.

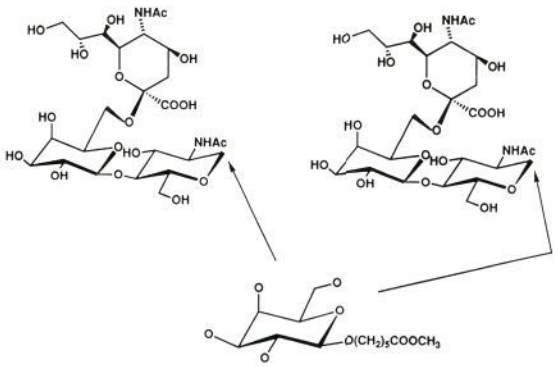

Figure 2. Synthetic target heptasaccharides containing two receptor ligands, namely, αDNeuAc(2→6)βDGal(1→4)βDGlcNAc fragments anchored to two of the four hydroxyls of the galactose containing the tether $(CH_2)_5COOCH_3$.

Finally, the deprotection of trisaccharides **9–14** to the completely deblocked trisaccharides **15–20** was achieved in multisteps following established procedures.[23–26] The structures of all of the deprotected trisaccharides **15–20** were confirmed from their NMR data (provided in the supplementary material).

Enzymatic Synthesis of Penta- and Heptasaccharides. The trisaccharides **15–20** containing two βGlcNAc units, when treated with slightly more than 2 equiv of UDP-galactose and bovine galactosyl transferase,[27] provided the two lactosamine-containing pentasaccharides **21–26**. About one-tenth of the recommended amounts[27] of manganese chloride and sodium cacodylate were used in these enzymic reactions to obtain the product free of these salts. We find the use of phosphate (pH 6.8, 200–400 mesh) instead of the conventional chloride resin column desirable to obtain high-purity products. The enzymic digalactosylation was over in 24 h for all six trisaccharides, and no monogalactosylated material was seen either by thin layer or by gel permeation chromatography. The structures of the six pentasaccharides were confirmed from their proton and carbon chemical shifts [^{1}H NMR spectra (Figure 1) and chemical shift assignments (Tables 3 and 4) are provided in the supplementary material].

These six pentasaccharides were then treated with CMP-NeuAc and Galβ1,4GlcNAc α2,6-sialyltransferase for 24 h according to the published methods.[28,29] In addition to the disialylated hep-

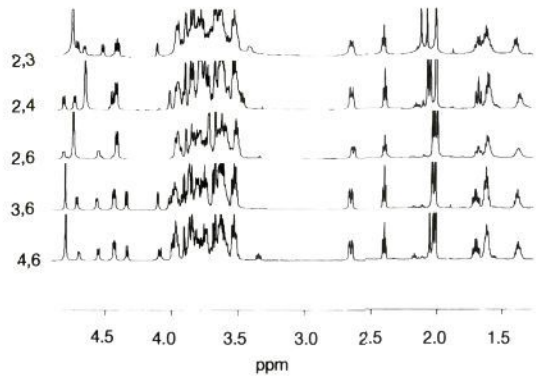

Figure 3. Proton NMR spectra (600 MHz) of the synthetic heptasaccharides containing two αDNeuAc(2→6)βDGal(1→4)βDGlcNAc fragments attached to the 2,3-, 2,4-, 2,6-, 3,6-, and 4,6-positions of the reducing end galactose (compounds **27–31**, respectively; see Table I).

tasaccharides, small amounts of monosialylated product (even though addition of more enzymes would have converted the monosialosides to the desired disialoside, this was not done due to the limited availability of the sialyltransferase) and free sialic acids were also obtained. These could be conveniently removed by absorption of the total products on a phosphate resin column. Elution with 5 mM phosphate buffer provided the monosialylated product followed by free sialic acid. Subsequent elution with 50 mM phosphate buffer provided highly pure disialylated material followed by cytidine monophosphate.

The enzymatic sialylation was very good for the 2,4-, 2,6-, 3,6-, and 4,6-pentasaccharides (**22**, **23**, **25**, and **26**, respectively). In the case of 2,3-pentasaccharide **21**, the reaction was sluggish after the addition of the first sialic acid. Even though these could be completely converted to the disialoside by the repeated addition of sialyltransferase, it was not attempted as the amount of the material obtained was sufficient for biological evaluation. In the case of 3,4-pentasaccharide **24**, the reaction stopped after the first addition of sialic acid with no traces of disialoside in the reaction. Thus, we prepared five heptasaccharides **27–31**, respectively[29b] (Table I). The 600-MHz ^{1}H NMR spectra of these disialosides are shown in Figure 3.

NMR of the Tri-, Penta-, and Heptasaccharides. Table I shows a list of the tri-, penta-, and heptasaccharides for which detailed NMR studies have been carried out. Except for the heptasaccharides (Tables II and III), the data for the remainder of the compounds are presented in the supplementary material. In general, for the GlcNAc units, 1D TOCSY measurements[30] with Gaussian-shaped selective pulses[31,32] at different spin mixing times provided spin–spin information transfer from the well-separated anomeric hydrogen signals to the entire pyranose ring hydrogens and this enabled complete proton chemical shift assignments. To establish the linkage position of the two GlcNAc residues to the reducing galactose unit, 1D ROESY for the trisaccharides (NOESY measurements at 600 MHz were not useful for the trisaccharides) and 1D and 2D NOESY measurements for the penta- and heptasaccharides were used. For the galactose units, 1D TOCSY measurements from anomeric hydrogen signals enabled chemical shift assignments up to H-4. The chemical shifts of H-5 of galactose were obtained from 1D TOCSY methods by selective excitation of H-4 signals or by NOE from the anomeric hydrogen. The chemical shift one of the galactose C-6 hydrogen (H-6*S*) signals was obtained by the observation of NOE from the corresponding H-4. For the sialic acid units, TOCSY and NOESY measurements were useful to establish chemical shifts of hydrogens up to H-6. ^{1}H–^{13}C correlated and relayed spectra were useful for obtaining all of the carbon chemical shifts and

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Table II. ^{1}H Chemical Shifts of the Heptasaccharides
αDNeuAc^d(2→6)βDGal^c(1→4)βDGlcNAc(^{b}1→x)[αDNeuAc$^{d'}$(2→6)Gal$^{c'}$(1→4)βDGlcNAc$^{b'}$(1→y)]βDGal^{a}O(CH$_2$)$_5$COOCH$_3$[a]

unit		x = 2, y = 3 (27)	x = 2, y = 4 (28)	x = 2, y = 6 (29)	x = 3, y = 6 (30)	x = 4, y = 6 (31)
αDNeuAc^d	H-3eq	2.68 (5.0, 12.4)	2.68 (4.7, 12.3) [0.65]	2.67 (4.6, 12.2) [0.54]	2.68 (4.7, 12.4) [0.73]	2.68 (4.6, 12.4) [1.38]
	H-3ax	1.72 (12.4)	1.71 (12.3) [0.63]	1.72 (12.2) [0.58]	1.73 (12.4) [0.72]	1.73 (12.4) [0.74]
	H-4	3.66	3.66	3.66	3.66 (10.0)	3.66
	H-5	3.81 (10.1)	3.80 (10.0)	3.80	3.82 (10.1)	3.80 (10.2)
	H-6	3.71	3.70	3.70	3.72	3.71 (1.5)
	H-7	3.57	3.56	3.56	3.55	3.56
	H-8	3.89	3.89	3.89	3.90	3.88
	H-9A	3.88	3.88	3.88	3.89	3.89
	H-9B	3.64	3.64	3.64	3.65	3.64
	NAc	2.04	2.04	2.03	2.04	2.04
βDGal^c	H-1	4.45 (7.8)	4.45 (7.9) [1.30]	4.45 (8)	4.46 (8.0) [1.57]	4.46 (8.0) [1.56]
	H-2	3.54 (9.7)	3.54 [1.8]	3.54 (10)	3.55 (9.9)	3.55 (9.8)[b]
	H-3	3.66 (3.1)	3.67	3.68 (2.7)	3.68 (3.5)	3.68 (3.5)
	H-4	3.93	3.93	3.93	3.93	3.93
	H-5	3.83	3.82	3.83	3.84	3.83
	H-6A	3.99	3.99	4.00	4.01	3.99
	H-6B	3.56	3.56	3.56	3.56	3.55
βDGlcNAc^b	H-1	4.74 (8.2)	4.85 (8.5) [1.54]	4.85 (8.0) [1.58]	4.74 (8.2) [1.72]	4.72 (8.4) [1.81]
	H-2	3.71	3.76	3.76	3.80	3.76
	H-3	3.79	3.76	3.76	3.79	3.76
	H-4	3.61 (9.9)	3.62	3.62	3.67	3.66
	H-5	3.44	3.53	3.58	3.61	3.56
	H-6A	3.99 (2.2, 12.2)	3.96 (<2, 12.3)	3.98 (<2, 12.4)	3.96 (<2, 12.2)	3.98 (1.6, 12.2)
	H-6B	3.85 (3.0)	3.83 (5.2)	3.83 (5.4)	3.86 (4.5)	3.85 (5.0)
	NAc	2.15[b]	2.10[b]	2.07[b]	2.06[b]	2.08[c]
αDNeuAc$^{d'}$	H-3eq	2.67 (5.0, 12.4)	2.67 (4.6, 12.3) [0.65]	2.67 (4.6, 12.2) [0.54]	2.68 (4.7, 12.4) [0.73]	2.68 (4.6, 12.4)
	H-3ax	1.71 (12.4)	1.71 (12.4) [0.63]	1.71 (12.2) [0.58]	1.72 (12.4) [0.72]	1.72 (12.4) [0.74]
	H-4	3.66	3.66	3.66	3.66 (10.0)	3.66
	H-5	3.81 (10.1)	3.80 (10.0)	3.80	3.81 (10.1)	3.80 (10.2)
	H-6	3.71	3.70	3.70	3.72	3.71 (1.5)
	H-7	3.57	3.56	3.56	3.55	3.56
	H-8	3.89	3.89	3.89	3.90	3.88
	H-9A	3.88	3.88	3.88	3.89	3.89
	H-9B	3.64	3.64	3.64	3.65	3.64
	NAc	2.04	2.04	2.03	2.04	2.04
βDGal$^{c'}$	H-1	4.43 (8.0)	4.45 (7.9) [1.31]	4.45 (8.0)	4.46 (8.0) [1.54]	4.45 (8.0) [1.56]
	H-2	3.57 (9.7)	3.54 [1.8]	3.54 (10)	3.55 (9.9)	3.54 (9.8)[b]
	H-3	3.66 (3.1)	3.67	3.68 (2.7)	3.68 (3.50)	3.68 (3.5)
	H-4	3.93	3.93	3.93	3.93	3.93
	H-5	3.83	3.82	3.83	3.84	3.83
	H-6A	3.99	3.99	4.00	4.01	3.99
	H-6B	3.56	3.56	3.56	3.56	3.55
βDGlcNAc$^{b'}$	H-1	4.69	4.76 (7.8) [1.58]	4.59 (8.3) [1.28]	4.59 (8.4) [1.57]	4.57 (8.0) [1.51]
	H-2	3.81	3.79	3.76	3.76	3.75
	H-3	3.81	3.78	3.76	3.77	3.75
	H-4	3.70	3.63	3.63	3.64	3.66 (9.5)
	H-5	3.56	3.60	3.63	3.65	3.61
	H-6A	3.93 (<2, 12.2)	3.99 (<2, 12.2)	4.00 (<2, 12.2)	4.01 (<2, 12.5)	4.00 (2.2, 12)
	H-6B	3.86 (4.5)	3.88 (5.3)	3.83 (4.9)	3.83 (4.4)	3.85 (5.5)
	NAc	2.10[b]	2.08[b]	2.05[b]	2.06[b]	2.05[c]
βDGal^a	H-1	4.55 (8.0)	4.48 (7.8) [1.32]	4.45 (8.0)	4.37 (8.0) [1.39]	4.35 (8.0) [1.56]
	H-2	3.84 (9.7)	3.50 (9.9) [1.94]	3.63	3.55 (9.5)	3.37 (9.5)
	H-3	3.99 (3.5)	3.82 (3.5)	3.70 (3.5)	3.71 (3.6)	3.72 (3.0)
	H-4	4.14	4.05 (0.6) [1.38]	3.85	4.13 [1.68]	4.02
	H-5	3.67	3.65	3.76	3.78	3.78
	H-6A	3.85	3.75	4.00	4.04	4.11 [1.41]
	H-6B	3.73	3.66	3.77	3.78	3.79
(CH$_2$)$_5$COOCH$_3$	H-1A	3.91	3.87	3.88	3.89	3.87
	H-1B	3.69	3.67	3.67	3.64	3.61
	H$_2$-2	1.65	1.63	1.66	1.65	1.64
	H$_2$-3	1.41	1.39	1.42	1.41	1.40
	H$_2$-4	1.66	1.64	1.65	1.65	1.64
	H$_2$-5	2.43	2.42	2.43	2.43	2.42
	OCH$_3$	3.70	3.71	3.71	3.70	3.70

[a] The hydrogen chemical shifts are expressed relative to HOD (4.83 ppm, ∂ acetone 2.23 ppm). The vicinal hydrogen–hydrogen coupling constants in hertz and the proton spin-lattice relaxation times (T_1) in seconds are shown in the parentheses and brackets, respectively. [b,c] These assignments may be reversed.

for assigning those hydrogen chemical shifts that could not be obtained directly.

In general, the chemical shifts of H-1 of the GlcNAc unit attached to the 2-position of βDGal^a occurred at 4.8 ppm, those of the units attached to the 3- and 4-positions at 4.7 ppm, and those from units at 6 of the galactose occurred at 4.5–4.6 ppm, and this trend was seen as long as the two GlcNAc units were not adjacent to each other. Addition of a galactose to the 4-position of GlcNAc, as seen in the case of pentasaccharides **21–26**, had only a marginal effect on the remainder of hydrogens. The addition of the terminal sialic acids, as seen in the heptasaccharides **27–31**, generally deshielded the GlcNAc H-1 by about 0.03 ppm, as reported by Vliegenthart et al.[33] One exception to this was the 2,3-heptasaccharide **27**, where both of the GlcNAc H-1 signals were shielded by about 0.1 ppm (Table II). The chemical shifts of the galactose hydrogens among the pentasaccharides **21–26**

Table III. ^{13}C Chemical Shifts of the Heptasaccharides αDNeuAc^{d}(2→6)βDGal^{c}(1→4)βDGlcNAc^{b}(1→x)[αDNeuAc$^{d'}$(2→6)βDGal$^{c'}$(1→4)βDGlcNAc$^{b'}$(1→y)]βDGal^{a}O$(CH_2)_5COOCH_3$[a]

unit		x = 2, y = 3 (**27**)	x = 2, y = 4 (**28**)	x = 2, y = 6 (**29**)	x = 3, y = 6 (**30**)	x = 4, y = 6 (**31**)
αDNeuAc^{d}	C-2	100.8		100.8	101.0	100.8
	C-3	40.7	40.8	40.6	40.8 [0.16]	40.8
	C-4	68.8	68.9	68.8	68.9 [0.31]	68.9
	C-5	52.5	52.5	52.5	52.5 [0.31]	52.6
	C-6	73.2	73.2	73.2	73.2 [0.31]	73.2
	C-7	69.0	69.1	69.0	69.1 [0.28]	69.1
	C-8	72.4	72.4	72.3	72.4 [0.34]	72.4
	C-9	63.3	63.3	63.3	63.3 [0.23]	63.3
	NAc	22.7	22.7	22.7	22.7	22.7
βDGal^{c}	C-1	104.1	104.1	104.2	104.2 [0.30]	104.1
	C-2	71.3	71.4	71.4	71.4 [0.30]	71.4
	C-3	73.0	73.1	73.1	73.1 [0.30]	73.1
	C-4	69.0	69.1	69.0	69.0 [0.28]	69.1
	C-5	74.3	74.3	74.3	74.3 [0.28]	74.3
	C-6	63.9	64.0	63.9	64.0 [0.17]	64.0
βDGlcNAc^{b}	C-1	99.8	101.7	101.9	103.1 [0.30]	102.4
	C-2	56.4	55.9	55.9	55.7 [0.26]	55.7
	C-3	72.7	73.0	73.1	73.0 [0.29]	73.2
	C-4	81.6	81.4	81.5	81.1 [0.30]	81.3
	C-5	75.3	75.1	75.2	75.0 [0.28]	74.9
	C-6	60.8	61.2	61.2	60.8 [0.17]	61.0
	NAc	23.6	23.4[b]	23.0	23.1 [0.88][b]	23.1[b]
αDNeuAc$^{d'}$	C-2	100.8		100.8	101.0	100.8
	C-3	40.7	40.8	40.6	40.8 [0.16]	40.8
	C-4	68.8	68.9	68.8	68.9 [0.30]	68.9
	C-5	52.5	52.5	52.5	52.5 [0.31]	52.6
	C-6	73.2	73.2	73.2	73.2 [0.31]	73.2
	C-7	69.0	69.1	69.0	69.1 [0.28]	69.1
	C-8	72.4	72.4	72.3	72.4 [0.34]	72.4
	C-9	63.3	63.3	63.3	63.3 [0.23]	63.3
	NAc	22.7	22.7	22.7	22.7	22.7
βDGal1→4$^{c'}$	C-1	104.4	104.1	104.2	104.2 [0.31]	104.2
	C-2	71.3	71.4	71.4	71.4 [0.30]	71.4
	C-3	73.0	73.1	73.1	73.1 [0.30]	73.1
	C-4	69.0	69.1	69.0	69.0 [0.28]	69.1
	C-5	74.3	74.3	74.3	74.3 [0.28]	74.3
	C-6	63.8	64.0	63.9	64.0 [0.17]	64.0
βDGlcNAc$^{b'}$	C-1	102.2	102.2	101.7	101.8 [0.26]	101.9
	C-2	56.2	55.8	55.4	55.5 [0.28]	55.4
	C-3	72.8	73.0	73.1	73.1 [0.32]	73.2
	C-4	80.9	81.4	81.4	81.4 [0.27]	81.4
	C-5	75.2	74.9	75.0	75.1 [0.28]	75.1
	C-6	60.6	61.2	61.0	61.0 [0.18]	61.0
	NAc	23.2[b]	23.1[b]	23.0[b]	23.0 [1.2][b]	23.0[b]
βDGal^{a}	C-1	101.3	101.6	101.9	103.2 [0.30]	103.1
	C-2	74.7	78.9	79.1	70.3 [0.29]	71.5
	C-3	81.5	74.3	73.5	82.9 [0.27]	73.4
	C-4	69.7	76.6	69.5	69.2 [0.26]	77.3
	C-5	75.0	74.6	73.8	74.0 [0.29]	73.3
	C-6	61.3	61.2	69.2	70.0 [0.13]	70.5
$(CH_2)_5COOCH_3$	CH_2-1	70.6	70.5	70.8	70.7 [0.31]	70.5
	CH_2-2	29.0	29.0	29.1	29.0 [0.36]	29.0
	CH_2-3	25.2	25.2	25.4	25.3 [0.56]	25.3
	CH_2-4	24.6	24.7	24.7	24.7 [0.69]	24.7
	CH_2-5	34.3	34.3	34.3	34.3 [0.88]	34.3
	OCH_3	52.7	52.7	52.8	52.7 [2.5]	52.8

[a] The carbon chemical shifts are expressed relative to 1,4-dioxane using the deuterium lock of the spectrometer, which set the chemical shift of dioxane at 66.9 ppm. The spin–lattice relaxation times (T_1) in seconds are given in brackets for the heptasaccharide **30**. [b] These assignments may be reversed.

and galactose and sialic acid hydrogens among the heptasaccharides **27–31** were nearly identical and in agreement with the published values for the *N*-acetyllactosamine and αDNeuAc(2→6)βDGal(1→4)βDGlcNAc derivatives.[28,33]

In the carbon spectra of the trisaccharides **15–20**, except for the chemical shifts of C-1 and C-2 of the GlcNAc units (which are dependent on the nature of the aglycon of Gal[a]), those of the remainder of the carbon atoms of the two GlcNAc units were nearly the same (Table 1, supplementary material). However, when the two GlcNAc units are placed on adjacent carbon atoms, as seen in **15** and **18**, then these units interact and this is reflected in their carbon chemical shifts. The magnitudes of deshielding for the glycosylated and the adjacent carbon atoms of the Gal[a] agreed with those published.[34] However, in the case of the trisaccharide **18**, there was an unusual deshielding for C-3[a] (83.5 ppm, compare this with 81.4 ppm in compound **15**) indicating steric crowding in this 3,4-trisaccharide. This interaction may result in the alteration of the normal glycosidic torsion angles[35] or the glycosidic oxygen bond angle. This deshielding effect was seen in the pentasaccharide **24** also. The ^{13}C chemical shifts for the lactosamine core in the pentasaccharides **21–23**, **25**, and **26** (Table III) and for the αDNeuAc(2→6)βDGal(1→4)βDGlcNAc

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Table IV. Inter- and Intra-ring Hydrogen NOEs at Different Mixing Times in the 1D NOESY Experiments for the Pentasaccharides βDGal^{c}(1→4)βDGlcNAc^{b}(1→x)[βDGal$^{c'}$(1→4)βDGlcNAc$^{b'}$(1→y)]βDGal^{a}OR (**21–26**)

compound	hydrogen experiencing NOE	mixing time, ms 100	200	300	400	calcd dist from H-1^{b}, Å[a]	hydrogen experiencing NOE	mixing time, ms 100	200	300	400	calcd dist from H-1$^{b'}$, Å[a]
		From H-1^{b}						From H-1$^{b'}$				
x = 2, y = 3 (**21**)	H-3^{b} + H-2^{a}	1.08	1.85	2.56	3.13	2.8[b]	H-3$^{b'}$	1.29	1.81	2.30	2.67	
	H-5^{b}	1.08	1.94	2.51	2.94		H-5$^{b'}$	1.50	3.29	5.32	7.33	
	H-3^{a}	0.23	0.37	0.52	0.65	3.2	H-3^{a}	2.64	5.88	7.17	8.57	2.2
	H-1^{a}	0.16	0.26	0.33	0.45	3.3	H-4^{a}	0.02	0.55	0.80	1.35	3.2[c]
x = 2, y = 4 (**22**)	H-5^{b}	0.17	0.33	0.40	0.52		H-5$^{b'}$[d]	2.85	6.09	7.61	9.34	
	H-2^{a}	0.42	0.76	0.96	1.13	2.1	H-3^{a}	0.21	0.13	0.22	0.43	4.1
	H-3^{a}	0.11	0.21	0.33	0.31	2.6	H-4^{a}[d]	2.11	3.84	5.05	5.84	2.6
								From H-4^{a}				
							H-1$^{b'}$	1.12	1.32	2.01	2.84	2.4
							H-5^{a}	0.69	1.22	1.56	1.82	
								From H-1$^{b'}$				
x = 2, y = 6 (**23**)	H-3^{b}	2.73	3.25	2.19	2.00		H-3$^{b'}$	4.84	2.66	1.98	2.46	
	H-5^{b}	1.81	1.50	2.11	2.78		H-5$^{b'}$	0.65	1.21	1.62	1.81	
	H-2^{a}	0.73	1.86	2.67	3.37	2.4	H-2$^{b'}$ + H-6R^{a}	0.06	0.71	0.51	0.81	
							H-6S^{a}	0.15	0.24	0.40	0.33	3.2
x = 3, y = 4 (**24**)	H-5^{b}	1.04	1.87	2.46	2.83		H-5$^{b'}$	1.69	2.10	3.07	3.77	
	H-3^{a}		0.96	1.11	1.45	2.8	H-3$^{b'}$	0.28	0.36	0.74	0.94	
	H-4^{a}		0.22	0.35	0.43	3.5	H-4^{a}	1.27	2.34	3.09	3.62	2.4
		From H-4^{a}						From H-4^{a}				
	H-1^{b}	0.15	0.30	0.44	0.57	3.2	H-1$^{b'}$	1.36	2.44	3.20	3.76	2.2
	H-3^{a}		1.83	1.30	1.73		H-5^{a}	0.52	1.44	1.71	2.09	
		From H-1^{b}						From H-1$^{b'}$				
x = 3, y = 6 (**25**)	H-3^{b}	0.27	1.53	1.49	1.32		H-3$^{b'}$	1.16	2.09	2.26	2.70	
	H-5^{b}	1.27	2.10	2.66	3.00		H-5$^{b'}$	0.51	1.00	1.30	1.57	
	H-3^{a}	5.45	3.88	3.72	3.64	2.1	H-2$^{b'}$ + H-6R^{a}	1.06	1.19	2.35	3.42	
	H-4^{a}	0.43	0.52	0.14	0.54	3.0	H-6S^{a}	0.16	0.25	0.32	0.42	3.0
								From H-4^{a}				
							H-1^{b}	0.26	0.28	0.34	0.35	3.0
							H-3^{a}	0.89	1.60	1.52	1.50	
							H-5^{a} + H-6R^{a}	1.26	2.13	2.83	3.36	
							H-6S^{a}	0.19	0.87	1.34	1.65	2.9
								From H-1$^{b'}$				
x = 4, y = 6 (**26**)	H-3^{b}	1.86	2.41	2.85	2.66		H-3$^{b'}$	2.09	2.04	2.47	2.17	
	H-5^{b}		1.35	1.97	2.35		H-5$^{b'}$	0.36	1.18	1.73	1.88	
	H-4^{a}	1.27	2.28	3.14	3.50	2.2	H-6R^{a}[e]		4.08	3.65	4.70	2.0
							H-6S^{a}	0.13	0.30	0.44	0.54	2.9

[a] The internuclear distance was calculated using H-5 of GlcNAc (H-1–H-5 = 2.4 Å) as the internal standard. [b] Internuclear distance to H-2^{a}. This was done by subtracting the expected NOE for H-3^{b} (based on that observed for the other intrahydrogen H-5^{b}). [c] Due to very weak NOE at 100 ms, it was not included in the calculation. [d] The overall larger NOE values for all of the hydrogens is due to the negative contribution of the overlapping HOD signal to the selectively saturated anomeric hydrogen signal. [e] Partly overlapped with hydrogen signals for H-2$^{b'}$.

Table V. Inter- and Intra-ring Hydrogen NOEs at Different Mixing Times in the 1D NOESY Experiments for the Heptasaccharides αDNeuAc^{d}(2→6)βDGal^{c}(1→4)βDGlcNAc^{b}(1→x)[αDNeuAc$^{d'}$(2→6)βDGal$^{c'}$(1→4)βDGlcNAc$^{b'}$(1→y)]βDGal^{a}OR (**27** and **30**)

compound	hydrogen experiencing NOE	mixing time, ms 50	100	200	300	400	800	calcd dist from H-1^{b}, Å[a]	hydrogen experiencing NOE	mixing time, ms 50	100	200	300	400	800	calcd dist from H-1$^{b'}$ Å[a]
			From H-1^{b}								From H-1$^{b'}$					
x = 2, y = 3 (**27**)	H-3^{b}		2.98	4.55		7.10	8.74		H-3$^{b'}$		1.82	2.71		4.05	5.10	
	H-5^{b}		3.41	6.80		9.42	11.0		H-5$^{b'}$		2.02	3.69		5.39	6.33	
	H-1^{a}		0.56	1.47		2.55	2.78	3.2	H-3^{a}		2.31	2.80		5.55	6.01	2.3
	H-2^{a}		2.78	3.91		5.91	7.49	2.6	H-4^{a}		0.17	0.27		0.56	0.89	3.8
x = 3, y = 6 (**30**)	H-5^{b}	0.93	1.78	3.13	4.05	4.74	5.15		H-5$^{b'}$	0.98	2.20	3.87	5.03	5.55	6.96	
	H-2^{a}	0.12	0.07	0.02	0.23	0.29	0.44	4.5	H-3$^{b'}$ + H-6R^{a}	2.51	5.40	8.76	10.60	13.50	16.60	2.2[b]
	H-3^{a}	1.77	3.33	5.91	6.94	7.91	7.89	2.2	H-4^{a}		0.07	0.18	0.32	0.44	0.98	4.3
	H-4^{a}	0.26	0.58	1.04	1.44	1.75	2.49	3.0	H-6S^{a}	0.36	0.68	1.25	1.70	1.90	2.28	2.9
			From H-4^{a}								From H-4^{a}					
	H-3^{a}		2.17	3.05		4.86			H-5^{a} + H-6R^{a}		2.16	3.65		5.93		3.2
	H-1^{b}		0.42	0.86		1.44		3.2	H-6S^{a}		1.01	1.99		2.92		2.7

[a] The internuclear distance was calculated using H-5 of GlcNAc (H-1–H-5 = 2.4 Å) as the internal standard. [b] Internuclear distance to H-6R^{a}. This was done by subtracting the expected NOE for H-3$^{b'}$ (based on that observed for the other intrahydrogen H-5$^{b'}$).

sequences in the heptasaccharides **27–31** were essentially the same as those published,[28,36,37] indicating very little interaction with the reducing galactose end.

Nuclear Overhauser Enhancement Measurements on Penta- and Heptasaccharides. In order to evaluate the conformational properties of these penta- and heptasaccharides, one- and two-dimensional NOESY experiments were carried out. The results are reported in Tables IV and V. Since the proton and carbon chemical shifts of the βDGal(1→4)βDGlcNAc fragments among the pentasaccharides and the αDNeuAc(2→6)βDGal(1→4)-βDGlcNAc fragments among the heptasaccharides were the same as those of the monovalent structures, the conformational properties involving these sequences were assumed to be the same as those published.[37,38] Therefore, our investigation was centered

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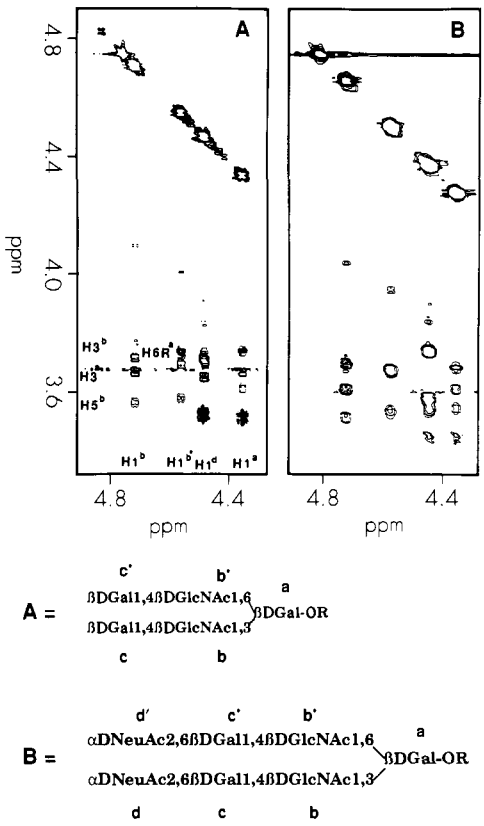

Figure 4. Segments of the 2D contour maps (600 MHz, 200 ms mixing time) of the pentasaccharide **25** (A) and the corresponding heptasaccharide **30** (B) showing the NOESY cross peaks from the anomeric hydrogens.

around the glycosidic linkages containing the two GlcNAc's and the Gal^a units in order to evaluate how the two trisaccharide receptor determinants are oriented relative to each other for interacting with the hemagglutinins.

The 2D NOESY spectra of the 3,6-penta- and 3,6-heptasaccharides (**25** and **30**, respectively, Figure 4) clearly indicate the good quality of the Overhauser enhancements from the anomeric to intra- and inter-ring hydrogens. Similar results were obtained for the 2,3-penta- and 2,3-heptasaccharides (**21** and **27**, respectively) and 2,4-penta- and 2,4-heptasaccharides (**22** and **28**, respectively) as well (Figures 2 and 3 of the supplementary material). Importantly, Figure 4 shows that the conformations around the GlcNAc^b, GlcNAc$^{b'}$, and Gal^a units in the penta- and the corresponding heptasaccharides are nearly identical. Thus, in Figure 4A,B, NOEs from H-1^b to the two intra-ring hydrogens (H-5^b, δ = 3.60 ppm in **25** and 3.61 ppm in **30**; H-3^b, δ = 3.75 ppm in **25** and 3.79 in **30**) and the aglyconic hydrogen (H-3^a, δ = 3.70 ppm in **25** and 3.71 in **30**) and NOEs from H-1$^{b'}$ to H-5$^{b'}$ (δ = 3.61 ppm in **25** and 3.65 ppm in **30**) and H-3$^{b'}$ (δ = 3.72 ppm in **25** and 3.77 ppm in **30**) and the aglyconic hydrogens (H-6*S*^{a}, δ = 3.77 ppm in **25** and 3.78 in **30**) are observed.

In order to quantify the NOE data and to estimate useful inter-ring hydrogen distances around the GlcNAc$^{b \text{ and } b'}$ and Gal^a linkages, 1D NOESY measurements at different mixing times using Gaussian-shaped selective pulses were used for the pentasaccharides **21–26** (Table IV) and for the two heptasaccharides **27** and **30** (Table V). The NOE buildup curves for the penta- (Figure 4, supplementary material) and heptasaccharides were linear below 200 ms. Assuming the r^{-6}-th distance dependence of hydrogen NOEs,[39] the H–H distances in these penta- and heptasaccharides could be calculated from a known internal standard. For example, with GlcNAc^b or GlcNAc$^{b'}$, the two intra-ring hydrogens H-3 or H-5 could be used as internal standards for evaluating unknown inter-ring hydrogens. In Tables IV and V, we have used H-5 of GlcNAc's as the internal standard, since the signals for these hydrogens appeared well separated from other overlapping hydrogens. To calculate the inter-ring hydrogen distances from weak NOEs, the NOE data was extrapolated to zero mixing times according to method of Baleja et al.[40] in order to minimize the errors in distance estimation, especially for the hydrogens separated by more than 3 Å.

For the 2,3-pentasaccharide **21**, NOEs from H-1^b to intra-ring hydrogens H-3^b and H-5^b and to the inter-ring H-2^a, H-1^a, and H-3^a were seen. Since the chemical shifts of H-2^a and H-3^b were nearly degenerate (δ H-2^a = 3.82, H-3^b = 3.81 ppm), the NOE for H-2^a was obtained by subtracting the expected NOE for H-3^b (based on that observed for the other intra-ring hydrogen H-5^b) from the total signal, and this value was used to calculate the H-2^a–H-1^b distance (2.8 Å, Table IV). In the case of the glycosidic linkage involving GlcNAc$^{b'}$, NOEs from H-1$^{b'}$ to two inter-ring hydrogens H-3^a and H-4^a were observed. On this basis, the H-1$^{b'}$–H-3^a distance was calculated as 2.2 Å (Table IV).

As compared to **21**, the H-1^b–H-2^a and H-1^b–H-3^a distance in 2,4-pentasaccharide **22** is shorter (2.1 and 2.6 Å, respectively) and the H-1^b is closer to H-3^a than to H-1^a (Table IV). For **22**, large NOEs from H-1$^{b'}$ to H-4^a, as well as from H-4^a to H-1$^{b'}$, were seen, and the H-1$^{b'}$–H-4^a distance estimated from either of the hydrogens was in good accordance with the expectation based on calculated molecular models (Table IV; also see the supplementary material).

For the 2,6-pentasaccharide **23**, we have, in addition to the rigid GlcNAc1,2 linkage, a more flexible 1,6 linkage. At 200-ms mixing times and above, NOEs from H-1$^{b'}$ to H-6*R*^{a} and H-6*S*^{a} were seen. (The assignments of H-6*R*^{a} and H-6*S*^{a} were made on the basis of NOEs from H-4^a. The signal of H-6*S*^{a} at 4.00 ppm exhibited strong NOE from H-4^a, as opposed to H-6*R*^{a}.) However, the NOE for H-6*R*^{a} could not be quantified for distance measurements, as its signals extensively overlapped with those of H-2$^{b'}$ (δ H-6*R*^{a} = 3.75, δ H-2$^{b'}$ = 3.76 ppm), which also exhibited NOE from H-1$^{b'}$.

One-dimensional NOEs for the other pentasaccharides **24–26** showed similar intense NOEs from the anomeric to the corresponding aglyconic hydrogens indicating close proximity of these two hydrogens (Table IV).

For the two heptasaccharides **27** and **30**, the magnitudes of NOEs from the GlcNAc anomeric hydrogens to Gal^a hydrogens (Table V) are similar to those observed for the corresponding asialo pentasaccharides **21** and **25**, suggesting that their conformational properties around the GlcNAc$^{b,b'}$ and Gal^a linkages are nearly identical.

Calculations of the NeuAc End Distance Distribution. Monte Carlo (MC) and HSEA calculations were performed for the penta- and heptasaccharides listed in Table I, in an effort to determine the end distance distribution between the C-2 carbons of the NeuAc units. The procedures used in these calculations were similar to those described in the literature,[41,42] except that the GEGOP program[43] and the MC simulations[44] were used to evaluate the dynamics of the conformational preferences. For the sialosidic and lactosamine linkages, the conformational preferences from the published reports[37,38] were used as the starting points in the MC calculations. The resulting inter-ring hydrogen distances around the GlcNAc$^{b,b'}$ and Gal^a linkages have been compared with those calculated from NOE measurements (Table 5, supplementary material). These results show, especially for less than 3 Å inter-ring hydrogen distances, a good agreement

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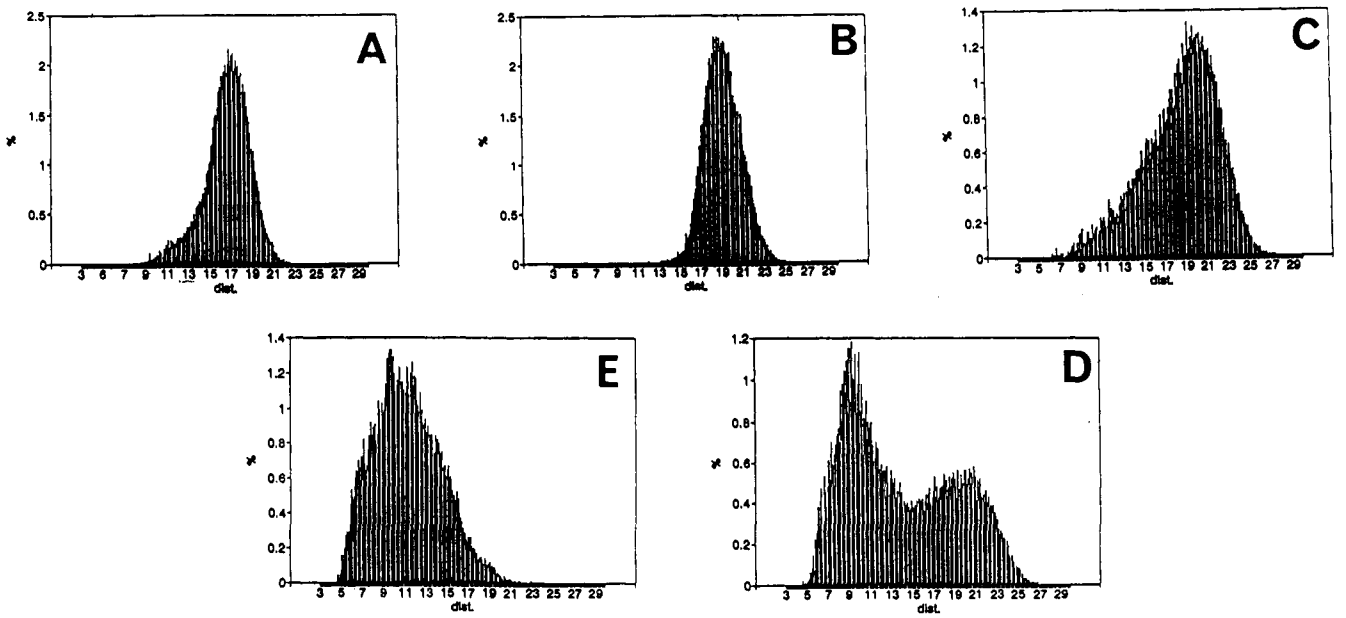

Figure 5. Distribution of conformer population (vertical axis) as a function of distance (horizontal axis, in 0.1-Å steps) between the anomeric carbons of the two end sialic acids of the heptasaccharides **27** (A), **28** (B), **29** (C), **30** (D), and **31** (E), as indicated by the Monte Carlo sampling methods (see the Experimental Section).

between the NOE-based values and those obtained either from the minimum energy conformation or from the MC averaged distances (both at 300 and 600 K). For the 1,6 linkages, a better agreement between the NOE-based distances and MC simulations performed at 600 K is seen, as a result of the sampling of all ω angles. Particularly noteworthy to our investigation was that the distance distribution between the C-2 to C-2 of the end sialic acids (Figure 5) is centered around 17–19 Å in the 2,3-, 2,4-, and 2,6-disialosides (compounds **27**, **28**, and **29**, respectively) to about 9–10 Å in the case of the 3,6- and 4,6-disialosides (compounds **30** and **31**). Similar results were obtained when we compared the C-5 to C-5 or C-8 to C-8 atoms of the two end sialic acid residues. As shown in the next section, these two latter compounds exhibited the greatest inhibitory potencies, as compared to the other disialosides.

Inhibition of Viral Adsorption to Resialylated Erythrocytes by Synthetic Disialosides. The synthetic bivalent sialosides were tested as inhibitors for the adsorption of influenza virus of human isolates to resialylated erythrocytes according to the procedure by Pritchett et al.[9] The erythrocytes used in this assay were enzymatically sialylated to contain αDNeuAc(2→6)βDGal(1→4)βDGlcNAc sequences and are only partially sialylated in order to evaluate the binding potencies of weakly binding sialosides. The results are shown in Table VI. Weakly binding methyl αDNeuAc was used as a reference compound which shows 50% inhibition at 1.90 mM concentration. (Under these conditions, sialyl 2,6-lactosamine derivative LSTc was a 1.7 times more potent inhibitor than the methyl sialoside (see reference to Prichett et al.).) We also prepared a monosialoside αDNeuAc(2→6)βDGal(1→4)-βDGlcNAc(1→2){βDGal(1→4)βDGlcNAc(1→4)}βDGalO-$(CH_2)_5COOCH_3$ as another reference monosialoside, which contained only one trisaccharide receptor determinant. It can be seen that this is a 2-fold more potent inhibitor as the methyl sialoside. However, when the second sialic acid was added to this, the inhibitory potency dropped 1.5-fold, indicating the detrimental effect of the addition of the second sialic acid. The binding potencies increased from 2,3-disialoside **27** onward with the 3,6- and 4,6-disialosides (**30** and **31**, respectively) exhibiting 10- and 8.4-fold better inhibition. However, the binding dropped again in the case of 2,6-disialoside **29**.

These results together with the MC simulations indicate that the proper placement of the two sialic acids may be important for efficient binding to the hemagglutinin molecules. In general, disialosides with an end distance of about 9 Å (Figure 5) appear to bind the best.

Table VI. Inhibition of Influenza Virus Adsorbtion to Erythrocytes by Cluster Sialosides

compound	concentration for 50% inhibition,[a] mM	relative potency
Me αDNeuAc	1.9	1.0
2,3-disialoside **27**	0.25	7.2
2,4-disialoside **28**	1.3	1.5
2,6-disialoside **29**	0.38	4.8
3,6-disialoside **30**	0.18	10
4,6-disialoside **31**	0.23	8.4
BSA–3,6-disialoside **33**	<0.12	>14.4
byproduct of **28**[b]	0.95	1.9

[a] Inhibition of influenza virus (A/Aichi/2/68) to resialylated erythrocytes was examined as described by Pritchett et al. (*Virology* **1987**, *160*, 502–506). [b] The structure of the monosialoside is tentatively assigned as αDNeuAc(2→6)βDGal(1→4)βDGlcNAc(1→2)[βDGal(1→4)βDGlcNAc(1→4)]βDGalO$(CH_2)_5COOCH_3$.

In preliminary experiments, we attached the pentasaccharide **25** to bovine serum albumin (BSA),[18] which was then sialylated with 2,6-sialyltransferase and CMP-NeuAc. This conjugate exhibited 50% inhibition at concentrations less than 0.25 mM. Considering the fact that the BSA–pentasaccharide conjugate is only partially sialylated, the increased binding potency of the protein-bound sialoside conjugate promises that the preparation of a macromolecule containing a 3,6-disialoside should provide very good inhibitors for influenza-mediated hemagglutination of erythrocytes.

Discussion

The importance of multivalent cell surface glycosides in mediating cell adhesion by protein receptor molecules (lectins) has been elegantly demonstrated over the years by Lee and coworkers.[11,45] It appears[7,14–16] that such cooperative binding phenomena may also occur when influenza virus hemagglutinins bind to the sialyl oligosaccharides of cell surface glycoproteins and glycolipids. To examine this further for the eventual design of synthetic inhibitors, we undertook the synthesis of a series of heptasaccharides containing two sialic acid recognition units with a systematic distance variation between them. We recognized that the lactosamine core holding the sialic acid may be important

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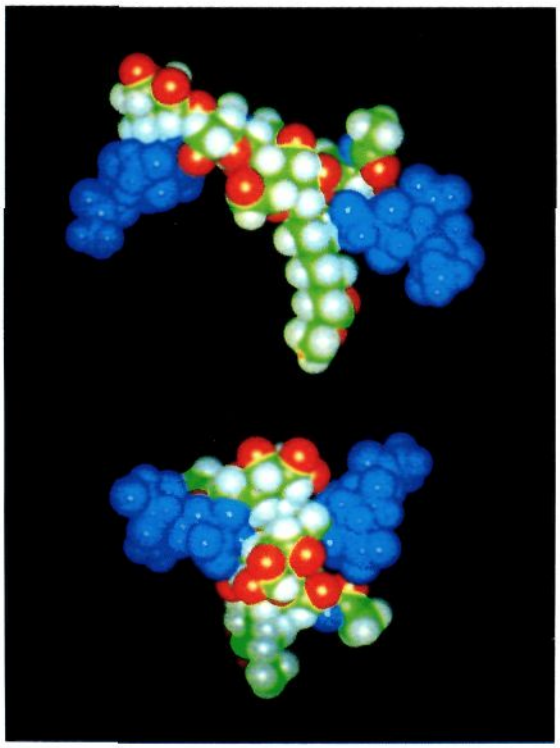

Figure 6. CPK models corresponding to the lowest energy conformers obtained by the GEGOP procedures (see the Experimental Section) for the heptasaccharides **28** (A) and **30** (B).

in the binding event by holding the sialic acids in the proper orientation.[37]

Even though the heptasaccharides are complex and are a synthetic challenge, we were able to synthesize them by combined chemical and enzymatic methods in quantities sufficient for biological evaluations. Furthermore, we have attached a tether to all of these heptasaccharides so that these molecules can be attached to proteins,[18] can be polymerized as has been demonstrated by Roy et al.[13] and by Spaltenstein and Whitesides,[14] and can be attached to hydrophobic residues such as 1-naphthylmethyl groups that have been shown to dramatically increase the binding potencies to hemagglutinins.[17]

In addition, we have been able to subject these penta- and heptasaccharides to detailed NMR investigation in order to elucidate the structure–activity relationships. As can be seen from Figure 3, the proton NMR spectra are very complex due to extensive overlap of methine hydrogen signals in the region between 3 and 4 ppm and due to the presence of identical αDNeuAc(2→6)βDGal(1→4)βDGlcNAc sequences. Using modern NMR pulse techniques, complete proton and carbon chemical shifts were established for these molecules.

Comparison of the proton and carbon chemical shifts of the two disaccharide fragments, namely, αDNeuAc(2→6)βDGal units in the heptasaccharides, with those published[37] indicated that the conformational properties around the sialoside linkages in these bivalent structures should be in the "bent" conformation as proposed in the literature.[37] This was also evident from the 2D NOESY spectra of the heptasaccharides. Therefore, we could estimate the sialic acid end distances by determining the conformational properties around the glycosidic linkage involving the two GlcNAc's (units b and b′) and the galactose (unit a) and in conjunction with the MC simulations.

The molecular models thus constructed for the heptasaccharides **27–30** suggest that the end sialic acid distances can vary from ~19 Å in 2,4-heptasaccharide **28** to ~9 Å in 3,6- and 4,6-sialosides (Figure 5). On the basis of the models proposed by Glick et al.,[15] these two distances are too short for these molecules to engage in intermolecular binding to the adjacent hemagglutinin molecules of the influenza virus (Figure 1). However, these appear well poised to interact intramolecularly to any of the two sites of the hemagglutinin trimer. (The increased binding potency of the synthetic sialosides may be also due to factors other than intramolecular binding to HA. This is likely to be solved through the crystal structure of the oligosaccharide–protein complex or probably by extensive NMR methods (see ref 10).)

A possible explanation for the differential binding potencies of the heptasaccharides **28** and **30** can be forwarded on the basis of their sialic acid disposition. CPK models corresponding to the minimum energy conformations show that in **28** the two sialic acids point to opposite directions, whereas these are projected in the same plane in compound **30** (Figure 6). Similar features are seen in the other potent 4,6-heptasaccharide as well, indicating that an end distance of about 9 Å may be a requirement for optimum binding to the virus. On this basis and the published reports,[14,16] we propose that macromolecules (polymers) containing polyvalent sialosides containing either the 3,6- or 4,6-heptasaccharide ligands should be capable of binding to the influenza virus hemagglutinins both intra- and intermolecularly and thus may prove to be potent virus inhibitors. Efforts to polymerize these materials are under way and these will be reported subsequently.

Experimental Section

Unless specified, all reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). 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). Elemental analyses were performed in Galbraith Laboratories, Inc., Knoxville, TN, or in Quantities Technologies Inc., Bound Brook, NJ. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at ambient temperatures. The structural identities were established by NMR methods.

^{1}H NMR spectra were recorded on a Bruker AMX-600, AM-600 (600 MHz), or AM-500 (500 MHz) or a GE NMR QE-300 or QE-500 spectrometer. Unless specified, all of the measurements were taken at 295 K. The hydrogen and carbon chemical shifts in $CDCl_3$ are expressed relative to tetramethylsilane. For solutions of compounds in deuterium oxide or deuterated methanol, the hydrogen chemical shifts are expressed relative to the HOD signal (4.83 ppm at 295 K), which sets the chemical shifts for acetone at 2.23 ppm. The chemical shift of the HOD proton signal is temperature sensitive. A standard solution of 5% acetone in D_2O was used for spectral calibration before each measurement, and the HOD signal was set relative to the internal acetone signal at 2.23 ppm. Thus at 295 K, the chemical shift of the HOD signal was established as 4.83 ppm. It was also noted that the HOD signal shifted upfield by 0.014 ppm for a 1 K increase in temperature.) The carbon chemical shifts are expressed relative to external TMS using the deuterium lock of the spectrometer, which sets the chemical shifts of 1,4-dioxane at 66.9 ppm.

Proton spin–lattice relaxation time (T_1) measurements were carried out at 600 MHz by using the inversion–recovery technique. ^{13}C spectra were recorded at 295 K with the GE OMEGA500 instrument operating at 125.74 MHz. Carbon spin–lattice relaxation time (T_1) measurements were carried out with the same instrument by using the inversion–recovery technique, and the T_1 values were determined from eight delays (VD = 0.0, 0.01, 0.06, 0.10, 0.16, 0.24, 0.4, and 1.0 s, 1600 transients per delay with a 6-s delay between acquisitions) and a fully relaxed spectrum (with a 5-s delay time between 180° and 90° pulses, spectral width = 256.5 ppm, pulse width (90°) = 17 μs, data size = 32 K points) using a three-point fitting routine. Two-dimensional (2D) ^{13}C–^{1}H correlation spectra were obtained on a Bruker AM600 using inverse ^{1}H detection and the heteronuclear multiple quantum coherence (HMQC) *filtration sequence described by Lerner and Bax.*[46] *Suppression of* ^{1}H–^{12}C resonances was aided by preceding the sequence with a bilinear rotation decoupling (BIRD) sequence[47] allowing 0.5 s for recovery. Decoupling of ^{13}C during acquisition used a GARP sequence with a radio frequency field strength of 4100 Hz. Phase sensitivity in ω_1 was achieved by TPPI,[48] and timing of the first increment was adjusted to allow linear back-prediction of the first point for optimum base line flatness.[49] Typical data sets used 512 t_1 increments and 1024 real t_2 data. Data were windowed in both t_1 and t_2 with a moderately shifted squared cosine bell and zero-filled to a final real matrix size of $1K(\omega_1) \times 2K(\omega_2)$.

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Relayed C–H correlation spectra were obtained with the same basic sequence and processing considerations, but an MLEV-17 spin lock was inserted before data acquisition.[46,50]

One-dimensional TOCSY and 1D NOESY experiments were performed at 600 MHz according to the published report.[32]

The computer modeling of oligosaccharides were performed using the HSEA-based GESA[42] and GEGOP[43] programs on Ardent Titan, Silicon Graphics Iris, or Indigo. This procedure is an interactive exchange between GESA local or global minima and Monte Carlo calculations[44] using GEGOP. The Monte Carlo calculations performed 300 000–500 000 steps starting from the global minimum conformation with acceptance ratios between 20 and 50%.

Preparations of Monosaccharides 1–8. See the supplementary material.

5-(Methoxycarbonyl)pentyl 4,6-*O*-Benzylidene-2,3-bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside (9). Compound **3** (700 mg) was dissolved in dry nitromethane (CH_3NO_2, 15 mL) containing 4-Å molecular sieves (5.0 g), silver trifluoromethanesulfonate (AgOTf, 957 mg) and *s*-collidine (0.45 mL) and stirred under nitrogen at −25 °C. A solution of 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranosyl bromide[20] (1.85 g) in CH_2Cl_2 (5 mL) was added dropwise. After 10 min, the reaction mixture was gradually warmed up to room temperature and left stirring for 3 h. Additional AgOTf (686 mg), collidine (0.322 mL), and the bromide (1.29 g in 5 mL of CH_3NO_2) were added, and the reaction was continued for another 16 h. It was then diluted with CH_2Cl_2 and filtered over a pad of Celite. The filtrate was washed with water, 5% sodium thiosulfate, 1 M ice cold HCl, and saturated $NaHCO_3$ solution. The solution was dried over anhydrous magnesium sulfate, filtered, and concentrated. Chromatography of the crude product on a column of silica gel using ethyl acetate:hexane:ethanol = 20:20:1 as eluant gave the protected trisaccharides (1.7 g) (this material had about 10% with 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranose, which had near-identical mobility as **9**; this was removed in subsequent steps) and pure **9** (0.36 g): $[\alpha]^{25}_D$ +24.2 ± 2° (*c* 0.99, MeOH); ^1H NMR and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{60}H_{66}O_{26}N_2$: C, 58.54; H, 5.37; N, 2.28. Found: C, 58.04; H, 5.38; N, 2.15.

5-(Methoxycarbonyl)pentyl 3-*O*-Allyl-6-*O*-(*tert*-butyldimethylsilyl)-2,4-bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside (10). Compound **4** (3.18 g) in dry CH_3NO_2 (70 mL) was glycosylated with of 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranosyl bromide (6.37 g) in CH_3NO_2 (30 mL) in the presence of 4-Å molecular sieves (15.0 g), AgOTf (3.30 g), and *s*-collidine (1.50 mL) as described for **9**. After 24 h, additional AgOTf (0.516 g), collidine (0.26 mL), and the bromide (1.0 g) were added, and the reaction was continued for another 3 h. The reaction mixture was processed as described above for **9**. Chromatography of the crude product on a column of silica gel (ethyl acetate:hexane = 2:3 at the start to 3:2 at the end) gave the protected trisaccharides **10** (3.45 g). Two disaccharide fractions (1.75 and 1.1 g, as evidenced by ^1H NMR) were also obtained. No attempt was made to convert these to the trisaccharide **10**: mp 87.5 °C; $[\alpha]^{25}_D$ −8.6 ± 2° (*c* 1.04, $CHCl_3$); ^1H NMR and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{62}H_{80}O_{26}SiN_2$: C, 57.40; H, 6.17; N, 2.16. Found: C, 57.26; H, 6.14; N, 2.16.

5-(Methoxycarbonyl)pentyl 2,6-Bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-β-D-glucopyranosyl)-3,4-*O*-isopropylidene-β-D-galactopyranoside (11). Compound **5** (0.7 g) in dry CH_3NO_2 (15 mL) was glycosylated with of 2-deoxy-2-phthalimido-3,4,6-tri-*O*-α,β-D-glucopyranosyl bromide (1.85 g) in CH_3NO_2 (5 mL) in the presence of 4-Å molecular sieves (5.0 g), AgOTf (0.96 g), and *s*-collidine (0.45 mL) as described for **9**. After 3 h, the reaction mixture was processed as described above for **9**. Chromatography of the crude product on a column of silica gel (ethyl acetate:hexane = 2:3) gave the protected trisaccharides **11** (1.56 g): mp 121 °C; $[\alpha]^{25}_D$ +31.3 ± 2° (*c* 0.99, $CHCl_3$); ^1H NMR and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{56}H_{66}O_{26}N_2$: C, 56.85; H, 5.58. Found: C, 55.05; H, 5.57.

5-(Methoxycarbonyl)pentyl 2,6-Di-*O*-allyl-3,4-bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside (12). Compound **6** (1.67 g) in dry CH_3NO_2 (40 mL) was glycosylated with 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranosyl bromide (6.64 g) in CH_2Cl_2 (10 mL) in the presence of 4-Å molecular sieves (5.0 g), AgOTf (3.34 g), and *s*-collidine (1.10 mL) as described for **9**. After 16 h, the reaction mixture was processed. Purification on a column of silica gel (ethyl acetate:hexane = 3:2) gave a compound homogeneous on TLC. Proton NMR of the product indicated it to be the protected trisaccharide **12** (1.51 g) containing about 10% of 2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-D-glucopyranose (as this has TLC mobility identical to **12**): ^1H NMR and ^{13}C NMR, see supplementary material.

To further characterize **12** and for elemental analysis, the allyl groups of **12** were removed (described below for **18**) and the resultant product [5-(methoxycarbonyl)pentyl 3,4-bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside] was separated from the 2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-D-glucopyranose impurity. This product was found to be pure by NMR and suitable for optical rotation and elemental analysis. **12**: $[\alpha]^{25}_D$ +36.6 ± 2° (*c* 1.04, MeOH); ^1H NMR (CD_3OD) δ 7.71–7.80 (m, phthalimido hydrogens), 5.96 (d, *J* = 8.6 Hz, H-1^b), 5.94 (dd, *J* = 9.3, 10.7 Hz, H-3^b), 5.68 (dd, *J* = 9.3, 10.5 Hz, H-3^b), 5.58 (d, *J* = 8.6 Hz, H-1$^{b'}$), 5.33 (dd, *J* = 9.3, 10.0 Hz, H-4$^{b'}$), 5.06 (dd, *J* = 9.0, 10.3 Hz, H-4^b), 4.68 (dd, *J* = 4.2, 12.5 Hz, H-6^{b}a), 4.29–4.19 (m, 5 H, H-6′b, H-2^b, H-2$^{b'}$, H-6$^{b'}$a, H-4^a), 4.13 (dd, *J* = 1.7, 12.2 Hz, H-6$^{b'}$b), 4.00 (m, 2 H, H-5^b and H-5$^{b'}$), 3.94 (d, *J* = 7.8 Hz, H-1^a), 3.71–3.62 (m, 3 H, H-6^{a}a,b, one of OCH_2), 3.60 (s, COOCH_3), 3.42 (dd, *J* = 6.35, 7.33 Hz, H-5^a), 3.37 (dd, *J* = 3.2, 10.0 Hz, H-3^a), 3.31 (m, one of OCH_2 of aglycon), 2.87 (dd, *J* = 7.8, 10.0 Hz, H-2^a), 2.23 (t, *J* = 7.6 Hz, CH_2COO), 2.21, 2.08, 2.05, 2.03, 1.88, 1.80 (6 × s, CH_3COO), 1.50, 1.43, 1.23 (6 H, hydrogens of pentyl group); ^{13}C NMR (CD_3OD) δ 176.0, 172.4, 172.3, 171.8, 171.6, 171.4, 171.2, 169.8, 168.9, 168.6, 168.55, 135.6, 135.3, 135.2, 133.1, 132.7, 130.0, 124.3, 124.2, 104.7, 102.5, 99.1, 86.3, 75.7, 75.5, 73.3, 72.54, 72.51, 71.9, 70.9, 70.4, 70.3, 69.58, 69.56, 63.6, 63.0, 62.6, 55.9, 55.8, 52.0, 34.6, 30.1, 26.4, 25.7, 21.2, 20.68, 20.66, 20.61, 20.4, 20.3. Anal. Calcd for $C_{53}H_{62}O_{26}N_2$: C, 55.69; H, 5.43; N, 2.45. Found: C, 55.14; H, 5.44; N, 2.28.

5-(Methoxycarbonyl)pentyl 2,4-Di-*O*-benzoyl-3,6-bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside (13). Compound **7** (1.50 g) in dry CH_3NO_2 (80 mL) was glycosylated with 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranosyl bromide (3.0 g) in CH_3NO_2 (20 mL) in the presence of 4-Å molecular sieves (3.5 g), AgOTf (1.57 g), and *s*-collidine (0.7 mL), as described for **9**. After 16 h, other portions of AgOTf (0.39 g) and *s*-collidine (0.2 mL) and a solution of the bromide (0.75 g) in CH_3NO_2 (20 mL) were added to the cooled (−28 °C) reaction mixture. After the addition, the reaction mixture was allowed to warm up to room temperature and was processed after 3 h. Chromatography of the crude product on a column of silica gel (ethyl acetate:hexane:acetonitrile = 3:6:2) gave the protected trisaccharide **13** (1.60 g): mp 119.6–123.1 °C; $[\alpha]^{25}_D$ +50.9 ± 2° (*c* 1.00, $CHCl_3$); ^1H NMR and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{67}H_{70}O_{28}N_2$: C, 59.55; H, 5.18; N. 2.07. Found: C, 59.03; H, 5.19; N, 2.06.

5-(Methoxycarbonyl)pentyl 2,3-Di-*O*-allyl-4,6-bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-acetyl-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside (14). Compound **8** (7.50 g) in dry CH_3NO_2 (250 mL) was glycosylated with 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-α,β-D-glucoopyranosyl bromide (20.2 g) in CH_2Cl_2 (100 mL) in the presence of 4-Å molecular sieves (100 g), AgOTf (10.4 g), and *s*-collidine (4.7 mL), as described for **9**. After 16 h, other portions of AgOTf (2.86 g) and the bromide (5.50 g) in CH_3NO_2 (20 mL) were added. Processing of the reaction mixture after 2 h and purifying by chromatography on a column of silica gel (ethyl acetate:hexane:ethanol = 20:20:1) gave the protected trisaccharide **14** (13.90 g): mp 108.6 °C; $[\alpha]^{25}_D$ +23.4 ± 2° (*c* 0.98, $CHCl_3$); ^1H NMR and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{59}H_{70}O_{26}N_2$: C, 57.93; H, 5.73. Found: C, 57.30; H, 5.83.

General Methods for Deprotection and Purification of Sugars. The *O*-acetate groups were removed under Zemplen's conditions with sodium methoxide (NaOMe, 0.5 M) and methanol (MeOH), and the reaction mixture was neutralized with H^+ (sulfonic acid) resin. The phthalimido groups were removed by refluxing the de-O-acetylated trisaccharides with hydrazine in refluxing MeOH. The O-acetylations were carried out with pyridine–acetic anhydride in the presence of catalytic amounts of 4-(*N*,*N*-dimethylamino)pyridine (DMAP). Bio Gel purifications were carried out by dissolving the deprotected oligosaccharides (≤100 mg) in deionized water (5 mL) and applying them to a column of Bio Gel P2 (200–400 mesh, 500 mL) eluted and equilibrated with water. Column fractions (7.5 mL) were monitored for absorption at 214 nm, and the active fractions were pooled, concentrated under reduced pressure to 10 mL, and lyophilized.

5-(Methoxycarbonyl)pentyl 2,3-Bis-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (15). Compound **9** (1.70 g) was

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de-O-acetylated (MeOH (50 mL), NaOMe (200 μL), 24 h) followed by the removal of the phthalimido groups (MeOH (50 mL), hydrazine (0.38 mL), 8 h). After acetylation (pyridine (50 mL), acetic anhydride (8 mL), DMAP (10 mg), 16 h), the product was purified by chromatography on a column of silica gel using ethyl acetate:hexane:ethanol = 5:5:1 as eluant to obtain the N-acetylated derivative of **9** (799 mg). This was dissolved in MeOH (20 mL) containing *p*-toluenesulfonic acid (50 mg) and refluxed for 15 min. The solution was cooled, neutralized with triethylamine, and concentrated to dryness. The residue was dissolved in CH_2Cl_2 (75 mL) and washed with water, 1 M ice cold HCl, and saturated $NaHCO_3$ solution. The solution was dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was redissolved in pyridine–acetic anhydride (2:1, 15 mL). After 16 h, the product was worked up as described above and purified by high-pressure liquid chromatography using ethyl acetate:hexane:ethanol = 6:5:1 as eluant to obtain an amorphous product (peracetylated **15**, 645 mg): $[\alpha]^{25}_D$ +14.1 ± 2° (*c* 1.06, MeOH); ^1H NMR and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{45}H_{66}O_{26}N_2$: C, 51.43; H, 6.29; N, 2.67. Found: C, 50.61; H, 6.28; N, 3.03. The peracetylated derivative of **15** (645 mg) was deacetylated with MeOH (20 mL) and NaOMe (125 μmol) to obtain the trisaccharide **15** (330 mg) as a colorless powder: $[\alpha]^{25}_D$ −15.1 ± 2° (*c* 1.01, H_2O); ^1H and ^{13}C NMR (D_2O), see Tables 1 and 2 of the supplementary material. Anal. Calcd for $C_{29}H_{50}O_{18}N_2$: C, 48.74; H, 7.0; N, 3.9. Found: C, 46.04; H, 7.17; N, 4.23.

5-(Methoxycarbonyl)pentyl 2,4-Bis-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (16). The *O*-allyl and the *O*-tBDMS groups of compound **10** (3.1 g) were removed as described for **7** (supplementary material), and the residue was acetylated with pyridine–acetic anhydride (2:1, 15 mL) containing DMAP (10 mg). After 48 h, the product was worked up and purified on a column of silica gel using ethyl acetate:hexane = 1:1 as eluant to obtain a colorless solid (the allyl and the *tert*-butyldimethylsilyl groups of **10** have been replaced with acetates, 2.58 g): ^1H NMR ($CDCl_3$) δ 8.03–7.73 (m, phthalimido hydrogens), 5.80 (dd, *J* = 9.2, 10.7 Hz, H-3^b), 5.54 (dd, *J* = 9.1, 10.9 Hz, H-3$^{b'}$), 5.22 (d, *J* = 8.1, H-1^b), 5.13 (dd, *J* = 9.2, 10.2 Hz, H-4^b), 5.00 (dd, *J* = 9.1, 10.2 Hz, H-4$^{b'}$), 4.69 (d, *J* = 8.4, H-1$^{b'}$), 4.58 (dd, *J* = 2.9, 10.2 Hz, H-3^a), 4.31 (dd, *J* = 8.2, 10.7 Hz, H-2^b), 4.21 (dd, *J* = 2.9, 12.1 Hz), 4.21 (d, *J* = 7.3, H-1^a), 4.20 (dd, *J* = 5.2, 12.1 Hz), 4.08 (dd, *J* = 8.2, 10.7 Hz), 3.94 (broad d, *J* = 3.0 Hz, H-4^a), 3.81 (dd, *J* = 3.2, 12.1 Hz), 3.74 (m, H-5^b), 3.69 (s, $COOCH_3$), 3.62 (m, 1 H, OCH_2C), 3.52 (broad t, *J* = 6.3 Hz, H-5^a), 3.43 (dd, *J* = 7.4, 10.3 Hz, H-2^a), 3.37 (m, 1 H, OCH_2C), 3.12 (m, H-5$^{b'}$), 2.34 (t, CH_2COO), 2.15, 2.09, 2.03, 2.02, 2.00 (2×), 1.87, 1.80 (8 × CH_3CO), 1.61, 1.43, 1.33 (6 H, aglyconic hydrogens).

The above residue was de-O-acetylated (MeOH (50 mL), NaOMe (0.5 mL), 16 h), and the phthalimido groups were then removed (MeOH (125 mL), hydrazine (0.65 mL), 24 h). The residue was then stirred in MeOH (50 mL) and acetic anhydrie (2.5 mL). After 10 min, Et_3N (1 mL) and acetic anhydride (5 mL) were added, and the mixture was stirred at room temperature for 3 h. The solution was concentrated to dryness, redissolved in MeOH (100 mL) containing H^+ resin, stirred for 3 h, and filtered. The filtrate was concentrated to dryness, and the residue was acetylated (pyridine (50 mL), acetic anhydride (15 mL), DMAP (10 mg), 16 h). Purification of the crude product on a column of silica gel using ethyl acetate:CH_2Cl_2:ethanol = 10:10:1 as eluant gave a colorless solid (the peracetylated derivative of compound **16**, 1.3 g). This was deactylated with MeOH (50 mL) and NaOMe (250 μmol) to obtain the trisaccharide **16** (169 mg) as a colorless powder: $[\alpha]^{25}_D$ −15.9 ± 2° (*c* 1.02, H_2O); ^1H and ^{13}C NMR (D_2O), see Tables 1 and 2 of the supplementary material. Anal. Calcd for $C_{29}H_{50}O_{18}N_2$: C, 48.74; H, 7.0; N, 3.9. Found: C, 44.19; H, 7.00; N, 3.82.

5-(Methoxycarbonyl)pentyl 2,6-Bis-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (17). To an ice cold solution of compound **11** (24.0 g) in CH_2Cl_2 (400 mL) was added 90% aqueous trifluoroacetic acid (115 mL), and the mixture was stirred. After 45 min at ice bath temperature, the reaction mixture was washed twice with water and with saturated $NaHCO_3$ solution. Chromatography of the crude product on a column of silica gel using ethyl acetate:hexane:ethanol = 10:10:1 as eluant gave a colorless solid (the isopropylidene group of **11** has been removed, 17.8 g): mp 164–165 °C; $[\alpha]^{25}_D$ +24.8 ± 2° (*c* 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.82 and 7.73 (m, phthalimido hydrogens), 5.83 (t, *J* = 10.3 Hz, H-3^b), 5.73 (t, *J* = 9.9 Hz, H-3$^{b'}$), 5.54 (d, *J* = 8.5 Hz, H-1^b), 5.39 (d, *J* = 8.5 Hz, H-1$^{b'}$), 5.17 and 5.08 (2 × t, *J* = 9.9 Hz, H-4^b and H-4$^{b'}$), 4.12 (d, *J* = 7.7 Hz, H-1^a), 3.68 (s, $COOCH_3$), 2.28 (t, CH_2COO), 2.09, 2.07, 2.02 (2 ×), 1.85 and 1.84 (6 × CH_3CO), 1.55, 1.34, 1.23 (6 H, hydrogens of pentyl group); ^{13}C NMR ($CDCl_3$) δ 173.8, 170.4, 169.8, 169.28, 169.25, 167.9, 167.4, 134.3, 134.0, 131.8, 131.5, 123.5, 123.4, 101.3, 98.6 and 98.2, 80.5, 72.7, 72.4, 72.2, 72.0, 70.9, 70.8, 69.3, 69.0, 68.3, 67.8, 62.2, 62.1, 55.0, 54.7, 51.3, 34.0, 29.3, 25.5, 24.7, 20.6, 20.49, 20.47, 20.31, 20.26. Anal. Calcd for $C_{53}H_{62}O_{26}N_2$: C, 55.69; H, 5.43; N, 2.45. Found: C, 55.20; H, 5.44; N, 2.32.

A portion of the above residue (13.3 g) was de-O-acetylated (MeOH (300 mL), NaOMe (5 mL), 2 h) followed by the removal of the phthalimido groups (MeOH (500 mL), hydrazine (2.2 mL), 16 h). It was then concentrated to a dry residue and coevaporated with pyridine to remove traces of hydrazine. The residue was dissolved in MeOH (400 mL) followed by the addition of acetic anhydride (10 mL). After 20 min, acetic anhydride (20 mL) and Et_3N (4 mL) were added, and the reaction mixture was stirred at room temperature for 24 h. A white precipitate (product) was obtained. This was filtered, and the solid was washed with MeOH. It was acetylated (pyridine (100 mL), acetic anhydride (40 mL), DMAP (200 mg), 5 h) and then purified on a column of silica gel using ethyl acetate:CH_2Cl_2:ethanol = 10:10:1 as eluant to get a colorless solid (peracetylated derivative of **17**, 10.0 g): $[\alpha]^{25}_D$ −21.9 ± 2° (*c* 1.00, MeOH); ^1H and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{45}H_{66}O_{26}N_2$: C, 51.43; H, 6.29; N, 2.67. Found: C, 50.98; H, 6.40; N, 3.09. This product was de-O-acetylated (MeOH (300 mL), NaOMe (2.5 mL), 24 h) to obtain a colorless product **17** (5.91 g): $[\alpha]^{25}_D$ −17.8 ± 0.8° (*c* 1.09, H_2O); ^1H and ^{13}C NMR (D_2O), see Tables 1 and 2 of the supplementary material. Anal. Calcd for $C_{29}H_{50}O_{18}N_2 \cdot H_2O$: C, 47.54; H, 7.10. Found: C, 47.01; H, 7.12.

5-(Methoxycarbonyl)pentyl 3,4-Bis-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (18). The allyl protecting groups of compound **12** (0.94 g) were removed with an iridium catalyst as described for **7** (supplementary material), and the resultant product was acetylated with pyridine–acetic anhydride and purified by HPLC (ethyl acetate:hexane:ethanol = 10:10:1) to obtain an amorphous material (290 mg; the allyl groups of **12** have been replaced with acetyl groups): ^1H NMR ($CDCl_3$) δ 7.85–7.60 (m, phthalimido hydrogens), 5.90 (d, *J* = 8.4 Hz, H-1$^{b'}$), 5.84 (dd, *J* = 9.0, 10.7 Hz, H-3$^{b'}$), 5.61 (dd, *J* = 9.3, 10.4 Hz, H-3^b), 5.52 (d, *J* = 8.5 Hz, H-1^b), 5.28 (t, *J* = 9.5 Hz, H-4^b), 5.13 (dd, *J* = 9.2, 9.8 Hz, H-4$^{b'}$), 4.53 (dd, *J* = 5.2, 12.5 Hz), 4.48 (dd, *J* = 8.0, 10.2 Hz, H-2^a), 4.33 (dd, *J* = 8.5, 10.5 Hz, H-2^b), 4.31 (broad d, exhibits NOE from H-1$^{b'}$, H-4^a), 3.90 (m, H-5^b and H-5$^{b'}$), 3.59 (s, $COOCH_3$), 3.50 (dd, *J* = 2.7, 9.9 Hz, exhibits NOE from H-1^b, H-3^a), 3.40 and 3.18 (m, OCH_2C), 2.17 (m, CH_2COO, CH_3CO), 2.08, 2.01, 1.85, 1.83, (CH_3CO), 1.47, 1.30, 1.10 (6 H, hydrogens of pentyl group); ^{13}C NMR ($CDCl_3$) δ 174.0, 170.7, 170.6, 170.4, 170.2, 169.9, 169.3, 167.7, 167.3, 134.1, 133.7, 131.5, 131.2, 123.8, 123.5, 123.3, 100.4, 99.9, 96.7, 81.4, 73.2, 72.1, 71.8, 71.2, 70.9, 70.6, 69.3, 68.7, 68.2, 67.3, 64.0, 62.0, 61.9, 54.4, 54.2, 51.3, 33.8, 28.9, 25.1, 24.5, 20.8, 20.7, 20.63, 20.57, 20.4, 20.3.

This product was then de-O-acetylated (MeOH (20 mL), NaOMe (0.25 mL), 8 h) followed by the removal of the phthalimido groups (MeOH (10 mL), hydrazine (0.070 mL), 16 h), and the resultant product was acetylated and purified by HPLC (silica gel, ethyl acetate:hexane:ethanol = 5:5:1 eluant) to obtain the peracetylated derivative of **18** (180 mg): mp 110.3 °C; $[\alpha]^{25}_D$ −21.2 ± 2° (*c* 1.03, MeOH); ^1H and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{45}H_{66}O_{26}N_2$: C, 51.43; H, 6.29; N, 2.67. Found: C, 50.92; H, 6.30; N, 2.78.

This material (120 mg) was de-O-acetylated (NaOMe (0.25 mL), MeOH (20 mL), 60 °C, 48 h) and purified by gel permeation chromatography (Bio Gel) to obtain a colorless product **18** (59 mg): $[\alpha]^{25}_D$ −7.6 ± 2° (*c* 1.03, H_2O); ^1H and ^{13}C NMR, see Tables 1 and 2 of the supplementary material).

5-(Methoxycarbonyl)pentyl 3,6-Bis-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (19). Compound **13** (1.6 g) was de-O-acetylated (MeOH (80 mL), NaOMe (1 mL), 16 h), the phthalimido groups were then removed (MeOH (60 mL), hydrazine (0.27 mL), 24 h), and the resulting compound was acetylated (pyridine-acetic anhydride, 4:1 (50 mL), DMAP (100 mg), 16 h) and purified by HPLC using ethyl acetate:hexane:acetonitrile = 2:1:2 to obtain two trisaccharide fractions (residue 1 = 144 mg; residue 2 = 380 mg). Residue 1: $[\alpha]^{25}_D$ +3.8 ± 2° (*c* 1.00, MeOH); ^1H and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{45}H_{66}O_{26}N_2$: C, 51.43; H, 6.29; N, 2.67. Found: C, 50.79; H, 6.32; N, 2.97.

Both residues 1 and 2 were the desired protected trisaccharides, except that the residue 1 had a benzoate group at the 2 position of the reducing galactose, while residue 2 was the peracetylated material. This was also confirmed when residues 1 and 2 were deacylated with MeOH (20 mL) and NaOMe (2.5 mmol for residue 1, 24 h, room temperature, and 5 mmol for residue 2, 48-h reaction time at room temperature) to obtain the trisaccharide **18** (240 mg of combined yield from residues 1 and 2): $[\alpha]^{25}_D$ −10.1 ± 2° (*c* 1.06, H_2O); ^1H and ^{13}C NMR (D_2O), see Tables 1 and 2 of the supplementary material.

5-(Methoxycarbonyl)pentyl 4,6-Bis-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (20). The allyl groups of compound **14** (13.9 g) were removed as described above for **7** (supplementary material). The residue was then dissolved in pyridine–acetic anhydride

(4:1, 125 mL) containing DMAP (100 mg). After 24 h, the product was worked up as described above. Chromatograpy of the crude product on a column of silica gel using ethyl acetate:hexane:ethanol = 10:10:1 as eluant gave a colorless solid [5-(methoxycarbonyl)pentyl 2,3-di-*O*-acetyl-4,6-bis-*O*-(2-deoxy-3,4,6-tri-*O*-acetyl-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside, 13.0 g]: ^{13}C NMR ($CDCl_3$) δ 174.0, 170.9, 170.7, 170.55, 170.48, 170.09, 170.06, 169.5, 169.4, 168.1, 134.3, 134.0 131.3, 123.6, 100.3, 98.0, 97.9, 74.7, 73.7, 72.5, 71.7, 71.6, 70.7, 70.0, 69.3, 68.83, 68.75, 68.4, 68.2, 61.7, 61.5, 54.6, 54.2, 51.4, 33.9, 28.9, 25.3, 24.6, 20.8, 20.6, 20.4, 20.3.

The above residue was de-O-acetylated (MeOH (300 mL), NaOMe (5 mL), 16 h), followed by the removal of the phthalimido groups (MeOH (700 mL), hydrazine (3.40 mL), 18 h) and acetylation of the product (pyridine (200 mL), acetic anhydride (75 mL), DMAP (100 mg), 16 h). Purification by chromatography on a column of silica gel using ethyl acetate:CH_2Cl_2:ethanol = 5:5:1 gave an amorphous material (peracetylated **20**, 3.1 g): $[\alpha]^{25}_D$ −6.2 ± 2° (*c* 1.03, MeOH); ^1H and ^{13}C NMR, see supplementary material. Anal. Calcd for $C_{45}H_{66}O_{26}N_2$: C, 51.43; H, 6.29. Found: C, 50.64; H, 6.33.

This product was deacetylated (MeOH (150 mL), NaOMe (3 mL), 16 h), and the residue was dissolved in water and lyophilized. Purification on Bio Gel gave a colorless solid (1.02 g): $[\alpha]^{25}_D$ −24.1 ± 2° (*c* 1.05, H_2O); ^1H and ^{13}C NMR (D_2O), see Tables 1 and 2 of the supplementary material.

5-(Methoxycarbonyl)pentyl 2,3-Bis-*O*-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (21). A solution of compound **15** (72 mg, 100.8 μmol), uridine diphosphogalactose (UDP-galactose, 190 mg, 309 μmol, Sigma Chemical Company, St. Louis, MO), bovine galactosyltransferase (EC 2.4.1.22, 6.2 U), and BSA (3 mg) in 30 mM sodium cadodylate buffer (7 mL, pH 7.0) containing manganese chloride (40 μmol) was incubated at 37 °C for 22 h. The reaction mixture was diluted to 20 mL with deionized water, applied on a column of AG 1-X2 (Bio Rad, 200–400 mesh, phosphate form, pH 6.8), and eluted with deionized water (100 mL). The solution was concentrated to dryness, and the residue was dissolved in 5 mL of deionized water, applied on a column of Bio Gel P-2 (400 mesh), eluted, and equilibrated with water. Fractions (7.5 mL) that showed UV absorption at 220 nm were examined by TLC using ethyl acetate:ethanol:water = 2:1:1 and were pooled and lyophilized to obtain a colorless solid (74 mg, 71 μmol). The stuctural identity of **21** was unambiguously assigned by ^1H and ^{13}C NMR (see Tables 3 and 4 of the supplementary material).

5-(Methoxycarbonyl)pentyl 2,4-Bis-*O*-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (22). A solution of compound **16** (28 mg, 39.2 μmol), UDP-galactose, (84 mg, 136.6 μmol), bovine galactosyltransferase (5 U), and bovine serum albumin (3 mg) in 30 mM sodium cacodylate buffer (3 mL, pH 7.0) containing manganese chloride (40 μmol) was incubated at 37 °C for 22 h. The reaction mixture was diluted to 20 mL and purified as described for **21**. The yield of product **22** was 32 mg (30.7 μmol). The structural identity of **22** was unambiguously assigned by ^1H and ^{13}C NMR (see Tables 3 and 4 of the supplementary material).

5-(Methoxycarbonyl)pentyl 2,6-Bis-*O*-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (23). A solution of compound **17** (72 mg, 100.8 μmol), UDP-galactose, (190 mg, 309.0 μmol), bovine galactosyltransferase (6 U), and bovine serum albumin (3 mg) in 30 mM sodium cacodylate buffer (7 mL, pH 7.0) containing manganese chloride (40 μmol) was incubated at 37 °C for 22 h. The reaction mixture was diluted to 20 mL and purified as described for **21**. The yield of product **23** was 95 mg (91.3 μmol). The structural identity of **23** was unambiguously assigned by ^1H and ^{13}C NMR (see Tables 3 and 4 of the supplementary material).

5-(Methoxycarbonyl)pentyl 3,4-Bis-*O*-[(β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (24). A solution of compound **18** (60 mg, 84 μmol), UDP-galactose, (145 mg, 235 μmol), bovine galactosyltransferase (6.25 U), and bovine serum albumin (3 mg) in 30 mM sodium cacodylate buffer (5.25 mL, pH 7.0) containing manganese chloride (40 μmol) was incubated at 37 °C for 22 h. The reaction mixture was diluted to 20 mL and purified as described for **21**. The yield of product **24** was 47 mg (45.2 μmol). The structural identity of **24** was unambiguously assigned by ^1H and ^{13}C NMR (see Tables 3 and 4 of the supplementary material).

5-(Methoxycarbonyl)pentyl 3,6-Bis-*O*-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (25). A solution of compound **19** (85 mg, 119 μmol), UDP-galactose, (190 mg, 309 μmol), bovine galactosyltransferase (6.25 U), and bovine serum albumin (3 mg) in 30 mM sodium cacodylate buffer (7 mL, pH 7.0) containing manganese chloride (40 μmol) was incubated at 37 °C for 22 h. The reaction mixture was diluted to 20 mL and purified as described for **21**. The yield of product **25** was 114 mg (109.6 μmol). The structural identity of **25** was unambiguously assigned by ^1H and ^{13}C NMR (see Tables 3 and 4 of the supplementary material).

5-(Methoxycarbonyl)pentyl 4,6-Bis-*O*-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (26). A solution of compound **20** (72 mg, 100.8 μmol), UDP-galactose (190 mg, 309 μmol), bovine galactosyltransferase (6.25 U), and bovine serum albumin (3 mg) in 30 mM sodium cacodylate buffer (7 mL, pH 7.0) containing manganese chloride (40 μmol) was incubated at 37 °C for 22 h. The reaction mixture was diluted to 20 mL and purified as described for **21**. The yield of product **26** was 98 mg (94.2 μmol). The structural identity of **26** was unambiguously assigned by ^1H and ^{13}C NMR (see Tables 3 and 4 of the supplementary material).

General Procedure for the Preparation and Purification of Sialosides. The pentasaccharides (**21–26**) and CMP-NeuAc were dissolved in 0.1 M sodium cacodylate buffer (pH 6.5, 1 mL) containing Triton-CF 54 (0.1%) and bovine serum albumin (2 mg). The Galβ1,4GlcNAc α2,6-sialyltransferase was added, and the solution was incubated at 37 °C for 24 h. The reaction mixture was then diluted to 13 mL and applied on a column (1.5 × 9 cm) of Dowex 1-X2 (PO_4^{2-}) form, 200–400 mesh). The column was washed with distilled water (175 mL) and then eluted with 5 mM sodium phosphate buffer (pH 6.8). Fractions (5 mL) that showed UV absorbance at 214 nm were assayed for sialic acid by the periodate–resorcinol procedure.[55] When no more sialic acid or its derivatives eluted out (as evidenced by the absence of UV absorption at 214 nm), the elution buffer was changed to 50 mM phosphate buffer (pH 6.8) and the desired disialoside starts to elute (as evidenced by the periodate–resorcinol procedure). These fractions containing the disialoside product, which eluted after free sialic acid, were pooled and concentrated to a dry residue. The product was then redissolved in 2 mL of water and applied on a column (1.6 × 24 cm) of Sephadex G-15 (Sigma), equilibrated and eluted with water. The fractions (1.5 mL) containing the sialyl oligosaccharide, as evidenced by the periodate–resorcinol procedure, were measured for conductivity to exclude the contamination by salts, pooled, and lyophilized. The yields of the products were determined by the sialic acid estimation using the periodate–resorcinol procedure.

5-(Methoxycarbonyl)pentyl 2,3-Bis-*O*-[(5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid)-yl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (27). Compound **21** (16.0 mg, 15.3 μmol), CMP-NeuAc (25 mg, 39.2 μmol), and 2,6-sialyltransferase (296 mU) were incubated, and the product was isolated as described in the general procedure to give 2.1 μmol of product **27**. For complete structural characterization by NMR, see Tables II and III.

5-(Methoxycarbonyl)pentyl 2,4-Bis-*O*-[(5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid)-yl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (28). Compound **22** (17.5 mg, 16.8 μmol), CMP-NeuAc (37 mg, 58.0 μmol), and 2,6-sialyltransferase (500 mU) were incubated, and the product was isolated as described in the general procedure to give 7.6 μmol of product **28**. From 5 mM phosphate eluted fractions, 2.1 μmol of the monosialoside was obtained. For complete structural characterization by NMR, see Tables II and III.

5-(Methoxycarbonyl)pentyl 2,6-Bis-*O*-[(5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid)-yl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (29). Compound **23** (17.0 mg, 16.3 μmol), CMP-NeuAc (25 mg, 39.2 μmol), and 2,6-sialyltransferase (296 mU) were incubated, and the product was isolated as described in the general procedure to give 6.4 μmol of product **29**. For complete structural characterization by NMR, see Tables II and III.

5-(Methoxycarbonyl)pentyl 3,6-Bis-*O*-[(5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid)-yl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (30). Compound **25** (12.0 mg, 11.5 μmol), CMP-NeuAc (37 mg, 58.0 μmol), and 2,6-sialyltransferase (500 mU) were incubated, and the product was isolated as described in the general procedure to give 14.4 mg of product **30**. For complete structural characterization by NMR, see Tables II and III.

5-(Methoxycarbonyl)pentyl 4,6-Bis-*O*-[(5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid)-yl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (31). Compound **26** (12.0 mg, 11.5 μmol), CMP-NeuAc (37 mg, 58.0 μmol), and 2,6-sialyltransferase (500 mU) were incubated, and the product was isolated as described in the general procedure to give 5.5 μmol of the product. For complete structural characterization by NMR, see Tables II and III.

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Preparation of the BSA–30 Conjugate. A solution of compound **25** (75 mg) in MeOH (10 mL) containing hydrazine (3 mL) was refluxed for 2.5 h, concentrated to a dry residue, redissolved in 5 mL of water, and applied on a column of Bio Gel P2 (200–400 mesh) equilibrated and eluted with water. Fractions (8 mL) containing the product (fraction nos. 59–69) were pooled and concentrated to a dry residue (75 mg).

The above product (10.4 mg, 10 μmol) in dry DMF (1 mL) was cooled to −30 °C. A solution of hydrochloric acid (40 μmol) in DMF (100 μL) and *tert*-butyl nitrite (15 μmol) in DMF (100 μL) was added. After 60 min at temperatures between −20 and −30 °C, a solution of sulfamic acid (5 μmol) in DMF (100 μL) was added, and the solution was maintained at −30 °C for 10 min. BSA (13 mg) in sodium borate–$KHCO_3$ buffer (pH 9.0, 5 mL) was cooled in an ice bath, and the above DMF reaction mixture was added dropwise. After gentle shaking at 4 °C for 16 h, the solution was diluted (to 10 mL) and dialyzed against deionized water for 3 days; the deionized water was replaced every 12 h. The solution was lyophilized, and the residue was dissolved in a buffer (pH 6.5, 2 mL) containing sodium cacodylate (200 μmol), CMP-NeuAc (37 mg), triton (20 μg), and Galβ1,4GlcNAc α2,6-sialyltransferase (500 mU) and incubated for 24 h. The reaction mixture was then applied on a column of sephadex G-50 (fine) equilibrated and eluted with water. The fractions containing the product (UV absorption at 220 nm) were pooled and lyophilized (7.2 mg).

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Supplementary Material Available: Listings of the NMR data for the mono- and trisaccharide intermediates and the tri- and pentasaccharides, the 1D and 2D NOESY proton spectra of the pentasaccharides, and the NOE buildup curve for a pentasaccharide (26 pages). Ordering information is given on any current masthead page.

Conformation and Reactivity of Anomeric Radicals

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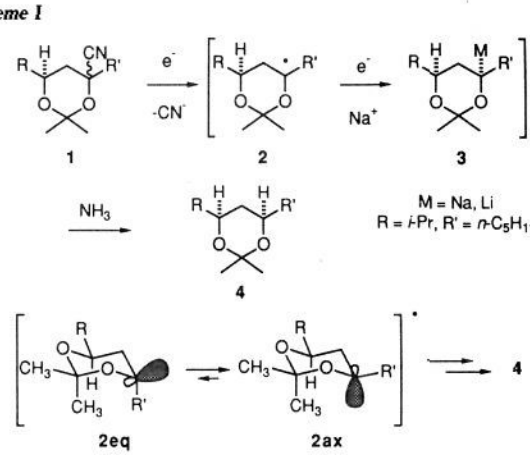
Abstract: Reductive decyanations of 2-cyanotetrahydropyran derivatives with sodium in ammonia show a strong preference for axial protonation. For the 2-cyanotetrahydropyran **6**, the selectivity is 119:1, and for the cyanooxadecalin **13**, which is sterically biased against axial hydrogen introduction, the ratio is 1.78:1. These stereochemical outcomes and ab initio calculations of the intermediate radical conformations are consistent with the following mechanistic model: reductive decyanation proceeds through the pyramidal, axial radical to give the configurationally stable, contrathermodynamic axial anion which is protonated with retention of configuration. Anomeric carbohydrate radicals have been described as nearly planar on the basis of electron spin resonance (ESR) spectroscopic studies, and that observation appears to be inconsistent with this model. Ab initio calculations show that though the pyramidalization at the radical center is small, the potential surface for pyramidalization is very asymmetric. Examination of the energy surface for the 2-tetrahydropyranyl radical **17** shows a 3.46 kcal/mol energy difference at UMP2/6-31G*//6-31G* on going from the axial ($\theta = -139.5°$) to the equatorial ($\theta = 149.5°$) conformer. The UMP2/6-31G*//6-31G* calculated energy differences between axial and equatorial conformers for tetrahydropyranyl radical **18** and oxadecalinyl radical **22** are qualitatively consistent with the experimentally observed reductive decyanation product ratios. The semiempirical methods AM1 and PM3 poorly model anomeric stabilization in radicals and are not useful for predicting radical conformations. Calculations show that introduction of an electron-withdrawing substituent, fluorine, in the equatorial 3-position of the tetrahydropyran radical **21c** flattens the radical center and makes the boat conformation **21b** more accessible, in good agreement with the substituent effects found in ESR studies of carbohydrate radicals.

Introduction

Radical intermediates play an ever increasing role in modern synthetic chemistry, and the stereochemistry of radical reactions is an area of considerable interest.[3] Substituted 2-tetrahydropyranyl radicals are particularly intriguing because the anisotropic interactions of the radical center with the adjacent oxygen atom dominate the stereochemical outcome of these radical reactions. These 2-tetrahydropyranyl radicals are important intermediates in a number of stereoselective transformations including radical-mediated synthesis of C-glycosides,[4] preparation of 2-deoxy-β-glycosides,[5] and synthesis of axial (2-tetrahydropyranyl)lithium[6] and 1-glycosyllithium[7] reagents. In each of these examples axial addition to the chair conformation of an anomeric radical predicts

Scheme I



the stereochemical outcome. For example, axial (2-tetrahydropyranyl)lithium reagents are produced by reductive lithiation of 2-(phenylthio)tetrahydropyrans, and the explanation given invokes the greater stability of the intermediate pyramidal axial radicals than of the equatorial radicals.[8] A similar explanation was

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