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Molecular design of N-linked tetravalent glycosides bearing *N*-acetylglucosamine, *N,N′*-diacetylchitobiose and *N*-acetyllactosamine: Analysis of cross-linking activities with WGA and ECA lectins

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ABSTRACT

Two types of nonspacer- and spacer-N-linked tetravalent glycosides bearing *N*-acetylglucosamine (GlcNAc), *N,N′*-diacetylchitobiose [$(GlcNAc)_2$] and *N*-acetyllactosamine (LacNAc) were designed and prepared as glycomimetics. The interactions of wheat germ (*Triticum vulgaris*) agglutinin (WGA) and coral tree (*Erythrina cristagalli*) agglutinin (ECA) with a series of tetravalent glycosides and related compounds were studied using a hemagglutination inhibition assay, a precipitation assay, double-diffusion test, and an optical biosensor based on surface plasmon resonance (SPR). The tetravalent glycosides were found to be capable of binding and precipitating the lectins as tetravalent ligands. Strong interactions with WGA, due to a combination of multivalency effects and spacer effects, were observed for tetravalent glycosides bearing flexible tandem GlcNAc. The chelate effect leads to large rate enhancement for the tetravalent system with favorable orientation of ligands. Our simple strategy produced multivalent glycosides with strong cross-linking activity for lectin as a specific coagulant.

1. Introduction

The cross-linking properties of a variety of plant and animal lectins with multivalent carbohydrates and glycoproteins have been reviewed.[1–4] These studies show that a number of lectins form cross-linked complexes with branched chain oligosaccharides,[5,6] glycopeptides,[7,8] and glycoproteins.[9,10] High affinity lectin ligands are of great interest in the cross-linking activities of such carbohydrate-mediated processes. The generation of high affinity ligands, however, is not trivial because the interaction of individual carbohydrate epitopes with lectin is, in many cases, weak and undiscriminating. The binding affinity can be dramatically increased by clustering lectin binding sites and carbohydrate recognition units.[11–15] The observation that binding affinity increases exponentially with the number of binding sites has been termed the glycoside cluster effect.[16,17] In general, monovalent carbohydrate–protein interactions often occur with low binding affinities ($K_d \sim 10^{-3}$ M).[18] However, multivalent interactions have several advantages over monomeric ones and are often used by nature to control a wide variety of cellular processes. With this in mind, multivalent carbohydrate analogs for high affinity binding to target lectins have been designed.[19–24] It is becoming increasingly clear that multivalency is a powerful design approach to increase the binding strength of synthetic ligands. Considering that strong binding is required for practical application of interference strategies, the synthesis and evaluation of multivalent carbohydrates is a topic of increasing prominence. We recently designed and prepared O- and N-linked divalent glycosides bearing GlcNAc and LacNAc, which are capable of precipitating WGA.[25,26] The multivalency effect can lead to truly large rate enhancement, even for systems of low valency, such as a divalent system.[27] Our studies using divalent glycosides were subsequently extended to tetravalent glycosides. We are interested in developing an efficient synthetic route to multivalent glycosides, for glycomimetics, as they tend to have enhanced affinity due to their multivalency for specific lectins. Here we describe a simple strategy to design tetravalent glycosides of GlcNAc, $(GlcNAc)_2$ and LacNAc, which have an enhanced multivalent effect due to a scaffold structure when bound to WGA and ECA. Using these tetravalent glycosides, interactions with lectins were analyzed using a hemagglutination inhibition assay, a precipitation assay, double-diffusion test, and surface plasmon resonance analysis.

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2. Results

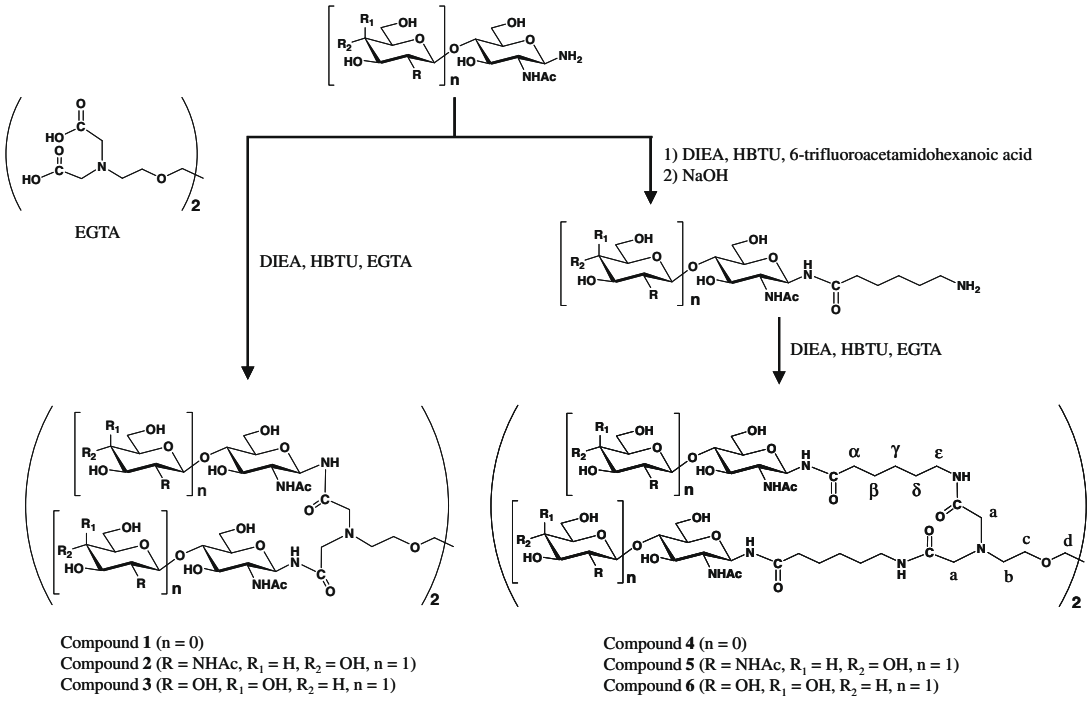
2.1. Convenient synthetic route to new types of tetravalent glycosides

Two types of N-linked tetravalent glycosides bearing GlcNAc, $(GlcNAc)_2$ and LacNAc were designed and prepared using ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate (EGTA) as a precursor, which is widely used as a calcium-specific chelator, Scheme 1. Nonspacer-N-linked tetravalent glycosides were firstly prepared as follows. The *N*-β-mono- and diglycosylamines bearing GlcNAc, $(GlcNAc)_2$ and LacNAc prepared by our method[25,28,29] were directly condensed with tetra-acetate EGTA in DMSO containing HBTU and DIEA. The tetravalent glycosides **1**, **2** and **3** were purified by chromatography on a Bio-Gel P-2 column and a charcoal-Celite column, producing yields of 55%, 26% and 23%, respectively, based on the amount of EGTA added (Scheme 1). For an alternative design, we made a spacer-N-linked tetravalent glycoside by insertion of a spacer group between the sugar and EGTA. Thus, the corresponding *N*-β-glycosylamines were first condensed with the carboxyl group of 6-trifluoroacetamidohexanoic acid as in Scheme 1. The resulting spacer-N-linked glycosides were then deacylated to the corresponding amino group by alkali treatment, and the amino group was reacted with EGTA, as described above. Targets **4**, **5** and **6** were purified by chromatography on a Bio-Gel P-2 column and an ODS column producing high yields of 71%, 74% and 67%, respectively, based on the amount of EGTA added. The tetravalent glycosides were elucidated by ^1H and ^{13}C NMR analyses, as described previously.[25,26] Structures of tetravalent glycosides bearing $(GlcNAc)_2$ were used as a reference in the analysis of ^1H NMR spectra. In the ^1H NMR spectra of **2** and **5**, two types of glycosidic proton signals were clearly observed in the lower field with larger coupling constants (**2**, δ 5.11, $J_{1,2}$ 9.8 Hz, H-1 and δ 4.63, $J_{1',2'}$ 8.6 Hz, H-1′; **5**, δ 5.06, $J_{1,2}$ 9.0 Hz, H-1 and δ 4.62, $J_{1',2'}$ 8.5 Hz, H-1′). In ^{13}C NMR spectra, two types of glycosidic signals were also characterized by the lower field (**2**, δ 104.2, C-1′β and δ 81.0, C-1β; **5**, δ 104.2, C-1′β and δ 81.1, C-1β). The spectra showed only the separated and overlapping resonances corresponding to respective sugar moieties. This simplicity suggests that these four sugar moieties can be superposed upon each other. ESI-MS analysis of tetra-headed glycosides **1**, **2**, **3**, **4**, **5** and **6** showed molecular ions at *m/z* 1211.5, 2023.8, 1859.7, 1663.8, 2477.4 and 2312.3, respectively, arising from the $[M+Na]^+$ ions. These results indicate that the resulting tetravalent glycosides consist exclusively of stereoregular sugars with tetravalent units. These compounds were soluble in water and the solubilities were 5–10%.

2.2. Hemagglutination inhibition assay

The interaction abilities of a series of tetravalent glycosides related to GlcNAc were compared with those of $(GlcNAc)_2$, $(GlcNAc)_3$ and divalent N^1,N^7-di-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-pimeamide $[(GlcNAc)_2\text{-Pim-}(GlcNAc)_2]$, which were used as control samples and are potent inhibitors of the hemagglutination of WGA (Table 1A). In the hemagglutination inhibition assay, a multivalency effect was observed for **2**, **4** and **5**, but not for **1** bearing GlcNAc, when compared with $(GlcNAc)_2$, $(GlcNAc)_3$ and divalent $(GlcNAc)_2\text{-Pim-}(GlcNAc)_2$. Compounds **2**, **4** and **5** acted as inhibitors and their activities were $4 \sim 8 \times 10^3$ fold higher than the reducing sugars $(GlcNAc)_2$ and $(GlcNAc)_3$ and 2–520 fold higher than divalent $(GlcNAc)_2\text{-Pim-}(GlcNAc)_2$. Compound **5** was the most effective inhibitor at a low concentration among the tetravalent glycosides: IC_{50} was 0.18 μM. Replacement of **4**, which has only one GlcNAc, with **5**, which has two GlcNAc, markedly increased the inhibition, producing a 260-fold higher affinity. Inhibition by spacer-linked **5** was also 32-fold higher than the inhibition by nonspaced-analog **2**, and 4–8-fold higher than that of glycoproteins ovalbumin and asialofetuin.

In contrast, strong multivalency effects were not much using ECA (Table 1B). Inhibitions by **3** and **6**, bearing LacNAc, was 2–8-fold than the inhibition by divalent LacNAc-Pim-LacNAc. However,



Scheme 1. Synthesis of nonspacer- and spacer-N-linked tetravalent glycosides.

Table 1
Inhibition of WGA- and ECA-mediated hemagglutination by tetravalent glycosides

Inhibitors	IC_{50}[a] (μM)
(A) WGA	
1	>4000
2	5.86
3	>4000
4	46.9
5	0.18
6	ND[b]
$(GlcNAc)_2$	750
$(GlcNAc)_3$	375
$(GlcNAc)_2$-Pim-$(GlcNAc)_2$	93.8
Ovalbumin	1.46
Asialofetuin	0.73
(B) ECA	
3	12
6	5.9
LacNAc	94
LacNAc-Pim-LacNAc	47
Asialofetuin	0.092
Fetuin	0.37

[a] Minimum concentration required for complete inhibition of hemagglutination.
[b] ND: Not determined.

inhibition by **3** and **6** were 64–130-fold lower when compared with asialofetuin.

2.3. Precipitation analysis

Brewer et al. have shown that lectins can bind to and precipitate a variety of naturally occurring oligosaccharides and glycopeptides.[5,7] The quantitative precipitation assays were performed using WGA and ECA. Various concentrations of lectin (8–256 μM) and tetravalent glycosides (0.025–12.8 mM) were mixed on a 96-well microplate. When each tetravalent glycoside was added to the lectin solution under appropriate conditions, a precipitate formed within a few minutes, as shown in Figure 1. The precipitate can be inhibited or reversed by chitin oligomer $(GlcNAc)_n$ (n = 2–3) and LacNAc as specific hapten sugars. Figure 2A shows precipitin curves for WGA in the presence of **1**, **2**, **4** and **5**, compared with divalent $(GlcNAc)_2$-Pim-$(GlcNAc)_2$. The concentration of **4** and **5** at the equivalence point (region of maximum precipitation) of the precipitin curve for 128 μM of WGA was near 125 μM, which shows that the stoichiometry of binding of **4** and **5** to WGA are 1:1 in both cases. In contrast, the concentration of **2** at the equivalence point was in the region of 0.5–1.25 μM, although the value was not accurately determined under the present conditions. The ratio of these concentrations gives the stoichiometry of binding of the glycoside to lectin.[30] Compound **1**, which showed far less reactivity than other glycosides, precipitated only about 15% of WGA at the equivalent zone, compared to approximately 100%, 100% and 85% by **2**, **5** and **4**, respectively. Experiments using ECA showed it gave only weak turbidity with compound **6** at room temperature and much stronger precipitation was observed at 4 °C; studies were therefore performed at this temperature (Fig. 2B).[31] The ratios of concentrations of **3** and **6** to ECA at the equivalence points were shown to be 1:5 and 1:1.25, respectively. As a result, the reaction was observed between tetravalent **1**, **2**, **4**, **5** and WGA, but not between **3** and **6**. In contrast, ECA reacted with **3** and **6**, but not with **1**, **2**, **4** and **5**.

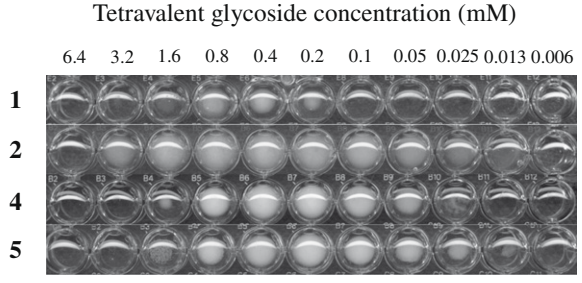


Figure 1. Photographs of precipitate formed by addition of tetravalent glycosides to WGA–PBS solution.

2.4. Double diffusion test

Figure 3 shows the results of double diffusion reactions using tetravalent glycosides **1**–**6** with WGA and ECA lectins. In general, precipitate is formed by the reaction between two specific high molecular weight compounds, such as lectin-glycoprotein and lectin-polysaccharide.[32,33] Interestingly, sharp precipitin bands are seen between the central well with WGA and the surrounding wells containing tetravalent glycosides **2**, **4** and **5** (Fig. 3A). Precipitin bands were not observed with **1**, **3** and **6**. Weak precipitin bands were observed between the central well with ECA and the surrounding wells containing **3** and **6** bearing LacNAc at 4 °C (Fig. 3B). These reactivities correlate well with the results of the hemagglutination inhibitory assay.

2.5. SPR analysis

Interaction of tetravalent glycosides non-spaced **1** and **2**, and spacer-linked **4** and **5** with WGA were analyzed using a BIAcore 2000. WGA was immobilized onto the surface of a sensor chip using the amine coupling method. The affinity of interaction was determined in solution by co-injecting an equilibrium mixture of a fixed amount of WGA with a variable amount of tetravalent glycoside onto surface-bound WGA. The surface was regenerated at the end of each cycle using 50 mM H_3PO_4. Figure 5 shows sensorgrams of binding between immobilized WGA and free-WGA. As anticipated, injection of a solution of WGA without nonspacer-N-linked tetravalent glycoside (i.e., negative control) resulted in no change of response units (RU). However, as the concentration of tetravalent glycosides **1**, **2**, **4** and **5** increased (i.e., from 0.1 μM to 0.1 mM) there was an increase in RU. Upon reaching the maximum RU, a decrease followed. The effect of the difference in multivalency was further examined by comparison of the tetravalent glycosides with chitin-oligomers. In the sensorgrams, response units (RU) increased linearly with increasing concentrations of the oligomers. As anticipated, these results indicate that the binding between WGA and $(GlcNAc)_2$/$(GlcNAc)_3$ on the sensor chip is promoted by the glycosides. Based on these results, Kd values of $(GlcNAc)_2$ and $(GlcNAc)_3$ were recorded as 1.6×10^{-4} and 1.5×10^{-5}, respectively.[25] Unfortunately, SPR analysis was not possible for target tetravalent glycosides because of unusual behavior on the sensorgram. The sensorgrams of tetravalent glycosides displayed behavior quite different from those of chitin-oligomers. Thus, co-injections with tetravalent glycosides showed a marked increase in RU in the region of 0.001 and 0.1 mM with maximal values at 0.1 mM. Upon reaching the maximum RU, there followed a sharp decrease. These compounds appear to effectively bind and promote the cross-linking of WGA in solution, rather than inhibiting the binding of WGA to the immobilized WGA. As the concentrations of these compounds were increased, they inhibited the binding of the clustered WGA to the surface WGA. The dissociation rate at maximal RU (0.1 mM) of **2** and **5** bearing tandem GlcNAc is qualitatively shown to be much slower than that of **1** and **4** bearing single GlcNAc. Based on the sensorgrams of Figure 5, we plotted the RUs at 180 s, which corresponds to the cross-linking maximum responses for the tetravalent glycosides to the surface-based WGA, against the glycoside concentration, as shown in Figure 4, although some of the sensorgrams did not reach equilibrium. Our main purpose was to prove the ability of WGA to act as a tet-

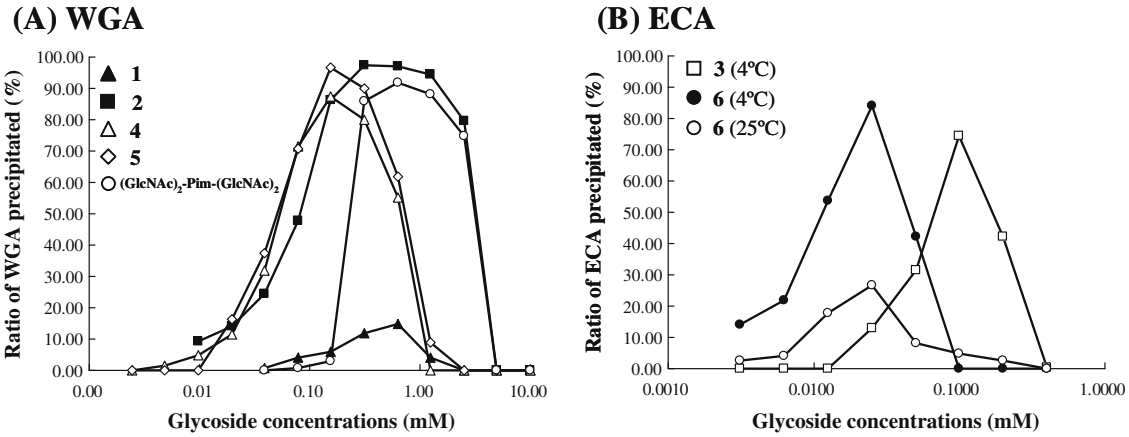


Figure 2. Precipitin curves for the precipitation of WGA (A) and ECA (B) with tetravalent glycosides. The percentage of precipitated WGA or ECA was calculated by subtracting the amount of WGA/ECA in the supernatant from the total amount of WGA/ECA, respectively.

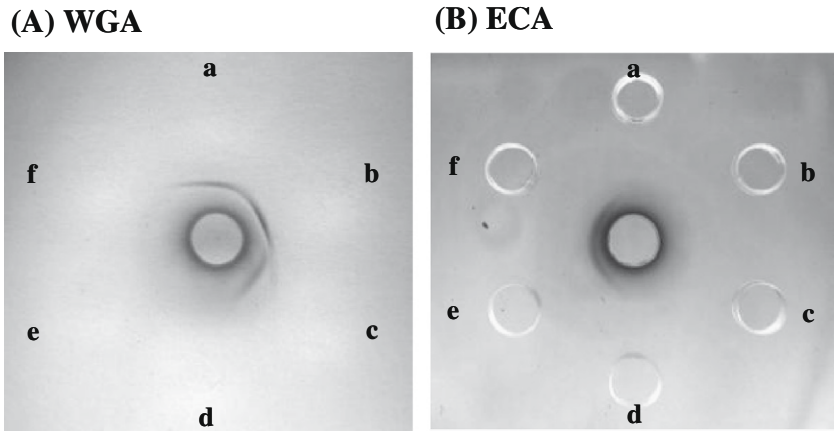


Figure 3. Double diffusion reaction of tetravalent glycosides with WGA (A) and ECA (B) in agar gel. Lectin solutions were put in the center wells. Portions of tetra-headed glycoside solutions were added to the peripheral wells. (a) **5**; (b) **2**; (c) **4**; (d) **1**; (e) **6**; (f) **3**.

ravalent ligand through SPR analysis. In this case, the amount corresponded to 6700 RU of WGA (Fig. 5A and B) immobilized onto the surface, and 8100 RU in Figure 5C–H. Divalent GlcNAc-Pim-GlcNAc was also plotted and compared with the tetravalent glycosides as a control sample. The tetravalent glycosides **2**, **4** and **5** produced maximum RU at approximately 0.1 mM, in contrast with the divalent glycoside, which showed maximum RU at 1 mM. The maximal RU of spacer-linked **4** and **5** was greater than that of nonspacer-linked **1** and **2**. The results of the SPR analysis are in agreement with the results of the precipitation analysis.

3. Discussion

We have recently reported a synthetic method for generating spacer-N-linked divalent glycosides bearing GlcNAc and $(GlcNAc)_2$ with different spacer groups. The divalent glycosides were shown to be capable of precipitating WGA and jack bean (*Canavalia ensiformis*) agglutinin (Con A) as divalent ligands.[25–27] They were even able to achieve divalency in systems of low valency. These results encouraged us to prepare multivalent carbohydrate analogs for high affinity binding to target lectins. We designed two types of nonspacer- and spacer-N-linked tetravalent glycosides to increase the binding strength with specific lectins. The efficient glycosylation protocol using EGTA as a precursor, described here, was a key step in the present study. In a first attempt, the *N*-β-mono- and diglycosylamines were directly coupled to precursor EGTA, producing nonspacer-N-linked tetravalent glycosides **1**, **2** and **3**, in which the scaffolds are directly connected by an amido group, but have fewer rotatable bonds. In another attempt, the glycosides bearing a pentylamido group in a glycon moiety were coupled to EGTA in a similar manner, producing spacer-N-linked tetravalent glycosides **4**, **5** and **6** with possible rotatable bonds. Our synthetic methods are easy and efficient in the synthesis of tetravalent gly-

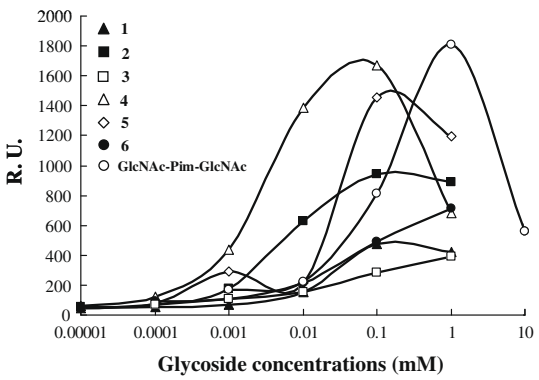


Figure 4. Cross-linking formation of WGA with tetravalent glycosides on the sensor chip. WGA corresponding to 6700 response units was immobilized onto the sensor chip.

cosides with stereoregular sugars through tetravalent carboxyl groups.

The resulting tetravalent glycosides were used in the analysis of WGA and ECA: Interactions were measured using four methods: hemagglutination inhibition assay, precipitation assay, double-diffusion test, and biosensor analysis. In the hemagglutination inhibition assay with WGA, a multivalent effect was observed for tetravalent **2**, **4** and **5**, when compared with the divalent $(GlcNAc)_2$-Pim-$(GlcNAc)_2$. A strong multivalency effect was detected in **5** at a low concentration (IC_{50} 0.18 μM). Inhibition by flexible tetravalent **5**, bearing tandem GlcNAc**,** was much higher than the analog **4**, bearing a single GlcNAc and rigid nonspaced **2**, bearing tandem GlcNAc. The inhibition activity of the tetravalent glycosides increased in the order of **5** > **2** > **4** > **1**. It is worth noting that

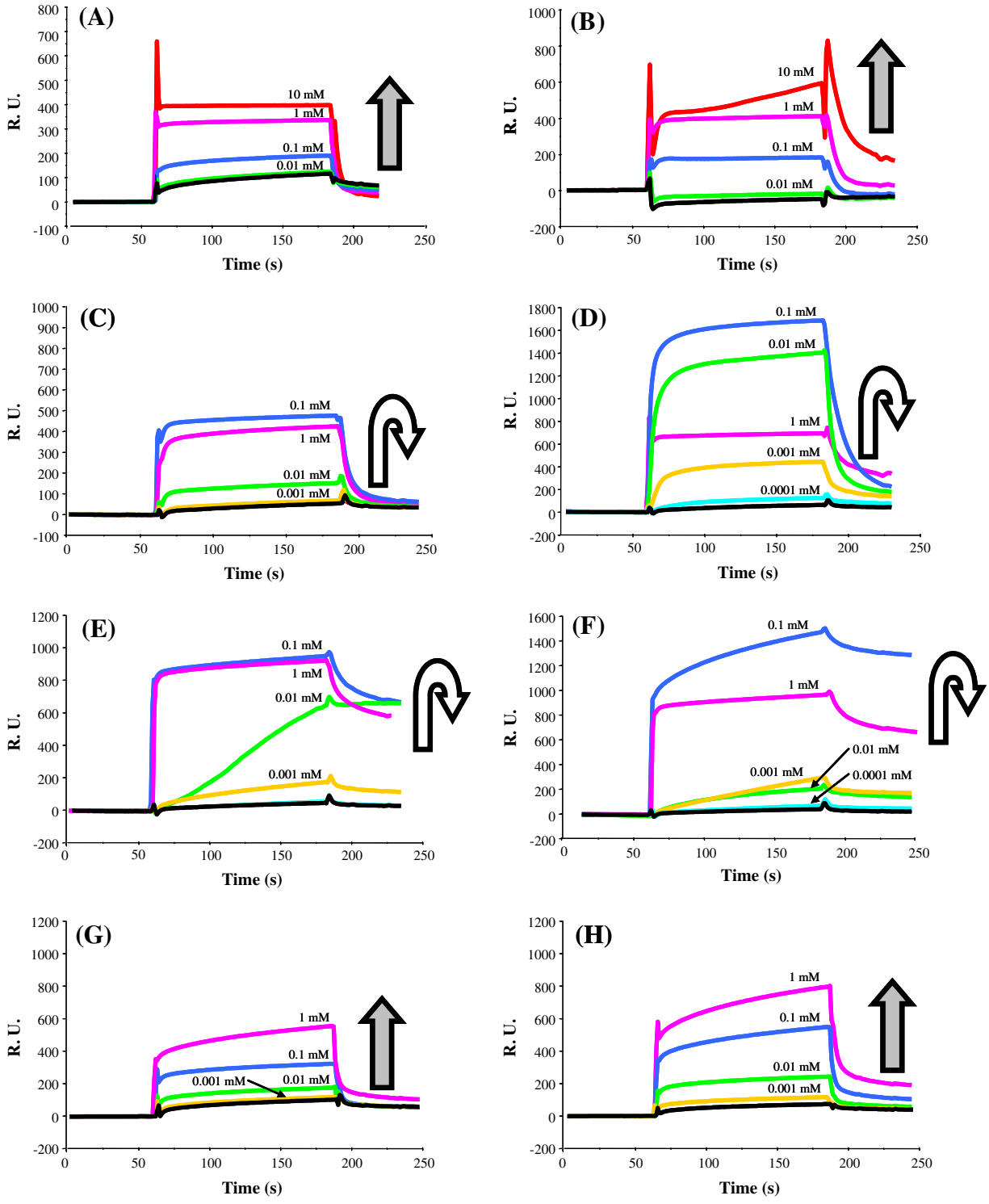


Figure 5. Sensorgrams showing the interactions of the glycosides with WGA. WGA corresponding to 8100 (A and B) or 6700 (C–H) response units was directly immobilized onto the sensor chip. WGA was co-injected with glycoside: (A) chitobiose, (B) chitotriose, (C) **1**, (D) **4**, (E) **2**, (F) **5**, (G) **3**, and (H) **6** (Color lines show increased and decreased RU, or injected without glycoside as a negative control (black line)).

5, despite its low molecular weight, increases binding by a factor of 4–8 when compared with ovalbumin and asialofetuin. In contrast, multivalency effects were not much detected in ECA. The minimal inhibitory concentrations of the lectin mediated hemagglutination of ECA were: 12 μM for **3**, 5.9 μM for **6** and 47 μM for $(GlcNAc)_2$-Pim-$(GlcNAc)_2$. The relative binding affinity of **6** was 130 times less than the binding affinity of asialofetuin. It suggests that ECA has a small multivalency enhancement ability, when comparing with WGA. This would be due to a difference of the binding mode on sugar sequence. ECA acts in an *exo*-manner on LacNAc sequence, while WGA both in *endo* and *exo*-manner on GlcNAc sequence.[34] We have already reported the ECA is much less subject to the cluster effect by glycopolymer than WGA.[32]

In the quantitative precipitation assay, tetravalent glycosides were shown to be capable of precipitating WGA/ECA as tetravalent ligands. The result is consistent with the sugar specificities of the corresponding lectins reported for naturally occurring glycoproteins, glycolipids and oligosaccharides. The precipitin profiles (Figs. 1 and 2) are similar to antigen-antibody,[30] lectin-polysaccharide,[33] and lectin-glycopeptide[5] and complex-type oligosaccharide-Con A precipitin curves[8] which suggests similar multivalent interactions between the tetravalent glycosides and WGA/ECA, despite the low molecular weights as ligands. The precipitin profile between flexible **4**/**5** with spacer and WGA was shown to proceed stoichiometrically, compared with rigid **1** and **2** with no spacer. Compounds **4** and **5** behaved tetravalently in WGA binding.[5] Similar precipitation experiments with spacer-linked **6** showed that it is divalent for ECA binding. No precipitates were observed between divalent glycoside and ECA. The results suggested the spacer-linked glycosides effectively bind and promote cross-linking of WGA in solution, rather than inhibiting the binding of lectin. Precipitation of WGA with **2**, **4** and **5** occurs at room temperature, whereas precipitation of ECA with **3** and **6**, which bind more weakly, requires a lower temperature (4 °C). In general, the precipitation data for ligand with lectin reflects the relative affinities of carbohydrate for the proteins. The binding activities of lectins, including WGA and ECA, produced cross-linked complexes with the tetravalent glycosides. The results were supported by the double diffusion test. However, ECA has a much smaller multivalency enhancement than WGA. The present results are easily predictable by previous our reports. Because we have already reported that artificial glycopolymers bearing $(GlcNAc)_2$ unit shows a strong interaction with WGA, because of a cluster effect of multivalent oligosaccharide chains in the polymer, whereas ECA is much less subject to cluster effect by glycopolymer bearing LacNAc unit.[35,36] SPR competition binding assays were used to monitor the effect of tetravalent glycosides on the interaction of soluble WGA with surface-bound WGA. The dissociation rate of **2** and **5** at maximal RU bearing tandem GlcNAc was much slower than that of **1** and **4** bearing single GlcNAc. The dissociation rates can be summarized as: **5** > **2** ≫ **4** > **1**. The slow down of the dissociation rate enhances binding affinity, and is due to multivalency effects.[15] This relationship is consistent with the hemagglutination inhibition activity mention above. Thus, it indicates that the binding affinity is not only enhanced by the existence of a spacer on the tetravalent glycosides, but also a sugar length. In our study, RU increased as the concentration of the tetravalent glycosides increased from 0.1 μM to 0.1 mM. Thus, the tetravalent glycosides promoted, rather than inhibited binding of WGA to a surface-bound WGA. Our data suggests co-injection of tetravalent glycoside and tetravalent WGA as analyte results in simultaneous cross-linking. The cross-linking complexes presumably bind to the surface-bound WGA through unbound tetravalent glycosides. Compounds **4** and **5**, with flexible spacer, clearly show maximum binding at 0.1 mM and divalent GlcNAc-Pim-GlcNAc displayed maximum binding at 1 mM. The results of SPR analysis corresponded with the results of the precipitation assay. Burke et al. used SPR to show the trivalent mannose macrocycle, which is more potent than the corresponding monovalent derivative, functions by cross-linking Con A in solution.[37] This is consistent with our results.

Our results show flexible tetravalent **5** has particularly high-affinity for WGA. Gour and Verma with the help of AFM (atomic force microscope) imaging demonstrated strong, complex interactions between a trivalent mannose conjugate and Con A.[38] Their results suggest the orientation of a tetra-headed glycoside as ligand was highly compatible with the formation of cross-linked complexes, which is the likely cause of binding enhancement for this class of lectin. The chelate effect leads to large rate enhancement in tetravalent systems, with favorable orientation of ligands. In the chelation mechanism, neighboring binding sites are simultaneously occupied by ligands bridged by suitable spacers.[39] Typically, binding of the first ligand is thought to reduce the entropic barriers for binding the second and further ligands. The chelate effect can lead to a large rate enhancement for the tetravalent system of **5**, so that the spacer is optimal in terms of flexibility. On closer inspection, it was found that binding enhancement was due to a combination of multivalency effects and spacer effects.

4. Conclusion

We designed two types of nonspacer- and spacer-N-linked tetravalent glycosides to increase the binding strength of synthetic ligands for WGA and ECA. Spacer-N-linked tetravalent glycoside bearing flexible tandem GlcNAc (**5**) showed a strong multivalency effect for WGA, as studied through lectin-ligand interactions. Our results suggest that even small synthetic conjugates could act as potential ligands for lectin binding, producing similar multivalent interactions with glycoprotein. Future studies on the interaction of synthetic multivalent glycoside conjugates with lectins for the generation of cross-linking complexes are planned and are expected to complement other biophysical studies of lectin-multivalent carbohydrate interactions.

5. Experimental

5.1. Materials

GlcNAc was purchased from Sigma–Aldrich (St. Louis, MO, USA). $(GlcNAc)_2$ and $(GlcNAc)_3$ were a kind gift from Yaizu Suisan Kagaku Industry Co., Ltd (Shizuoka, Japan). LacNAc, N^1,N^7-di-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-pimeamide (GlcNAc-Pim-GlcNAc) and N^1,N^7-di-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-pimeamide [$(GlcNAc)_2$-Pim-$(GlcNAc)_2$] were prepared by our previously described methods.[25] WGA and ECA were purchased from J-OIL MILLS, Inc. (Yokohama, Japan). CM-5 sensor chips and the amine coupling kit, containing *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride (EDC) and ethanolamine-hydrochloride, were purchased from Biacore AB (Uppsala, Sweden). All other chemicals were obtained from commercial sources.

5.2. Analytical methods

HPLC analysis was carried out using an Asahipak NH2-P50 4-E column (4.6 × 250 mm, Shodex, Japan) with a JASCO Intelligent system liquid chromatograph and detection at 210 nm. The bound material was eluted with 75% or 80% CH_3CN at a flow rate of 1.0 mL/min at 40 °C. FAB-mass analysis was carried out in positive ion mode using a JEOL JMS DX-303HF mass spectrometer

coupled to a JEOL DA-800 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1000 were employed. The ESI-MS spectra were measured on a JMS-T100LC mass spectrometer. ^{1}H NMR spectra were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. Chemical shifts are expressed in δ relative to sodium 3-(trimethylsilyl) propionate as an external standard.

5.3. Synthesis of nonspacer-N-linked tetravalent glycosides

β-*N*-Acetylglucosaminylamine (GlcNAc β-NH_2), β-*N*,*N*′-diacetylchitobiosylamine [$(GlcNAc)_2$ β-NH_2] and β-*N*-acetyllactosaminylamine (LacNAc β-NH_2) were prepared from GlcNAc, $(GlcNAc)_2$ and LacNAc by our previously described methods[25,28,29] and used for synthesis of tetravalent glycosides as follows.

5.3.1. Nonspacer-N-linked tetravalent GlcNAc glycoside (1)

After EGTA (38 mg, 0.1 mmol) was dissolved in DMSO (1.0 mL), *N*-ethyldiisopropylamine (0.35 mL, 2.0 mmol), HBTU (455 mg, 1.2 mmol) and triethylamine (0.6 mL) were added to the solution. The mixture was allowed to preactivate for approximately 30 min. In a separate vial, GlcNAc β-NH_2 (110 mg, 0.5 mmol) was dissolved in DMSO (0.6 mL). The solution was added to the activated solution. The mixture was allowed to react at room temperature with constant stirring for 72 h. After which, the reaction mixture was directly loaded onto a Bio-Gel P-2 column (6.0 × 90 cm). The column was developed with H_2O at a flow rate of 1.4 mL/min and a fraction size of 15 mL/tube. Fractions 38–43 were concentrated and dissolved in 5 mL of 10% EtOH and then loaded onto a charcoal-Celite column (2.8 × 30 cm) equilibrated with 10% EtOH and washed with 0.5 L of 10% EtOH. Subsequently, the adsorbed portion was eluted with a linear gradient of 15–65% EtOH in a total volume of 1 L, at a flow rate of 3.0 mL/min, and a fraction size of 20 mL/tube. An aliquot from fractions 18–28 was then concentrated and lyophilized: compound **1** was obtained in a total yield of 55% (66 mg), based on the initial amount of EGTA. HR-ESI-MS: *m/z* 1211.51428 $[M+Na]^+$ (calcd for $C_{46}H_{80}N_{10}Na_1O_{26}$, 1211.51429); ^{1}H NMR (D_2O, 500 MHz, 30 °C): δ 5.13 (d, 4H, $J_{1,2}$ 9.8 Hz, H-1), 3.91 (dd, 4H, $J_{5,6b}$ 1.4, $J_{6a,6b}$ 12.5 Hz, H-6b), 3.88 (t, 4H, $J_{1,2}$ 9.8, $J_{2,3}$ 9.8 Hz, H-2), 3.77 (dd, 4H, $J_{5,6a}$ 4.9, $J_{6a,6b}$ 12.5 Hz, H-6a), 3.69–3.65 (8H, H-3, H-d), 3.62 (4H, H-c), 3.55 (m, 4H, $J_{4,5}$ 9.8, $J_{5,6a}$ 4.9, $J_{5,6b}$ 1.4 Hz, H-5), 3.52 (t, 4H, $J_{3,4}$ 9.8, $J_{4,5}$ 9.8 Hz, H-4), 3.43 (8H, H-a), 2.81 (4H, H-b), 2.02 (s, 12H, C*H*$_3$CONH–); ^{13}C NMR (D_2O, 125 MHz, 30 °C): δ 177.4 (CH_3*C*ONH–), 177.3 (–N*H*C*O*– of EGTA), 81.1 (C-1), 80.5 (C-5), 77.0 (C-3), 72.44 (C-4), 72.35 (C-d), 71.4 (C-c), 63.4 (C-6), 60.8 (C-a), 57.2 (C-2), 56.4 (C-b), 24.9 (*C*H$_3$CONH–).

5.3.2. Nonspacer-N-linked tetravalent $(GlcNAc)_2$ glycoside (2)

The desired compound **2** was synthesized from EGTA (38 mg, 0.1 mmol) and $(GlcNAc)_2$ β-NH_2 (212 mg, 0.5 mmol) in a manner similar to that used to prepare compound **1**. Compound **2** was obtained in a total yield of 27% (53 mg), based on the initial amount of EGTA. ESI-MS: *m/z* 2023.8 $[M+Na]^+$; ^{1}H NMR (D_2O, 500 MHz, 30 °C): δ 5.11 (d, 4H, $J_{1,2}$ 9.8 Hz, H-1), 4.63 (d, 4H, $J_{1',2'}$ 8.6 Hz, H-1′), 3.95 (4H, H-6′b), 3.92 (t, 4H, $J_{1,2}$ 9.8, $J_{2,3}$ 9.8 Hz, H-2), 3.87–3.76 (16H, H-3, H-6b, H-2′, H-6′a), 3.70–3.58 (24H, H-4, H-5, H-6a, H-3′, H-c, H-d), 3.55–3.48 (8H, H-4′, H-5′), 3.41 (8H, H-a), 2.80 (4H, H-b), 2.09 (s, 12H, C*H*$_3$CONH′–), 2.01 (s, 12H, C*H*$_3$CONH–); ^{13}C NMR (D_2O, 125 MHz, 30 °C): δ 177.41 (CH_3*C*ONH′–), 177.39 (CH_3*C*ONH–), 177.3 (–N*H*C*O*– of EGTA), 104.2 (C-1′), 81.8 (C-4), 81.0 (C-1), 79.1 (C-5), 78.8 (C-5′), 76.3 (C-3′), 75.6 (C-3), 72.6 (C-4′), 72.3 (C-d), 71.5 (C-c), 63.4 (C-6′), 62.8 (C-6), 60.9 (C-a), 58.4 (C-2′), 56.6 (C-2), 56.4 (C-b), 25.0 (*C*H$_3$CONH′–), 24.9 (*C*H$_3$CONH–).

5.3.3. Nonspacer-N-linked tetravalent LacNAc glycoside (3)

The desired compound **3** was synthesized from EGTA (38 mg, 0.1 mmol) and LacNAc β-NH_2 (172 mg, 0.5 mmol) in a manner similar to that used to prepare compound **1**. Compound **3** was obtained in a total yield of 23% (42 mg), based on the initial amount of EGTA. HR-ESI-MS: *m/z* 1859.72486 $[M+Na]^+$ (calcd for $C_{70}H_{120}N_{10}Na_1O_{46}$, 1859.72558); ^{1}H NMR (D_2O, 500 MHz, 30 °C): δ 5.14 (d, 4H, $J_{1,2}$ 9.8 Hz, H-1), 4.51 (d, 4H, $J_{1',2'}$ 7.6 Hz, H-1′), 3.98–3.92 (12H, H-2, H-6b, H-4′), 3.88–3.74 (24H, H-3, H-4, H-6a, H-5′, H-6′b, H-6′a), 3.71–3.66 (12H, H-5, H-3′, H-d), 3.62 (4H, H-c), 3.58 (dd, 4H, $J_{1',2'}$ 8.0, $J_{2',3'}$ 9.8 Hz, H-2′), 3.43 (s, 8H, H-a), 2.81 (4H, H-b), 2.02 (s, 12H, C*H*$_3$CONH–); ^{13}C NMR (D_2O, 125 MHz, 30 °C): δ 177.4 (CH_3*C*ONH–), 177.2 (–N*H*C*O*– of EGTA), 105.7 (C-1′), 81.1 (C-1), 80.9 (C-4), 79.4 (C-5), 78.2 (C-5′), 75.6 (C-3), 75.3 (C-3′), 73.8 (C-2′), 72.4 (C-d), 71.5 (C-c), 71.4 (C-4′), 63.9 (C-6′), 62.8 (C-6), 60.9 (C-a), 56.7 (C-2), 56.4 (C-b), 24.9 (*C*H$_3$CONH–).

5.4. Synthesis of spacer-N-linked tetravalent glycosides

5.4.1. Spacer-N-linked tetravalent GlcNAc glycoside (4)

Spacer-N-linked glycoside was prepared from GlcNAc β-NH_2 as follows. After 6-trifluoroacetamidohexanoic acid (454 mg, 2.0 mmol) was dissolved in DMSO (0.6 mL), DIEA (2.1 mL, 12 mmol) and HBTU (1.14 g, 3.0 mmol) were added to the solution. The mixture was allowed to preactivate for approximately 10 min. In a separate vial, GlcNAc β-NH_2 (528 mg, 2.4 mmol) was dissolved in DMSO (1.3 mL). The solution was heated slightly to dissolve the monosaccharide. After the solution was cooled to room temperature, the monosaccharide solution was added to the activated solution. The mixture was allowed to react at room temperature with constant stirring for 3 h. After the reaction mixture was concentrated to a solid, it was dissolved in 5 mL of $CHCl_3/CH_3OH/H_2O$ = 8/2/0.26 and then loaded onto a Silica Gel 60 N column (3.0 × 60 cm). The column was developed with the same solvent at a flow rate of 10 ml/min and a fraction size of 25 mL/tube. Fractions 73–110 were pooled and concentrated. *N*-(ε-Trifluoroacetamidocaproyl)-β-*N*-acetylglucosaminylamine was obtained in a total yield of 71% (504 mg). Next, *N*-(ε-trifluoroacetamidocaproyl)-β-*N*-acetylglucosaminylamine (100 mg, 0.23 mmol) was dissolved in 1.0 M NaOH (1 mL). After the mixture was incubated for 10 min at room temperature, it was loaded onto a Sephadex G-25 column (2.5 × 55 cm) equilibrated with water at a flow rate of 0.4 mL/min and fraction size of 3.0 mL/tube. An aliquot from fractions 44–50 was concentrated and lyophilized. *N*-(ε-aminocaproyl)-β-*N*-acetylglucosaminylamine was obtained at a total yield of 99% (76 mg). FAB-MS: *m/z* 334 $[M+H]^+$ (matrix: glycerol); ^{1}H NMR (D_2O, 500 MHz, 25 °C): δ 5.06 (d, 1H, $J_{1,2}$ 9.8 Hz, H-1), 3.88 (dd, 1H, $J_{5,6b}$ 1.9, $J_{6a,6b}$ 12.2 Hz, H-6b), 3.81 (t, 1H, $J_{1,2}$ 9.8, $J_{2,3}$ 9.8 Hz, H-2), 3.75 (dd, 1H, $J_{5,6a}$ 4.6, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.61 (t, 1H, $J_{2,3}$ 9.8, $J_{3,4}$ 9.8 Hz, H-3), 3.51 (m, 1H, $J_{4,5}$ 9.8, $J_{5,6a}$ 4.6, $J_{5,6b}$ 1.9 Hz, H-5), 3.48 (t, 1H, $J_{3,4}$ 9.8, $J_{4,5}$ 9.8 Hz, H-4), 2.97 (t, 2H, H-ε), 2.28 (t, 2H, H-α), 2.00 (s, 3H, C*H*$_3$CONH–), 1.66 (q, 2H, H-δ), 1.60 (q, 2H, H-β), 1.36 (q, 2H, H-γ); ^{13}C NMR (D_2O, 125 MHz, 25 °C): δ 180.3 (–N*H*C*O*–), 177.4 (CH_3*C*ONH–), 81.2 (C-1), 80.4 (C-5), 77.0 (C-3), 72.3 (C-4), 63.3 (C-6), 57.2 (C-2), 42.0 (C-ε), 38.2 (C-α), 29.3 (C-δ), 27.8 (C-γ), 27.3 (C-β), 24.8 (*C*H$_3$CONH–). The resulting spacer-N-linked glycoside bearing GlcNAc was used for obtaining the tetravalent glycoside. After EGTA (38 mg, 0.1 mmol) was dissolved in DMSO (0.9 mL), *N*-ethyldiisopropylamine (0.17 mL, 1.5 mmol), HBTU (455 mg, 1.2 mmol) and triethylamine (0.6 mL) were added to the solution. The mixture was allowed to preactivate for approximately 30 min. In a separate vial, *N*-(ε-aminocaproyl)-β-*N*-acetylglucosaminylamine (150 mg, 0.45 mmol) was dissolved in DMSO (0.7 mL). The solution was then added to the activated solution. The mixture was allowed to react at room temperature with constant stirring for 72 h. The reaction mixture was then directly

loaded onto a Bio-Gel P-2 column (6.0 × 90 cm). The column was developed with H_2O at a flow rate of 1.4 mL/min and a fraction size of 15 mL/tube. Fractions 48–55 were concentrated and dissolved in 5 mL of 15% MeOH and then loaded onto an ODS column (2.5 × 60 cm) equilibrated with 15% MeOH and washed with 1 L of 15% MeOH. Subsequently, the adsorbed portion was eluted with a linear gradient of 15–60% MeOH in a total volume of 2 L, at a flow rate of 2.0 mL/min, and a fraction size of 20 mL/tube. An aliquot from fractions 92–96 was then concentrated and lyophilized: compound **4** was obtained in a total yield of 26% (42 mg), based on the initial amount of EGTA. HR-ESI-MS: *m/z* 1663.85005 $[M+Na]^+$ (calcd for $C_{70}H_{124}N_{14}Na_1O_{30}$, 1663.85054); ^1H NMR (D_2O, 500 MHz, 30 °C): δ 5.07 (d, 4H, $J_{1,2}$ 9.8 Hz, H-1), 3.90 (dd, 4H, $J_{5,6b}$ 1.9, $J_{6a,6b}$ 12.5 Hz, H-6b), 3.83 (t, 4H, $J_{1,2}$ 9.8, $J_{2,3}$ 9.8 Hz, H-2), 3.77 (dd, 4H, $J_{5,6a}$ 4.6, $J_{6a,6b}$ 12.5 Hz, H-6a), 3.67–3.64 (8H, H-c, H-d), 3.64 (t, 4H, $J_{2,3}$ 9.8, $J_{3,4}$ 9.8 Hz, H-3), 3.53 (m, 4H, $J_{4,5}$ 9.8, $J_{5,6a}$ 4.6, $J_{5,6b}$ 1.9 Hz, H-5), 3.50 (t, 4H, $J_{3,4}$ 9.8, $J_{4,5}$ 9.8 Hz, H-4), 3.31 (8H, H-a), 3.23 (t, 8H, H-ε), 2.82 (4H, H-b), 2.29 (8H, H-α), 2.02 (s, 12H, C*H*$_3$CONH–), 1.60 (q, 8H, H-β), 1.54 (q, 8H, H-δ), 1.31 (q, 8H, H-γ); ^{13}C NMR (D_2O, 125 MHz, 30 °C): δ 180.3 (–NHCO–), 177.3 (CH_3CONH–), 176.0 (–NHCO– of EGTA), 81.2 (C-1), 80.5 (C-5), 77.0 (C-3), 72.5 (C-d), 72.4 (C-4), 71.4 (C-c), 63.4 (C-6), 61.4 (C-a), 57.5 (C-b), 57.2 (C-2), 41.7 (C-ε), 38.5 (C-α), 31.0 (C-δ), 28.4 (C-γ), 27.7 (C-β), 24.9 (*C*H$_3$CONH–).

5.4.2. Spacer-N-linked tetravalent $(GlcNAc)_2$ glycoside (5)

N-(ε-Aminocaproyl)-β-*N*,*N*-diacetylchitobiosylamine was synthesized from 6-trifluoroacetamidohexanoic acid (182 mg, 0.8 mmol) and $(GlcNAc)_2$ β-NH_2 (338 mg, 0.8 mmol) in a manner similar to that used to prepare *N*-(ε-aminocaproyl)-β-*N*-acetylglucosaminylamine. *N*-(ε-Aminocaproyl)-β-*N*, *N*′-diacetylchitobiosylamine was obtained in a total yield of 70%. HR-ESI-MS: *m/z* 537.27662 $[M+H]^+$ (calcd for $C_{22}H_{41}N_4O_{11}$, 537.27718); ^1H NMR (D_2O, 500 MHz, 30 °C): δ 5.07 (d, 1H, $J_{1,2}$ 9.5 Hz, H-1), 4.62 (d, 1H, $J_{1',2'}$ 8.5 Hz, H-1′), 3.94 (1H, H-6′b), 3.88–3.84 (2H, H-2, H-6b), 3.80–3.75 (3H, H-2′, H-3, H-6′a), 3.69–3.65 (2H, H-4, H-6a), 3.61–3.57 (2H, H-3′, H-5), 3.54–3.50 (2H, H-4′, H-5′), 2.96 (t, 2H, H-ε), 2.30 (t, 2H, H-α), 2.08 (s, 3H, C*H*$_3$CONH′–), 2.01 (s, 3H, C*H*$_3$CONH–), 1.69–1.59 (4H, H-δ, H-β), 1.37 (q, 2H, H-γ); ^{13}C NMR (D_2O, 125 MHz, 30 °C): δ 180.3 (–NHCO–), 177.4 (CH_3CONH′–, CH_3CONH–), 104.2 (C-1′), 81.7 (C-4), 81.1 (C-1), 79.1 (C-5), 78.8 (C-5′), 76.3 (C-3′), 75.6 (C-3), 72.6 (C-4′), 63.4 (C-6′), 62.8 (C-6), 58.4 (C-2′), 56.7 (C-2), 42.1 (C-ε), 38.3 (C-α), 29.7 (C-δ), 27.8 (C-γ), 27.4 (C-β), 25.0 (*C*H$_3$CONH′–), 24.8 (*C*H$_3$CONH–). The resulting *N*-(ε-aminocaproyl)-β-*N*,*N*′-diacetylchitobiosylamine (215 mg, 0.4 mmol) was coupled with EGTA (38 mg, 0.1 mmol) in a manner similar to that used to prepare compound **4**. Compound **5** was obtained in a total yield of 39% (95 mg), based on the initial amount of EGTA. ESI-MS: *m/z* 2477.4 $[M+Na]^+$; ^1H NMR (D_2O, 500 MHz, 30 °C): δ 5.06 (d, 4H, $J_{1,2}$ 9.0 Hz, H-1), 4.62 (d, 4H, $J_{1',2'}$ 8.5 Hz, H-1′), 3.94 (4H, H-6′b), 3.88–3.84 (8H, H-2, H-6b), 3.80–3.76 (12H, H-3, H-2′, H-6′a), 3.69–3.58 (24H, H-4, H-5, H-6a, H-3′, H-c, H-d), 3.55–3.47 (8H, H-4′, H-5′), 3.30 (8H, H-a), 3.23 (t, 8H, H-ε), 2.82 (4H, H-b), 2.27 (8H, H-α), 2.09 (s, 12H, C*H*$_3$CONH′–), 2.01 (s, 12H, C*H*$_3$CONH–), 1.59 (8H, H-β), 1.54 (8H, H-δ), 1.30 (8H, H-γ); ^{13}C NMR (D_2O, 125 MHz, 30 °C): δ 180.3 (–NHCO–), 177.4 (CH_3CONH′–), 177.3 (CH_3CONH–), 176.0 (–NHCO– of EGTA), 104.2 (C-1′), 81.7 (C-4), 81.1 (C-1), 79.0 (C-5), 78.8 (C-5′), 76.3 (C-3′), 75.6 (C-3), 72.6 (C-4′), 72.5 (C-d), 71.4 (C-c), 63.4 (C-6′), 62.8 (C-6), 61.4 (C-a), 58.4 (C-2′), 57.5 (C-b), 56.6 (C-2), 41.7 (C-ε), 38.5 (C-α), 31.0 (C-δ), 28.4 (C-γ), 27.6 (C-β), 25.0 (*C*H$_3$CONH′–), 24.9 (*C*H$_3$CONH–).

5.4.3. Spacer-N-linked tetravalent LacNAc glycoside (6)

N-(ε-Aminocaproyl)-β-*N*-acetyllactosaminylamine was synthesized from 6-trifluoroacetamidohexanoic acid (41 mg, 0.18 mmol) and LacNAc β-NH_2 (70 mg, 0.18 mmol) in a manner similar to that used to prepare *N*-(ε-aminocaproyl)-β-*N*-acetylglucosaminylamine. *N*-(ε-Aminocaproyl)-β-*N*-acetyllactosaminylamine was obtained in a total yield of 74%. FAB-MS: *m/z* 496 $[M+H]^+$ (matrix: glycerol); ^1H NMR (D_2O, 500 MHz, 25 °C): δ 5.08 (d, 1H, $J_{1,2}$ 9.8 Hz, H-1), 4.47 (d, 1H, $J_{1',2'}$ 7.6 Hz, H-1′), 3.93 (dd, 1H, $J_{5,6b}$ 1.9, $J_{6a,6b}$ 12.2 Hz, H-6b), 3.92 (1H, H-4′), 3.86 (t, 1H, $J_{1,2}$ 9.8, $J_{2,3}$ 9.8 Hz, H-2), 3.84 (dd, 1H, $J_{5,6a}$ 4.6, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.80–3.71 (5H, H-3, H-4, H-5′, H-6′a, H-6′b), 3.66 (dd, 1H, $J_{2',3'}$ 10, $J_{3',4'}$ 3.4 Hz, H-3′), 3.65 (1H, H-5), 3.54 (dd, 1H, $J_{1',2'}$ 7.6, $J_{2',3'}$ 10 Hz, H-2′), 2.93 (t, 2H, H-ε), 2.28 (t, 2H, H-α), 1.99 (s, 3H, C*H*$_3$CONH–), 1.63 (q, 2H, H-δ), 1.59 (q, 2H, H-β), 1.34 (q, 2H, H-γ); ^{13}C NMR (D_2O, 125 MHz, 25 °C): δ 180.4 (–NHCO–), 177.4 (CH_3CONH–), 105.6 (C-1′), 81.1 (C-1), 80.6 (C-4), 79.3 (C-5), 78.2 (C-5′), 75.6 (C-3), 75.3 (C-3′), 73.8 (C-2′), 71.4 (C-4′), 63.9 (C-6′), 62.6 (C-6), 56.7 (C-2), 42.1 (C-ε), 38.3 (C-α), 29.7 (C-δ), 27.8 (C-γ), 27.4 (C-β), 24.8 (*C*H$_3$CONH–). The resulting *N*-(ε-aminocaproyl)-β-*N*-acetyllactosaminylamine (139 mg, 0.28 mmol) was coupled with EGTA (27 mg, 0.07 mmol) in a manner similar to that used to prepare compound **4**. Compound **6** was obtained in a total yield of 33% (52 mg), based on the initial amount of EGTA. ESI-MS: *m/z* 2312.3 $[M+Na]^+$; ^1H NMR (D_2O, 500 MHz, 30 °C): δ 5.10 (d, 4H, $J_{1,2}$ 9.5 Hz, H-1), 4.51 (d, 4H, $J_{1',2'}$ 8.0 Hz, H-1′), 3.97–3.95 (8H, H-4′, H-6b), 3.90–3.85 (8H, H-2, H-6a), 3.83–3.74 (20H, H-3, H-4, H-5′, H-6′b, H-6′a), 3.70–3.67 (12H, H-5, H-3′, H-d), 3.64 (4H, H-c), 3.58 (dd, 4H, $J_{1',2'}$ 8.0, $J_{2',3'}$ 10 Hz, H-2′), 3.31 (s, 8H, H-a), 3.23 (t, 8H, H-ε), 2.82 (4H, H-b), 2.28 (8H, H-α), 2.02 (s, 12H, C*H*$_3$CONH–), 1.60 (q, 8H, H-β), 1.54 (q, 8H, H-δ), 1.31 (q, 8H, H-γ); ^{13}C NMR (D_2O, 125 MHz, 30 °C): δ 180.3 (–NHCO–), 177.3 (CH_3CONH–), 176.0 (–NHCO– of EGTA), 105.7 (C-1′), 81.1 (C-1), 80.9 (C-4), 79.3 (C-5), 78.2 (C-5′), 75.6 (C-3), 75.4 (C-3′), 73.8 (C-2′), 72.5 (C-d), 71.4 (C-4′, C-c), 63.9 (C-6′), 62.7 (C-6), 61.4 (C-a), 57.5 (C-b), 56.8 (C-2), 42.7 (C-ε), 38.5 (C-α), 31.0 (C-δ), 28.4 (C-γ), 27.6 (C-β), 24.9 (*C*H$_3$CONH–).

5.5. Hemagglutination inhibition assay

The hemagglutination inhibition assay was carried out using 96-well microtiter plates as described previously.[29] Phosphate-buffered saline (PBS, pH 7.4) was used as a dilution buffer. WGA or ECA lectin suspension (2^2 hemagglutination titers in 0.02 mL of PBS) was added to each well containing the oligosaccharides and di-/tetravalent glycosides (4000–0.5 μM) or glycoproteins (125–0.06 μM) in a twofold serial dilution in dilution buffer. After incubation for 40 min at 4 °C, 0.04 mL of 0.4% (v/v) rabbit suspension erythrocytes was added to the plates, and allowed to settle for 40 min at 4 °C. The maximum dilution of the samples showing complete inhibition of hemagglutination was defined as the hemagglutination inhibition titer.

5.6. Precipitation analysis

Various concentrations of WGA/ECA and glycosides dissolved in PBS were mixed with an equal volume (total volume: 50 μL) on 96-well microtiter plate, which was then incubated at room temperature for 1 h. The resulting precipitate was visually observed. As a following step, precipitation assays with WGA/ECA were performed by UV-detection of WGA/ECA measurement of glycoside in supernatant. Various concentrations of glycoside solutions (50 μL) were added to 128 μM of WGA/ECA solution (50 μL) in a microtube. The solution was then centrifuged at 8000*g* for 10 min to remove precipitated material. The supernatant was diluted and analyzed by measuring the absorbance at 280 nm. Precipitated WGA/ECA was calculated from a standard curve.

5.7. Double diffusion test in agar

Four percent agar (3.0 g) was dissolved in 9.0 mL of 100 mM sodium phosphate-buffer (pH 7.2) containing 0.85% NaCl, to give a concentration of 0.9%; two drops of 1% sodium azide was then added. The solution was poured into a glass dish (90 mm, i.d.) to form a layer 3–4 mm thick. Wells were made with a steel puncher. Aqueous WGA or ECA lectins [0.2 or 0.4 mM in 10 mM PBS (pH 7.4), 13.5 μL] and 13.5 μL of a tetravalent glycoside solution [0.5 mM in 10 mM PBS (pH 7.4)] were placed in the central and peripheral wells, respectively, with a syringe. Reactions with WGA were incubated for 3 h at room temperature, and with ECA for 8 h at 4 °C. Gels were stained with 0.5% Amido Black 10B in 7.5% acetic acid and washed with 7.5% acetic acid.

5.8. Surface plasmon resonance analysis

SPR was recorded using a BIAcore 2000 (Biacore AB). WGA was directly immobilized onto the chip to confirm cross-linking of tetravalent glycosides with WGA. After chip activation with 0.1 M NHS and 0.4 M EDC, WGA in 10 mM sodium acetate buffer (pH 5.0) at a concentration of 0.5 mg/mL was passed through the flow cells at a rate of 10 μL/min. Upon immobilization of approximately 6700 or 8100 response units, the chip was capped by exposure to 1 M ethanolamine. A control lane which was not activated by NHS and EDC was used as a reference. All analyses were performed by eluting with HBS-P buffer (10 mM HEPES, 150 mM NaCl, 0.005% surfactant P20 [pH 7.4]) at a flow rate of 10 μL/min at 25 °C. The mixed solution of various concentrations of glycosides and WGA (16 μM) in 200 μL of HBS-P buffer was prepared and then incubated for 1 h before injection. An aliquot of the solution (20 μL) was then injected over the immobilized chip. The chip was regenerated by the injection of 5 μL of 50 mM of H_3PO_4, followed by HBS-P. Each sensorgram was obtained by subtracting the reference cell: a buffer only injection, without glycoside, was performed. All analyses were performed at least twice to verify reproducibility. The RUs at 180 s, corresponding to the cross-linking maximum responses for the tetravalent glycosides to the surface-bound WGA, were checked and plotted against glycoside concentrations.

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