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Efficient synthesis of spacer-N-linked double-headed glycosides carrying *N*-acetylglucosamine and *N*,*N*′-diacetylchitobiose and their cross-linking activities with wheat germ agglutinin

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Abstract—We describe here an efficient synthetic route to spacer-N-linked double-headed glycosides via a simple two-step procedure. *N*-Acetylglucosamine (GlcNAc) and *N*,*N*′-diacetylchitobiose [$(GlcNAc)_2$] were treated with ammonia and the resulting *N*-β-glycosylamines were coupled to a series of dicarboxylic acids. Condensation with each dicarboxylic acid proceeded stereoselectively to give the corresponding β-N-linked double-headed glycoside without the need for any protection/deprotection steps. Interaction of the resulting N-linked double-headed glycosides with wheat germ agglutinin (WGA) were then investigated using a precipitation assay and an optical biosensor based on surface plasmon resonance (SPR). Spacer-N-linked double-headed glycosides bearing GlcNAc and $(GlcNAc)_2$ were found to be capable of binding and precipitating WGA as divalent ligands. However, the length of the spacer groups between the two terminal sugar residues was found to greatly influence the cross-linking activities with the lectin.

Keywords: Double-headed glycoside; *N*-Acetylglucosamine; N-Linked; WGA; Cross-linking; Precipitation

1. Introduction

Designing multivalent carbohydrate analogs for high affinity binding to target lectins is considerably popular.[1–5] The cross-linking properties of a variety of plant and animal lectins with multivalent carbohydrates and glycoproteins have been reviewed.[6,7] These studies show that a number of lectins form cross-linked complexes with branched chain oligosaccharides,[8–10] glycopeptides,[11–13] glycoproteins[14–17] and polysaccharides.[18,19] The mechanism of binding between lectin and glycoprotein or glycopeptide has also been intensively examined. Binding studies show that some lectins recognize the saccharide chip or internal to the carbohydrate chain, whereas others recognize the saccharide on the base of carbohydrate chains.[20,21] Accordingly, carbohydrate epitopes are present in multivalent arrays at the cell membrane where they can serve as highly effective and specific ligands.[22,23] Bhattacharyya et al. reported that even low molecular weight oligomannose and bisected complex oligosaccharides bind to concanavalin A (Con A) to precipitate the lectin.[11–13] We recently designed and prepared O-linked divalent glycosides bearing GlcNAc, which is capable of precipitating wheat germ agglutinin (WGA), as the first report of the enzymatic synthesis of divalent glycosides.[24] The divalent glycoside can act as superior ligands because of their ability to cluster target lectins. As a result of these studies and because of the abundance and biological importance of 2-acetamido sugars, we were interested in developing an efficient synthetic route to N-linked GlcNAc-divalent

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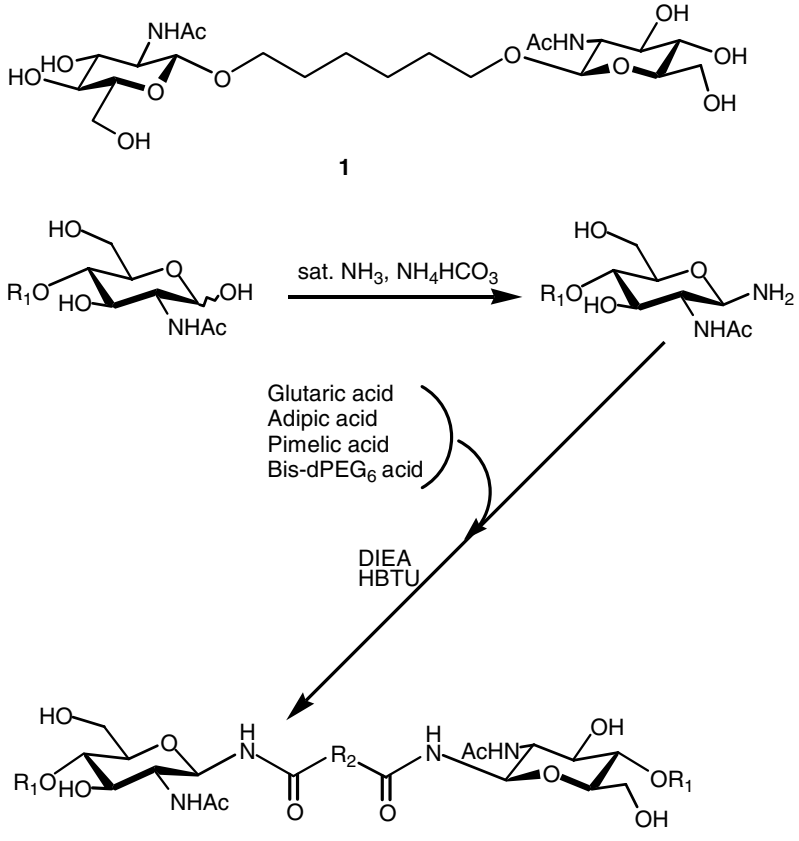
glycosides as a glycomimetic. In this report, we refer to divalent glycosides as 'double-headed glycosides'.

The present paper describes the development of an efficient synthetic method for generating spacer-N-linked double-headed glycosides bearing GlcNAc and $(GlcNAc)_2$ with different spacer groups. The interaction of these double-headed glycosides as divalent ligands with WGA was then analyzed using a precipitation assay and surface plasmon resonance analysis.

2. Results and discussion

2.1. Synthesis of double-headed glycoside

We recently reported an enzymatic method for preparing spacer-O-linked double-headed glycosides bearing GlcNAc [GlcNAc-Hx-GlcNAc (**1**), hexan-1,6-diyl bis-(2-acetamido-2-deoxy-β-D-glucopyranoside)] by chitinolytic enzyme-mediated transglycosylation (Scheme 1).[24] The enzyme catalyzes the direct transfer of GlcNAc residue to a primary diol acceptor. The double-headed glycoside was shown to bind, cross-link and precipitate WGA. These results encouraged us to prepare spacer-N-linked double-headed GlcNAc-glycosides as ligands that possess cross-linking activity with a target lectin. As represented in Scheme 1, an amino function was introduced into the anomer position of GlcNAc and $(GlcNAc)_2$ with ammonia according to the method of Hiratake and co-workers.[25] The resulting *N*-β-acetylglucosaminylamine and *N*,*N*′-β-diacetylchitobiosylamine were then condensed with one of the three dicarboxylic acids ($n = 3$, 4 or 5) and 3,6,9,12,15-pentaoxaheptadecane-1,17-dicarboxylic acid (Bis-dPEG$_6$-acid™) with HBTU and diisopropyl ethanolamine (DIEA) in DMSO. The N-glycosylation proceeded stereoselectively



2: R_1 = H, R_2 = -$(CH_2)_3$-
3: R_1 = H, R_2 = -$(CH_2)_4$-
4: R_1 = H, R_2 = -$(CH_2)_5$-
5: R_1 = H, R_2 = -$(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2$-
6: R_1 = GlcNAc residue, R_2 = -$(CH_2)_4$-
7: R_1 = GlcNAc residue, R_2 = -$(CH_2)_5$-
8: R_1 = GlcNAc residue, R_2 = -$(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2$-

Scheme 1. Structure of O-linked double-headed glycoside **1** and the synthetic method of N-linked double-headed glycosides **2**–**8**.

within a few hours to give only the β-glycoside without the need for any protection and deprotection steps. The yields of **2**, **3**, **4**, and **7** were 50–60%, based on the amount of dicarboxylic acid added. The yields of **5** and **8** were somewhat higher (60–85%). The target compounds were purified by chromatography on a single silica gel column and their structures were determined by ^{1}H and ^{13}C NMR absorptions on the basis of two-dimensional C–H COSY techniques. The difference in the length of dicarboxylic acid as spacer did not significantly affect the yields of the condensation reactions. These compounds were soluble in water and DMSO. This method affords an easy and efficient synthesis of spacer-linked double-headed glycosides bearing GlcNAc and $(GlcNAc)_2$ from the corresponding glycosylamine.

2.2. Interaction of double-headed glycosides with WGA

2.2.1. Precipitation analysis. The interaction of the resulting spacer-N-linked double-headed glycosides carrying GlcNAc or $(GlcNAc)_2$ with WGA was analyzed by two different methods: precipitation and biosensor analysis. O-Linked double-headed glycoside (GlcNAc-Hx-GlcNAc, **1**), which has the ability of precipitating WGA as a divalent ligand, was used as a control. The ability of the synthetic N-linked double-headed glycosides (**2**–**8**) to bind to WGA was first analyzed by precipitation analyses. WGA (256 μM) and double-headed glycoside (0.025–12.8 mM) were mixed on a 96-well microplate. When each double-headed GlcNAc- and $(GlcNAc)_2$-glycoside was added to the WGA solution under appropriate conditions, **2**, **3**, **4**, **6** and **7** formed a precipitate within a few minutes, whereas **5** and **8** with the same spacer did not. However, the presence of chitooligosaccharide [$(GlcNAc)_2$ and $(GlcNAc)_3$] prevented the formation of precipitates with **2**, **3**, **4**, **6** and **7**. Furthermore, the precipitates formed with **2**, **3**, **4**, **6** and **7** dissolved upon the addition of the oligomer. These results indicate that the double-headed GlcNAc glycosides bind specifically to WGA. Precipitin curves for WGA in the presence of double-headed glycosides **2**, **3**, and **4** were prepared by measuring the lectin concentration in the supernatant (Fig. 1a).

The ability to form a precipitate was compared with that of spacer-O-linked double-headed glycoside **1**. The concentration of **2**, **3** or **4** at the equivalence point (region of maximum precipitation) of the precipitin curve for 128 μM of WGA was about 1 mM. By contrast, the concentration of **1** at the equivalence point was in the region of 0.1–2.5 mM, although the value was not accurately determined under the present conditions. Figure 1b compares the precipitin curves of **3** and **6** bearing GlcNAc or $(GlcNAc)_2$, respectively, with the same spacer group. These results clearly show that the addition of GlcNAc to **3** increases the amount of precipitate obtained in the assay. On the other hand, the addition of GlcNAc to **4** did not substantially increase the amount of precipitate generated in the assay (Fig. 1c).

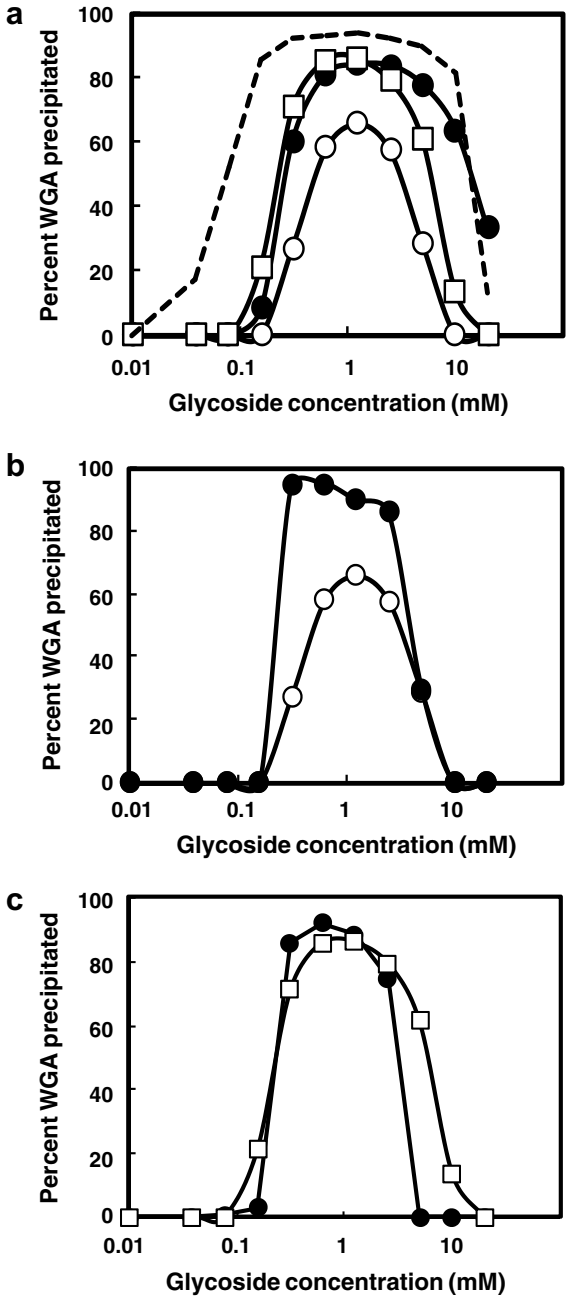


Figure 1. Precipitin curves for the precipitation of WGA with N-linked double-headed glycosides. The percentage of WGA precipitated was calculated by subtracting the amount of WGA in the supernatant from the total amount of WGA. (a) GlcNAc-N-linked double-headed glycosides **2**–**4** precipitated WGA. The curve corresponding to O-linked double-headed glycoside **1** is shown by a dashed line for comparison. ●, **2**; ○, **3**; □, **4**. (b) GlcNAc- and $(GlcNAc)_2$-N-linked double-headed glycosides bearing $-(CH_2)_4-$ on the spacer precipitated WGA. ○, **3**; ●, **6**. (c) GlcNAc- and $(GlcNAc)_2$-N-linked double-headed glycosides bearing $-(CH_2)_5-$ on the spacer precipitated WGA. □, **4**; ●, **7**.

Precipitation analysis showed that N-linked double-headed GlcNAc glycosides specifically bind to WGA in an analogy with O-linked double-headed GlcNAc glycosides. However, the effect of the spacer-N-linked double-headed GlcNAc glycosides on the interaction with the lectin was somewhat weaker than that of O-linked double-headed GlcNAc glycoside **1**. Our results demonstrate that double-headed analogs with flexible spacer groups (O-linked) between the two GlcNAc residues possess higher affinity for the lectin compared to those bearing an inflexible spacer group (N-linked) through an NHCO linkage. Furthermore, the length of the spacer group between the two GlcNAc residues appears to affect the ability to precipitate WGA. The maximum amount of precipitate generated with double-headed glycosides increased 30–33% when the spacer unit contained an odd number of methylene groups (**2** and **4**) compared to a corresponding glycoside with an even number of methylene groups in the spacer unit (**3**). On the contrary, double-headed glycosides **5** and **8** with an extended spacer (PEG) group did not form a precipitate in the assay. We have already reported that O-linked double-headed glycoside bearing triethylene glycol spacer is also capable of precipitating WGA in analogy with **1**.[24] From these results, the length of the spacer might be important in allowing the molecule to

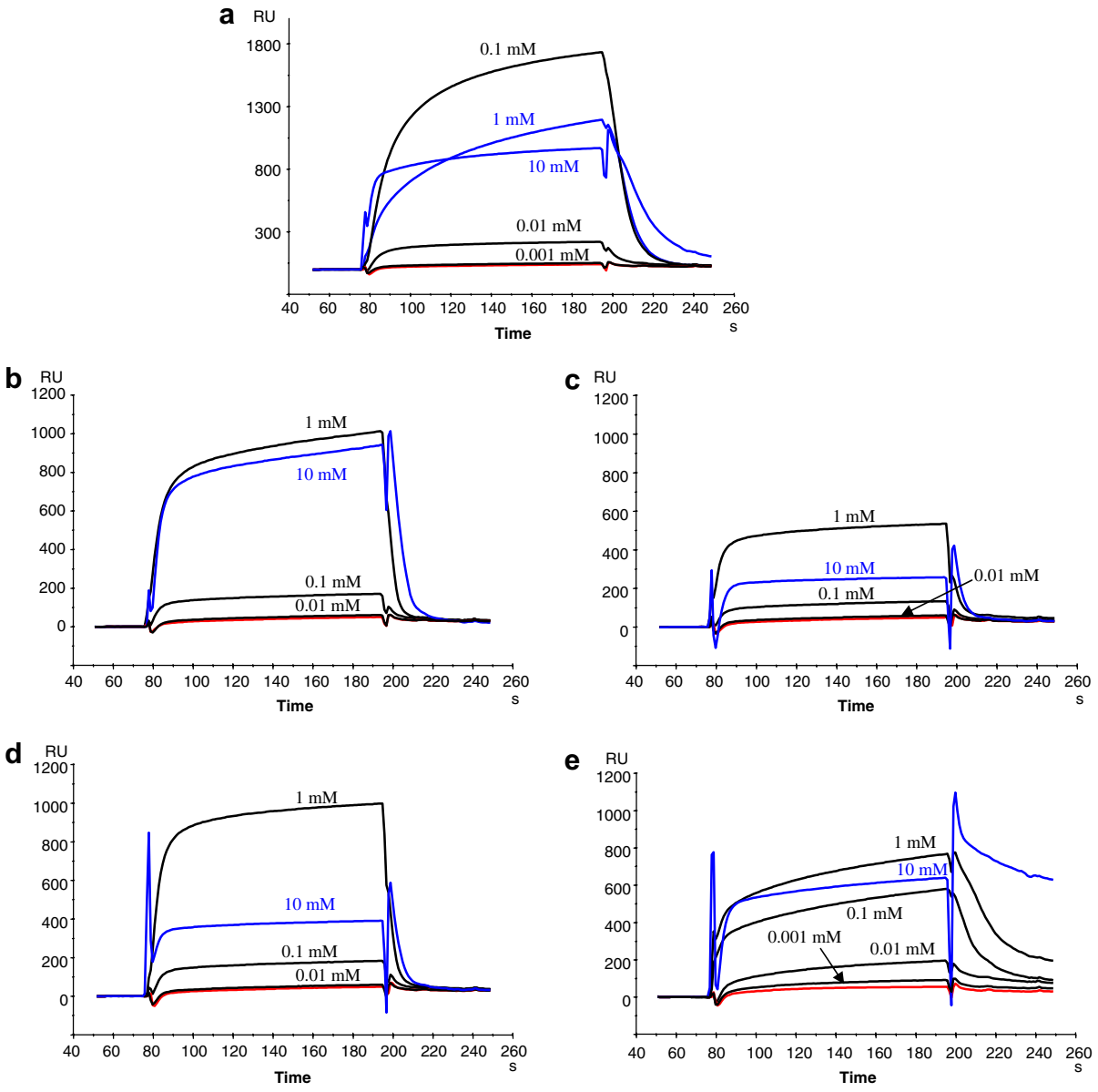


Figure 2. Sensorgrams showing the interactions of the glycosides with WGA. WGA corresponding to 5700 response units was directly immobilized onto the sensor chip. WGA was co-injected with glycoside: (a) **1**, (b) **2**, (c) **3**, (d) **4** and (e) **6** (Black and blue lines show increased and decreased RU, respectively), or injected without glycoside as a negative control (red line).

adopt a favorable orientation during interaction with the GlcNAc binding site. It has been reported that the binding and cross-linking properties of lectins are sensitive to the degree of flexibility and spacing between the carbohydrate epitopes of the analogs,[26,27] which is entirely consistent with the present results.

2.2.2. SPR analysis. We have already reported that by SPR analysis, O-linked double-headed glycoside **1** promotes, rather than inhibits, binding of WGA to surface-bound asialofetuin on a sensor chip.[24] This result is dramatically different from that of the corresponding monovalent glycoside, which inhibits the binding of the lectin. In the present work, an SPR competition binding assay[28–30] was used to monitor the effect of synthetic N-linked double-headed glycosides on the interaction of soluble WGA with a surface-bound WGA, which was directly immobilized onto the surface of a sensor chip using the amine coupling method. Cross-linking of N-linked double-headed glycosides with WGA in solution was monitored by co-injecting an equilibrium mixture of a fixed amount of WGA with a variable amount of glycoside onto a surface-bound WGA. The surface was regenerated at the end of each cycle using 50 mM H_3PO_4. Figure 2 shows sensorgrams of binding between immobilized WGA and free-WGA. As anticipated,

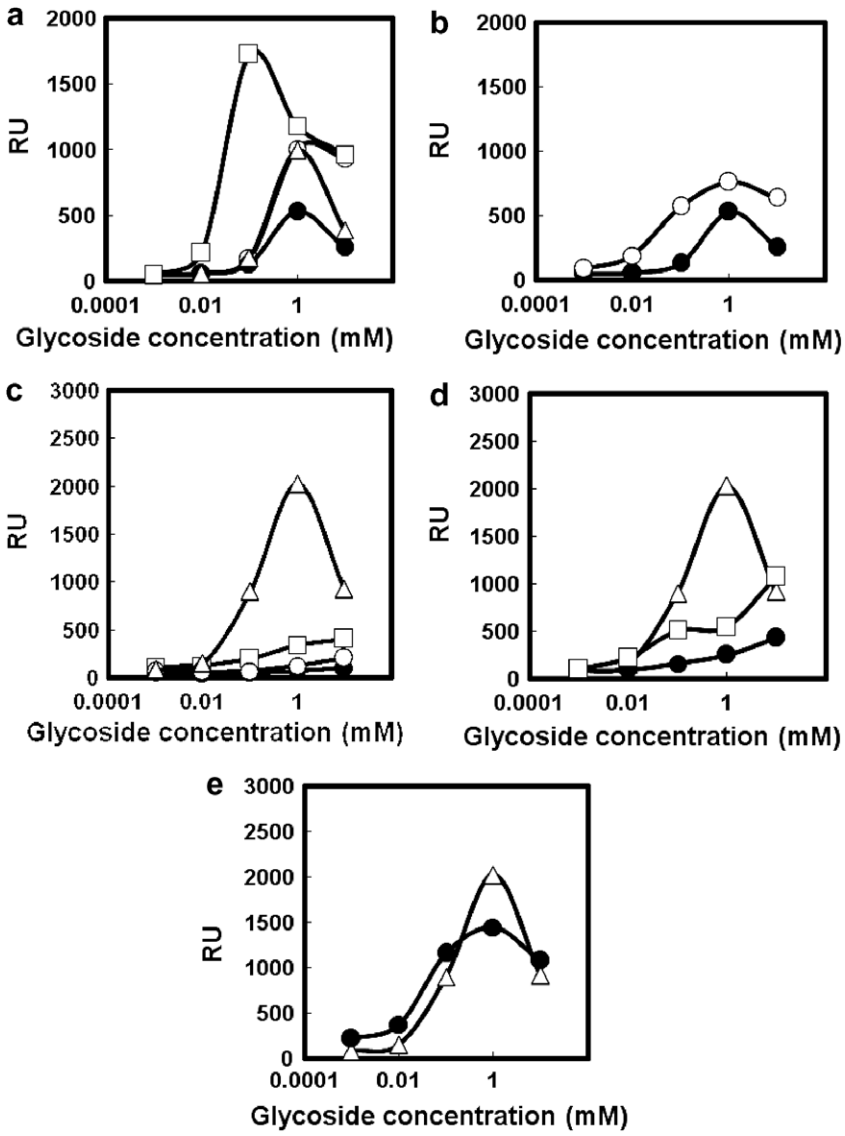


Figure 3. Cross-linking formation of WGA with N-linked double-headed glycosides on the sensor chip. In case of (a) and (b), WGA corresponding to 5700 response units was immobilized onto the sensor chip. In case of (c), (d) and (e), WGA corresponding to 8700 response units was immobilized onto the sensor chip. (a) GlcNAc-(N and O)-linked double-headed glycosides **1**–**4**. □, **1**; ○, **2**; ●, **3**; △, **4**. (b) GlcNAc- and $(GlcNAc)_2$-N-linked double-headed glycosides bearing $-(CH_2)_4-$ on the spacer. ●, **3**; ○, **6**. (c) Reducing sugars and O-linked mono-headed glycoside control. □, $(GlcNAc)_2$; ○, GlcNAc-Hx; ●, GlcNAc; △, **4**. (d) GlcNAc- and $(GlcNAc)_2$-N-linked double-headed glycosides bearing PEG spacer. □, **8**; ●, **5**; △, **4**. (e) GlcNAc- and $(GlcNAc)_2$-N-linked double-headed glycosides bearing $-(CH_2)_5-$ on the spacer. △, **4**, ●, **7**.

injection of a solution of WGA in the absence of double-headed glycoside (i.e., negative control) resulted in no change of response units (RU). However, as the concentration of N-linked double-headed glycosides (**2**, **3** or **4**) increased (i.e., from 0.001 mM to 1 mM) there was an increase in RU (Fig. 2b–e). Upon reaching the maximum RU, a decrease followed.

A similar sensorgram profile was also seen for the O-linked double-headed glycoside **1**, although in this case there was a marked increase in RU between 0.001 and 0.1 mM, compared to that observed for **2**, **3** and **4** (Fig. 2a). Thus, 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 are increased further, they inhibit the binding of the clustered WGA to the surface WGA. These compounds also act as inhibitors at the appropriate stoichiometry by the precipitation assay mentioned above. Based on the sensorgrams of Figure 2, we plotted the RUs at 190 s, which corresponds to the cross-linking maximum responses for the double-headed glycosides to the surface-bound WGA, against glycoside concentration (as shown in Fig. 3), although some of the sensorgrams did not reach equilibrium. Our main purpose was to prove its ability to act as a divalent ligand through SPR analysis. In this case, the amount corresponded to 5700 RU of WGA (Figs. 3a and b) immobilized onto the surface, while 8700 RU in Figures 3c–e. GlcNAc, $(GlcNAc)_2$ and mono-headed glycoside GlcNAc-Hx (6-hydroxyhexyl-2-acetamido-2-deoxy-*O*-β-D-glucopyranoside) were also injected as a control and then plotted (Fig. 3c). These compounds were not seen on the top of the peak as in the case of **1** and N-linked double-headed glycosides, although they seemed to show a slight increase of RU. These results suggest that these control samples cannot cross-link surface WGA and free WGA. The cross-linking activity with the GlcNAc double-headed glycosides increased in the order of **1** > **2** = **4** > **3**. The maximal RU of compound **6**, bearing $(GlcNAc)_2$, was 30% greater than that of **3**, bearing a single GlcNAc with the same spacer unit (Fig. 3b). In contrast, the maximal RU of compound **7** bearing $(GlcNAc)_2$ was 30% smaller than that of **4** bearing GlcNAc (Fig. 3e). The binding activity corresponds to the results from the precipitation analysis.

Our data suggest that co-injection of double-headed glycoside and tetravalent WGA as analyte results in simultaneous cross-linking. The cross-linking complexes presumably also bind to the surface-bound WGA through unbound double-headed glycoside. Burke et al. have found that trivalent mannose macrocycle, which is more potent than the corresponding monovalent derivative, functions by cross-linking Con A in solution by SPR.[31] When double-headed glycosides bearing GlcNAc and $(GlcNAc)_2$ with a more extended PEG spacer (**5** and **8**) were applied to SPR, **5** did not show a top of the peak, but **8** seemed to show a little top of the peak at 0.1 mM. Compound **8** also reached 1000 RU at higher concentrations. However, in a precipitation assay, they are not capable of precipitating WGA as divalent ligands as mentioned above. This suggests that the increase in RU at higher concentrations (10 mM) might be caused by the equimolar binding of glycosides to the surface WGA. This result shows that the length of the spacer group between two terminal residues greatly influences the cross-linking activity.

In conclusion, we have developed a practical synthetic method to obtain spacer-N-linked double-headed glycosides. The double-headed glycosides were shown to bind and cross-link WGA as divalent ligands. The present findings indicate that the spacing and the flexibility of the spacer units in divalent carbohydrates affect the structures of cross-linking complex with WGA. These results have important implications for the interaction of lectins with multivalent carbohydrate receptors in biological systems.

3. Experimental

3.1. General methods

A O-linked double-headed glycoside **1** (GlcNAc-Hx-GlcNAc) was prepared enzymatically as described previously.[24] GlcNAc was purchased from Sigma–Aldrich (St Louis, MO). $(GlcNAc)_2$ was a kind gift from Yaizu Suisan Kagaku Industry Co., Ltd (Shizuoka, Japan). Bis-$dPEG_6$-acid was purchased from Nakalai Tesque, Inc (Kyoto, Japan). WGA was purchased from J-OIL MILLS, Inc (Yokohama, Japan). Sensor chips CM-5 and the amine coupling kit containing NHS, EDC and ethanolamine-hydrochloride for interaction studies, were purchased from Biacore AB (Uppsala, Sweden). All other chemicals were obtained from commercial sources.

3.2. Analytical methods

HPLC was carried out using a Shodex Asahipak NH2P-50 4E column (∅ 4.6 × 250 mm, Showa Denko K.K., Chiba, Japan) in a Jasco Gulliver Series liquid chromatograph with an UV-975 intelligent UV–vis detector. Bound material was eluted from the column using a H_2O–CH_3CN (ratio 20:80 or 25:75) at a flow rate of 1.0 mL/min at 40 °C. The eluent was monitored at a wavelength of 210 nm. ^1H and ^{13}C NMR spectra of each sample in D_2O were recorded on a JEOL JNM-LA 500 spectrometer at 30 °C. Chemical shifts were expressed in δ relative to sodium 3-(trimethylsilyl) propionate (TPS) as an external standard. 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 mass resolution of 1000 was employed.

3.3. Synthesis of double-headed glycosides

3.3.1. N^1,N^5-Di-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-glutaramide (2). GlcNAc was converted to 2-acetamido-2-deoxy-β-D-glucopyranosyl amine with ammonia by Hirateke's method.[25] The resulting product was coupled with glutaric acid as follows. DIEA (0.43 mL, 2.5 mmol) and HBTU (379 mg, 1 mmol) were added to 0.3 mL of DMSO containing glutaric acid (60 mg, 0.45 mmol), followed by addition of 1.5 mL of DMSO containing *N*-acetylglucosamine (220 mg, 1 mmol). The mixture was incubated at room temperature and analyzed by TLC with $CHCl_3$–CH_3OH–H_2O (6:4:1) by the orcinol–sulfuric acid method. After 3 h, the solution was charged onto a silica gel column (∅ 2.9 × 62 cm). The column was developed with $CHCl_3$–CH_3OH–H_2O (6:4:1) into 20 mL fractions at a flow rate of 10 mL/min. Each fraction was analyzed by TLC with the same solvent. Tubes 60–100 were concentrated and lyophilized to afford compound **2** in a 56.6% yield based on the amount of glutaric acid added (138 mg). ^{1}H NMR (D_2O): δ 5.07 (d, 2H, $J_{1,2}$ 9.8 Hz, H-1), 3.88 (dd, 2H, H-6_b), 3.82 (dd, 2H, H-2), 3.76 (dd, 2H, H-6_a), 3.61 (t, 2H, H-3), 3.53 (m, 2H, H-5), 3.49 (t, 2H, H-4), 2.27 (m, 4H, CH_2-α), 2.00 (s, 6H, CH_3CO–), 1.83 (m, 2H, CH_2-β); ^{13}C NMR (D_2O): δ 179.2 (–CH_2*C*ONH–), 177.4 (CH_3*C*ONH–), 81.1 (C-1), 80.4 (C-5), 77.0 (C-3), 72.3 (C-4), 63.3 (C-6), 57.1 (C-2), 37.6 (*C*H_2-α), 24.8 (*C*H_3CONH–), 24.3 (*C*H_2-β); FAB-mass: *m*/*z* 537 $[M+H]^+$.

3.3.2. N^1,N^6-Di-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-apidamide (3). The desired compound **3** was synthesized from adipic acid (66.3 mg, 0.45 mmol) and 2-acetamido-2-deoxy-β-D-glucopyranosyl amine (220 mg, 1 mmol) in a manner similar to that used to prepare compound **2**. The product was obtained in a high yield (143 mg, 57.3% based on the amount of adipic acid added). ^{1}H NMR (D_2O): δ 5.06 (d, 2H, $J_{1,2}$ 9.8 Hz, H-1), 3.89 (dd, 2H, H-6_b), 3.81 (dd, 2H, H-2), 3.76 (dd, 2H, H-6_a), 3.61 (t, 2H, H-3), 3.53 (m, 2H, H-5), 3.49 (t, 2H, H-4), 2.29 (m, 4H, CH_2-α), 2.00 (s, 6H, CH_3CO–), 1.55 (m, 4H, CH_2-β); ^{13}C NMR (D_2O): δ 180.0 (–CH_2*C*ONH–), 177.4 (CH_3*C*ONH–), 81.1 (C-1), 80.4 (C-5), 77.0 (C-3), 72.3 (C-4), 63.3 (C-6), 57.1 (C-2), 38.1 (*C*H_2-α), 27.2 (*C*H_2-β), 24.8 (*C*H_3CONH–); FAB-mass: *m*/*z* 551 $[M+H]^+$.

3.3.3. N^1,N^7-Di-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-pimeamide (4). The desired compound **4** was synthesized from pimelic acid (72.6 mg, 0.45 mmol) and 2-acetamido-2-deoxy-β-D-glucopyranosyl amine (220 mg, 1 mmol) in a manner similar to that used to prepare compound **2**. The product was obtained in a high yield (140.4 mg, 55.0% based on the amount of pimelic acid added). ^{1}H NMR (D_2O): δ 5.05 (d, 2H, $J_{1,2}$ 9.8 Hz, H-1), 3.88 (dd, 2H, H-6_b), 3.81 (dd, 2H, H-2), 3.75 (dd, 2H, H-6_a), 3.60 (t, 2H, H-3), 3.52 (m, 2H, H-5), 3.48 (t, 2H, H-4), 2.26 (m, 4H, CH_2-α), 2.00 (s, 6H, CH_3CO–), 1.57 (m, 4H, CH_2-β), 1.26 (m, 2H, CH_2-γ); ^{13}C NMR (D_2O): δ 180.5 (–CH_2*C*ONH–), 177.4 (CH_3*C*ONH–), 81.1 (C-1), 80.4 (C-5), 77.0 (C-3), 72.3 (C-4), 63.3 (C-6), 57.1 (C-2), 38.3 (*C*H_2-α), 30.3 (*C*H_2-γ), 27.5 (*C*H_2-β), 24.9 (*C*H_3CONH–); FAB-mass: *m*/*z* 565 $[M+H]^+$.

3.3.4. N^1,N^{17}-Di-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,6,9,12,15-pentaoxaheptadecanediamide. The desired compound **5** was synthesized from Bis-dPEG$_6$-acid (20.0 mg, 0.059 mmol) and 2-acetamido-2-deoxy-β-D-glucopyranosyl amine (29 mg, 0.13 mmol) in a manner similar to that used to prepare compound **2**. The product was obtained in a high yield (37.7 mg, 85.6% based on the amount of Bis-dPEG$_6$-acid added). ^{1}H NMR (D_2O): δ 5.09 (d, 2H, $J_{1,2}$ 9.8 Hz, H-1), 3.88 (dd, 2H, H-6_b), 3.83 (dd, 2H, H-2), 3.78 (m, 2H, –OC*Hb*$_2$$CH_2$CONH–), 3.72 (dd, 2H, H-$6_a$), 3.70–3.65 (m, 18H, –OCH_2CH_2O–, –OC*Ha*$_2$$CH_2$CONH–), 3.62 (t, 2H, H-3), 3.53 (m, 2H, H-5), 3.49 (t, 2H, H-4), 2.57 (t, 4H, –OCH $_2$CH_2CONH–), 2.01 (s, 6H, CH_3CO–); ^{13}C NMR (D_2O): δ 177.6 (–OCH_2CH_2*C*ONH–), 177.5 (CH_3*C*ONH–), 81.1 (C-1), 80.4 (C-5), 77.0 (C-3), 72.3 (C-4, –O*C*H_2*C*H_2O–), 69.2 (–O*C*H_2CH_2CONH–), 63.3 (C-6), 57.1 (C-2), 38.7 (–OCH_2*C*H_2CONH–), 24.8 (*C*H_3CONH–); FAB-mass: *m*/*z* 743 $[M+H]^+$.

3.3.5. N^1,N^6-Di-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-adipamide (6). $(GlcNAc)_2$ was also converted to β-*N*,*N*′-diacetylchitobiosyl amine by the method mentioned above. The desired compound **6** was synthesized from adipic acid (45.2 mg, 0.31 mmol) and β-*N*,*N*′-diacetylchitobiosyl amine (230 mg, 0.54 mmol) in a manner similar to that used to prepare compound **2**. The product (90.4 mg) was obtained in a yield of 30.5% based on the amount of adipic acid added. ^{1}H NMR (D_2O): δ 5.05 (d, 2H, $J_{1,2}$ 9.5 Hz, H-1), 4.59 (d, 2H, $J_{1',2'}$ 8.3 Hz, H-1′), 3.92 (dd, 2H, H-$6'_b$), 3.86–3.82 (4H, H-6_b, H-2), 3.77–3.73 (6H, H-3, H-$6'_a$, H-$2'_a$), 3.68–3.64 (4H, H-6_a, H-4), 3.59–3.55 (4H, H-5, H-3′), 3.49–3.45 (4H, H-4′, H-5′), 2.27 (m, 4H, CH_2-α), 2.07 (s, 6H, CH'_3CO–), 1.99 (s, 6H, CH_3CO–), 1.53 (m, 4H, CH_2-β); ^{13}C NMR (D_2O, 125 MHz): δ 179.9 (–CH_2*C*ONH–), 177.4 (CH_3*C*ONH–, CH_3*C*′ONH–), 104.2 (C-1′), 81.6 (C-4), 81.0 (C-1), 79.0 (C-5), 78.7 (C-5′), 76.2 (C-3′), 75.6 (C-3), 72.5 (C-4′), 63.3 (C-6′), 62.7 (C-6), 58.4 (C-2′), 56.6 (C-2), 38.1 (*C*H_2-α), 27.1 (*C*H_2-β), 24.9

(CH_3CONH–, $C'H_3CONH$–); FAB-mass: m/z 957 $[M+H]^+$.

3.3.6. N^1,N^7-Di-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-pimeamide (7). The desired compound **7** was synthesized from pimelic acid (33.6 mg, 0.21 mmol) and β-*N*,*N*′-diacetylchitobiosyl amine (190 mg, 0.45 mmol) in a manner similar to that used to prepare compound **2**. The product (101 mg) was obtained in a yield of 49.5% based on the amount of pimelic acid added. ^{1}H NMR (D_2O): δ 5.05 (d, 2H, $J_{1,2}$ 9.8 Hz, H-1), 4.59 (d, 2H, $J_{1',2'}$ 8.2 Hz, H-1′), 3.92 (dd, 2H, H-6′$_b$), 3.87–3.83 (4H, H-6$_b$, H-2), 3.77–3.74 (6H, H-3, H-6′$_a$, H-2′), 3.68–3.63 (4H, H-6$_a$, H-4), 3.59–3.56 (4H, H-5, H-3′), 3.53–3.46 (4H, H-4′, H-5′), 2.25 (m, 4H, CH_2-α), 2.07 (s, 6H, CH'_3CO–), 2.00 (s, 6H, CH_3CO–), 1.56 (m, 4H, CH_2-β), 1.26 (m, 4H, CH_2-γ); ^{13}C NMR (D_2O): δ 180.5 (–CH_2CONH–), 177.4 (CH_3CONH–, CH_3C'ONH–), 104.2 (C-1′), 81.6 (C-4), 81.0 (C-1), 79.0 (C-5), 78.7 (C-5′), 76.3 (C-3′), 75.6 (C-3), 72.5 (C-4′), 63.4 (C-6′), 62.7 (C-6), 58.4 (C-2′), 56.6 (C-2), 38.3 (CH_2-α), 30.3 (CH_2-γ), 27.4 (CH_2-β), 24.9 (CH_3CONH–, $C'H_3CONH$–); FAB-mass: m/z 971 $[M+H]^+$.

3.3.7. N^1,N^{17}-Di-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,6,9,12,15-pentaoxaheptadecanediamide (8). The desired compound **8** was synthesized from Bis-dPEG$_6$-acid (16.9 mg, 0.05 mmol) and β-*N*,*N*′-diacetylchitobiosyl amine (46.6 mg, 0.11 mmol) in a manner similar to that used to prepare compound **2**. The product (34.5 mg) was obtained in a yield of 60.0% based on the amount of Bis-dPEG$_6$-acid added.; ^{1}H NMR (D_2O): δ 5.08 (d, 2H, $J_{1,2}$ 9.5 Hz, H-1), 4.60 (d, 2H, $J_{1',2'}$ 8.6 Hz, H-1′), 3.92 (dd, 2H, H-6′$_b$), 3.88–3.82 (4H, H-6$_b$, H-2), 3.79–3.63 (30H, H-6$_a$, H-4, H-3, H-6′$_a$, H-2′, –OCHb_2CH$_2$CONH–, –OCHa_2CH$_2$CONH–, –OCH_2CH_2O–), 3.60–3.56 (4H, H-5, H-3′), 3.51–3.46 (4H, H-4′, H-5′), 2.56 (t, 4H, –OCH$_2$CH_2CONH–), 2.07 (s, 6H, CH'_3CO–), 2.01 (s, 6H, CH_3CO–); ^{13}C NMR (D_2O, 125 MHz): δ 177.6 (–CH_2CONH–), 177.4 (CH_3CONH–, CH_3C'ONH–), 104.2 (C-1′), 81.6 (C-4), 81.0 (C-1), 79.0 (C-5), 78.7 (C-5′), 76.3 (C-3′), 75.6 (C-3), 72.5 (C-4′), 72.4 (–OCH_2-CH_2O–), 63.3 (C-6′), 62.7 (C-6), 58.4 (C-2′), 56.6 (C-2), 38.7 (–OCH$_2C$H$_2$CONH–), 24.9 (CH_3CONH–, $C'H_3CONH$–); FAB-mass: m/z 1149 $[M+H]^+$.

3.4. Binding assay with WGA

3.4.1. Precipitation analysis. Various concentrations of WGA and glycosides dissolved in PBS were mixed with an equal volume (total volume: 40 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 were performed by UV-detection of WGA measurement of glycoside in supernatant. Various concentrations of glycoside solutions (50 μL) were added to 128 μM of WGA solution (50 μL) in a microtube. After incubation at room temperature for 1 h the solution was centrifuged at 8000*g* for 10 min to remove precipitated material. The supernatant was diluted and analyzed by measuring the absorbance at 280 nm. The precipitated WGA was then calculated from a standard curve. The dissociation of WGA–glycoside precipitates was induced by addition of $(GlcNAc)_n$ ($n = 2$–3) as hapten sugars. We measured the amount of hapten sugar required to dissociate the WGA–glycoside precipitate. Precipitates were formed by mixing of 256 μM of WGA (10 μL) and 2.5 mM of glycoside solutions (10 μL) on 96-well microtiter plate. Various concentrations of hapten sugar solutions (20 μL) were added to the well and the minimum dissociation concentration of hapten sugar was determined by observation.

3.4.2. 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 double-headed glycosides with WGA. After chip activation with 0.1 M of NHS and 0.4 M of 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, the chip was capped by exposure to 1 M ethanolamine yielding a signal of approximately (1) 5700 and (2) 8700 response units. The control lane was used without treating. 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. 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 that of reference cell and buffer injection without glycoside was performed. All analyses were performed at least two times to verify reproducibility. The RUs at 190 s, corresponding to the cross-linking maximum responses for the double-headed glycosides to the surface-bound WGA were checked and plotted against glycoside concentrations.

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Supplementary data

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