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Enzymatic Synthesis of Spacer-Linked Divalent Glycosides Carrying *N*-Acetylglucosamine and *N*-Acetyllactosamine: Analysis of Cross-Linking Activities with WGA

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Divalent glycosides carrying *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyllactosamine (LacNAc) were designed and prepared as glycomimetics. First, hexan-1,6-diyl *bis*-(2-acetamido-2-deoxy-β-D-glucopyranoside) (GlcNAc-Hx-GlcNAc) and 3,6-dioxaoct-1,8-diyl *bis*-(2-acetamido-2-deoxy-β-D-glucopyranoside) (GlcNAc-Doo-GlcNAc) were enzymatically synthesized by transglycosylation of an *N,N′N″,N‴*-tetraacetylchitotetraose [$(GlcNAc)_4$] donor with a primary diol acceptor, utilizing a chitinolytic enzyme from *Amycolatopsis orientalis*. The resulting divalent glycosides were further converted to the respective hexan-1,6-diyl bis-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside] (LacNAc-Hx-LacNAc) and 6-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-hexyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (LacNAc-Hx-GlcNAc), and respective 3,6-dioxaoct-1,8-diyl bis-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside] (LacNAc-Doo-LacNAc) and 8-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3,6-dioxaoctyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (LacNAc-Doo-GlcNAc) by galactosyltransferase. The interaction of wheat germ agglutinin (WGA) with a series of divalent glycosides and related compounds were studied using a biosensor based on surface plasmon resonance (SPR) and by precipitation analysis. Our results demonstrated that divalent glycosides carrying GlcNAc on both sides and GlcNAc and LacNAc on each side are capable of precipitating WGA as divalent ligands, but that the corresponding monovalent controls and divalent glycosides carrying LacNAc on both sides are unable to precipitate the lectin and bind as univalent ligands.

Key words: Amycolatopsis orientalis, cross-linking, divalent glycoside, enzymatic synthesis, WGA.

Abbreviations: AP, alkaline phosphatase; Con A, concanavalin A; Doo, 3,6-dioxaocto-1,8-diyl; EDC, *N*-ethyl-*N′*-(dimethylaminopropyl)carbodiimide; Gal, D-Galactose; β-GalT, β1,4-galactosyltransferase; GlcNAc, *N*-acetyl-D-glucosamine; $(GlcNAc)_2$, *N,N′*-diacetylchitobiose; $(GlcNAc)_3$, *N,N′,N″*-triacetylchitotriose; $(GlcNAc)_4$, *N,N′,N″,N‴*-tetraacetylchitotetraose; Hx, hexan-1,6-diyl; K_D, dissociation constant; LacNAc, *N*-acetyllactosamine; MIC, minimum inhibitory concentrations; NHS, *N*-hydroxysuccimide; *p*NP, *p*-nitrophenyl; RU, resonance unit; SPR, surface plasmon resonance; TPS, sodium 3-(trimethylsilyl)-propionate; WGA, wheat germ (*Triticum vulgaris*) agglutinin.

The cross-linking properties of a variety of plant and animal lectins with multivalent carbohydrates and glycoproteins have been reviewed (*1*, *2*). Studies show that a number of lectins form cross-linked complexes with branched chain oligosaccharides (*3–5*), glycopeptide (*6–8*) and glycoproteins (*9–12*). The structural features of multivalent ligands influence their activities and the mechanism by which they function (*13*, *14*). Accordingly, carbohydrate epitopes are present in multivalent arrays at the cell membrane where they can serve as highly effective and specific ligands (*15*, *16*). In general, monovalent ligands of low molecular weight can interact with oligomeric lectin, but cannot span multiple binding sites within the lectin. Binding affinities for monovalent protein–carbohydrate interactions are often weak. Bhattacharyya *et al*. reported that even low molecular weight oligomannose and bisected complex type oligosaccharides bind to concanavalin A (Con A) to precipitate the lectin (*6–8*). Burke *et al*. have reported that a synthetic trivalent mannose ligand linked to a macrocyclic core functions by clustering Con A in solution (*17*). Because of the abundance and biological importance of 2-acetamido sugars, we were interested in developing a convenient enzymatic synthesis of divalent *N*-acetylglucosamine (GlcNAc)-glycosides with an acyclic spacer. Lehmann *et al*. have reported the chemical synthesis of the divalent GlcNAc-glycoside with a 10-carbon spacer as a binding model with two separate binding sites for the outer GlcNAc residues (*18*, *19*). However, the method for obtaining the divalent glycoside involves elaborate

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procedures for protection, glycosylation and deprotection. The use of glycosidases, such as *N*-acetylhexosaminidase [EC 3.2.1.52], for divalent glycoside synthesis is attractive because it may allow direct transfer of a GlcNAc residue to a primary diol acceptor.

This paper describes the enzymatic synthesis of spacer-linked divalent glycosides carrying GlcNAc and *N*-acetyllactosamine (LacNAc) and the interaction of the divalent glycosides as divalent ligands with wheat germ agglutinin (WGA) using a hemagglutination inhibition assay, precipitation analyses and surface plasmon resonance (SPR).

MATERIALS AND METHODS

Materials—Amycolatopsis orientalis IFO 12806T was obtained from the Institute of Fermentation, Osaka (Japan). Crude chitinolytic enzyme was prepared as follows (*20*). The culture filtrate from *A. orientalis* was brought to 80% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation at 9,000*g* for 30 min, dissolved in deionized water. The crude enzyme solution was dialysed, lyophilized and then used for synthesis of glycosides without further purification. GlcNAcβ-*p*NP, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ and LacNAc were kind gifts from Yaizu Suisan Kagaku Industry Co., Ltd (Shizuoka, Japan). UDP-Gal was kindly supplied by Yamasa Shoyu Co., Ltd (Chiba, Japan). All other chemicals were obtained from commercial sources.

Analytical Methods—HPLC was carried out using a Shodex Asahipak NH2P-50 4E column (Φ 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. Elution of the column was performed with H_2O/CH_3CN of 20/80, at a flow rate of 1.0 mL/min at 40°C and wavelength of 210 nm. ^{1}H- 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.

Enzyme Assay—β-*N*-Acetylhexosaminidase activity was assayed as follows: a mixture containing 100 μL of GlcNAcβ-*p*NP (5 mM), 50 μL of 100 mM sodium acetate buffer (pH 5.5) and 100 μL of an appropriate concentration of the crude enzyme was incubated at 40°C for 12 min and stopped by addition of 250 μL of 1.0 M Na_2CO_3. The amount of liberated *p*-nitrophenol was determined by measuring the absorbance at 405 nm using a microplate reader (Biolumin 960, Amersham Pharmacia, Uppsala, Sweden). One unit was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol from GlcNAcβ-*p*NP per minute at 40°C.

Preparation of GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc—$(GlcNAc)_4$ (831 mg, 1 mmol) and 1,6-hexanediol (59.1 mg, 0.5 mmol) were dissolved in 9 mL of sodium-acetate buffer (50 mM, pH 6.7), followed by addition of the crude enzyme powder (1 mL, 5 U/mL). The mixture was incubated at 40°C for 45 h. The reaction was terminated by heating in boiling water for 10 min. The solution was charged onto a charcoal-Celite column (Φ 4.5 × 45 cm) equilibrated with 10% ethanol. The column was developed with 5.0 L of 10% ethanol, and then bound material was eluted with a linear gradient of 10–50% ethanol (6L) at a flow rate of 4.0 mL/min. Fractions of 50 mL were collected. The absorbance of the eluent was monitored at 210 nm and analysed by TLC with $CHCl_3/CH_3OH/H_2O$ (6:4:1) by the orcinol-sulfuric acid method. The eluate was divided into three fractions (F-1; tubes 67–73, F-2; tubes 74–81, F-3; tubes 91–95). F-2 was concentrated and dissolved in $CHCl_3/CH_3OH/H_2O$ (6:4:1) and then charged onto a silicagel column (Φ 2.0 × 27 cm). The column was developed with the same solvent into 15 mL fractions at a flow rate of 10 mL/min. Each fraction was analysed by TLC with $CHCl_3/CH_3OH/H_2O$ (6:4:1) by the above method. Tubes 9–19 were concentrated and lyophilized to afford GlcNAc-Hx-GlcNAc (54.8 mg, 10.5% based on the amount of donor added). F-1 was concentrated and lyophilized without rechromatography to afford GlcNAc-Hx (72.6 mg, 22.6% based on the amount of donor added). In a similar manner, F-3 was applied to a silicagel column as described earlier. Tubes 7–11 were concentrated and lyophilized to afford $(GlcNAc)_2$-Hx (7.4 mg, 1.4% based on the amount of donor added).

GlcNAc-Doo-GlcNAc was prepared from $(GlcNAc)_4$ (831 mg, 1 mmol) and triethylene glycol (700 μL, 5 mmol) with crude enzyme powder (1 mL, 5 U/mL) in a manner similar to that used to prepare GlcNAc-Hx-GlcNAc. The product GlcNAc-Doo-GlcNAc was obtained in a yield of 4.7% (26.4 mg) based on the amount of donor added. GlcNAc-Doo and $(GlcNAc)_2$-Doo were also obtained in a yield of 69.0% (244 mg) and 0.75% (4.2 mg) based on the amount of donor added, respectively.

GlcNAc-Hx-GlcNAc. FAB-mass: *m/z* 525 $[M+H]^+$; ^{1}H-NMR (D_2O, 500 MHz): δ4.51 (d, 2H, $J_{1,2}$ 8.5 Hz, H-1), 3.92 (dd, 2H, H-6b), 3.88 (m, 2H, H-αb), 3.75 (dd, 2H, H-6a), 3.67 (dd, 2H, H-2), 3.59 (m, 2H, H-αa), 3.54 (m, 2H, H-3), 3.49–3.42 (4H, H-5, H-4), 2.03 (s, 6H, C<u>H</u>$_3$CONH-), 1.55 (m, 4H, H-β), 1.32 (m, 4H, H-γ).

GlcNAc-Hx. FAB-mass: *m/z* 322 $[M+H]^+$; ^{1}H-NMR (D_2O, 500 MHz): δ4.50 (d, 1H, $J_{1,2}$ 8.6 Hz, H-1), 3.92 (dd, 1H, H-6b), 3.89 (m, 1H, H-αb), 3.74 (dd, 1H, H-6a), 3.68 (dd, 1H, H-2), 3.63–3.56 (3H, H-αa, H-ζ), 3.53 (m, 1H, H-3), 3.49–3.42 (2H, H-4, H-5), 2.04 (s, 3H, C<u>H</u>$_3$CONH-), 1.60–1.51 (4H, H-β, H-ε), 1.39–1.30 (4H, H-γ, H-δ).

$(GlcNAc)_2$-Hx. FAB-mass: *m/z* 525 $[M+H]^+$; ^{1}H-NMR (D_2O, 500 MHz): δ4.59 (d, 1H, $J_{1',2'}$ 8.3 Hz, H-1′), 4.50 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1), 3.94–3.45 (16H, H-6b, H-6a, H-5, H-4, H-3, H-2, H-6′b, H-6′a, H-5′, H-4′, H-3′, H-2′, H-αb, H-αa, H-ζ), 2.07 (s, 3H, C<u>H</u>$_3$CONH-), 2.03 (s, 3H, C<u>H</u>$_3$CONH-), 1.59–1.52(4H, H-β, H-ε), 1.37–1.31 (4H, H-γ, H-δ).

GlcNAc-Doo-GlcNAc. FAB-mass: *m/z* 557 $[M+H]^+$, 579$[M+Na]^+$; ^{1}H NMR (D_2O, 500 MHz): δ4.57 (d, 2H, $J_{1,2}$ 8.6 Hz, H-1), 4.01 (m, 2H, H-αb), 3.93 (dd, 2H, H-6b), 3.80–3.63 (14H, H-6a, H-2, H-αa, H-β, H-γ), 3.56 (m, 2H, H-3), 3.46–3.44 (4H, H-5, H-4), 2.05 (s, 6H, C<u>H</u>$_3$CONH–).

GlcNAc-Doo. FAB-mass: *m/z* 354$[M+H]^+$, 376$[M+Na]^+$; ^{1}H NMR (D_2O, 500MHz): δ4.57 (d, 1H,

Table 1. **^{13}C-NMR chemical shifts of the synthetic glycosides in D_2O.**

Chemical shifts (δ) Compounds		C-1	C-2	C-3	C-4	C-5	C-6	*C*OCH$_3$	COCH$_3$	C-α	C-β	C-γ	C-δ	C-ε	C-ζ
GlcNAc-Hx-GlcNAc	GlcNAc	103.9	58.4	76.6	72.7	78.7	63.6	177.2	25.0	73.2	31.4	27.5			
GlcNAc-Hx	GlcNAc	103.9	58.4	76.7	72.7	78.7	63.6	177.2	25.0	73.2	31.3	27.5	27.6	34.1	64.6
(GlcNAc)$_2$-Hx	GlcNAc (n)	104.3	58.4	76.3	72.5	78.7	63.3	177.4	24.9						
	GlcNAc (r)	103.8	57.8	75.3	82.3	77.3	63.0	177.2	25.0	73.3	31.3	27.5	27.6	34.1	64.5
LacNAc-Hx-LacNAc	Gal	105.7	73.8	75.3	71.4	78.2	63.8								
	GlcNAc	103.8	57.9	75.3	81.3	77.6	62.9	177.1	25.0	73.3	31.4	27.5			
LacNAc-Hx-GlcNAc	GlcNAc	103.9	58.4	76.6	72.7	78.6	63.5	177.2	25.0						
	Gal	105.7	73.8	75.3	71.3	78.1	63.8								
	GlcNAc (in LacNAc)	103.8	57.9	75.3	81.2	77.6	62.9	177.2	25.0	73.2	31.4	27.5	27.5	31.4	73.3
GlcNAc-Doo-GlcNAc	GlcNAc	103.8	58.3	76.6	72.7	78.6	63.5	177.3	25.0	71.7	72.5	72.5			
GlcNAc-Doo	GlcNAc	103.8	58.3	76.7	72.7	78.7	63.6	177.4	25.0	71.7	72.5	72.5	72.3	74.5	63.2
(GlcNAc)$_2$-Doo	GlcNAc (n)	104.3	58.4	76.3	72.6	78.7	63.3	177.4	24.9						
	GlcNAc (r)	103.7	57.7	75.3	82.3	77.3	63.0	177.3	25.0	71.8	72.5	72.5	72.3	74.5	63.2
LacNAc-Doo-LacNAc	Gal	105.7	73.8	75.3	71.4	78.2	63.8								
	GlcNAc	103.8	57.9	75.3	81.3	77.6	62.9	177.3	25.0	71.8	72.5	72.5			
LacNAc-Doo-GlcNAc	GlcNAc	103.9	58.3	76.7	72.8	78.7	63.6	177.3	25.0						
	Gal	105.7	73.8	75.3	71.4	78.2	63.8								
	GlcNAc (in LacNAc)	103.8	57.9	75.3	81.3	77.6	62.9	177.3	25.0	71.8	72.5	72.5	72.3	74.5	63.2
LacNAc-Doo	Gal	105.7	73.8	75.3	71.4	78.2	63.8								
	GlcNAc	103.8	57.9	75.3	81.3	77.6	62.9	177.3	25.0	71.8	72.5	72.5	72.3	74.5	63.2

$J_{1,2}$ 8.6 Hz, H-1), 4.01 (m, 1H, H-αb), 3.93 (dd, 1H, H-6b), 3.80–3.63 (13H, H-6a, H-2, H-αa, H-β, H-γ, H-δ, H-ε,H-ζ), 3.55 (m, 1H, H-3), 3.46–3.44 (2H, H-5, H-4), 2.05 (s, 3H, CH$_3$CONH–).

(GlcNAc)$_2$-Doo. *m/z* 557[M + H]$^+$; ^{1}H NMR (D_2O, 500 MHz): δ4.59 (d, 1H, $J_{1',2'}$ 8.6 Hz, H-1′), 4.56 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.01 (m, 1H, H-αb), 3.92 (dd, 1H, H-6b), 3.85 (dd, 1H, H-6′b), 3.80–3.47 (21H, H-6a, H-5, H-4, H-3, H-2, H-6′a, H-5′, H-4′, H-3′, H-2′, H-αa, H-β, H-γ, H-δ, H-ε,H-ζ), 2.07 (s, 3H, CH$_3$CONH-), 2.04 (s, 3H, CH$_3$CONH–).

^{13}C NMR data of these compounds in D_2O are given in Table 1.

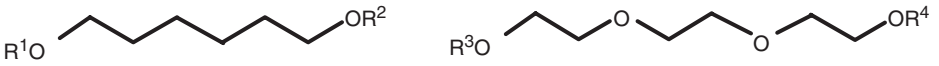
Preparation of LacNAc-Hx-LacNAc and LacNAc-Doo-LacNAc—GlcNAc-Hx-GlcNAc (42.0 mg, 0.08 mmol) and UDP-Gal (117 mg, 0.19 mmol) were dissolved in 80 mL of MES buffer (50 mM, pH 6.5) containing 10 mM of $MnCl_2$ and BSA (0.5 mg/mL), followed by addition of GalT (1.6 U) and AP (160 U). The mixture was incubated at 30°C for 8 h. The reaction was terminated by heating in boiling water for 10 min. The solution was loaded onto a Sep-Pak C18 column (bed volume, 35 mL) equilibrated in water. After the column was washed with 175 mL of water, the adsorbed material was eluted with 15% methanol. The eluted fraction was concentrated and dissolved in $CHCl_3/CH_3OH/H_2O$ (6:4:1) and then charged onto a silicagel column (Φ 2.0 × 27 cm) as described earlier. Tubes 37–59 were concentrated and lyophilized to afford LacNAc-Hx-LacNAc (6.7 mg, 9.8% based on the amount of acceptor added). Tubes 19–27 were concentrated and lyophilized to afford LacNAc-Hx-GlcNAc (9.4 mg, 17.1% based on the amount of acceptor added). LacNAc-Hx as a control glycoside was prepared by using a previously described method (*21*).

The desired LacNAc-Doo-LacNAc was enzymatically synthesized from GlcNAc-Doo-GlcNAc (44.5 mg, 0.08 mmol) and UDP-Gal (117 mg, 0.19 mmol) in a manner similar to that used to prepare LacNAc-Hx-LacNAc. The product LacNAc-Doo-LacNAc was obtained in a high yield (44.4 mg, 63.0% based on the amount of acceptor added). LacNAc-Doo-GlcNAc was also obtained in a yield of 25.4% (14.6 mg) based on the amount of acceptor added. Monovalent glycoside LacNAc-Doo was also synthesized from GlcNAc-Doo (42.4 mg, 0.12 mmol) and UDP-Gal (87.8 mg, 0.14 mmol) in a manner similar to that used to prepare LacNAc-Doo-LacNAc. The product was obtained in a high yield of 86.7% (53.6 mg) based on the amount of acceptor added.

LacNAc-Hx-LacNAc. FAB-mass: *m/z* 850 [M + H]$^+$; ^{1}H NMR (D_2O, 500 MHz): δ4.53 (d, 2H, $J_{1,2}$ 8.0 Hz, H-1), 4.47 (d, 2H, $J_{1',2'}$ 7.7 Hz, H-1′), 4.00-3.52 (28H, H-6b, H-6a, H-5, H-4, H-3, H-2, H-6′b, H-6′a, H-5′, H-4′, H-3′, H-2′, H-αb, H-αa), 2.03 (s, 6H, CH$_3$CONH–), 1.55 (m, 4H, H-β), 1.32 (m, 4H, H-γ).

LacNAc-Hx-GlcNAc. *m/z* 688 [M + H]$^+$; ^{1}H NMR (D_2O, 500 MHz): δ4.51 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1 of LacNAc), 4.49 (d, 1H, $J_{1,2}$ 8.6 Hz, H-1 of GlcNAc), 4.46 (d, 1H, $J_{1',2'}$ 7.7 Hz, H-1′ of LacNAc), 3.96–3.34 (22H, H-6b, H-6a, H-5, H-4, H-3, H-2 of GlcNAc, H-6b, H-6a, H-5, H-4, H-3, H-2 of LacNAc, H-6′b, H-6′a, H-5′, H-4′, H-3′, H-2′ of LacNAc, H-αb, H-αa, H-ζb,H-ζa), 2.02 (6H, CH$_3$CONH- of GlcNAc and LacNAc), 1.58–1.52 (4H, H-β, H-ε), 1.35–1.28 (4H, H-γ, H-δ).

LacNAc-Doo-LacNAc. FAB-mass: *m/z* 881 [M + H]$^+$; ^{1}H NMR (D_2O, 500 MHz): δ4.59 (d, 2H, $J_{1,2}$ 8.2 Hz, H-1), 4.48 (d, 2H, $J_{1',2'}$ 7.6 Hz, H-1′), 4.03–3.53 (36H, H-6b, H-6a, H-5, H-4, H-3, H-2, H-6′b, H-6′a, H-5′, H-4′, H-3′, H-2′, H-αb, H-αa, H-β, H-γ), 2.04 (s, 6H, CH$_3$CONH–).



Abbreviation	R^1	R^2	R^3	R^4
GlcNAc-Hx-GlcNAc	GlcNAc	GlcNAc		
GlcNAc-Hx	GlcNAc	H		
$(GlcNAc)_2$-Hx	$(GlcNAc)_2$	H		
LacNAc-Hx-LacNAc	LacNAc	LacNAc		
LacNAc-Hx-GlcNAc	LacNAc	GlcNAc		
LacNAc-Hx	LacNAc	H		
GlcNAc-Doo-GlcNAc			GlcNAc	GlcNAc
GlcNAc-Doo			GlcNAc	H
$(GlcNAc)_2$-Doo			$(GlcNAc)_2$	H
LacNAc-Doo-LacNAc			LacNAc	LacNAc
LacNAc-Doo-GlcNAc			LacNAc	GlcNAc
LacNAc-Doo			LacNAc	H

Fig. 1. **Abbreviation and structure of the synthetic glycosides.**

LacNAc-Doo-GlcNAc. FAB-mass: *m/z* 719 $[M+H]^+$; ^1H NMR (D_2O, 500 MHz): δ4.59 (d, 1H, $J_{1,2}$ 10.1 Hz, H-1 of LacNAc), 4.55 (d, 1H, $J_{1,2}$ 8.3 Hz, H-1 of GlcNAc), 4.48 (d, 1H, $J_{1',2'}$ 7.5 Hz, H-1′ of LacNAc), 4.01–3.41 (30H, H-6b, H-6a, H-5, H-4, H-3, H-2 of GlcNAc, H-6b, H-6a, H-5, H-4, H-3, H-2 of LacNAc, H-6′b, H-6′a, H-5′, H-4′, H-3′, H-2′ of LacNAc, H-αb, H-αa, H-β, H-γ,H-δ, H-ε,H-ζb, H-ζa), 2.04 (6H, C$\underline{H}_3$CONH– of GlcNAc and LacNAc).

LacNAc-Doo. FAB-mass: *m/z* 516 $[M+H]^+$; ^1H NMR (D_2O, 500MHz): δ4.59 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.48 (d, 1H, $J_{1',2'}$ 8.0 Hz, H-1′), 4.03–3.53 (24H, H-6b, H-6a, H-5, H-4, H-3, H-2, H-6′b, H-6′a, H-5′, H-4′, H-3′, H-2′, H-αb, H-αa, H-β, H-γ,H-δ, H-ε,H-ζ), 2.04 (s, 3H, C$\underline{H}_3$CONH–).

^{13}C NMR data of these compounds in D_2O are given in Table 1.

Hemagglutination Inhibition Assay—Erythrocyte suspension (20 μL) was added to 2-fold dilutions of lectins in 96-well microtiter U-plates and incubated for 40 min. The minimum concentration of lectin required for agglutination of erythrocytes was determined, and a 4-fold greater concentration was used for the following inhibition assay. Two-fold dilutions (20 μL) of chitin oligosaccharides and glycosides were prepared in 96-well plates. An aliquot (20 μL) of the lectin solution was added to each well and the plates were incubated at room temperature for 40 min. Agglutination of erythrocytes was carefully observed and the minimum concentrations of oligosaccharides and glycosides required to inhibit erythrocyte agglutination were determined.

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 8,000*g* for 10 min to remove precipitated material. The supernatant was diluted and analysed 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 $(GlcNAc)_n$ ($n = 1$–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.

SPR Analysis—SPR was recorded using a BIAcore 2000 (Biacore AB, Uppsala, Sweden). Asialofetuin was immobilized covalently *via* the primary amines to the carboxyl groups within a dextran layer on the sensor chip CM-5 according to the manufacturer's specifications. After chip activation with 0.1 M of NHS and 0.4 M of EDC, asialofetuin in 10 mM sodium acetate buffer (pH 4.8) 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 11,000 response units. 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. A 20 μL aliquot of WGA solution of varying concentration was injected over the immobilized chip without inhibitor in order to generate a calibration curve. WGA–glycoside interactions were investigated as follows. 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. A 20 μL aliquot of solution was then injected over the immobilized chip. The chip was regenerated by injection of 5 μL of 50 mM of

H_3PO_4, followed by HBS-P. The dissociation constant (K_D) was calculated by using BIAevaluation 3.1 software (Biacore AB, Sweden).

RESULTS

Enzymatic Synthesis of Divalent GlcNAc- and LacNAc-Glycosides—A crude chitinolytic enzyme preparation from *A. orientalis* was used for the synthesis of Hx-spacer-linked divalent glycoside carrying GlcNAc. The enzyme catalysed the synthesis of target divalent glycoside GlcNAc-Hx-GlcNAc together with monovalent glycosides GlcNAc-Hx and $(GlcNAc)_2$-Hx through *N*-acetylglucosaminyl transfer from $(GlcNAc)_4$ to 1,6-hexanediol. The transfer reaction led to the preferential formation of GlcNAc-Hx over GlcNAc-Hx-GlcNAc. In a similar manner, when triethylene glycol was used as an acceptor, the enzyme formed target GlcNAc-Doo-GlcNAc with monovalent glycosides GlcNAc-Doo and $(GlcNAc)_2$-Doo.

Enzymatic transformation into divalent LacNAc glycosides was carried out by addition of a Gal residue from UDP-Gal to the respective GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc by β-GalT from bovine milk. When GlcNAc-Hx-GlcNAc was used as an acceptor, the enzyme catalysed the synthesis of target LacNAc-Hx-LacNAc carrying LacNAc units on both sides together with LacNAc-Hx-GlcNAc carrying LacNAc and GlcNAc on each side (Fig. 2A). The maximum production of LacNAc-Hx-LacNAc was reached at 8 h. In a similar manner, with GlcNAc-Doo-GlcNAc as acceptor the enzyme formed target LacNAc-Doo-LacNAc carrying divalent LacNAc together with LacNAc-Doo-GlcNAc carrying LacNAc and GlcNAc on each side (Fig. 2B). In this case, LacNAc-Doo-LacNAc was formed in preference to LacNAc-Doo-GlcNAc.

HPLC analysis of the transglycosylation profile from UDP-Gal to GlcNAc-Hx-GlcNAc catalysed by β-GalT is shown in Fig. 2A. Only two transfer products of target LacNAc-Hx-LacNAc with LacNAc-Hx-GlcNAc were observed during the entire course of reaction, but the mode of formation differed from each other. LacNAc-Hx-GlcNAc was first produced and its formation obeyed the first-order rate law within ~4 h. In contrast, LacNAc-Hx-LacNAc formation was much slower and was observed only after 4 h. Maximum production of LacNAc-Hx-LacNAc was reached after 8 h, along with appreciable amounts of LacNAc-Hx-GlcNAc. This was also the case for the galactosylation from UDP-Gal to GlcNAc-Doo-GlcNAc for obtaining divalent glycosides LacNAc-Doo-LacNAc with LacNAc-Doo-GlcNAc (Fig. 2B). However, the divalent formation of LacNAc-Doo-LacNAc was somewhat different from that of LacNAc-Hx-LacNAc. Formation of LacNAc-Doo-LacNAc was much slower, although the amount increased markedly during the subsequent reaction. The relation between the yields of LacNAc-Doo-LacNAc and LacNAc-Doo-GlcNAc was reversed in the later stage of the reaction. Once the formation of LacNAc-Doo-GlcNAc had reached a maximum, its concentration varied little during the subsequent reaction.

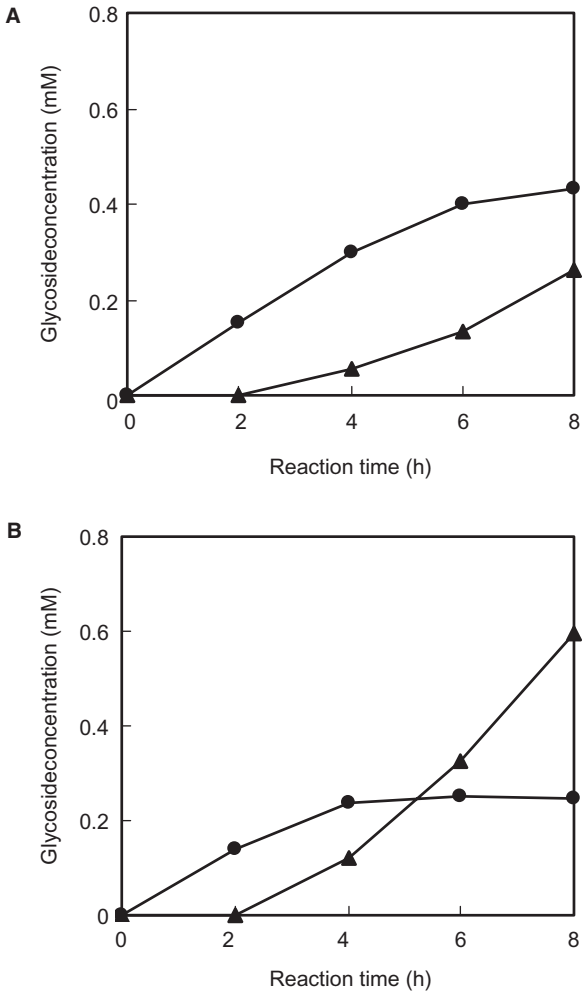


Fig. 2. **Time courses of the formation of divalent LacNAc glycosides by using βGalT from bovine milk.** A reaction mixture containing GlcNAc-Hx-GlcNAc or GlcNAc-Doo-GlcNAc and UDP-Gal in 50 mM MES buffer, pH 6.5, was incubated at 30°C. (A) GlcNAc-Hx-GlcNAc was used as a glycosyl acceptor. Filled triangle, LacNAc-Hx-LacNAc; filled circle, LacNAc-Hx-GlcNAc. (B) GlcNAc-Doo-GlcNAc was used as a glycosyl acceptor. Filled triangle, LacNAc-Doo-LacNAc; filled circle, LacNAc-Doo-GlcNAc.

Hemagglutination Inhibition Assay—Interaction of the synthetic glycosides with WGA was first analyzed by a hemagglutination inhibition assay. Inhibitory abilities of a series of synthetic monovalent and divalent glycosides related to GlcNAc were compared with those of GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ as control samples, which are potent inhibitors for hemagglutination with WGA. The monovalent glycosides [GlcNAc-Hx, $(GlcNAc)_2$-Hx, GlcNAc-Doo and $(GlcNAc)_2$-Doo] inhibited WGA with the same order of relative inhibition strength as that of the corresponding reducing sugars GlcNAc and $(GlcNAc)_2$, respectively (Table 2). In contrast, the affinities of WGA for the divalent glycosides GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc, carrying GlcNAc on both sides, were too weak to produce any detectable inhibition of hemagglutination even when the

Table 2. **Inhibiton of WGA-mediated hemagglutination by glycosides.**

Inhibitor	MIC (mM)
GlcNAc-Hx-GlcNAc	–
GlcNAc-Hx	25
$(GlcNAc)_2$-Hx	1.56
LacNAc-Hx-GlcNAc	–
GlcNAc-Doo-GlcNAc	–
GlcNAc-Doo	25
$(GlcNAc)_2$-Doo	0.78
LacNAc-Doo-GlcNAc	6.3
GlcNAc	50
$(GlcNAc)_2$	1.56
$(GlcNAc)_3$	0.20

Dash indicates no inhibition with 25 mM of glycoside.

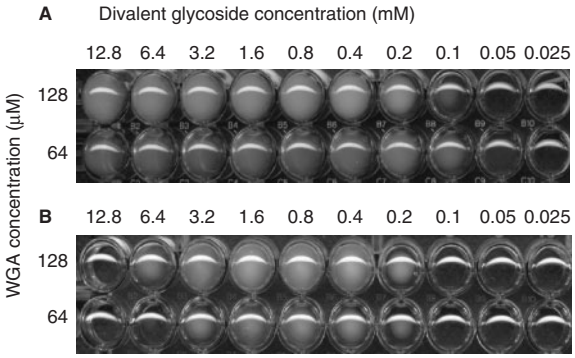


Fig. 3. **Photographs of precipitate formed by addition of divalent glycosides to WGA-PBS solution.** (A) Precipitate of GlcNAc-Hx-GlcNAc. (B) Precipitate of GlcNAc-Doo-GlcNAc.

concentration of ligand was 25 mM. In the same manner, the hemagglutination assay was subjected to a series of monovalent (LacNAc-Hx and LacNAc-Doo) and divalent glycosides (LacNAc-Hx-LacNAc and LacNAc-Doo-LacNAc) related to LacNAc, but they did not act as inhibitors. LacNAc itself did not inhibit at concentrations up to 50 mM. In the hetero divalent glycosides, LacNAc-Doo-GlcNAc acts as a weak inhibitor of hemagglutination, whereas LacNAc-Hx-GlcNAc does not.

Precipitation Analysis—Interaction of divalent glycosides with WGA was investigated by precipitation analysis. Various concentrations of WGA (8–256 μM) and divalent glycoside (0.025–12.8 mM) were mixed on a 96-well microplate. When each divalent glycoside (GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc) was added to WGA solution under appropriate conditions, a precipitate formed within a few minutes (Fig. 3). In each case, the precipitate was prevented from forming in the presence of $(GlcNAc)_2$ or $(GlcNAc)_3$ and dissolved upon addition of $(GlcNAc)_2$ or $(GlcNAc)_3$ (1 or 1 mM in both cases, respectively). Figure 4 shows precipitin curves for WGA in the presence of divalent glycosides GlcNAc-Hx-GlcNAc, LacNAc-Hx-GlcNAc, GlcNAc-Doo-GlcNAc and LacNAc-Doo-GlcNAc. The concentration of GlcNAc-Doo-GlcNAc at the equivalence point (region of maximum precipitation) of the precipitin curve for 128 μM of WGA

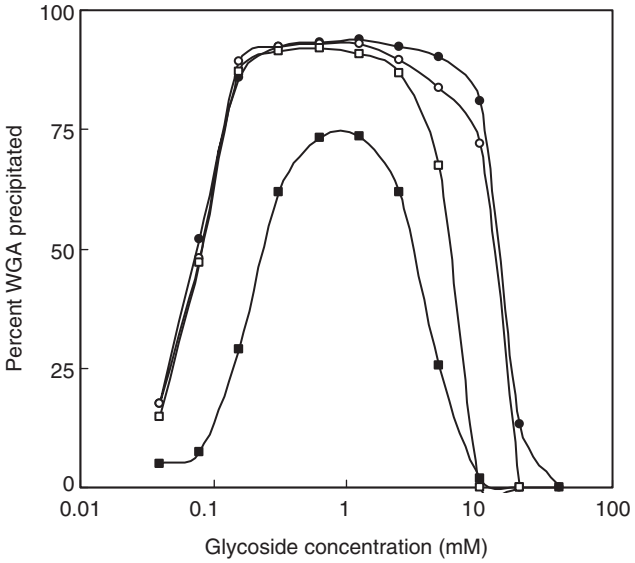


Fig. 4. **Precipitin curves for precipitation of WGA by divalent glycosides.** Percent WGA precipitated was calculated by subtracting WGA amount in supernatant from total WGA amount. Filled circle, GlcNAc-Hx-GlcNAc; open circle, LacNAc-Hx-GlcNAc; filled square, GlcNAc-Doo-GlcNAc; open square, LacNAc-Doo-GlcNAc.

was about 1 mM. Values for the other divalent glycosides were in the region of 0.1–2.5 mM, but could not be clearly determined under the present conditions. Monovalent glycosides and divalent glycosides carrying LacNAc on both sides (LacNAc-Hx-LacNAc and LacNAc-Doo-LacNAc) did not form a precipitate.

SPR Analysis—Interactions of divalent glycosides with WGA were analysed using BIAcore 2000. Asialofetuin was immobilized onto the surface of a sensor chip using the amine coupling method. The affinity of interaction was determined in solution by coinjecting an equilibrium mixture of a fixed amount of WGA with a variable amount of synthetic glycoside onto a surface-bound asiolofetuin. The surface was regenerated at the end of each cycle using 50 mM H_3PO_4. Figure 5 shows sensorgrams of interactions between WGA and monovalent and divalent glycosides. In the sensorgrams of monovalent glycosides, response units (RU) decrease linearly with increasing concentrations of glycoside (Fig. 5-2, 5-3, 5-6, 5-8, 5-9 and 5-12). As anticipated, these results indicate that the binding between WGA and bound-asialofetuin on the sensor chip is inhibited by the glycosides. The same phenomena were also observed when $(GlcNAc)_{1–3}$ were coinjected with WGA. In contrast, the sensorgrams of divalent glycosides GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc displayed behaviour quite different from those of monovalent glycosides. Coinjections with GlcNAc-Hx-GlcNAc or GlcNAc-Doo-GlcNAc showed a marked increase of RU between 1 μM–0.1 mM and 1 μM–1 mM with maximal values at 0.1 and 1 mM, respectively (Fig. 5-1 and 5-7). Upon reaching the maximum RU, there followed a sharp decrease. For LacNAc-Hx-GlcNAc and LacNAc-Doo-GlcNAc, no increase in RU was observed even at elevated

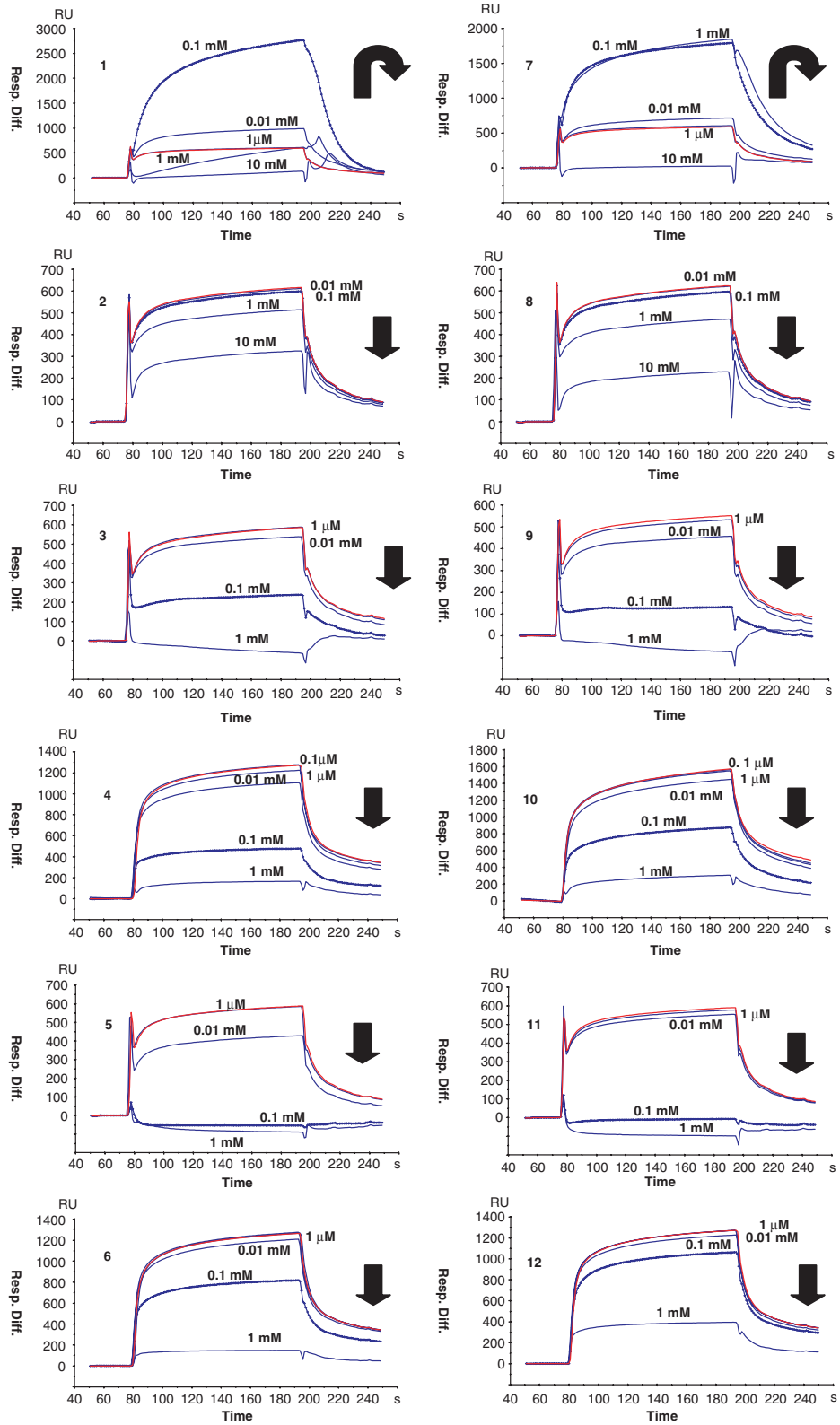


Fig. 5. **Sensorgrams showing the interactions of the glycosides with WGA.** Asialofetuin was immobilized onto the sensor chip. WGA was coinjected with glycoside (*Blue line*), or injected without glycoside as a control (*Red line*). +++; 0.1 mM glycoside. (1) GlcNAc-Hx-GlcNAc, (2) GlcNAc-Hx, (3) $(GlcNAc)_2$-Hx, (4) LacNAc-Hx-LacNAc, (5) LacNAc-Hx-GlcNAc, (6) LacNAc-Hx, (7) GlcNAc-Doo-GlcNAc, (8) GlcNAc-Doo, (9) $(GlcNAc)_2$-Doo, (10) LacNAc-Doo-LacNAc, (11) LacNAc-Doo-GlcNAc, (12) LacNAc-Doo.

Table 3. **Affinity parameter obtained from the interactions of glycosides with WGA by use of a biosensor.**

Glycoside	K_D (M)[a]	Glycoside	K_D (M)[a]
Glycoside			
GlcNAc-Hx-GlcNAc	–[b]	GlcNAc-Doo-GlcNAc	–[b]
GlcNAc-Hx	1.4×10^{-2}	GlcNAc-Doo	7.1×10^{-3}
$(GlcNAc)_2$-Hx	1.2×10^{-4}	$(GlcNAc)_2$-Doo	2.9×10^{-5}
LacNAc-Hx-LacNAc	8.4×10^{-6}	LacNAc-Doo-LacNAc	3.2×10^{-5}
LacNAc-Hx-GlcNAc	6.5×10^{-6}	LacNAc-Doo-GlcNAc	2.7×10^{-5}
LacNAc-Hx	4.7×10^{-5}	LacNAc-Doo	1.2×10^{-4}
Reducing sugar			
GlcNAc	5.3×10^{-2}	Lactose	1.5×10^{-1}
$(GlcNAc)_2$	1.6×10^{-4}	LacNAc	2.3×10^{-4}
$(GlcNAc)_3$	1.5×10^{-5}		

[a]Values were determined by the affinity solution method as described in the experimental section.
[b]Affinity parameters were not calculated because of the unusual increase of RU.

concentrations of glycoside (Fig. 5-5 and 5-11). Based on these results, K_D values of the glycosides were compared with those of the corresponding sugars, GlcNAc and $(GlcNAc)_2$ (Table 3). However, this analysis was not possible for target compounds GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc because of the unusual behaviour on the sensorgram.

DISCUSSION

We have developed the enzymatic synthesis of spacer-linked divalent glycosides carrying GlcNAc (GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc) by transglycosylation of a $(GlcNAc)_4$ donor with primary diol acceptors, utilizing chitinolytic enzyme from *A. orientalis*. Interestingly, glycosylation to bilateral hydroxyl groups in 1,6-hexanediol and triethylene glycol acceptors is greatly influenced by the structure of the acceptor. Our results demonstrate that GlcNAc-Hx-GlcNAc, with a non-polar spacer group possesses higher affinity for the enzyme compared with that of GlcNAc-Doo-GlcNAc, with a polar spacer group. This is the first report of chitinolytic enzyme-mediated transglycosylation for the direct synthesis of divalent GlcNAc glycosides. We have recently found that a cellulase from *Trichoderma reesei* catalyzed a condensation reaction between lactose and 1,6-hexanediol to produce 6-hydroxyhexyl β-lactoside (*21*). In this case, the glycosylation of 1,6-hexanediol was selective for the unilateral hydroxyl group and did not target the other hydroxyl group.

The resulting divalent GlcNAc glycosides (GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc) were easily transformed into target divalent LacNAc glycosides (LacNAc-Hx-LacNAc and LacNAc-Doo-LacNAc) and the glycosides carrying LacNAc and GlcNAc on each side (LacNAc-Hx-GlcNAc and LacNAc-Doo-GlcNAc) by galactosyltransferase, respectively. Galactosylation to the acceptor was strongly influenced by the spacer structure because the transformation into LacNAc-Doo-LacNAc from GlcNAc-Doo-GlcNAc showed much higher potency than that of LacNAc-Hx-LacNAc from GlcNAc-Hx-GlcNAc.

The resulting divalent glycosides carrying GlcNAc and LacNAc were used for analysing interaction with WGA by three methods: hemagglutination-inhibition assay, precipitation assay and a biosensor analysis. Each assay was repeated at least two times in order to verify reproducibility. In the hemagglutination inhibition assay, we observed that divalent GlcNAc glycosides GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc did not show any inhibition activity for WGA, while their corresponding monovalent glycosides acted as potent inhibitors. This result is consistent with the sugar specificities of WGA reported for naturally occurring glycoproteins, glycolipids and oligosaccharides (*22–24*). The unusual behaviour of the divalent glycosides was further studied by precipitation analyses. It is noteworthy that GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc, carrying GlcNAc on both sides, are capable of precipitating the lectin in addition to LacNAc-Hx-GlcNAc and LacNAc-Doo-GlcNAc, carrying GlcNAc and LacNAc on each side. These divalent glycosides specifically bind to WGA because their precipitation with the lectin can be inhibited or reversed by chitin oligosaccharides $(GlcNAc)_n$ $(n = 1–3)$ as specific hapten sugars. The present findings indicate that even low molecular weight ligand such as the divalent glycosides are capable of precipitating WGA, which has an ability to precipitate some glycoproteins (*25*).

The ability of each divalent glycoside to bind WGA was compared with that of the corresponding monovalent control glycoside. A surface plasmon resonance (SPR) competition binding assay was used to monitor the effect of synthetic glycosides on the interaction of soluble WGA with a surface-bound asialofetuin. The interaction of WGA with the divalent GlcNAc glycosides (GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc) is markedly different from that observed for the corresponding monovalent glycosides. Here, RU increased as the concentration of GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc increased from 1 μM to 0.1 mM and 1 μM to 1 mM, respectively (Fig. 5-1 and 5-7). Thus, the divalent glycosides (GlcNAc-Hx-GlcNAc and GlcNAc-Doo-GlcNAc) promote, rather than inhibit, binding of WGA to a surface-bound asialofetuin. The data suggest that the divalent glycoside binds to tetravalent WGA simultaneously. The clusters presumably bind to the immobilized asialofetuin ligand because the clustered WGA possess unoccupied carbohydrate binding sites that can interact with the surface. Burke *et al*. have found that trivalent mannose macrocycle, which is more potent than the corresponding

monovalent derivative, functions by clustering Con A in solution by SPR (*17*). This behaviour is entirely consistent with the present results. The accurate binding data of divalent GlcNAc glycosides with WGA can be evaluated by X-ray analysis (*1*, *26*) and isothermal titration microcalorimetry (*27*, *28*). In contrast, the sensorgrams of LacNAc-Hx-GlcNAc and LacNAc-Doo-GlcNAc, which are capable of precipitating WGA, were different from those of divalent GlcNAc glycosides (Fig. 5-5 and 5-11). These glycosides inhibit interaction between WGA and a surface-bound asialofetuin as is the case with the monovalent and divalent LacNAc glycosides (Fig. 5-4, 5-6, 5-10 and 5-12). Our data suggest that LacNAc moieties of the hetero-divalent glycoside compete with LacNAc on a surface-bound asialofetuin, which possesses triantennary terminal-LacNAc chains, and are involved with binding to WGA. As a result, LacNAc-Hx-GlcNAc and LacNAc-Doo-GlcNAc function as hapten sugars on SPR. We have previously reported that SPR enables detection of the binding of WGA to the surface-bound glycopeptides carrying side chains of LacNAc (*29*).

In conclusion, divalent glycosides carrying GlcNAc were synthesized in a single step by transglycosylation using a chitinolytic enzyme from *A. orientalis*. A Gal residue was subsequently added to the resulting glycosides by galactosylation. This is the first report of the enzymatic synthesis of divalent glycosides as glycomimetics. Despite the low molecular weight of the ligand, divalent glycosides carrying GlcNAc residues were shown to have the ability to precipitate WGA and to facilitate the formation of clusters in solution. Our results suggest that divalent glycosides can act as superior ligands because of their ability to cluster target lectins.

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