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Effect of shape, size, and valency of multivalent mannosides on their binding properties to phytohemagglutinins

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Clusters of di-, tri-, and tetra-antennary α-D-mannopyranosides were synthesized in good yields based on the coupling of amine-bearing mono- or trisaccharide {Man α(1→6)[Man α(1→3)]Man} haptens to poly-isocyanate or -isothiocyanate tethering cores. The relative binding properties of the resulting multivalent ligands were determined by turbidimetric and solid phase enzyme-linked lectin assays (ELLA) using plant lectins (phytohemagglutinins) Concanavalin A (Con A) and *Pisum sativum* (pea lectin) having four and two carbohydrate binding sites, respectively. Rapid and efficient cross-linking between tetravalent Con A and mannopyranosylated clusters were measured by a microtiter plate version of turbidimetric analyses. In inhibition of binding of the lectins to yeast mannan, the best tetravalent monosaccharide (30) and trisaccharide (31) inhibitors were shown to be 140 and 1155 times more potent inhibitors than monomeric methyl α-D-mannopyranoside against pea lectin and Con A, respectively. Compounds 30 and 31 were thus 35- and 289-fold more potent than the reference monosaccharide based on their hapten contents. As a general observation, the ligands bearing the Man α(1→6)[Man α(1→3)]Man trimannoside structures were found to be more potent inhibitors for Con A than the ligands having single mannoside residues, whereas pea lectin could not discriminate between the two types of ligands.

***Keywords*: mannoside clusters, Concanavalin A, pea lectin, cross-linking, valency**

Introduction

The syntheses and development of potent carbohydrate inhibitors is of prime interest to understand the different factors involved in protein-carbohydrate interactions. Features such as structure, shape, size, geometry, and valency have proved to be determinant factors in influencing the generally weak binding properties of carbohydrate ligands ($K_D \approx 0.1$–1 mM) [1]. To overcome these weak binding interactions, multivalent neoglycoconjugates [2, 3] ranging from clusters [4] and oligomers [5–7], to macromolecular polydispersed systems such as glycopolymers [8, 9] and glycodendrimers [10–13] have been generated to provide antiadhesins of higher affinity. A large number of these multivalent carbohydrate ligands demonstrated powerful inhibitory properties when tested against their specific animal or plant lectins. For instance, a glycodendrimer carrying twelve terminal α-sialoside residues was shown to be 182-fold more potent than its corresponding monomer when used in the inhibition of binding of the slug lectin *Limax flavus* to human orosomucoid [14]. Clusters containing as few as three N-acetylgalactosamine (GalNAc) [15] or galactose (Gal) [16] residues represent classical examples of the 'cluster effect' [17] used in the study of hepatic asialoglycoprotein receptors (ASGP-R). In these cases, thousand fold increases in binding affinity have been observed. Similar trends also prevail in the inhibition of influenza virus hemagglutination of human erythrocytes by polymers containing sialosides [8, 18]. Because of the potential toxicological and immunological drawbacks of glycopolymers for therapeutic interventions, smaller and better defined structures are desirable.

The sometime drastic increased inhibitory properties observed from these multivalent neoglycoconjugates are far from being clearly understood. As the higher associative forces that held these clusters to their reciprocally clustered receptors is not likely to originate from an intrinsic change in the individual binding affinities (K_D) (Roy R, Pagé D, Dimick S, Toone EJ, unpublished data), but rather from an overall cooperative binding forces (avidity), it becomes appealing to generate architecturally well defined 'glycoclusters'. One likely explanation for the improved binding of glycoclusters may reside in the overall structures of the cross-linked lattices which inevitably form when two

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multivalent ligands and receptors meet one each other (Figure 1A) [19]. In fact, there is accumulating evidences that divalent lectin and divalent carbohydrate interactions can lead to the formation of linear one-dimensional cross-linked complexes that are soluble [20]. Alternatively, insoluble multiply cross-linked three dimensional lattices usually form upon binding multivalent (and even divalent) carbohydrates to multivalent lectins [19].

Irrespective of the nature of the cross-linking interactions that occur upon binding, the increased cooperative associative forces must result in altered association/dissociation kinetics (k_{on}/k_{off}) [21]. These kinetics should also be dependent on the size, shape, and valency of the neoglycoconjugate clusters.

In order to shed some further light on these multiple carbohydrate-lectin interactions, our group has been involved in the design of dendritic and clustered neoglycoconjugates of defined shape, size, and valency [2–7, 14, 22]. Some particularly attractive targets are ligands bearing terminal mannoside residues because of the involvement of this sugar in many bacterial infections [23] and in host defense immune response [24]. For instance, both serum mannose-binding proteins (MBPs) [25, 26] and macrophages cells [27, 28] mediate the uptake of different pathogens *via* mannose-specific receptors [29, 30]. As many of these mammalian receptors or lectins have carbohydrate-recognition domains (CRDs) also organized as clusters [27, 28], it is important to develop different multivalent mannoside ligands for binding studies. Macromolecular mannopyranosylated systems such as glycopolymers [31, 32] and glycodendrimers [33, 34] have already demonstrated powerful binding properties to model plant lectins. Further refinements obtained through shapes and geometry optimizations by means of different tethering cores have produced analogous low-molecular weight mannopyranosylated ligands that also showed good inhibitory properties [35, 36].

Previous studies [37] done with fimbriated *E. coli* demonstrated the enhanced inhibitory potential of different oligomannoside residues bearing the so-called trimannoside core Man $\alpha(1 \rightarrow 6)$[Man $\alpha(1 \rightarrow 3)$]Man found on N-linked glycoproteins. Many mannose-specific lectins, including Con A, also bind N-glycan trimannoside structures with high affinity [1]. This enhanced binding character is the net result of the direct recognition of all three sugar units by the lectin *via* multiple hydrogen bondings (Figure 1B) [38, 39]. On the other hand, single mannoside residues binds only at the $\alpha(1 \rightarrow 6)$ binding site with much lower affinity [40]. Incorporation of this trisaccharide structure into multivalent ligands should therefore generate more potent ligands. As the design of such glycomimetic inhibitors could have potential applications as antiadhesins or cell-targeting vectors [41], we describe herein the synthesis of di-, tri-, and tetravalent mannopyranosylated ligands bearing mono or trisaccharide haptens, along with their inhibitory properties using the plant lectins Con A and *Pisum sativum* (PSA) as models.

Materials and methods

General methods

Melting points were determined on a Gallenkamp apparatus and are uncorrected. The ^1H and ^{13}C NMR spectra were obtained on a Brüker 500 MHz AMX NMR spectrometer. The proton chemical shifts (δ) are given relative to internal chloroform (7.24 ppm) for $CDCl_3$ solutions, to internal DMSO (2.49 ppm) for DMSO-d_6 solutions, and to internal HOD (4.65 ppm) for D_2O solutions. The carbon

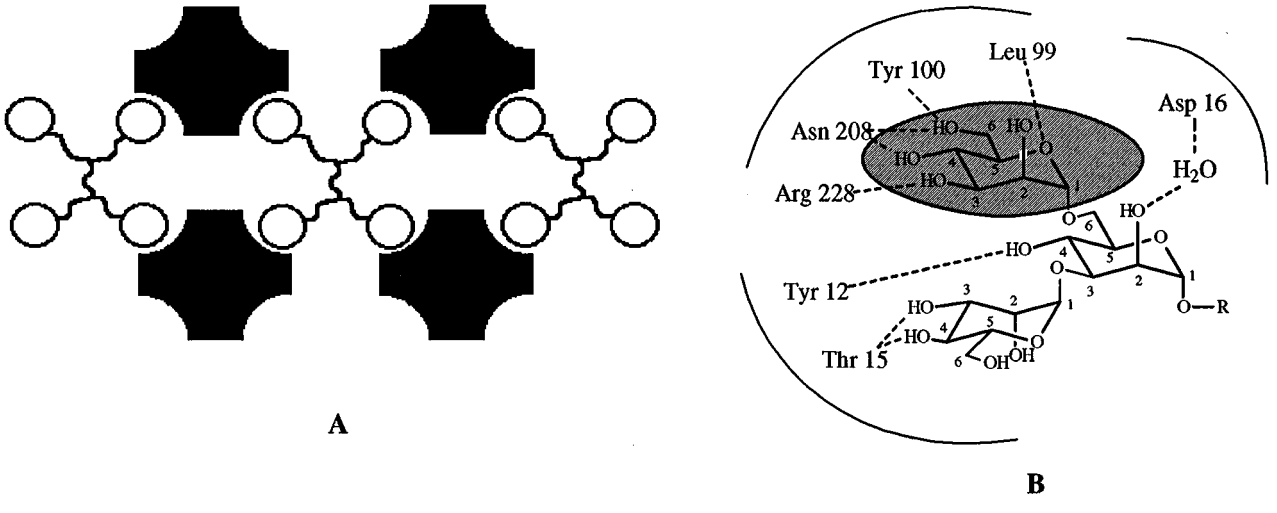


Figure 1. (A) Schematic representation of possible cross-linked lattices which can form upon binding tetravalent carbohydrate ligands to tetravalent Conc A. (B) Binding of Man $\alpha(1 \rightarrow 6)$Man $\alpha(1 \rightarrow 3)$]Man trimannosyl ligand to Con A: single mannoside residue binds at the same site than the $\alpha(1 \rightarrow 6)$ arm (shadowed) of the trimannoside. (Adapted from reference [38]).

chemical shifts are given relative to deuterochloroform (77.0 ppm) and DMSO-d_6 (39.5 ppm). The assignments were based on COSY, DEPT, and HMQC experiments. Optical rotations were measured on a Perkin-Elmer 241 polarimeter and were run at 23 °C. Mass spectra were recorded on a VG 7070-E spectrometer (CI ether) and Kratos Concept IIH for FABMS using glycerol matrix. Thin layer chromatography (TLC) was performed using silica gel 60 F-254 and column chromatography on silica gel 60. Absorbances for the ELLA tests and turbidimetric measurements were performed on a Dynatech MR 600 Microplate Reader. Methyl α-D-mannopyranoside was purchased from Aldrich. The lectins from *Canavalia ensiformis* (Con A) and *Concanavalin A*-peroxidase labeled, along with yeast mannan from *Saccharomyces cerevisiae* were purchased from Sigma (cat. nos C 2631, L 6397 and M 7504 respectively). Pea lectin (*Pisum sativum*) and horseradish peroxidase labeled pea lectin were obtained from EY Laboratories (cat. nos L-2701-10 and H-2701-1).

3-(2-aminoethylthio)propyl α-D-mannopyranoside (**2**)

Allyl mannoside **1** (31) (1.33g, 6.04 mmol) and cysteamine hydrochloride (570 mg, 5.03 mmol) were dissolved in degassed H_2O (35 ml) and the solution was irradiated ($\lambda = 254$ nm) in a quartz tube at 30 ° C for 3 h. The solution was lyophilized and the crude oily residue was purified by silica gel column chromatography using initially $CHCl_3$: MeOH / 5:1 (v/v) and then MeOH as eluents, giving compound **2** (1.53 g) as a colorless oil in 85% yield; $[\alpha]_D = +55.5°$ (c = 1.00, MeOH); ^{1}H NMR (D_2O) δ 4.68 (d, 1 H, $J_{1,2} = 1.7$ Hz, H-1), 3.75–3.40 (m, 8 H, H-2, H-3, H-4, H-5, H-6a, H-6b and Ha), 2.69 (t, 2 H, J = 6.6 Hz, Hd), 2.49 (m, 4 H, Hc and Hd), 1.72 (dd, 2 H, J = 7.5 Hz, Hb); ^{13}C NMR (D_2O) δ 100.5 (C-1), 73.6 (C-5), 71.4 (C-3), 70.8 (C-2), 67.5 (C-4), 61.7 (C-6), 40.3 (Ce), 33.8 (Cd), 32.9 (Cb), 28.4 (Cc); mass spectrum (CI) (rel. intensity) *m/z* 298.0 ($M^+ + 1$, 100% base peak).

5-azido-3-oxapentyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**5**)

A solution of freshly prepared acetobromomannose **3** (1.05 g, 2.58 mmol) in dry CH_3CN (10 ml) was added to a stirred mixture of 5-azido-3-oxapentanol **4** (0.37 ml, 2.82 mmol), $Hg(CN)_2$ (650 mg, 2.56 mmol), $HgBr_2$ (925 mg, 2.56 mmol) and 0.3 nm powdered molecular sieves in CH_3CN (8 ml). After 1 h of stirring at room temperature, CH_2Cl_2 (20 ml) was added, the mixture was filtered through Celite and the filtrate was evaporated under reduced vacuum. The syrupy residue was extracted with CH_2Cl_2 (10 ml, 4X), filtered, and the liquid extract was successively washed with 10% aq. KI, saturated $NaHCO_3$ and water. The solvent was filtered and evaporated under reduced pressure. The resulting syrup was purified by silica gel column chromatography using CH_2Cl_2: acetone/20:1 (v/v) to afford compound **3** as a colorless syrup (860 mg) in 75% yield; $[\alpha]_D = +24.3°$ (c = 1.03, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.32 (m, 3 H, H-2, H-3 and H-4), 4.89 (d, 1 H, H-1), 4.30 (dd, 1 H, $J_{5,6a} = 4.2$ Hz, $J_{6a,6b} = 10.1$ Hz, H-6), 4.08 (m, 1 H, H-5), 3.85 (dd, 1 H, $J_{5,6b} = 4.2$ Hz, H-6b), 3.48 (m, 6 H, CH_2O), 3.34 (t, 2 H, J = 5.1 Hz, CH_2N_3), 2.10–1.98 (m, 12 H, Ac); ^{13}C NMR ($CDCl_3$) δ 170.1, 169.5, 169.4 and 169.3 (C = O's), 97.2 (C-1), 69.0 (C-5), 68.2 (C-2), 66.8 (C-3), 65.6 (C-4), 62.0 (C-6), 50.2 (CH_2N_3), 20.4, 20.3 and 20.2 (2C) (Ac).

5-azido-3-oxapentyl α-D-mannopyranoside (**6**)

Compound **5** (860 mg, 1.86 mmol) was dissolved in MeOH (10 ml) containing 0.01M NaOMe (pH > 8.5). After 30 min of stirring at room temperature, the solution was neutralized with Amberlite IR 120 (H^+) resin, filtered and evaporated under reduced pressure to afford **6** as a colorless syrup (530 mg) in 97% yield which was used in the next step without further purification.

5-azido-3-oxapentyl 3,6-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**7**)

A mixture of **6** (200 mg. 0.68 mmol), $HgBr_2$ (615 mg, 1.7 mmol), $Hg(CN)_2$ (430 mg, 1.7 mmol) and 0.3 nm powdered molecular sieves (2 g) in dry CH_3CN (10 ml) was stirred for 30 min at room temperature. A solution of freshly prepared acetobromomannose **3** (770 mg, 1.7 mmol) in dry CH_3CN (10 ml) was then added dropwise and the mixture was further stirred at room temperature for 1 h. The mixture was then filtered through Celite, the solid material rinsed with MeOH (20 ml), and the filtrate was evaporated under reduced pressure. The syrupy residue was extracted with CH_2Cl_2 (15 ml, 4X), filtered and the filtrate was successively washed with 10% aq. KI, saturated $NaHCO_3$ and water. The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. Silica gel column chromatography of the residue using CH_2Cl_2: acetone/5:1 (v/v) afforded product **7** as a white solid foam (215 mg) in 34% yield; $[\alpha]_D = +69.0°$ (c = 1.45, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.30 (m, 6 H, H-2′, H-3′, H-4′, H-2″, H-3″, and H-4″), 5.13 (d, 1 H, H-1″), 4.98 (d, 1 H, H-1′), 4.85 (d, 1 H, H-1), 4.35 (m, 1 H, H-5″), 4.23 (m, 4 H, H-3, H-5, H-6a and H-6b), 3.66 (m, 6 H, CH_2O), 3.38 (t, 2 H, J = 5.1 Hz, CH_2N_3), 3.29 (d, 1 H, J = 3.5 Hz, OH-4), 2.95 (d, 1 H, J = 5.6 Hz, OH-2), 2.18-2.03 (M, 24 h, Ac); ^{13}C NMR ($CDCl_3$) δ 170.5-169.7 (C = O's), 99.8 (C-1), 99.1 (C-1″), 97.2 (C-1′), 81.5 (C-3), 71.5 (C-5), 69.9 (C-2), 69.6 (C-2′), 69.3 (C-2″), 69.1 (C-3′), 68.9 (C-3″), 68.9 (C-5′), 68.3 (C-5″), 66.4 (C-6), 66.0 (C-4′ and C-4″), 65.6 (C-4), 62.7, 62.4 (C-6′ and C-6″), 50.2 (CH_2N_3), 20.4, 20.8–20.2 (Ac); Anal. Calcd for $C_{75}H_{86}O_{17}N_4$ (1315.52): C, 47.85; H, 5.81; N, 4.41. Found: C, 47.46; H, 5.91; N, 4.28.

Signals are designed as follows: αMan, non-primed; Man α(1 → 3), single primed; Man α(1 → 6), double-primed.

5-azido-3-oxapentyl 3,6-di-*O*-(α-D-mannopyranoside)-α-D-mannopyranoside (**8**)

Compound **7** (215 mg, 0.34 mmol) was dissolved in MeOH (5 ml) containing 0.01 M NaOMe (pH > 8.5). After 30 min of stirring at room temperature, the solution was neutralized with Amberlite IR 120 (H^+) resin and filtered. Evaporation of the solvent under reduced pressure followed by lyophilization of the residue in water afforded compound **8** as a white powder (203 mg) in 97% yield; $[\alpha]_D = +83.3^\circ$ (c = 1.30, MeOH); ^{1}H NMR (D_2O) δ 5.18 (d, 1 H, $J_{1',2'} = 1.5$ Hz, H-1′), 4.98 (d, 1 H, $J_{1'',2''} = 1.5$ Hz, H-1″), 4.93 (d, 1 H, $J_{1,2} = 1.5$ Hz, H-1), 4.21 (dd, 1 H, $J_{2,3} = 3.2$ Hz, H-2), 4.14 (dd, 1 H, $J_{2',3'} = 3.2$ Hz, H-2′), 4.08 (dd, 1 H, $J_{5,6a} = 3.8$ Hz, $J_{6a,6b} = 11.8$ Hz, H-6a), 4.06 (dd, 1 H, $J_{2'',3''} = 4.06$ Hz, H-2″), 4.01 (dd, 1 H, $J_{3,4} = 8.6$ Hz, H-3), 3.98-3.72 (m, 19 H, H-3′, H-3″, H-4, H-4′, H-4″, H-5, H-5′, H-5″, H-6b, H-6a′, H-6a″, H-6b′, H-6b″ and CH_2O), 3.57 (t, 2 H, J = 5.1 Hz, CH_2N_3); ^{13}C NMR (D_2O), δ 101.8 (C-1′), 99.7 (C-1), 99.0 (C-1″), 78.1 (C-3), 72.8 (C-5′), 72.2 (C-5″), 70.6 (C-5), 70.2 (C-3″), 69.9 (C-3″), 69.6 (C-2), 69.1 (C-2″), 69.0 and 68.9 (*C*H_2O), 66.3 (2C), (C-4′ and C-4″), 66.2 (*C*H_2O), 65.2 (C-4), 64.9 (C-6), 60.5 (2C) (C-6′ and C-6″), 49.7 (*C*H_2N_3); FAB-MS (pos.) for $C_{22}H_{39}N_3O_{17}$ 617.23: found: 618.32 ($M^+ + 1$, 1.5% base peak).

5-amino-3-oxapentyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**9**)

Compound **8** (100 mg, 0.162 mmol) was dissolved in MeOH (10 ml) containing a catalytic amount of 10% Pd-C. Hydrogen was bubbled for 2 h at room temperature. The catalyst was then filtered through Celite and the filtrate was evaporated under reduced pressure to afford compound **9** as a white solid foam (95 mg) in 99% yield. This product was used for the next step without further purification.

Synthesis of divalent mannopyranosylated ligands **11** and **12**

Compounds **11** and **12** were synthesized following a similar procedure. Compound **2** or **9** (2.4 eq.) were dissolved in DMSO (1 ml) containing 1 drop of DIPEA. 1,4-diisocyanatobutane **10** (1 eq.) was then added and the solutions were stirred at room temperature for 1 hour. The DMSO solutions were then lyophilized with water, and the crude product were purified by size exclusion Biogel P-2 column affording compounds **11** and **12** in 69% and 71% yield respectively; compound **11**: $[\alpha]_D = +38.1^\circ$ (c = 1.00, H_2O); ^{1}H NMR (D_2O) δ 4.93 (d, 2 H, $J_{1,2} = 1.7$ Hz, H-1), 4.01 (dd, 2 H, $J_{2,3} = 3.4$ Hz, H-2), 3.95 (dd, 2 H, $J_{5,6a} = 1.9$ Hz, $J_{6a,6b} = 11.9$ Hz, H-6a), 3.91–3.82 (m, 6 H, H-3, H-6b and Ha), 3.73 (dd, 2 H, $J_{3,4} = 9.4$ Hz, H-4), 3.72 (m, 2 H, H-5), 3.68 (m, 2 H, Ha′), 3.39 (t, 4 H, J = 6.6 Hz, He), 3.18 (bs, 4 H, NHCH_2), 2.73 (m, 8 H, Hc and Hd), 1.97 (m, 4 H, Hb), 1.56 (t, 4 H, J = 2.7 Hz, $NHCH_2CH_2$); ^{13}C NMR (D_2O) δ 159.9 (C=O), 99.3 (C-1), 72.3 (C-5), 70.2 (C-3), 69.7 (C-2), 66.3 (C-4), 65.7 (Ca), 60.5 (C-6), 39.1 (NH*C*H_2), 38.9 (Ce), 31.1 (Cd), 28.2 (Cb), 27.5 (Cc), 26.2 ($NHCH_2CH_2$); FAB-MS (pos.) for $C_{28}H_{54}N_4O_{14}S_2$ 734.3: found 735.3 ($M^+ + 1$, 7.3% base peak); compound **12**: $[\alpha]_D = +63.3^\circ$ (c = 0.60, H_2O); ^{1}H NMR (D_2O) δ 5.19 (s, 2 H, H-1′), 4.97 (s, 2 H, H-1″), 4.92 (s, 2 H, H-1), 4.19 (d, 2 H, $J_{1,2} = 2.0$ Hz, H-2), 4.14 (d, 2 H, $J_{1',2'} = 1.6$ Hz, H-2′), 4.06 (m, 8 H, H-2″ and H-6a), 3.97–3.71 (m, 36 H, H-3, H-3′, H-3″, H-4, H-4′, H-4″, H-5, H-5′, H-5″, H-6b, H-6a′, H-6b′, H-6a″, H-6b″, Ha and Hb), 3.67 (t, 4 H, J = 5.4 Hz, Hc), 3.38 (t, 4 H, Hd), 3.18 (bs, 4 H, NHCH_2), 1.55 (bs, 4 H, $NHCH_2CH_2$); ^{13}C NMR (D_2O) δ 101.9 (C-1), 99.7 (C-1′), 99.0 (C-1″), 72.9 (C-5′), 72.2 (C-5″), 70.6 (C-5), 70.2 (C-3″), 69.9 (C-3′), 69.6 (C-2), 69.5 (C-2′), 69.4 (Cc), 69.1 (C-2), 68.9 (Cb), 66.4 (Ca), 66.3 (2C) (C-4′ and C-4″), 65.2 (C-4), 64.8 (C-6), 60.5, 60.6 (C-6′ and C-6″), 39.1 (2C) (NH*C*H_2 and Cd), 26.2 ($NHCH_2CH_2$).

Methyl 3,5-diaminobenzoate (**14**)

3,5-diaminobenzoic acid **13** (5.00 g, 32.9 mmol) was dissolved in MeOH (250 ml). Concentrated H_2SO_4 was added dropwise until the pH became acidic (pH ≈ 5.0) and the solution was refluxed for 2 days. The solution mixture was then neutralized with Amberlite IRA-400 (OH^-) resin, filtered and the solvent was evaporated under reduced pressure. The crude product was recrystallized (3X) from 99% EtOH, giving pure **14** (3.45g) in 65% yield; mp: 119–120 °C; ^{1}H NMR (DMSO-d_6) δ 6.46 (d, 2 H, J = 2.0 Hz, H-ortho), 6.06 (d, 1 H, J = 1.9 Hz, H-para), 5.20 (bm, 4 H, NH_2), 3.73 (s, 3 H, Me); ^{13}C NMR (DMSO-d_6) δ 167.1 (C=O), 148.9 (C-ipso), 130.6 (C-meta), 104.1 (2C) (C-ortho and C-para), 51.6 (Me); mass spectrum (EI) (rel. intensity) *m/z* 166.1 (M^+, 100%), 135.1 ($M^+ - OMe$, 30.6%).

Methyl 3,5-diisothiocyanatobenzoate (**15**)

Compound **14** (250 mg, 1.50 mmol) was dissolved into CH_2Cl_2 (25 ml) containing DIPEA (65 µl, 2.5 eq.). Thiophosgene (0.570 ml, 5 eq.) was added and the solution was stirred at room temperature for 45 min. The solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography using hexanes: EtOAc/3:1 (v/v) as eluent, giving pure **15** (360 mg) in 96% yield; mp: 95–96 °C; IR ($CHCl_3$) ν2054.8 cm^{-1} (N=C=S, b); ^{1}H NMR ($CDCl_3$) δ 7.73 (d, 2 H, J = 2.4 Hz, H-ortho), 7.18 (t, 1 H, J = 1.9 Hz, H-para), 3.92 (s, 3 H, Me);^{13}C NMR ($CDCl_3$) δ 164.6 (C=O), 139.3 (C-ipso), 133.3 (N=C=S), 133.1 (C-meta), 126.0 (C-para), 125.2 (C-ortho), 52.8 (Me); mass spectrum (EI) (rel. intensity) *m/z* 249.9 (M^+, 6.6%).

Synthesis of divalent mannopyranosylated ligands **16** to **19**

Divalent mannopyranosylated ligands **16** to **19** were synthesized following a similar procedure. Compounds **2** or

9 (2.4 eq.) were dissolved in DMSO (1 ml) containing 1 drop of DIPEA. Compound **15** (1 eq.) was then added and the solution was stirred at room temperature for 30 min. When the solutions were let to stirred overnight under the same conditions, hydrolysis of the methyl ester occurred giving products **17** and **19**. The DMSO solutions were lyophilized with water and the crude product were purified by size exclusion Biogel P-2 or P-4 columns giving compounds **16**, **17**, **18** and **19** in 94%, 93%, 56% and 53% yields respectively; compound **16**: $[\alpha]_D = +23.6^\circ$ (c = 0.50, MeOH); ^{1}H NMR (DMSO-d_6) δ 9.90 and 8.00 (bs, 2 H, NH′s), 7.83 (d, 3 H, H-ortho and H-para), 4.68 (d, 2 H, J = 3.3 Hz, OH-2), 4.59 (d, 2 H, $J_{1,2}$ = 1.2 Hz, H-1), 4.52 (d, 2 H, J = 5.8 Hz, OH-4), 4.40 (m, 4 H, OH-3 and OH-6), 3.83 (s, 3 H, Me), 3.68–3.61 (m, 8 H, H-6a, Ha and He), 3.58 (s, 2 H, H-2), 3.46–3.27 (m, 10 H, H-3, H-4, H-5, H-6b and Ha′), 2.70 (t, 4 H, J = 7.2 Hz, Hd), 2.59 (t, 4 H, J = 7.1 Hz, Hc), 1.79 (tt, 4 H, Hb); ^{13}C NMR (DMSO-d_6) d 180.4 (C=S), 165.8 (C=O), 139.8 (C-meta), 130.0 (C-ipso), 118.7 (C-para), 99.9 (C-1), 74.0 (C-5), 71.0 (C-3), 70.3 (C-2), 67.0 (C-4), 65.0 (Ca), 61.1 (C-6), 52.3 (Me), 43.5 (Ce), 29.8 (Cd), 29.3 (Cb), 27.8 (Cc); FAB-MS (pos.) for $C_{32}H_{52}N_4O_{14}S_4$ 844.2: found 845.3 ($M^+ + 1$, 0.5%); compound **17**: similar chemical shifts for both ^{1}H NMR and ^{13}C NMR except for H-ortho and H-para (7.70 ppm); FAB-MS (pos.) for $C_{31}H_{50}N_4O_{14}S_4$ 830.2: found 831.3 ($M^+ + 1$, 1.1%); compound **18**: $[\alpha]_D = +63.5^\circ$ (c = 1.00, H_2O); ^{1}H NMR (D_2O) δ 7.87 (s, 2 H, H-ortho), 7.72 (s, 1 H, H-para), 5.09 (s, 2 H, H-1′), 4.95 (d, 2 H, $J_{1'',2''}$ = 1.5 Hz, H-1″), 4.92 (s, 2 H, H-1), 4.14 (bs, 2 H, H-2), 4.10 (bs, 2 H, H-2′), 4.06 (m, 4 H, H-6a and H-2″), 4.01 (s, 3 H, Me), 3.95–3.71 (m, 44 H, H-3, H-3′, H-3″, H-4, H-4′, H-4″, H-5, H-5′, H-5″, H-6b, H-6a′, H-6b′, H-6a″, H-6b″, Ha, Hb, Hc and Hd); ^{13}C NMR (D_2O) d 179.6 (C=S), 167.1 (C=O), 131.3 (C-para), 123.0 (C-ortho), 101.9 (C-1), 99.7 (C-1′), 99.0 (C-1″), 78.2 (C-3), 72.8 (C-5′), 72.2 (C-5″), 70.6 (C-5), 70.2 (C-3″), 69.9 (C-3′), 69.6 (C-2), 69.5 (C-2′), 69.1 (C-2″), 69.0, 68.2 (Cb and Cc), 66.4 (Ca), 66.3 (2C) (C-4′ and C-4″), 65.2 (C-4), 64.8 (C-6), 60.5 (2C) (C-6′ and C-6″), 52.5 (Me), 43.9 (Cd); FAB-MS (pos.) for $C_{54}H_{88}N_4O_{36}S_2$ 1433.5: found 1433.5 (0.1%, $M^+ + 1$); compound **19**: $[\alpha]_D = +56.1^\circ$ (c = 1.20, H_2O); similar chemical shifts for both ^{1}H NMR and ^{13}C NMR except for H-ortho (7.64 ppm), H-para (7.61 ppm) and *C*OOH (172.8 ppm); FAB-MS (pos.) for $C_{52}H_{86}N_4O_{35}S_2$ 1418.4: found 1419.4 ($M^+ + 1$, 0.1%).

1,3,5-triisothiocyanatobenzene (**22**)

3,5-dinitroaniline **20** (100 mg, 0.55 mmol) was dissolved in MeOH (10 ml) containing a catalytic amount of 10% Pd-C. Hydrogen was bubbled for 1 h at room temperature. The catalyst was filtered through Celite and the solvent was evaporated under reduced pressure. The residue was then readily dissolved in acetone (10 ml) containing DIPEA (0.715 ml, 7.5 eq.) and thiophosgene (0.315 ml, 7.5 eq.) was added. The solution was stirred at room temperature for 30 min. The solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (twice) using hexanes: EtOAc/9:1 (v/v) as eluent, affording compound **22** (120 mg) in 88% yield; mp 60–61 °C; IR ($CHCl_3$) ν 2007.5 cm^{-1} (N=C=S, b); ^{1}H NMR ($CDCl_3$) δ 6.91 (s, 3 H, H-arom.); ^{13}C NMR ($CDCl_3$) δ 139.9 (N=*C*=S), 134.1 (*C*–H arom.), 121.1 (*C*–N=C=S); mass spectrum (CI) (rel. intensity) m/z 249.9 ($M^+ + 1$, 100%).

Synthesis of trivalent mannopyranosylated ligands **23** and **24**

Trivalent mannopyranosylated ligands **23** and **24** were synthesized following a similar procedure. Compounds **2** or **9** (3.6 eq.) were dissolved in DMSO (1 ml) containing 1 drop of DIPEA. Compound **22** (1 eq.) was added and the solution was stirred at room temperature for 45 min. The DMSO solutions were lyophilized with water and the products were purified by size exclusion Biogel P-2 columns, affording compounds **23** and **24** in 87% and 70% yields respectively; compound **23**: $[\alpha]_D = +20.0^\circ$ (c = 1.30, H_2O); ^{1}H NMR (D_2O) δ 7.23 (bs, 3 H, H-arom.), 4.93 (bs, 3 H, H-1), 4.02 (bs, 3 H, H-2), 4.01-3.87 (m, 12 H, H-3, H-6a, H-6b and Ha), 3.77 (dd, 3 H, $J_{3,4}$ = 9.6 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.68 (m, 6 H, H-5 and H-a′), 2.89 (bs, 6 H, Hd), 2.76 (bs, 6 H, Hc), 1.98 (bs, 6 H, Hb); ^{13}C NMR (D_2O) δ 178.9 (C=S), 119.3 (*C*-H arom.), 99.4 (C-1), 72.2 (C-5), 70.3 (C-3), 69.8 (C-2), 66.2 (C-4), 60.4 (C-6), 43.6 (Ce), 29.9 (Cd), 28.6 (Cb), 27.7 (Cc); FAB-MS (pos.) for $C_{42}H_{72}N_6O_{18}S_6$ 1140.3: found 1141.3 ($M^+ + 1$, 1.8%); compound **24**; $[\alpha]_D = +77.1^\circ$ (c = 1.00, H_2O); ^{1}H NMR (D_2O) δ 7.27 (s, 3 H, H-arom.), 5.11 (s, 3 H, H-1′), 4.96 (d, 3 H, $J_{1'',2''}$ = 1.6 Ha, H-1″), 4.92 (d, 3 H, $J_{1,2}$ = 1.5 Hz, H-1), 4.18 (dd, 3 H, H-2), 4.11 (d, 3 H, $J_{1',2'}$ = 1.7 Hz, $J_{2',3'}$ = 3.1 Hz, H-2′), 4.05 (m, 6 H, H-2″ and H-6a), 3.96–3.70 (m, 66 H, H-3, H-3′, H-3″, H-4, H-4′, H-4″, H-5, H-5′, H-5″, H-6b, H-6a′, H-6b′, H-6a″, H-6b″, Ha, Hb, Hc and Hd); ^{13}C NMR (D_2O) δ 179.5 (C=S), 119.3 (C-H arom.), 101.9 (C-1), 99.7 (C-1′), 99.0 (C-1″), 78.2 (C-3), 72.8 (C-5′), 72.2 (C-5″), 70.6 (C-5), 70.2 (C-3″), 69.9 (C-3′), 69.6 (C-2), 69.5 (C-2′), 69.2 (C-2″), 69.0, 68.3 (Cb and Cc), 66.4 (Ca), 66.3 (2C) (C-4′ and C-4″), 65.2 (C-4), 64.8 (C-6), 60.6, 60.5 (C-6′ and C-6″), 43.9 (Cd).

1,6-bis(3,5-dinitrobenzamido)hexane (**27**)

3,5-dinitrobenzoic acid (**25**) (500 mg, 2.36 mmol) was refluxed in $SOCl_2$ (5 ml) for 3 h under nitrogen. The solvent was then evaporated and coevaporated with toluene under reduced pressure. The resulting acyl chloride **26** was dissolved in dry CH_2Cl_2 to which was added dropwise a solution of hexamethylene diamine (100 mg, 0.86 mmol) and DIPEA (0.410 ml, 2.35 mmol) in CH_2Cl_2 over a period of 30 min at 0 °C. The solution was then let to stir at room temperature for 20 min or until the appearance of a yellowish precipitate. The precipitate was filtered and rinsed with

CH_2Cl_2, giving compound **27** (350 mg) in 81% yield; mp: 211–212 °C; ^{1}H NMR (THF-d_8), δ 9.06 (t, 2 H, H-para), 9.03 (d, 4 H, H-ortho), 8.39 (s, 2 H, NH), 3.45 (m, 4 H, NHCH_2), 1.67 (t, 4 H, J = 6.9 Hz, NHCH$_2$CH_2), 1.48 (tt, 4 H, J = 3.5 Hz, NHCH$_2$CH$_2$CH_2); ^{13}C NMR (THF-d_8) δ 163.2 (C=O), 149.7 (*C*-NO$_2$), 139.2 (*C*-C(O)NH), 127.9 (C-ortho), 121.2 (C-para), 40.8 (NH*C*H$_2$), 30.4 (NHCH$_2$*C*H$_2$), 27.3 (NHCH$_2$CH$_2$*C*H$_2$); mass spectrum (CI) (rel. intensity) *m/z* 504.9 ($M^+ + 1$, 100% base peak).

1,6-bis(3,5-diaminobenzamido)hexane (**28**)

Compound **27** (100 mg, 0.20 mmol) was dissolved in a mixture of THF: MeOH (1:1 v/v, 15 ml) containing a catalytic amount of 10% Pd-C. Hydrogen was bubbled for 3 h at room temperature. The catalyst was then filtered through Celite and the solvent was evaporated under reduced pressure. The product was recrystallized from 99% EtOH, giving compound **28** (61 mg) in 80% yield; mp: 111–112 °C; ^{1}H NMR (CD$_3$OD) δ 8.05 (m, 2 H, NH), 6.38 (ad, 2 H, H-para), 6.15 (ad, 4 H, H-ortho), 3.22 (dt, 4 H, NHCH_2), 1.50 (tt, 4 H, NH*C*H$_2$CH_2), 1.31 (at, 4 H, NHCH$_2$*C*H$_2$CH_2); ^{13}C NMR (CD$_3$OD) δ 171.5 (C=O), 149.8 (*C*-NH$_2$), 137.5 (*C*-C(O)NH), 106.2 (C-para), 105.7 (C-ortho), 40.8 (NH*C*H$_2$), 30.4 (NHCH$_2$*C*H$_2$), 27.7 (NHCH$_2$CH$_2$*C*H$_2$); FAB-MS (pos.) for $C_{20}H_{28}N_6O_2$ 384.2: found 385.2 ($M^+ + 1$, 1.5%).

1,6-bis(3,5-diisothiocyanatobenzamido)hexane (**29**)

Compound **28** (30 mg, 0.078 mmol) was suspended in acetone (8 ml) containing DIPEA (135 ml, 10 eq.). Thiophosgene (60 ml, 10 eq.) was added and the solution was stirred at room temperature for 15 min or until a distinct precipitate formed. The solution was concentrated and the precipitate was filtered and rinsed with acetone, giving **29** (27 mg) in 63% yield; mp: 257–258 °C; IR (KBr) ν 2064 cm^{-1} (N=C=S, b); ^{1}H NMR (DMSO-d_6) δ 8.62 (m, 2 H, NH), 7.79 (t, 2 H, H-para), 7.69 (d, 4 H, H-ortho), 3.22 (dt, 4 H, NHCH_2), 1.50 (tt, 4 H, NHCH$_2$CH_2), 1.31 (tt, 4 H, NHCH$_2$CH$_2$CH_2); FAB-MS (pos.) for $C_{24}H_{20}N_6O_2S_4$ 552.1: found 553.1 ($M^+ + 1$, 1.4%).

Synthesis of tetravalent mannopyranosylated ligands **30** and **31**

Tetravalent mannosylated ligands **30** and **31** were synthesized following a similar procedure. Compound **2** or **9** (4.8 eq.) were dissolved in DMSO (1 ml) containing 1 drop of DIPEA. Compound **28** (1 eq.) was added and the solutions were stirred at room temperature for 45 min. The DMSO solutions were lyophilized with water and the products were purified by size exclusion Biogel P-4 columns, affording products **30** and **31** in 71% and 72% yields respectively; compound **30**; $[\alpha]_D = +31.2^\circ$ (c = 1.00, DMSO); ^{1}H NMR (DMSO-d_6) δ 9.76, 8.36, 7.88 (3bs, 10 H, NH′s), 7.67 (bs, 2 H, H-para), 7.54 (bs, 4 H, H-ortho), 4.68–4.66 (m, 8 H, OH-2 and OH-5), 4.58 (bs, 4 H, H-1), 4.51 (d, 4 H, J = 5.9 Hz, OH-3), 4.40 (t, 4 H, J = 5.9 Hz, OH-6), 3.67–3.59 (m, 16 H, H-6a, He and Ha), 3.58 (bs, 4 H, H-2), 3.46–3.27 (m, 20 H, H-3, H-4, H-5, H-6b and Ha′), 3.22 (d, 4 H, NHCH_2), 2.69 (t, 8 H, J = 7.1 Hz, Hd), 2.58 (dt, 8 H, J = 6.8 Hz, Hc), 1.79 (dt, 8 H, Hb), 1.50 (bs, 4 H, NHCH$_2$CH_2), 1.32 (bs, 4 H, NHCH$_2$CH$_2$CH_2); ^{13}C NMR (DMSO-d_6) δ 180.6 (C=S), 165.6 (C=O), 139.1 (C-meta), 138.4 (C-ipso), 121.5 (C-para), 118.2 (C-ortho), 99.8 (C-1), 74.0 (C-5), 71.0 (C-3), 70.3 (C-2), 67.0 (C-4), 65.0 (Ca), 61.2 (C-6), 43.6 (Ce), 39.0 (NH*C*H$_2$), 29.8 (Cd), 29.3 (Cb), 29.1 (NHCH$_2$*C*H$_2$), 27.7 (Cc), 26.3 (NHCH$_2$CH$_2$*C*H$_2$); FAB-MS (pos.) for $C_{68}H_{112}N_{10}O_{26}S_8$ 1740.6: found 1741.7 ($M^+ + 1$, 0.2%); compound **31**: $[\alpha]_D = +57.4^\circ$ (c = 0.50, H_2O); ^{1}H NMR (D$_2$O) δ 7.66 (s, 2 H, H-para), 7.55 (s, 4 H, H-ortho), 5.10 (s, 4 H, H-1′), 4.95 (d, 4 H, $J_{1'',2''} = 1.6$ Hz, H-1″), 4.91 (s, 4 H, H-1), 4.16 (bs, 4 H, H-2), 4.05 (m, 8 H, H-6a and H-2″), 3.94–3.71 (m, 88 H, H-3, H-3′, H-3″, H-4, H-4′, H-4″, H-5, H-5′, H-5″, H-6b, H-6a′, H-6a″, H-6b′, H-6b″, Ha, Hb, Hc and Hd), 3.45 (bs, 8 H, NHCH_2), 1.70 (bs, 8 H, NHCH$_2$CH_2), 1.49 (bs, 8 H, NHCH$_2$CH$_2$CH_2); ^{13}C NMR (D$_2$O) δ 123.5 (C-para), 119.1 (C-ortho), 101.9 (C-1), 99.7 (C-1′), 99.0 (C-1″), 78.3 (C-3), 72.8 (C-5′), 72.2 (C-5″), 70.7 (C-5), 70.2 (C-3″), 69.9 (C-3′), 69.6 (C-2), 69.5 (C-2′), 69.2 (C-2″), 69.0, 68.3 (Cb and Cc), 66.4 (Ca), 66.2 (2C) (C-4′ and C-4″), 65.2 (C-4), 64.8 (C-6), 60.5 (2C) (C-6′ and C-6″), 43.8 (Cd), 39.4 (NH*C*H$_2$), 27.7 (NHCH$_2$*C*H$_2$), 25.3 (NHCH$_2$CH$_2$*C*H$_2$).

Enzyme linked lectin assay (ELLA)

Nunc microtitration plates were coated with yeast mannan (100 µl per well) diluted from a stock solution of 10 µg ml^{-1} in 0.01 M phosphate saline buffer (PBS, pH 7.3 containing 0.1 mM Ca^{+2} and 0.1 mM Mn^{+2}) at room temperature overnight. The wells were then washed three times with 300 µl per well of washing buffer (PBS containing 0.05% (v/v) Tween 20) (PBST). This washing procedure was repeated after each incubation throughout the assay. The wells were then blocked with 150 µl per well of 1% BSA/PBS for 1 h at 37 °C. After washing, the wells were filled with 100 µl per well of serial dilutions of *Concanavalin A*-peroxidase labeled (Con A-HRP) or *Pisum sativum* lectin-peroxidase labeled (PSA-HRP) from 10^{-1} to 10^{-5} mg ml^{-1} in PBS and incubated at 37 °C for 1 h. The plates were washed and 50 µl per well of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg per 4 ml) in citrate-phosphate buffer (0.2 M, pH 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 min by adding 50 µl per well of 1 M H_2SO_4 and the absorbances was measured at 410 nm relative to 570 nm. Blank wells contained citrate-phosphate buffer. The concentration of each lectin-enzyme conjugate that read an absorbance between 0.8–1.0 was used for inhibition experiments.

Inhibition experiments

The microtiter plates were coated overnight at room temperature with yeast mannan (100 µl of 10 µg ml^{-1} solution). The plates were then washed and blocked with BSA as described previously. The monomers and multivalent mannosylated ligands were used as stock solutions varying from 1 to 3 mg ml^{-1} of PBS. Each inhibitor was added in serial two-fold dilutions (60 µl per well) in PBS with 60 µl of the desired lectin-enzyme conjugate concentration on Nunclon (Delta) microtiter plates and incubated at 37 °C for 1 h. The above solutions (100 µl) were then transferred to the ligand-coated plates which were incubated for another hour at 37 °C. The plates were washed as described above and the ABTS substrate was added (50 µl per well). Color development was stopped after 20 min and the absorbances was measured at 410 nm relative to that at 570 nm. The data were plotted and analyzed using Graphpad Inplot Software, v. 4.03. The percent inhibitions were calculated as follows:

$$\% \text{ Inhibition} = (A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})})/A_{(\text{no inhibitor})} \times 100\%$$

IC_{50}'s were reported as the concentration required for 50% inhibition of the coating ligand. Each test was done in triplicate.

The relative differences in binding free energies ($\Delta\Delta G^{\circ}$) could be estimated (under specific conditions) according to the following equation: $-\Delta\Delta G^{\circ} = RT \ln (K_1/K_2)$ [42] where K_1 and K_2 are the amounts of inhibitors that provide 50% inhibition.

Turbidimetric analysis

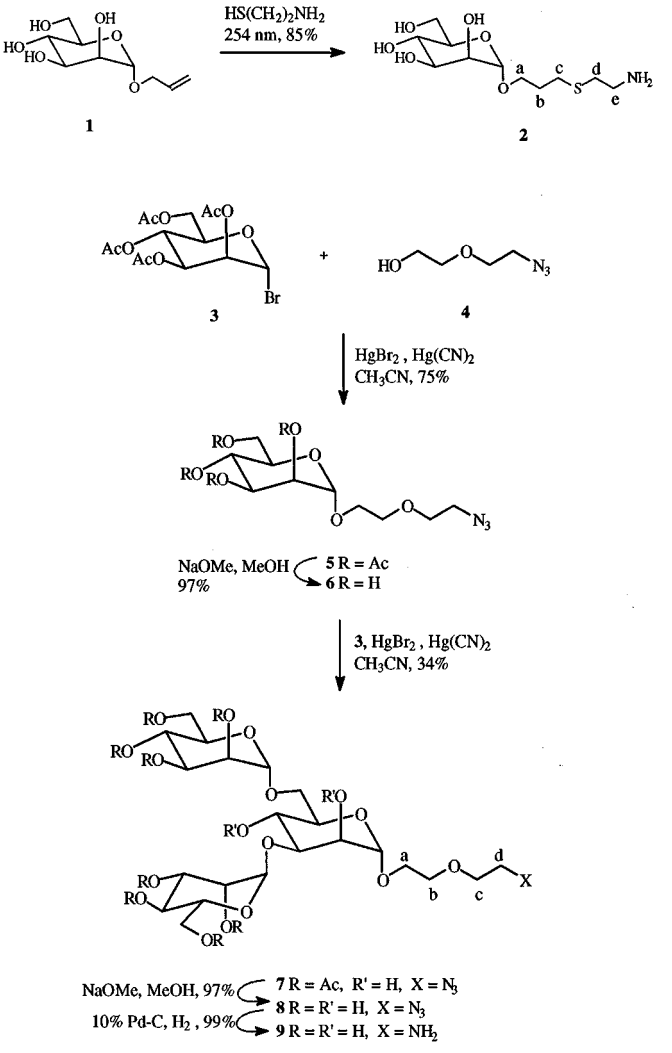
Turbidimetry experiments were performed in Linbro (Titertek) microtitration plates where 80 µl per well of stock lectin solution prepared from Con A (1 mg ml^{-1} PBS) was mixed with a volume corresponding to 10 nmol of hapten (trisaccharide) of stock solutions of inhibitors (1 mg ml^{-1} PBS). The solutions were diluted with PBS to obtain a final volume of 100 µl per well. The solutions were then incubated at room temperature for 2 h. The turbidity of the solutions was monitored by reading the absorbances at 490 nm at regular time intervals until no noticeable changes could be observed. Each test was done in triplicate.

Results and discussion

Synthesis of mannosylated ligands

All ligands were designed to share common structural features, their main differences residing in the nature of the terminal carbohydrate haptens (mono or trisaccharide) and the valency of the resulting ligand. The synthesis of the ligands was based upon the coupling of single or trimannoside residues bearing hydrophilic aglycons with terminal amino function to isocyanate or isothiocyanate cores *via* urea or thiourea linkages. This kind of procedure has previously been reported by our group [35, 36] and others and proved to create stable neoglycoconjugates.

The required α-D-mannopyranosylated monosaccharide precursor, having a heteroaliphatic spacer, was synthesized according to Scheme 1. Radical addition of cysteamine hydrochloride to known allyl α-D-mannopyranoside **1** [31] (H_2O, 254 nm, 35 °C, 3 h) provided 3-(2-aminoethylthio)propyl mannopyranoside **2** in 85% yield. It was desired that the trisaccharide hapten carry an aglycon with similar features to the one of compound **2**. Therefore, to a freshly prepared solution of acetobromomannose **3** (Scheme 1) was added 5-azido-3-oxapentanol **4** following a standard Koenigs-Knorr procedure ($Hg(CN)_2$, $HgBr_2$, CH_3CN, 3 m.s., 1 h) which afforded the 5-azido-3-oxapentyl α-D-mannopyranoside tetraacetate **5** in 75% yield. De-O-acetylation under standard Zemplén conditions (1 M NaOMe, MeOH, 30 min) afforded compound **6** in 97% yield.

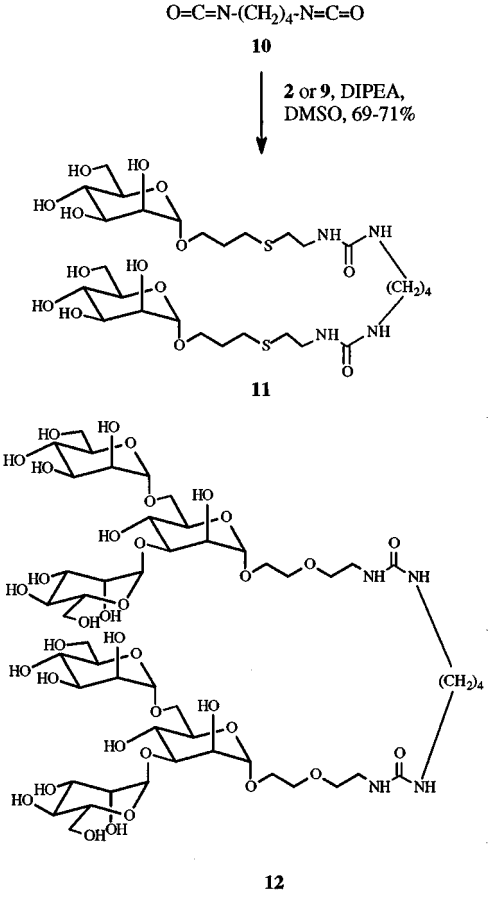


Scheme 1.

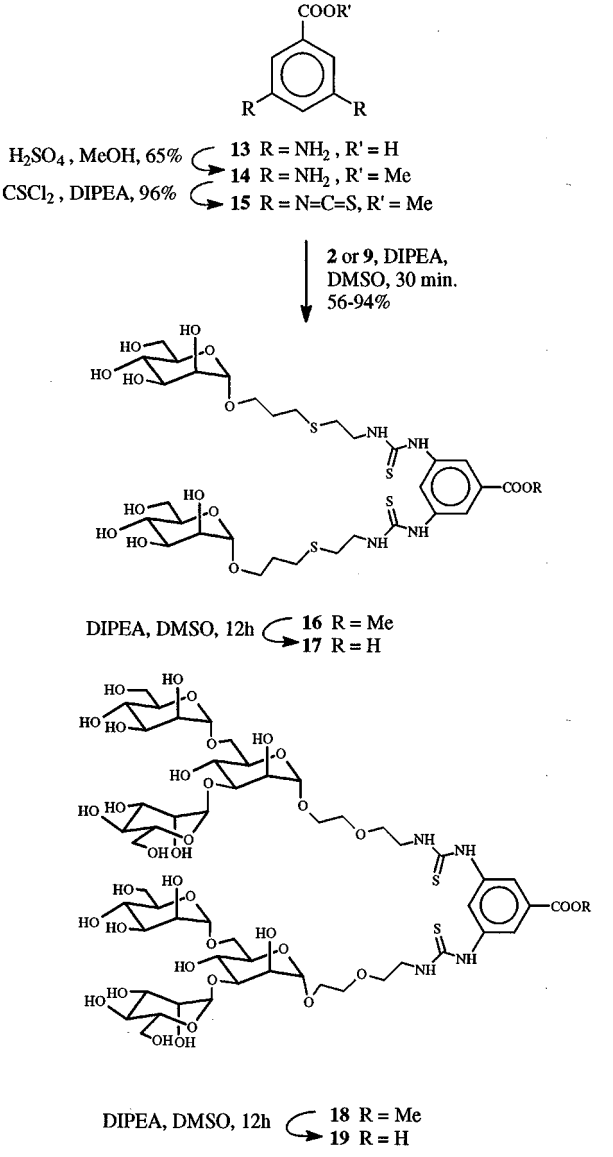
Many glycosylation procedures have been developed for the preparation of complex oligosaccharides [43]. However, the synthesis of complex N-glycans by classical chemical methods requires many steps involving time-consuming and delicate protecting group manipulations to produce, in this case, a mannose derivative with the 3- and 6-OH groups free for subsequent glycosylation. The overall yield of this sequential procedure is usually in the 30–40% range. We therefore used a one-step glycosylation procedure previously reported by Hindsgaul and co-workers [44]. Compound **6**, without any protecting groups, was glycosylated using classical Helferich glycosylation by adding 2.5 equivalents of acetobromomannose **3** along with $Hg(CN)_2$ and $HgBr_2$ as promoters (CH_3CN, 3 Å m.s., 1 h). The desired trisaccharide **7** could be easily isolated from the glycosylation mixture by simple silica gel column chromatography in 34% yield. Trimannopyranoside **7** was then de-O-acetylated under Zemplén conditions, followed by catalytic hydrogenation (H_2, 10% Pd-C, MeOH, 2 h) affording the corresponding amino-ending trimannopyranoside compound **9** in 97% yield.

Divalent ligands of different shapes were prepared to look at the effect of the ligand geometry on binding properties. Previous studies [35, 36] demonstrated these properties to be critical in the optimization of lectin-carbohydrate interactions. Therefore, to a solution of 1,4-diisocyanatobutane **10** (Scheme 2) in DMSO was added 2.4 equivalents of compound **2** or **9**, along with a catalytic amount of DIPEA. Lyophilization of the solutions, followed by purification by size exclusion chromatography (Biogel P-2) afforded divalent linear mannosylated ligands **11** and **12** in 69% and 71% yields respectively.

The other type of divalent clusters were centered around an aromatic tethering core to which the carbohydrate spacer arms were connected in both *meta* positions. This aromatic core (Scheme 3) was prepared by esterification of 3,5-diaminobenzoic acid **13** (MeOH, H_2SO_4, reflux, 2 days) to afford methyl ester **14** in 65% yield. Transformation of both aromatic amines into bis-isothiocyanate was



Scheme 2.

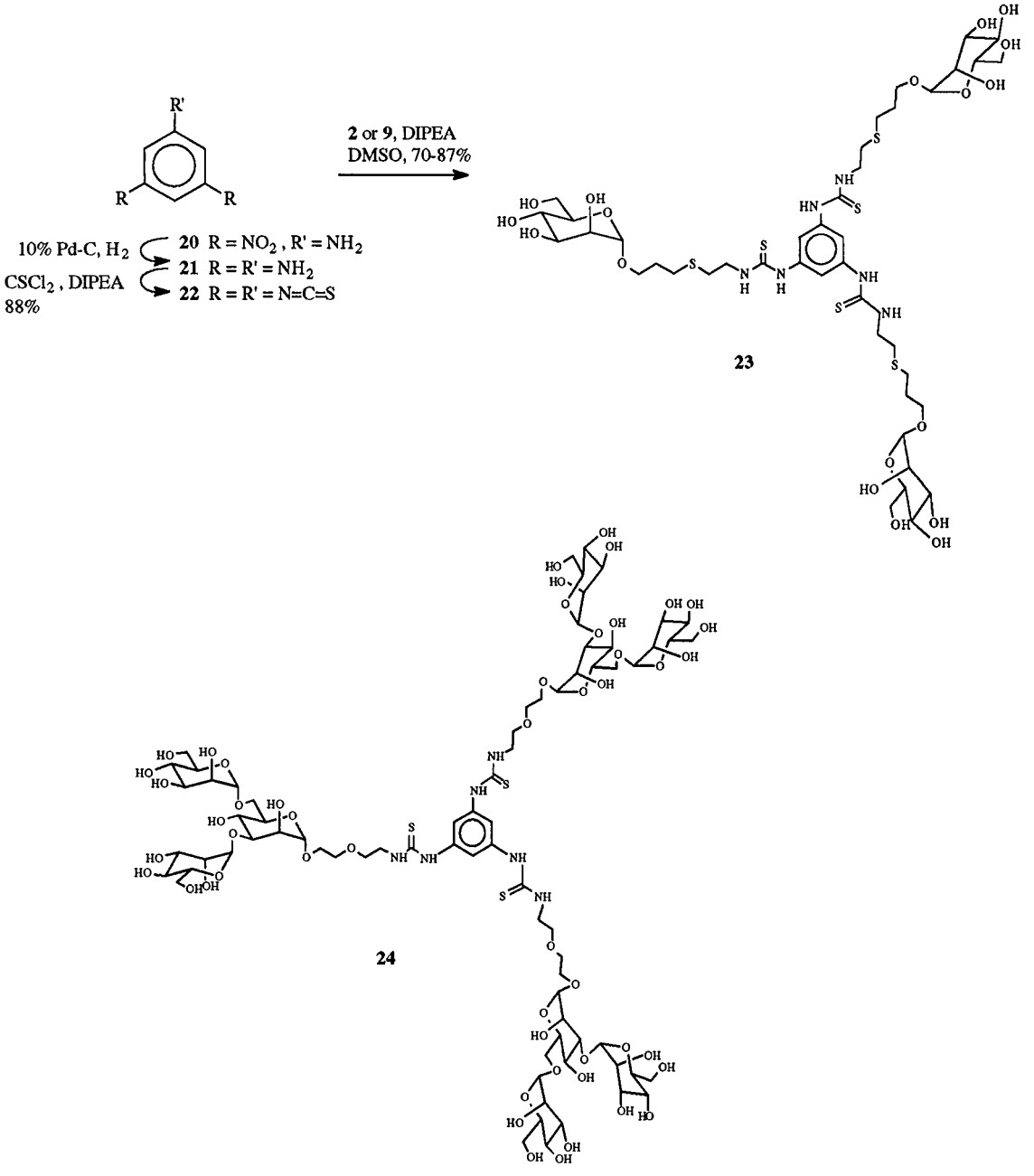


Scheme 3.

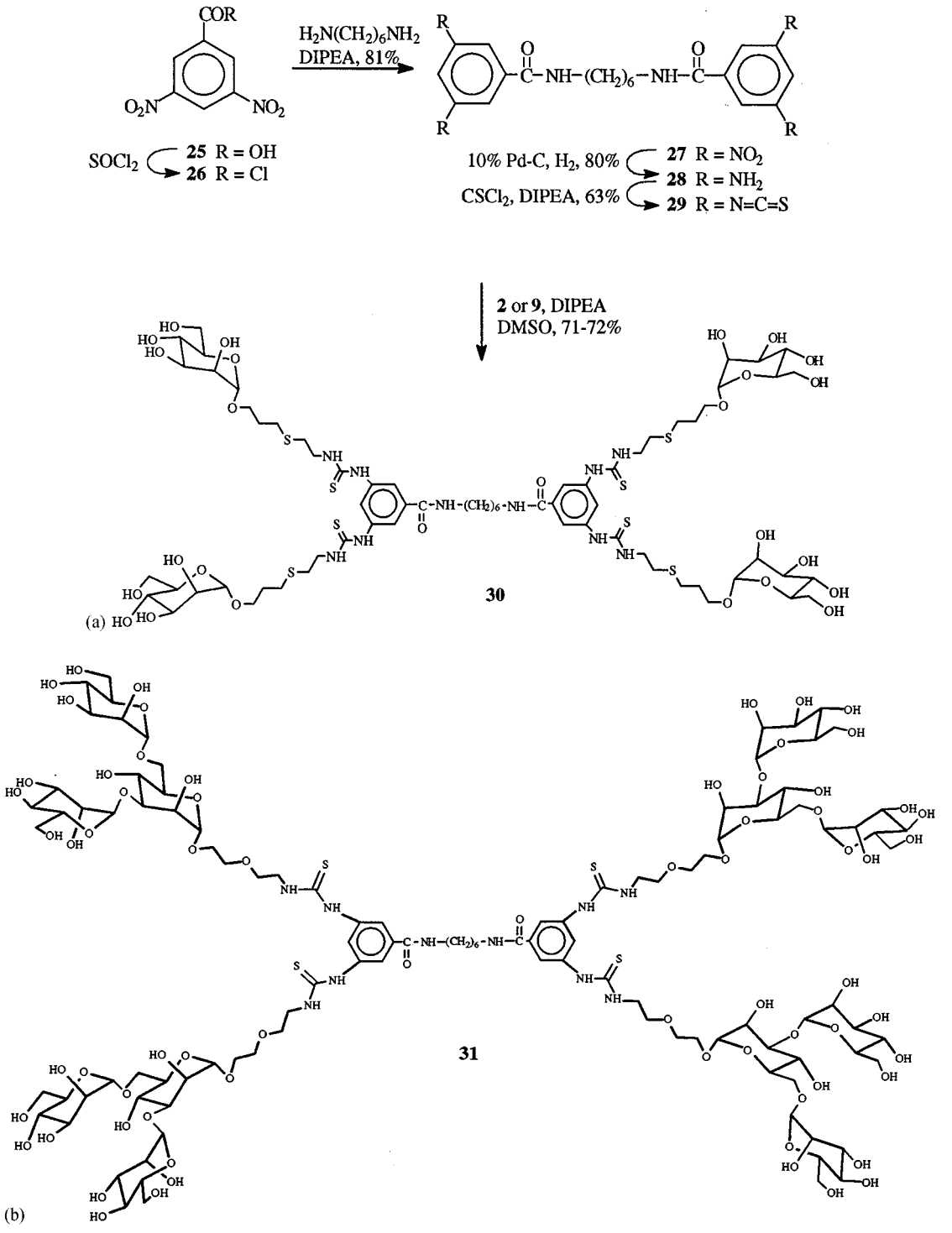
accomplished with thiophosgene ($CSCl_2$, CH_2Cl_2, DIPEA, 25 °C, 45 min) to provide **15** in 96% yield. Coupling mannopyranosylated amines **2** and **9** to diisothiocyanate **15** (2.4 eq., DMSO, DIPEA, 30 min) provided divalent ligands **16** and **18** in 94% and 56% yields. When the reaction mixtures were stirred overnight at room temperature, hydrolysis of the methyl ester was shown to occur, affording compounds **17** and **19** in 93% and 53% yields after purification.

Trivalent ligands **23** and **24** (Scheme 4) were also synthesized from an aromatic isothiocyanate tethering core. Catalytic hydrogenation of 3,5-dinitroaniline **20** (H_2, 10% Pd-C, MeOH, 1 h) afforded the corresponding triamine **21** which was readily reacted with thiophosgene ($CSCl_2$ 7.5 eq., acetone, DIPEA, 30 min) providing 1,3,5-triisothiocyanatobenzene **22** in 88% yield. Addition of monomers **2** and **9** (3.6 eq., DMSO, DIPEA, 45 min) afforded trivalent mannopyranosylated ligands **23** and **24** in 87% and 70% yields respectively.

Finally, the tetravalent mannopyranosylated clusters were prepared according to Scheme 5. 3,5-Dinitrobenzoic acid **25** was refluxed in $SOCl_2$ to form the corresponding acyl chloride **26** which was readily coupled with



Scheme 4.



Scheme 5.

hexamethylenediamine (CH_2Cl_2, DIPEA, 0 °C, 30 min) to produce tetramer **27** in 81% yield. Compound **27** was subjected to catalytic hydrogenation (H_2, 10% Pd-C, THF/MeOH, 3 h) affording the corresponding tetraamine **28** in 80% yield. Addition of thiophosgene (10 eq., acetone, DIPEA, 15 min) provided tetraisothiocyanate **29** in 63% yield. Coupling of monomers **2** and **9** (4.8 eq.) under the previously described conditions gave tetramannosylated ligands **30** and **31** in 71% and 72% yields respectively.

Mannopyranosylated ligands as potent inhibitors of binding

The efficiency of each of the ligands to inhibit the binding of yeast mannan to Con A and pea lectin was measured by ELLA. The results of the inhibition are shown in Figure 2 and Table 1. Methyl α-D-mannopyranoside (Me α-D-Man) was used as monomer for comparison purposes. The results with Con A clearly demonstrated the better binding properties of the ligands bearing the trimannoside {Man α(1 → 6) [Man α(1 → 3)]Man} structures. Trimannoside monomer **8** was close to 100 times more potent than Me α-D-Man. This is in accordance with the results previously reported by Brewer and co-workers [45] who found a similar value for Man α(1 → 6)[Man α(1 → 3)]Man α-OMe. As mentioned earlier, this enhanced binding character is the net result of the direct recognition of all three sugar units by the lectin [38, 39].

Divalent ligands **12**, **18** and **19** with IC_{50} values ranging from 1.8 to 7.0 μM were much more potent inhibitors than the corresponding ligands bearing monosaccharide haptens. Furthermore, compounds **18** and **19** were shown to be the best divalent ligands being 462 and 513 times more potent than Me α-D-Man, which represent a 231- and 257-fold increased in inhibitory potential based on per mannoside-hapten residues. These observations also underline the importance of the geometry of the ligands as divalent ligands **16** to **19** with the aromatic tethering core were better inhibitors than their corresponding divalent linear ligands **11** and **12**. Interestingly, when expressed on a per hapten basis, dimeric trimannoside **19** was slightly more potent than trimeric trimannoside **24** for both lectins (relative potency of 257:205 (Con A) and 5.4:5.2 (pea lectin), Table 1). These results further support the concept of well organized lattices and would indicate that one too many trimannoside ligands is 'floating free' and unbound in the trimer **24**.

Increase in the ligand valency also produced an increase in inhibitory potencies for both mono- and tri-saccharide ligands. The best overall inhibitor was shown to be tetramer **31** with an IC_{50} of 0.8 μM which constitutes greater than a 1155-fold increase in relative potency and 4.3 kcal mol^{-1} difference in binding energy when compared to Me α-D-Man. This IC_{50} value is even twice higher than the one previously reported for a poly-L-lysine glycodendrimer bearing as many as 16 terminal mannoside residues (IC_{50} = 1.6 μM) [33]. These values truly show the potential of low-molecular weight molecules as high affinity ligands.

The results of the inhibition of pea lectin (PSA) are also reported in Table 1. The values are much lower and in sharp contrast with those of Con A. The presence of the trimannoside structure did not seem to greatly affect the binding properties of the ligands. Stubbs *et al.* [46] previously reported that Man α(1 → 6)[Man α(1 → 3)]Man α-OMe binds to pea lectin with only slightly greater affinity than Me α-D-Man. The pea-trimannoside X-ray structure [47] showed that, as opposed to Con A, only a single mannose residue is found in the monosaccharide-binding site explaining the lack of any enhanced binding properties by the ligands. However, a modest enhancement of inhibitory properties were still obtained when the valency of the ligands was increased from 2 to 4.

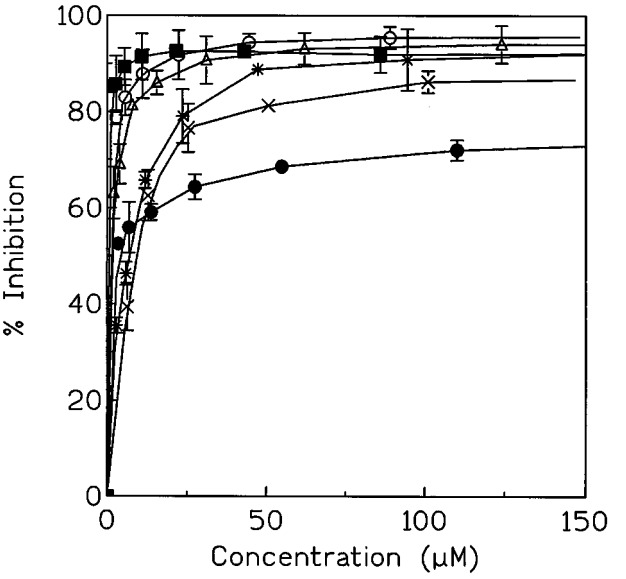


Figure 2. Results from the inhibition of binding of peroxidase-labeled Con A to yeast mannan by mannopyranosylated ligands **8** (X), **12** (*), **19** (○), **23** (●), **24** (△), and **31** (■).

Table 1. Results from the inhibition of binding of peroxidase-labeled Conc A and pea lectins to yeast mannan by multivalent mannopyranosylated ligands

Compound	*Con A*		*Pea lectin*	
	IC_{50} (μM)	*Relative potency*[a]	*IC_{50} (μM)*	*Relative potency*[a]
Me *α*-D-Man	924	1	3850	1
1	261	3.5	940	4.1
8	9.4	98.3	> 810[b]	—
11	> 680[b]	—	> 680[b]	—
12	7.0	132 (66)	> 378[b]	—
16	6.7	138 (69)	129	29.8 (14.9)
17	30.5	30.3 (15.2)	185	20.8 (10.4)
18	2.0	462 (231)	243	15.8 (7.9)
19	1.8	513 (257)	356	10.8 (5.4)
23	4.4	210 (70)	79.1	48.7 (16.2)
24	1.5	616 (205)	248	15.5 (5.2)
30	4.0	231 (57.8)	27.5	140 (35)
31	0.8	1155 (289)	73.5	52.4 (13.1)

[a] Value in parentheses are on a per-hapten basis.
[b] Gave between 28 and 38% inhibition.

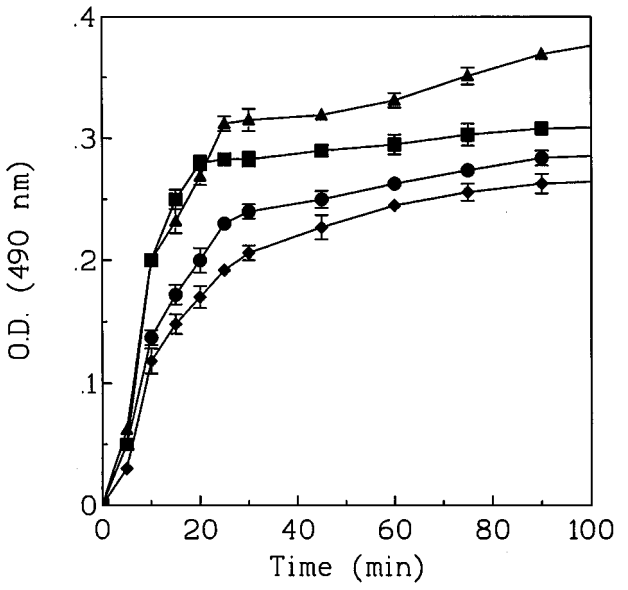


Figure 3. Time course of turbidimetric analyses of Con A with 10 nmol each of trisaccharide haptens in **12** (◆), **19** (△), **24** (●), **31** (■).

The huge difference of the binding affinities observed between the two lectins also resides in the fact that, at physiological pH, Con A exists as tetramers that facilitate the formation of a stable cross-linked lattice with mannosylated ligands (Figure 1A) [19], whereas the dimeric character of the pea lectin does not promote such a stable lattice. Further evidences were obtained by turbidimetric analyses. When the equivalent of 10 nmol of carbohydrate hapten was added to a solution of Con A, clear visible insoluble complexes could be readily seen after a few minutes of contact (Figure 3). No precipitation could be observed when the same experiment was repeated with pea lectin. The only ligand that enabled the formation of such a cross-linked lattice with pea lectin (not shown) was tetravalent ligand **30** which also showed to be the best inhibitor being 140 times more potent than Me α-D-Man.

Conclusion

Clusters of di-, tri-, and tetravalent mannopyranosylated ligands with mono- or tri-saccharide ending haptens were synthesized in good yields. They showed good inhibitory properties against model plant lectins in solid phase enzyme-linked lectin assays. Most interestingly, ligands bearing terminal Man α(1 → 6)[Man α(1 → 3)]Man residues were shown to be very potent inhibitor of Con A-yeast mannan interactions being up to 1150 more potent than Me α-D-Man. Such ligands may find potential applications as inhibitors of bacterial adherence since most fimbriated pathogens bind these structures with high affinity. Work is presently ongoing in order to reach these goals.

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