Functional Evaluation of Carbohydrate-Centred Glycoclusters by Enzyme-Linked Lectin Assay: Ligands for Concanavalin A

Maja Köhn,[c] Juan M. Benito,[c] Carmen Ortiz Mellet,*[a] Thisbe K. Lindhorst,*[b] and José M. García Fernández*[c]

*The affinities of the mannose-specific lectin concanavalin A (Con A) towards D-glucose-centred mannosyl clusters differing in the anomeric configuration of the monosaccharide core, nature of the bridging functional groups and valency, have been measured by a competitive enzyme-linked lectin assay. Pentavalent thioether-linked ligands (**5** and **7**) were prepared by radical addition of 2,3,4,6-tetra-O-acetyl-1-thio-α-D-mannopyranose to the corresponding penta-O-allyl-α- or -β-D-glucopyranose, followed by deacetylation. The distinct reactivity of the anomeric position in the D-glucose scaffold was exploited in the preparation of a tetravalent cluster (**10**) that keeps a reactive aglyconic group for further manipulation, including incorporation of a reporter group or attachment to a solid support. Hydroboration of the double bonds in the penta-O-allyl-α-D-glucopyranose derivative and replacement of the hydroxy groups with amine moieties gave a suitable precursor for the preparation of pentavalent and 15-valent mannosides through the thiourea-bridging reaction (**17** and **20**, respectively). The diastereomeric 1-thiomannose-coated clusters **5** and **7** were demonstrated to be potent ligands for Con A, with IC_{50} values for the inhibition of the Con A–yeast mannan association indicative of 6.4- and 5.5-fold increases in binding affinity (valency-corrected values), respectively, relative to the value for methyl α-D-mannopyranoside. The tetravalent cluster **10** exhibited a valency-corrected relative lectin-binding potency virtually identical to that of the homologous pentavalent mannoside **7**. In sharp contrast, replacement of the 1-thiomannose wedges of **5** with α-D-mannopyranosylthioureido units (**17**) virtually abolished any multivalent or statistic effects, with a dramatic decrease of binding affinity. The 15-valent ligand **20**, possessing classical O-glycosidic linkages, exhibited a twofold increase in lectin affinity relative to the penta-O-(thioglycoside) **5**; it is less efficient based on the number of mannose units. The results illustrate the potential of carbohydrates as polyfunctional platforms for glycocluster construction and underline the importance of careful design of the overall architecture in optimising glycocluster recognition by specific lectins.*

Introduction

Interactions between carbohydrate-binding proteins (lectins) and the oligosaccharide moieties of glycoprotein and glycolipid components of extracellular matrices and cell surfaces are involved in extensive cellular recognition processes including development, differentiation, morphogenesis, fertilisation, the immune response, implantation, cell migration and cancer metastasis.[1] A common feature of lectin binding to carbohydrate ligands is the intrinsic weak affinities at work, with association constants (K_a) typically in the mM range.[2] Presentation of the sugar epitopes as multiple copies on an appropriate scaffold (molecular, dendritic, polymeric) creates a multivalent display that can efficiently mimic the natural mode of affinity enhancement that arises from multiple interactions between the binding proteins and the carbohydrate ligands.[3] This concept, termed the multivalent or cluster effect,[4] has found application in the development of therapeutic agents designed to interfere with carbohydrate molecular recognition,[5] in carbohydrate-based anticancer drugs,[6] in vectorised drug or probe delivery[7] and in the targeted aggregation and clearance of pathogenic species.[8]The lectin-binding efficiency and specificity of glycoclusters have been found to be dependent not only on the epitope density but also on the nature of the core and on the geometrical characteristics of the multivalent assembly. In our laboratories, we have recently focussed on the use of carbohydrates as polyfunctional cores for glycocluster and glycodendrimer construction.[9,10] The possibility of selective fuctionalisation of the different hydroxy groups by well-established methodologies and control of the relative orientation of the branches by configurational and conformational bias makes these "full-carbohydrate glycoclusters" ideally suited for

[a] *Prof. C. Ortiz Mellet*
Departamento de Química Orgánica, Facultad de Química
Universidad de Sevilla
Apartado 553, 41071 Sevilla (Spain)
Fax: (+34) 954-624-960
E-mail: mellet@us.es

[b] *Prof. T. K. Lindhorst*
Institute of Organic Chemistry, Christiana Albertina University Kiel
Otto-Hahn-Platz 34, 24098 Kiel (Germany)
Fax: (+49) 431-880-7410
E-mail: tklind@oc.uni-kiel.de

[c] *M. Köhn, Dr. J. M. Benito, Dr. J. M. García Fernández*
Instituto de Investigaciones Químicas, CSIC
Américo Vespucio 49, Isla de la Cartuja, 41092 Sevilla (Spain)
Fax: (+34) 954-460-565
E-mail: jogarcia@iiq.csic.es

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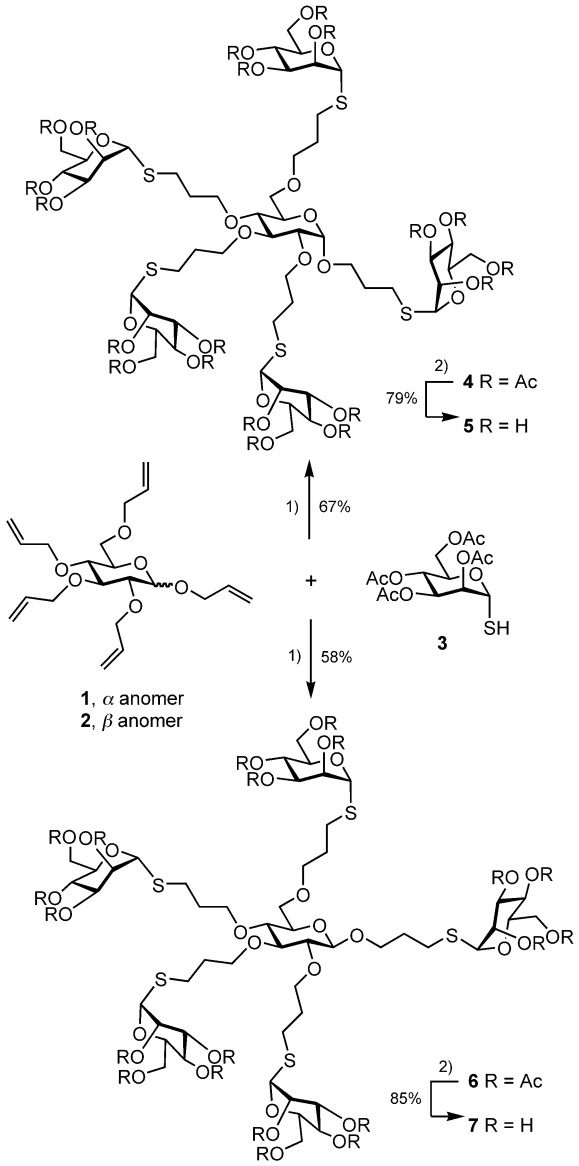
mapping the geometrical requirements for efficient lectin binding and for checking the current notions of multivalent carbohydrate–protein interactions. In this context, we now report on the preparation of a series of D-glucose-centred glycoclusters coated with α-D-mannopyranosyl units and the evaluation of their binding ability to the mannose-specific lectin concanavalin A (Con A) by a competitive enzyme-linked lectin assay (ELLA), with immobilised yeast mannan used as the reference ligand. Thioglycoside, glycosylthiourea and *O*-glycosidic derivatives have been comparatively assayed in order to pinpoint the influence of the bridging group in the recognition process. The effects of the D-glucopyranose core, the anomeric configuration and the glycocluster valency are also discussed.

Results and Discussion

Synthesis

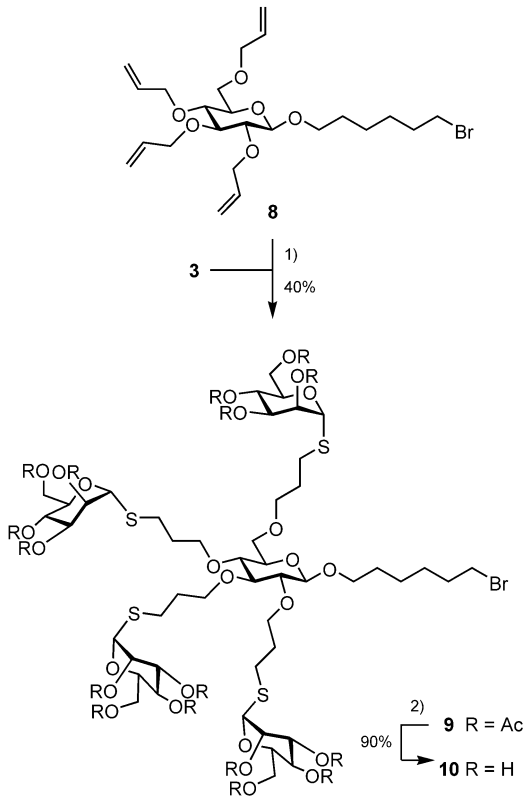
The known allyl 2,3,4,6-tetra-*O*-allyl-α- and -β-D-glucopyranosides **1**[9c] and **2**[5b] were chosen as the core building blocks for the preparation of pseudosymmetric pentabranched derivatives (Scheme 1). The versatile reactivity of the terminal olefin groups can be exploited in different ways to produce glycoclusters with a variety of linking functional groups. In this work we have purposely focussed on thioether and thiourea derivatives, since thioglycosides[11] and glycosylthioureas[12] rank amongst the most broadly used epitopes in multivalent neoglycoconjugate synthesis. Thus, photochemically promoted radical addition of 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside[13] (**3**) to **1** and **2** in methanol afforded the anti-Markovnikov adducts **4** and **6**,[14] respectively, which after conventional base-catalysed deacetylation, yielded the fully unprotected penta-*O*-(thiomannosides) **5** and **7** (Scheme 1). An analogous reaction sequence starting from 6-bromo-*n*-hexyl 2,3,4,6-tetra-*O*-allyl-β-D-glucopyranoside (**8**)[9c] afforded the tetravalent thiomannosyl ligand **10** bearing a functionalised tether at the anomeric position (Scheme 2). The presence of this reactive aglyconic group can be exploited for the incorporation of a fluorescent dye or for attaching the glycocluster to a polyfunctional scaffold or a solid support.[9c]



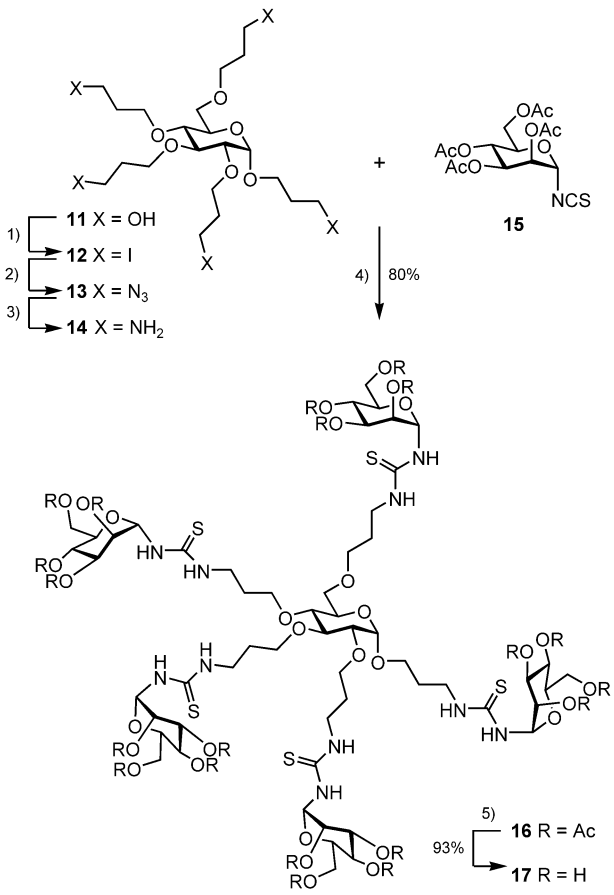
Scheme 1. *Pentavalent thiomannoside clusters **5** and **7**. 1)* hν *(254 nm), MeOH, 2 h; 2) NaOMe, MeOH.*

For the preparation of D-glucose-centred glycoclusters through the thiourea-bridging strategy, the penta-*O*-(3-hydroxypropyl) derivative **11**,[9d] resulting from hydroboration of **1**, was transformed into the corresponding penta-*O*-(3-iodopropyl) derivative **12** by reaction with the iodine/triphenylphosphine system in *N*,*N*-dimethylformamide (Scheme 3).[15] Nucleophilic displacement of the halogen atoms by azido groups ($\rightarrow$ **13**) and subsequent reduction with propanedithiol/triethylamine[16] led to the corresponding penta-*O*-(3-aminopropyl) D-glucoside derivative **14**. A coupling reaction of this pentaamino nucleophile with 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl isothiocyanate **15**[17] in pyridine provided the corresponding pentavalent α-D-mannopyranosylthiourea adduct **16** in 80% yield. The further deacetylation step with sodium methoxide in methanol was effected at 0 °C to avoid anomerisation at the mannopyranosylthiourea units.[18] Since precipitation of partially deprotected products occurred, a small amount of water was added at the final stage to ensure complete saponification of the ester groups. In this way, the target fully unprotected glycocluster **17** was obtained in virtually quantitative yield (Scheme 3).

In order to implement the above approach for the preparation of higher-valent D-glucose-centred glycoclusters, the coupling reaction of **14** with the isothiocyanate-armed trivalent mannosyl dendron **18**[19] was attempted (Scheme 4). However, a complex mixture of undersubstituted products was obtained in pyridine even after prolonged reaction times at 60 °C with a high excess of **18**. The pentameric thiourea adduct **19** could be obtained by carrying out the reaction in water/acetone at pH 8 (adjusted with sodium hydrogencarbonate), although some deacetylation occurred at this stage. Further deacetylation afforded the pentabranched 15-valent glycocluster **20** with α-D-mannopyranoside wedges (Scheme 4).



*Scheme 2. Tetravalent thiomannoside cluster **10**. 1) hν (254 nm), MeOH, 2 h; 2) NaOMe, MeOH.*



*Scheme 3. Pentavalent mannopyranosylthioureido cluster **17**. 1) I_2, triphenylphosphine, DMF, 80 °C, 68 %; 2) NaN_3, DMF, 90 °C, 16 h, 79 %; 3) 1,3-propanedithiol, Et_3N, MeOH, 48 h, 41 %; 4) pyridine, RT, 16 h; 5) NaOMe/MeOH, then water, 0 °C. DMF = N,N-dimethylformamide.*

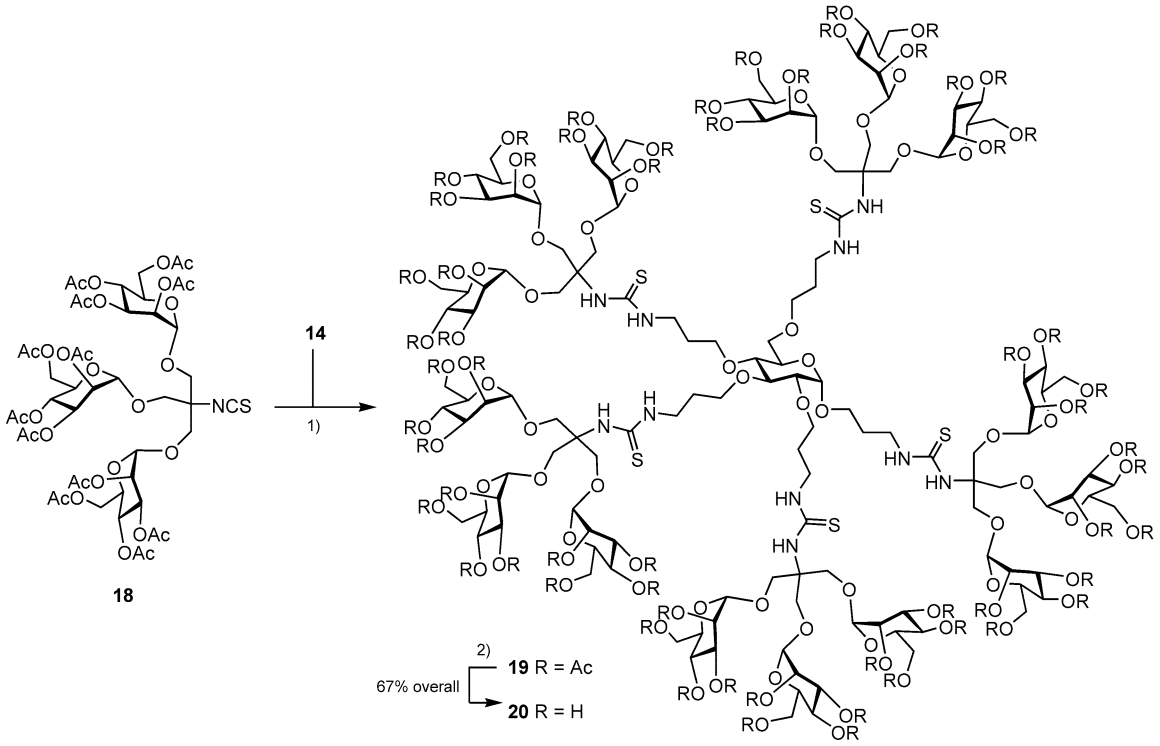
Both the radical addition of thiols to *O*-allyl derivatives and the thiourea-bridging reaction proved to be convenient strategies for the preparation of D-glucose-centred full-carbohydrate glycoclusters. It is worth mentioning that purification of the polyvalent glycoconjugates could be effected very efficiently at the acetylated or partially acetylated stage by simple column chromatography. After deacetylation, the final compounds were already obtained in high purity. In all cases, a final gel permeation chromatography (GPC) step was performed to obtain analytically pure homogeneous samples that were used in lectin-binding studies. The mass spectrometry and ^1H and ^{13}C NMR spectroscopy data were consistent with the proposed structures. In the case of the thiourea adducts, their NMR spectra recorded at room temperature exhibited significant line broadening due to the existence of slow rotation processes about the NH–C(=S) thiourea bonds.[20] Nevertheless, satisfactory spectra could be obtained at higher temperatures.

Binding of concanavalin A to D-glucose-centred polyvalent mannosides

The affinities of the prepared D-glucose-centred glycoclusters towards Con A were measured by ELLA.[21] ELLA measures the ability of a soluble saccharide to inhibit the association between a labelled lectin and a polymeric ligand fixed on the microtiter well (Con A labelled with horseradish peroxidase (HRP) and yeast mannan, respectively, in the present case). The concentration needed to achieve 50 % inhibition (IC_{50}) is then assumed to be inversely proportional to the lectin-saccharide binding free energy. Examination of literature data reveals, however, a relatively high dispersion of ELLA IC_{50} values for identical systems,[22] which makes a direct comparison of results difficult. To have a common reference with our former work, methyl α-D-mannopyranoside was included as a monovalent model compound in all of the series of measurements. The IC_{50} value obtained (865 μM) was virtually identical to that previously encountered (870 μM).[10a]

Results in triplicate were used for the plotting of the inhibition curves for each individual ELLA experiment. Typically, the IC_{50} values obtained from several independently performed tests were within ±15 %. Nevertheless, the relative inhibition potencies calculated from independent series of data were highly reproducible. The results are collected in Table 1.

The IC_{50} values of the pentavalent clusters **5** and **7** (27 and 31 μM, respectively), were indicative of strong Con A affinity enhancements relative to methyl α-D-mannopyranoside (6.4- and 5.5-fold on a per mannose basis). Since replacement of the aglyconic oxygen atom with a sulfur atom does not introduce significant structural changes in the glycosides,[23] it can



Scheme 4. *15-Valent mannopyranosyl cluster* ***20****. 1) Acetone/water (1:1), overnight; 2) NaOMe/MeOH, then water.*

Table 1. *ELLA data for binding inhibition of HRP-labeled Con A by* D*-glucose-centred polyvalent mannosides.*

Parameter	Compound				
	5	**7**	**10**	**17**	**20**
IC_{50} [μM]	27	31	40	640	18.5
relative potency[a]	6.4	5.5	5.4	0.27	3.1

[a] Per mol of mannopyranosyl residue relative to methyl α-D-mannopyranoside (IC_{50} = 865 μM).

be reasonably assumed that the observed increases in lectin affinity are associated with the multivalent presentation of the mannosyl ligands on the C_3-tethered α- or β-D-glucopyranoside scaffold, that is, the cluster effect. The tetravalent mannoside **10** exhibited a valency-corrected relative binding potency towards Con A that was virtually identical to that of the pentavalent derivative **7**, a result indicating that the presence of the four nonanomeric α-D-mannopyranosyl branches is sufficient to elicit a cluster effect, with the fifth branch still contributing to a statistic affinity increase.

Although relatively small, the difference in lectin-binding affinity between polyvalent mannosides differing in the anomeric configuration of the D-glucopyranoside scaffold was highly reproducible in a series of experiments, including different glycocluster preparations. Since Con A is known to bind α-D-glucopyranosides with a tenfold weaker affinity than the corresponding *manno* diastereomers,[16a] the possibility of an additional interaction between the core and the lectin in the case of the α-D-glucopyranoside-centred polymannoside **5** was considered. However, no inhibition of the Con A–yeast mannan association was observed in the presence of the penta-*O*-(3-hydroxypropyl) α-D-glucopyranoside derivative **11** at concentrations up to 5 mM. Most probably, the observed difference reflects a slightly more favourable disposition of the recognition epitopes in the α anomer than in the all-equatorial β anomer, in other words, an orientational effect.

Evaluation of the lectin-binding affinity of the penta-*O*-(α-D-mannopyranosylthioureido) cluster **17** by ELLA revealed a totally different scenario. The corresponding IC_{50} value (640 μM), in the same order as that for methyl α-D-mannopyranoside, indicated a significant decrease on valency-corrected relative potency (0.27), much lower than expected even from statistic considerations assuming identical thermodynamic parameters for the interaction between each individual mannopyranoside epitope and the lectin. Although a 40% decrease in binding affinity has been observed for monovalent α-D-mannopyranosylthioureas as compared with methyl α-D-mannopyranoside, multivalent mannopyranosylthiourea displays have been shown previously to exhibit a significant cluster effect upon interaction with Con A.[7a] It is therefore inferred that the observed result is a consequence of the radial disposition of the mannopyranosylthiourea wedges imposed by the rigid glucopyranose scaffold in the $^4C_1$ (D) chair conformation, in combination with the particular electronic and conformational properties of the semirigid thiourea segments. Actually, pseudoamide anomeric groups seem to impart a strong dependence of the glycocluster–lectin recognition properties upon the geometrical characteristics of the multivalent construct, in view of the dramatic differences recently observed between thioglyco-

side and glycosylthioureido- or glycosylamido-coated glycoclusters based on the cyclodextrin core.[10a, 11]

The 15-valent pentabranched mannoside **20**, with classical *O*-glycosidic bonds, was a very efficient inhibitor of the Con A–yeast mannan association ($IC_{50} = 18.5$ μM). However, the valency-corrected relative potency (3.1) is about half that of the pentavalent derivative **5**. Previous studies[23] of the Con A affinity of tris-(2-aminoethyl)methylamine-based mannosyl dendrons appended on achiral branching elements provided relative potencies of 3.2 and 16.0 for trivalent and hexavalent derivatives, respectively, while affinity enhancements of about fourfold (mannose molar basis) have been reported[19] for 1,3,5-benzenetricarboxylic acid-centred dendrimers with 9, 18 and 36 mannose residues from ELLA data. It seems, therefore, that a reduced number of binding epitopes (three to nine depending on the glycocluster architecture; four or less in this particular study) can display a maximum cluster effect, with further increases in valency resulting in an statistic binding enhancement, if any.

The origin of the cluster effect, which refers to a greater affinity enhancement than expected from the sum of the constitutive one-to-one interactions, remains controversial.[4a] Enthalpic stabilisation through the classical chelate effect is possible for glycoclusters with long enough spacers to expand different carbohydrate-binding sites in the lectin. An outstanding example, in connection with the present work, is the preparation of a remarkably efficient pentabranched ligand for cholera toxin by using D-glucose as a scaffold.[5b] Alternatively, entropy-driven precipitation of three-dimensional cross-linked complexes or clustering of the lectin in solution has been argued to be responsible.[24] As expected, such cross-linking interactions between multivalent lectins and polyvalent glycoconjugates are generally favoured for high-valent ligands with relatively long spacer arms. Provided that these requirements are fulfilled, the influence of the scaffold structure is rather limited.

Under ELLA conditions, however, aggregation phenomena are supposed to be impaired due to the presence of the 40 kD peroxidase protein.[21a] In fact, no precipitation was observed during incubation of lectin/ligand solutions at all the studied concentrations.[25] The significant affinity enhancements observed by this technique in our case support the existence of an aggregation-independent microcluster effect[26] which reaches a maximum at a limited number of haptens, is active even for rather short intersaccharide spacers and is highly sensitive to structural variations. A sliding mechanism or the existence of extending binding sites in the protein have been postulated previously to account for enhanced binding affinities between lectins and small glycoclusters that do not fit either to the chelate or the aggregation model.[2, 25a] Properly designed carbohydrate-centred glycoclusters might help to clarify the molecular basis of the microcluster effect. Moreover, optimisation of both the microcluster effect and cross-linking interactions (the macrocluster effect), for instance, by attaching the functionalised derivative **11** to a new scaffold, should result in much more efficient lectin ligands suitable for therapeutic applications. Work in that direction is currently underway in our laboratories.

Experimental Section

Materials and instruments: HRP-labelled concanavalin A (Sigma), mannan from *Saccharomyces cerevisiae* (Sigma) and all other chemicals were of the highest commercial purity and used as supplied. Optical rotations were measured at 20 °C in 1-cm or 1-dm tubes on a Perkin-Elmer 141 MC polarimeter. ^{1}H (and ^{13}C NMR) spectra were recorded at 300 (75.5) or 500 (125.7) MHz with Bruker 300 AMX, 500 AMX and 500 DRX machines. 1D TOCSY, 2D COSY, HMQC and HSQC experiments were used to assist with NMR signal assignments. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with Kieselgel 60 F_{254} (Merck), with visualisation by UV light and by charring with 10% H_2SO_4. Column chromatography was carried out with silica gel 60 (Merck, 230–400 mesh). FAB mass spectra were obtained with a Kratos MS-80 RFA instrument by using the following conditions: the primary beam consisted of Xe atoms with a maximum energy of 8 keV; the samples were dissolved in thioglycerol and the positive ions were separated and accelerated over a potential of 7 keV; NaI was added as cationising agent. MALDI-TOF mass spectra were recorded on a GSG System spectrometer operating in the positive-ion mode with an accelerating potential of 28 keV. Samples were dissolved in water at mM concentrations and mixed with a standard solution of 2,5-dihydroxybenzoic acid (DHB, 10 mg mL^{-1} in 10% aqueous EtOH) in 1:1 (*v*/*v*) relative proportions; the mixture (1 μL) was loaded onto the target plate and dried under vacuum immediately before acquisition. Elemental analyses were performed at the Instituto de Investigaciones Químicas (Sevilla, Spain).

3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosylthio)propyl 2,3,4,6-tetra-*O*-[3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)propyl]-α-D-glucopyranoside (4): A mixture of allyl 2,3,4,6-tetra-*O*-allyl-α-D-glucopyranoside[9c] (**1**; 15 mg, 0.04 mmol) and 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranose[13] (**3**; 210 mg, 0.58 mmol) in dry MeOH (2 mL) was irradiated at 250 nm under Ar for 2 h. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography by using petroleum ether/EtOAc/EtOH (2:4:0.1) as the eluent. Yield = 59 mg (67%); $[\alpha]_D = +105.9$ ($c = 1.0$, $CHCl_3$); $R_f = 0.29$ (petroleum ether/EtOAc/EtOH (2:4:0.1)).

3-(α-D-Mannopyranosylthio)propyl 2,3,4,6-tetra-*O*-[3-(α-D-mannopyranosylthio)propyl]-α-D-glucopyranoside (5): A solution of **4** (102 mg, 0.05 mmol) in MeOH (3 mL) was adjusted to pH 9 by addition of methanolic 1 M NaOMe solution. The mixture was stirred overnight at room temperature, neutralised with Amberlite IR-120(H^+) ion-exchange resin, filtered and concentrated. The resulting residue was purified by GPC (Sephadex G-25, water). Yield = 54 mg (79%); $[\alpha]_D = +61.2$ ($c = 0.53$, H_2O).

3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosylthio)propyl 2,3,4,6-tetra-*O*-[3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)propyl]-β-D-glucopyranoside (6): A mixture of allyl 2,3,4,6-tetra-*O*-allyl-β-D-glucopyranoside[5b] (**2**; 25 mg, 0.066 mmol) and **3** (360 mg, 0.99 mmol) in dry MeOH (2 mL) was irradiated at 250 nm under Ar for 1 h. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography by using petroleum ether/EtOAc/EtOH (2.5:4:0.1) as the eluent. Yield = 82 mg (58%); $[\alpha]_D = +91.9$ ($c = 1.2$, $CHCl_3$); $R_f = 0.33$ (petroleum ether/EtOAc/EtOH (2.5:4:0.1)).

3-(α-D-Mannopyranosylthio)propyl 2,3,4,6-tetra-*O*-[3-(α-D-mannopyranosylthio)propyl]-β-D-glucopyranoside (7): Compound **6** (43 mg, 0.02 mmol) was deacetylated with methanolic NaOMe for 17 h and purified as described above for the preparation of **5** from **4**. Yield = 23 mg (85%); $[\alpha]_D = +153.6$ ($c = 0.56$, H_2O).

6-Bromohexyl 2,3,4,6-tetra-*O*-[3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)propyl]-β-D-glucopyranoside (9): Compound **9** was obtained by irradiation (254 nm) of a mixture of 6-bromohexyl 2,3,4,6-tetra-*O*-allyl-β-D-glucopyranoside[9c] (**8**; 25 mg, 0.05 mmol) and **3** (218 mg, 0.60 mmol) in dry MeOH (2 mL) under Ar for 2 h, followed by purification of the resulting residue by column chromatography (petroleum ether/EtOAc/EtOH (2.5:4:0.1)). Yield = 39 mg (40%); R_f = 0.22 (petroleum ether/EtOAc/EtOH (2.5:4:0.1)); $[\alpha]_D$ = +69.2 (c = 1.45, $CHCl_3$).

6-Bromohexyl 2,3,4,6-tetra-*O*-[3-(α-D-mannopyranosylthio)propyl]-β-D-glucopyranoside (10): Compound **9** (34 mg, 0.017 mmol) was deacetylated by treatment with methanolic NaOMe for 6.5 h and purified as described above for the preparation of **5** from **4**. Yield = 20 mg (91%); R_f = 0.40 ($MeCN/H_2O/NH_4OH$ (9:3:1)); $[\alpha]_D$ = +126.0 (c = 0.3, H_2O).

3-Iodopropyl 2,3,4,6-tetra-*O*-(3-iodopropyl)-α-D-glucopyranoside (12): A solution of 3-hydroxypropyl 2,3,4,6-tetra-*O*-(3-hydroxypropyl)-α-D-glucopyranoside[9d] (**11**; 97 mg, 0.20 mmol), triphenylphosphine (690 mg, 2.63 mmol) and I_2 (533 mg, 2.10 mmol) in DMF (10 mL) was stirred at 80 °C for 3 h, then concentrated to one third of the starting volume. Methanol (3 mL) was added and the solution was adjusted to pH 9 by addition of NaOMe, stirred for 30 min and neutralised with Amberlite IR-120 (H^+) ion-exchange resin. The resin was filtered off and washed with methanol, the combined filtrates were concentrated and the residue was purified by column chromatography (petroleum ether/EtOAc (6:1)) to give **12** as a yellow oil. Yield = 139 mg (68%); $[\alpha]_D$ = +32.5 (c = 1.0, $CHCl_3$); R_f = 0.47 (petroleum ether/EtOAc (4:1)).

(3-Azidopropyl) 2,3,4,6-tetra-*O*-(3-azidopropyl)-α-D-glucopyranoside (13): A mixture of **12** (134 mg, 0.13 mmol) and NaN_3 (171 mg, 2.63 mmol) in dry DMF (5 mL) was vigorously stirred at 90 °C for 16 h. The reaction mixture was cooled and extracted with diethyl ether/toluene/water (2:1:1; 2×5 mL). The organic phase was washed with water, dried ($MgSO_4$), concentrated and purified by silica gel column chromatography (petroleum ether/EtOAc (2:1)). Yield = 61 mg (79%); $[\alpha]_D$ = +51.2 (c = 0.75, $CHCl_3$); R_f = 0.20 (petroleum ether/EtOAc (2:1)).

3-Aminopropyl 2,3,4,6-tetra-*O*-(3-aminopropyl)-α-D-glucopyranoside (14): Et_3N (280 μL, 2.0 mmol) and propane-1,3-dithiol (202 μL, 2.0 mmol) were added to a solution of **13** (122 mg, 0.20 mmol) in freshly distilled MeOH (4 mL). The reaction mixture was stirred at room temperature under N_2 for 72 h, then water (10 mL) was added and the aqueous solution was washed with CH_2Cl_2 (2× 25 mL). Compound **14** was obtained as a white hygroscopic lyophilisate and used in the next steps without further purification. Yield = 76 mg (82%).

3-[*N*′-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)thioureido]-propyl 2,3,4,6-tetra-*O*-{3-[*N*′-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)thioureido]propyl}-α-D-glucopyranoside (16): 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl isothiocyanate[17] (**15**; 113 mg, 0.29 mmol) was added to a solution of **14** (18 mg, 0.039 mmol) in pyridine (6 mL). The mixture was stirred at room temperature overnight, then concentrated and purified by column chromatography with EtOAc as the eluent to give **16** as an amorphous solid. Yield = 75 mg (80%); $[\alpha]_D$ = +40.7 (c = 1.5, $CHCl_3$); R_f = 0.12 (EtOAc).

3-[*N*′-(α-D-Mannopyranosyl)thioureido]propyl 2,3,4,6-tetra-*O*-{3-[*N*′-(α-D-mannopyranosyl)thioureido]propyl}-α-D-glucopyranoside (17): Methanolic NaOMe (1 M, 0.3 mL) was added to a solution of **16** (70 mg, 29 μmol) in MeOH (3 mL) at 0 °C. After 5 min a white precipitate appeared and the suspension was stirred for 10 min. Water (0.5 mL) was then added and the clear solution was stirred at 0 °C for 15 min, then neutralised with Amberlite IR-120 (H^+) ion-exchange resin and further demineralised with Duolite MB6113 (H^+, OH^-) mixed ion-exchange resin. Solvents were removed and the resulting residue was purified by GPC (Sephadex G-25, water). Yield = 42 mg (93%); $[\alpha]_D$ = +28.0 (c = 0.6, H_2O).

3-{*N*′-[Tris(α-D-Mannopyranosyloxymethyl)methyl]thioureido}-propyl 2,3,4,6-tetra-*O*-{3-{*N*′-[tris(α-D-mannopyranosyloxymethyl)methyl]-thioureido}propyl}-α-D-glucopyranoside (20): A solution of the pentaamine derivative **14** (20 mg, 0.043 mmol) and tris(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyloxymethyl)methyl isothiocyanate (**18**; 277 mg, 0.24 mmol) in water/acetone (1:1, 2 mL) was adjusted to pH 8 with aqueous $NaHCO_3$ and stirred at room temperature for 16 h, then concentrated. The residue was purified by silica gel column chromatography (petroleum ether/CH_2Cl_2/MeOH (1:7:0.3)) to give a mixture of the pentameric adduct **19** and partially deacetylated compounds, as seen from TLC. The mixture was dissolved in MeOH (2 mL) and adjusted at pH 9 by addition of 1 M methanolic NaMeO. After 5 min at room temperature, a white precipitate appeared and the resulting suspension was stirred for 10 min. Water (1 mL) was added and the clear solution was stirred for 4 h, then neutralised with Amberlite IR-120 (H^+) ion-exchange resin and further demineralised with Duolite MB6113 (H^+, OH^-) mixed ion-exchange resin. Compound **20** was isolated as a white lyophilisate after purification by GPC (Sephadex G-25, water). Yield = 106 mg (67%); $[\alpha]_D$ = +43.2 (c = 0.5, H_2O).

Enzyme-linked lectin assay (ELLA): Nunc-Inmuno plates (MaxiSorp) were coated overnight with yeast mannan (100 μL per well, diluted from a stock solution of 10 μg mL^{-1} in 0.01 M phosphate-buffered saline (PBS) containing 0.1 mM Ca^{+2} and 0.1 mM Mn^{+2} (pH 7.3)) at room temperature. The wells were then washed with washing buffer (3×300 μL, PBS containing 0.05% (*v*/*v*) Tween 20). The washing procedure was repeated after each incubation throughout the assay. The wells were then blocked with 1% BSA/PBS (150 μL per well) for 1 h at 37 °C. After washing, the wells were filled with serial dilutions of peroxidase-labeled concanavalin A (Con A–HRP; 100 μL) from 10^{-1}–10^{-5} mg mL^{-1} in PBS and incubated at 37 °C for 1 h. The plates were washed and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (50 μL per well, 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 1 M H_2SO_4 (50 μL per well) and the absorbances were measured at 415 nm. Blank wells contained citrate-phosphate buffer. The concentration of lectin–enzyme conjugate that read an absorbance between 0.8 and 1.0 was used for inhibition experiments.

Inhibition experiments: The microtiter plates were coated overnight at room temperature with yeast mannan (100 μL per well, 10 μg mL^{-1}). The wells were then washed and blocked with BSA as described above. The inhibitors were used as stock solutions of 5–7 mg mL^{-1} of PBS. Each inhibitor was added in a serial of twofold dilutions (60 μL per well) in PBS with Con A-HRP (60 μL) at the desired concentration on Nunclon (Delta) microtiter plates and incubated for 1 h at 37 °C. The above solutions (100 μL) were then transferred to the mannan-coated microplates which were incubated for another hour at 37 °C. The plates were washed and the ABTS substrate was added (50 μL per well). Colour development was stopped after 20 min and the absorbances were measured. The percentages of inhibition were calculated as given in Equation (1), where A = absorbance.

$$\%\,\text{Inhibition} = (A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})})/A_{(\text{no inhibitor})} \times 100 \qquad (1)$$

IC_{50} values were reported as the concentration required for 50% inhibition of the Con A–yeast mannan association.

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