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Comparative studies on lectin–carbohydrate interactions in low and high density homo- and heteroglycoclusters†

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A versatile synthetic procedure to construct series of high- and low-density homo- and heteroglycoclusters is reported. The binding properties of these synthetic multivalent glycoconjugates to concanavalin A (Con A), a model lectin, have been assessed by using a range of competitive and non-competitive binding assays including enzyme-linked lectin assays (ELLA), isothermal titration microcalorimetry (ITC) and surface plasmon resonance (SPR). In all cases, highly dense glycoclusters showed a substantial amplification of the lectin-binding strength in comparison with low-density counterparts. Interestingly, highly-dense glycoligand presentations, regardless of their homo- or heteroglycoligand pattern, furnished similar Con A binding properties, supporting the existence of a synergic effect (*heterocluster effect*) due to secondary interactions of "non-active" structural motifs in the presence of a certain density of "active" glycoligands.

Introduction

Molecular recognition of carbohydrates represents a research area with strong potential in drug discovery and biotech applications.[1] Thus, protein–carbohydrate interactions play central roles in many biological and pathological processes, such as cell development, differentiation, morphogenesis, fertilization, immune response, implantation, cell migration, and cancer metastasis.[2] Carbohydrate-binding proteins (lectins) are extensively involved in processes associated with cell proliferation and cell death, just to mention a few examples.[1,3,4] The ability to control these events with selective small molecular inhibitors/effectors offers enormous potential in glycobiology.[5] Because these binding events are linked to well-defined cellular responses, one might expect them to be highly potent and specific. Notwithstanding, most saccharide ligands bind to their protein receptors only weakly, with association constants (K_a) typically in the mM range. Nature has circumvented this problem through a multivalent presentation of the binding motifs, thus leading to affinities exceeding those expected from the simple addition of individual interactions. This phenomenon, first noted by Lee and co-workers,[6] and referred to as the "cluster" or "multivalent" glycoside effect,[7] has found a wide range of applications in biology and medicine.[3,5,8,9]

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Multivalent ligands can interact with receptors *via* many possible mechanisms such as the chelate effect, subsite binding, steric stabilization, statistical rebinding, or receptor clustering and, therefore, thorough understanding of biological systems is a complex task.[10] Synthetic multivalent glycoconjugates with well-defined structures have become powerful tools for the elucidation of the basis ruling carbohydrate recognition events and the design of novel glycodrugs.[3,8,11] Typically, these systems incorporate several copies of single sugar structural motifs on an appropriate scaffold (molecular, dendritic, polymeric). However, despite efficiently imitating functional ligand multivalent presentation in terms of valency and density, these models barely reflect the inherent heterogeneity of biological systems, therefore systematically underestimating its potential contribution. In fact, it has been observed that variations in the expression levels of a particular sugar motif in a heterogeneous environment may affect not only the individual binding affinity of a given protein receptor but also its selectivity.[12] However, only a few procedures that allow the construction of well-defined glycoclusters[13] and glycopolymers[14] from different sugar moieties have been described. Additionally, the generation and deconvolution of combinatorial libraries of di-, tri- and tetra-valent heteroglycoclusters have also been reported.[15,16]

Model systems to address binding events involving heterogeneous carbohydrate displays should comply with the need for polyvalency and high density and, simultaneously, allow a total control of the relative proportions and presentation of the different structural motifs. In this context, we and others have recently reported an efficient synthetic procedure for the construction of multi-antennary homo- and heteroglycoclusters based on a β-cyclodextrin (βCD) core (Fig. 1).[17,18] Preliminary binding affinity studies with the α-D-mannopyranose specific plant lectin concanavalin A (Con A) as model receptor by enzyme-linked lectin assay (ELLA)[19] showed that the mixed-type α-Man–β-Glc heteroglycoclusters, in spite of displaying a lower number

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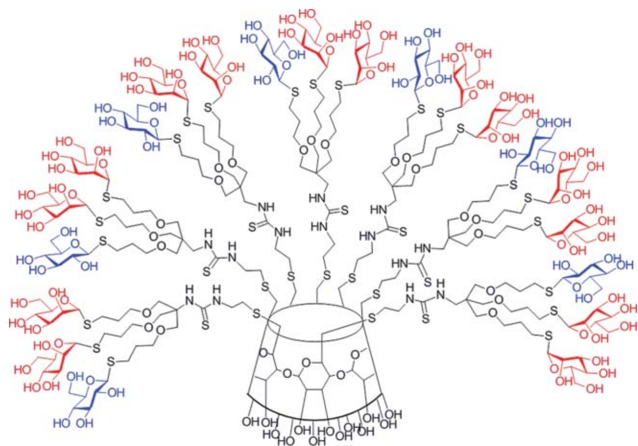

Fig. 1 Representative βCD-centered α-Man–β-Glc heteroglycocluster.

of putative selective binding motifs, exhibited Con A-binding affinities similar or even higher as compared to homogeneous α-mannosylated conjugates.[17] Such unexpected behaviour could not be explained in terms of a difference in effective binding motif concentration[20] and pointed to the existence of a "*heterocluster effect*", a term that refers to the increase in the intrinsic binding affinity of a carbohydrate ligand towards a lectin in the presence of a second sugar that itself is not a ligand for this lectin (intrinsic affinity means on a per-ligand basis, keeping the same overall topology and preventing contributions to binding from aggregation phenomena).

The nature of this effect remains uncertain and seems to be strongly dependent on the overall architecture. To get a deeper insight into this matter, we have now prepared a series of molecularly well-defined homo- and heteromultivalent glycoclusters. Low- and high-density central cores have been used to discriminate the influence of a pointed heterogeneity and a repetitive multiheterogeneity in a space-oriented platform. A comparative analysis of the performance of the different compounds towards Con A, an extensively studied mannose-binding lectin,[21] by a set of complementary techniques, namely ELLA,[19,22] isothermal titration calorimetry (ITC),[21,23] and surface plasmon resonance (SPR)[24] is reported.

Results and discussion

To evaluate how heterogeneity affects specific recognition of multivalent α-mannosides by Con A, the design of a set of suitable heteroglycoclusters featuring structurally diverse yet well-defined molecular architectures is mandatory. For such purpose, we have envisioned the synthesis of a series of multivalent displays ranging from fully homogeneous in the putative α-D-mannopyranoside ligand to fully homogeneous in β-D-glucopyranoside residue, with all intermediate combinations among them. β-Glucopyranosides have previously been shown to be unrecognized by Con A in control experiments.[25] Two different scaffolds were selected to prepare low-density and high-density multivalent systems, namely C-6-substituted methyl α-D-glucopyranoside and per-(C-6)-substituted-βCD, respectively. The contribution of these scaffolds to Con A recognition was shown to be negligible.[15,26] In both cases, a cysteaminyl spacer was incorporated to warrant accessibility of the coating binding motifs to recognition events. The homology between both core molecules and spacers was purposely chosen to detect any possible influence of the scaffold in the binding affinities. A modular strategy that takes advantage of the radical addition of thiols to double bonds[27–29] has been developed for the sequential incorporation of the desired monosaccharide motifs. A critical advantage of the methodology is that it allows sampling compounds with varied, yet perfectly defined densities of the constitutive sugars in an overall architecture that favours a highly compact packing by orientational bias. Dendrons themselves constitute interesting models to assess lectin binding but, more importantly, they can be assembled on the appropriate scaffold to attain a controlled ligand density.

Synthesis of homo- and heteroglycodendrons

To test the potential of the radical addition of thiosugars to double bonds for the construction of molecularly diverse structures, the stepwise addition of per-*O*-acetyl-1-thio-α-D-mannopyranose (**2**)[30] and per-*O*-acetyl-1-thio-β-D-glucopyranose (**3**)[31] to the triallylated pentaerythritol derivative **1**[32] was first explored. The choice of these glycoligands is motivated by the fact that α-D-mannopyranosyl residues are specifically recognised by Con A whereas β-D-glucopyranosyl derivatives are not. Previous studies had shown that α-D-thiomannopyranosyl ligands bind to Con A lectin with similar affinities to α-D-mannopyranosides and with higher affinities than N-linked analogues.[28b]

Homoglycodendrons **4** and **7** were prepared by UV-promoted radical addition of **2** and **3**, respectively, to the triallyl derivative **1**. Alternatively, azo-bis(isobutyronitrile) (AIBN) was used as radical starter. Anti-Markonikov addition led to adducts **4** and **7** with complete regioselectivity. Conventional deacetylation of these compounds yielded the corresponding trivalent unprotected derivatives **TriMan-OH** and **TriGlc-OH**. Triflyl activation of the primary hydroxyl group of **4** and **7**, followed by azide displacement (→ **5** and **8**) and isothiocyanation using the triphenylphosphine–carbon disulfide (TPP–CS_2)[33] system led to the corresponding isothiocyanate-armed dendrons **6** and **9**, respectively (Scheme 1).

Interestingly, the radical addition reaction of thiosugars **2** and **3** to **1** could be experimentally controlled by modulating the number of equivalents of the thiols in order to preferentially obtain the diaddition products **10** and **13** in good yields (Scheme 1). The diaddition products were further used to prepare the heterogeneous glycodendrons **14** and **17** by addition of the diastereomeric thiosugars. A reaction sequence analogous to that above discussed for the preparation of **6** and **9** allowed the synthesis of the mixed α-Man–β-Glc isothiocyanate-armed glycodendrons **12**, **16** and **19** (Scheme 1). Conventional deacetylation of **10**, **14** and **17** yielded the corresponding unprotected pentaerythritol-centered heteroglycoligands **DiMan-OH**, **DiManGlc-OH**, and **ManDiGlc-OH**.

As a monovalent control compound for binding studies, the mono-*S*-mannopyranosyl ligand (**Man-OH**) bearing a linear spacer similar to that present in the pentaerythritol derivatives **TriMan-**, **TriGlc-**, **DiMan-**, **DiManGlc-** and **ManDiGlc-OH** was elaborated (Scheme 2). The synthesis of **Man-OH** was achieved by radical addition of the per-*O*-acetyl-1-thiomannose **2** to allyl 2-hydroxypropyl ether **20**, obtained by selective allylation of propane-1,3-diol with allyl bromide in THF (40% yield), followed by conventional deacetylation. Additionally, the

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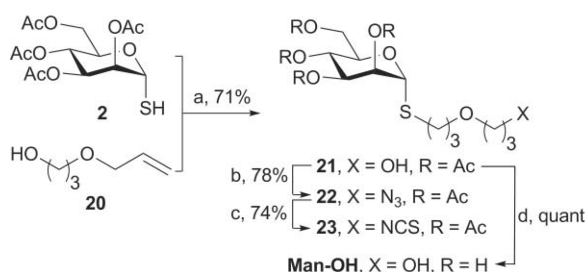
Scheme 1 Synthesis of homo- and heterogeneous glycodendrons. Reagents and conditions: a, $h\nu$ (250 nm), MeOH, Ar, RT; b, i) Tf_2O, CH_2Cl_2, Py, N_2, –25 °C, 20 min; ii) NaN_3, DMF, RT, 3 h; c, TPP, CS_2, dioxane, Ar, RT, 24 h; d, NaMeO, MeOH, RT, 1 h, then Amberlite IR 120 (H^+).



Scheme 2 Synthesis of mono-*S*-mannosyl ligand. Reagents and conditions: a, AIBN, dioxane, Ar, 75 °C, 45 min; b, i) TsCl, CH_2Cl_2, DMAP, RT, 24 h; ii) NaN_3, DMF, 80 °C, 4 h; c, TPP, CS_2, dioxane, RT, 16 h; d, NaMeO, MeOH, RT, 1 h, then Amberlite IR 120 (H^+).

monomannosylated isothiocyanate-functionalized building block **23** was prepared by tosylation of compound **21**, azide displacement (→ **22**), and TPP–CS_2-mediated isothiocyanation in good overall yield (Scheme 2).

Synthesis of methyl α-D-glucopyranoside and β-CD scaffolded homo- and heteroglyconjugates

Previous attempts to access hyperbranched cyclodextrin-centered conjugates by direct nucleophilic addition of per-(6-amino-6-deoxy)-βCD to isothiocyanates proved unsatisfactory.[17] Probably, the proximity of the nucleophilic centres in the cyclooligosaccharide structure results in strong steric hindrance and poor accessibility. To avoid this problem, we have used instead the cysteaminyl derivatives **24** (see Supporting Information) and **25**[17] as amine-armed cores to construct our low- and high-density glycoclusters, respectively. Compounds **24** and **25** were readily obtained by displacement of the corresponding 6-bromo-6-deoxy

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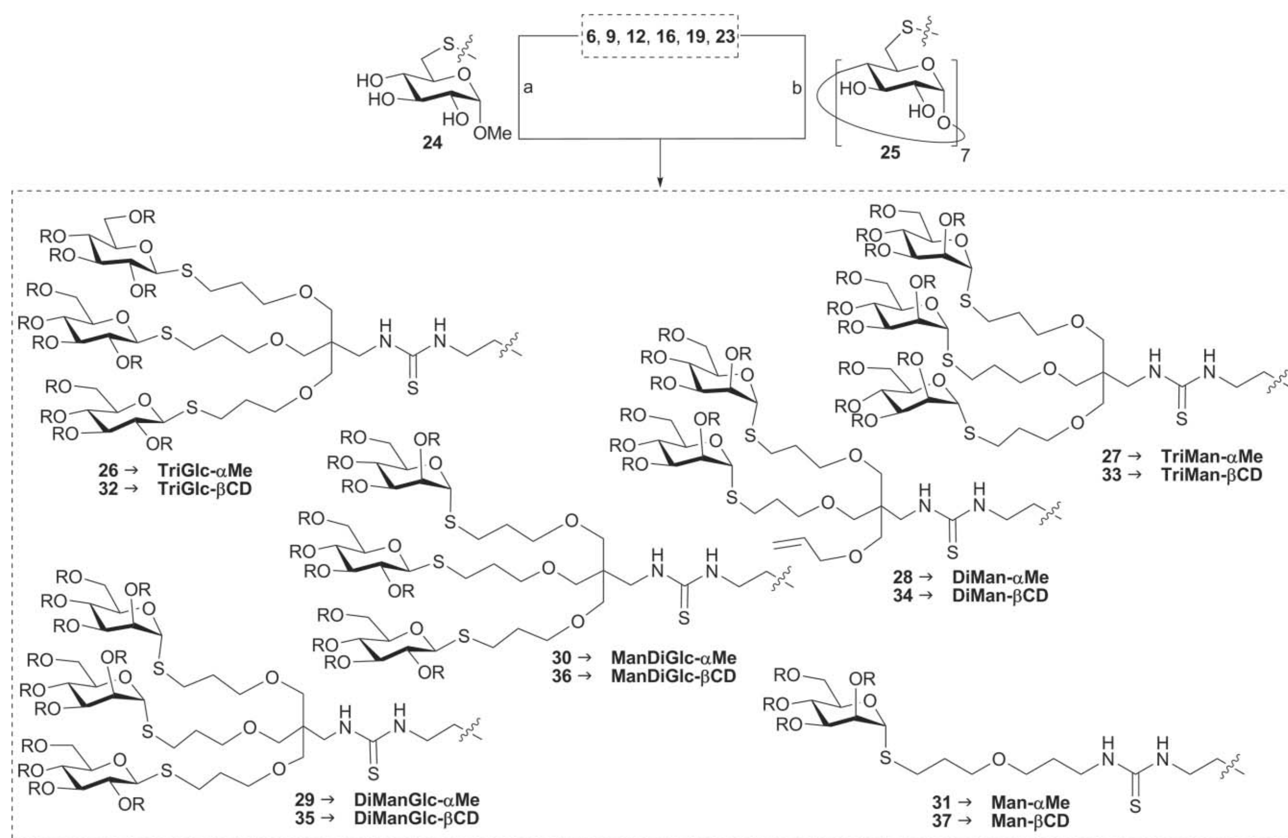
derivatives by cysteamine. Coupling reaction with isothiocyanate-armed homo- and hetero-glycodendrons by exploiting the thiourea forming reaction[34] let us obtain a collection of homogeneous and heterogeneous glycoclusters. Scheme 3 summarizes the synthesis of the glycoconjugate library, based on a two-step strategy involving the base-catalyzed amine–isothiocyanate coupling followed by acetyl group cleavage (mixed Zemplen *trans*-esterification–saponification). TLC- and NMR-monitoring of the reaction mixtures evidenced outstanding chemical yields for the amine–isothiocyanate coupling both in the αMe and βCD series, though chromatographic purification of the high molecular weight hemiacetylated intermediates in the βCD series handicapped the final yields in certain cases. Final acetyl cleavage quantitatively furnished the target multivalent glycoconjugates split into two separate batches: the low density glucopyranosyl adducts (αMe) and the high density CD-centered (βCD) constructs. The homogeneity and purity of all structures were confirmed by mass spectrometry (MS), NMR spectroscopy and microanalysis. ^{1}H and ^{13}C NMR spectra of the final fully unprotected conjugates in D_2O showed the typical line broadening associated with restricted rotation at the pseudoamide NH–C(=S) bonds,[35] which remained evident in the hyperbranched βCD derivatives also at elevated temperatures (333–353 K). The drastic decrease of motion at the central core region of the macromolecular conjugates provokes an increase in the relaxation time for the corresponding carbon atoms that translates into much lower intensities in the ^{13}C NMR spectra in comparison with the carbons of the external glycoligands. Nevertheless, both the ^{13}C NMR and ^{1}H NMR spectra were consistent with the expected C_7 symmetry for homogeneously C-6 substituted βCD-centered clusters. In the case of the homoglycoconjugates, proton spectra showed two spin systems at the characteristic sugar spectral region, one corresponding to the cyclodextrin and another to the coating monosaccharide. For heteroglycoclusters, three different spin systems were distinguished (see *e.g.*, Fig. 2).

The location of the trivalent dendrons at the primary face of the truncated-cone βCD platform provides a well defined topology in which binding motifs are oriented towards the same space region, providing a high-density glycoside surface. A three-dimensional view of compound **DiManGlc-βCD** is presented in Fig. 3 (MACROMODEL 6.0, MM2*). It reveals an external coat composed by α-Man and β-Glc monomers that mimics highly dense and heterogeneous regions of the cell surface glycocalix. The glycosidic crown spans to a maximum of 47 Å, shorter than the distance between two adjacent recognition sites in the Con A tetramer, therefore preventing any possible contribution associated to the chelate effect.[10]

Evaluation of concanavalin A binding capabilities

The binding affinity of the library of multivalent glycoligands synthesized against the tetrameric plant lectin concanavalin A (Con A) was extensively evaluated by enzyme-linked lectin assay



Scheme 3 Synthesis of α-D-glucopyranoside and β-CD scaffolded glycoconjugates. Reagents and conditions: a, i) Py, Et_3N, RT, 16 h; ii) NaMeO, MeOH, then Amberlite IR 120 (H^+); b, i) H_2O, acetone pH 8 ($NaHCO_3$), RT; ii) NaMeO, MeOH, H_2O, then Amberlite IR 120 (H^+), Duolite MB 6113 (H^+, OH^-).

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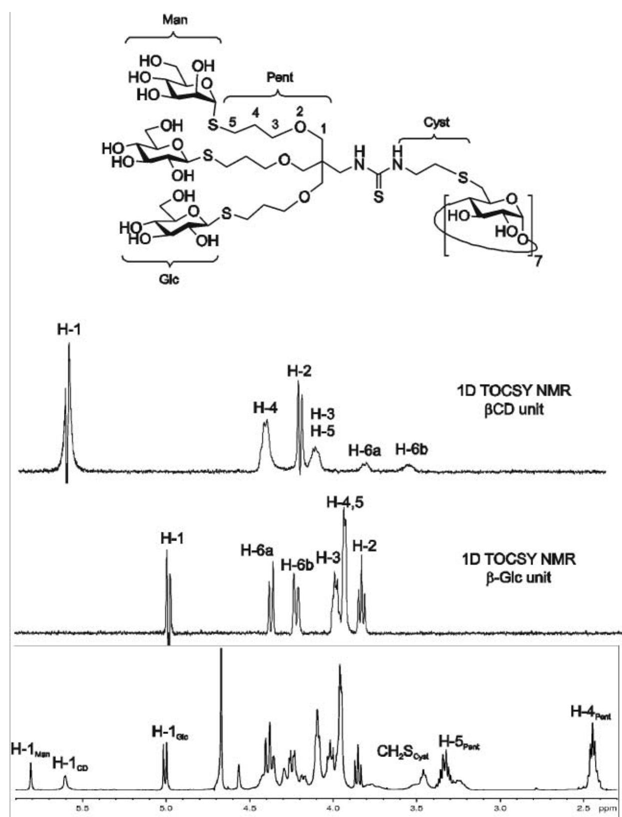

Fig. 2 ^{1}H NMR (500 MHz, D_2O, 353 K) of compound **ManDiGlc-βCD**. The 1D TOCSY spectra of βCD and β-Glc units are included.

(ELLA). In addition, isothermal titration microcalorimetry (ITC) and surface plasmon resonance (SPR) measurements were carried out on the hyperbranched glycoclusters **TriMan-βCD**, **DiMan-βCD**, **DiManGlc-βCD** and **ManDiGlc-βCD**.

Enzyme-linked lectin assays (ELLA)

ELLA measures the ability of a soluble ligand to inhibit the association between a labelled lectin and a polymeric ligand attached to the microtiter well (horseradish peroxidase-labelled Con A and yeast mannan, respectively, in the present case). The

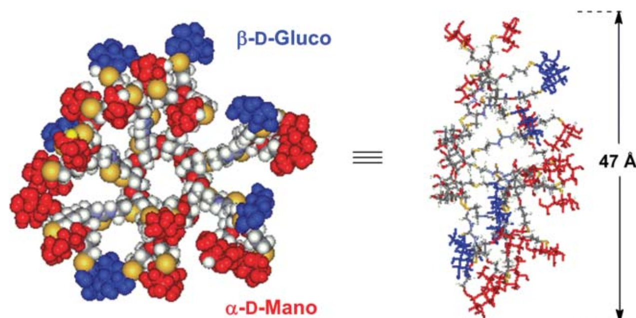

Fig. 3 3D view of heteromultivalent conjugate **DiManGlc-βCD**.

concentration needed to achieve 50% inhibition (IC_{50}) is then assumed to be inversely proportional to the lectin-saccharide free energy of binding. The experimental ELLA data are summarized in Table 1. In general, results are in agreement with the known Con A specificity and the expected increase in affinity with α-Man ligand valency. In fact, a substantial amplification of the binding affinity was observed for homogeneous α-Man clusters when monosaccharide units were presented in triads, in agreement with previous results.[15,24] Conversely, trivalent β-Glc conjugates **TriGlc-OH** and **TriGlc-αMe** were not recognised by Con A in the concentration range explored (up to *ca.* 4 mM). Additionally, comparative analysis of the IC_{50} data discarded significant contributions to the binding energy ascribable to the scaffolds. Furthermore, the presence of β-Glc monomers in low density heteroclusters is irrelevant regarding Con A binding to the α-Man ligands.

Highly dense glycoclusters featured a completely different scenario. The presence of β-Glc residues significantly contributes to the enhancement of lectin affinity in this highly-dense architecture. Though **TriMan-βCD** still features two and three fold better affinity than heterovalent **DiManGlc-βCD** and **ManDiGlc-βCD**, respectively, on a relative potency (mannose molar) basis this difference tends to level (7.51, 4.75 and 6.86, respectively, Table 1). The fact that the IC_{50} value for **DiMan-βCD** (14 α-Man residues and 7 allyl groups) is higher than that of **TriMan-βCD** indicates that the presence of allyl groups does not contribute to any significant level to the recognition process and, more importantly,

Table 1 Inhibition of yeast mannan–Con A binding measured by ELLA

Compound	Man units	Glc units	IC_{50} (μM)[a]	Relative potency	Relative potency (molar)
Man-OH	1	0	800	1	1
ManDiGlc-OH	1	2	605	1.32	1.32
DiMan-OH	2	0	319	2.51	1.25
DiManGlc-OH	2	1	275	2.91	1.45
TriMan-OH	3	0	46	17.3	5.77
ManDiGlc-αMe	1	2	790	1.10	1.10
DiManGlc-αMe	2	1	69	12.5	6.30
TriMan-αMe	3	0	53	16.3	5.43
Man-βCD	7	0	67	12.9	1.84
ManDiGlc-βCD	7	14	18	48.1	6.86
DiMan-βCD	14	0	76	11.4	0.81
DiManGlc-βCD	14	7	13	66.5	4.75
TriMan-βCD	21	0	5.5	157.8	7.51

[a] Relative values are compared to methyl-α-D-Man (IC_{50} 865 μM).[36] The IC_{50} are mean values obtained from at least three estimations. Errors are estimated in the range ±15%.

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Table 2 Thermodynamic parameters of CD-centered glycocluster–Con A binding determined by ITC (n.d.: not determined)

Compound	Man units	Glc units	$-\Delta G°$ kJ/mol	$-\Delta H°$ kJ/mol	$T\Delta S°$ kJ/mol
TriGlc-βCD	0	21	no binding detected		
ManDiGlc-βCD	7	14	32.4[a]	8.3	24.1
DiMan-βCD	14	0	21.1[b]	*n.d.*	*n.d.*
DiManGlc-βCD	14	7	32.3[c]	71.4	−39.1
TriMan-βCD	21	0	33.3[d]	152.8	−119.1

[a] Association constant values, calculated according to the Gibbs equation, are 4.8×10^5, [b] 5.0×10^3, [c] 4.6×10^5, [d] 6.9×10^5.

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supports that the glycosidic nature of the secondary ligand is necessary to boost the affinity towards Con A.

Since the presence of the horseradish peroxidase (HPR) label used in ELLA is supposed to prevent cross-linking processes, unless very long spacer arms are used,[7,17] these hyperbranched βCD probably interact with a single binding site in the lectin. Consequently, the observed affinity enhancement for heterovalent derivatives cannot be ascribed to aggregation. It is possible that weak secondary interactions,[11,37] either in the recognition site of the primary ligand or proximal to this region, operate when the α-Man and β-Glc motifs are closely packed together in a highly dense surface.

Isothermal titration microcalorimetry assays (ITC)

To get thermodynamic information that could reliably support the implication of β-Glc residues in the reinforcement of the α-Man–Con A interaction, ITC measurements were carried out. In these experiments soluble glycodendrons were titrated against a solution containing Con A, and the heat evolved or absorbed during binding was measured as a function of ligand concentration. HRP-labelled Con A was also used in these experiments in order to fully imitate the ELLA binding process. Deconvolution of these data yielded the binding constant, enthalpy of binding, and the stoichiometry of the interaction. The binding constant is easily related to the free energy of association, and the entropy is calculated through subtraction. The results confirmed the 1 : 1 stoichiometry, in agreement with the absence of precipitation.

The obtained thermodynamic data are collected in Table 2. Paralleling ELLA assays, heterogeneous α-Man–β-Glc clusters featured free energies of binding 50% higher than that found for the homo-14-valent conjugate **DiMan-βCD**. The 21-valent **TriMan-βCD**, **DiManGlc-βCD** and **ManDiGlc-βCD** displayed rather similar $\Delta G°$ values. However the enthalpic and entropic contributions to $\Delta G°$ were significantly different. Data are indicative of partial enthalpy–entropy compensation between glycocluster analogues having different mannose valency. Thus, glycocluster **ManDiGlc-βCD** compensates a lower enthalpic contribution with higher entropy of binding, suggesting that a sliding mechanism, promoted by the presence of the β-Glc ligand, is operating. The bind and slide (internal diffusion) model allows, for instance, a small fraction of bound lectin molecules to dynamically move from carbohydrate to carbohydrate epitope in globular and linear glycoproteins, and has been suggested to be a general mechanism of binding of ligands to biopolymers.[23b] In our system, partial entropy–enthalpy compensation might be at the origin of the observed heterocluster effect. Actually, **ManDiGlc-βCD** binding to Con A is, basically, an entropically driven process. On the other hand, the gain in enthalpy of binding when the α-Man valency increases, keeping unchanged the total sugar content, is balanced by the unfavourable entropy that characterises specific association processes in aqueous solutions.[19a] The active role of β-Glc was confirmed by the dramatic decrease in the free energy of binding of compound **DiMan-βCD** as compared with that of **DiManGlc-βCD**, having identical α-Man valency. The heterocluster effect implies a synergy between the primary (α-Man) and the secondary ligand (β-Glc) since the homogeneous derivative **TriGlc-βCD** did not bind to the lectin.

Surface plasmon resonance (SPR) experiments

SPR detection offers several unique advantages in comparison to other techniques. Both kinetic and thermodynamic parameters can be monitored in real time using minor amounts of materials without requiring specific labelling of the interacting partners.[38,39] To determine association constants (K_a) of our ligands with Con A, SPR measurements were carried out in which the lectin was immobilized on the chip surface.[38] While the opposite configuration, *i.e.* using immobilized glycoligands, has obvious advantages in terms of sensitivity, in our case that would have required the modification of the glycoclusters without altering the overall architecture, which implies a non-evident synthetic strategy.

The poor lectin affinity of low-density glycoclusters was translated into very weak K_a (data not shown). For this reason, no reliable data could be obtained in these cases, except for the compounds displaying the trivalent homomannosides (**TriMan-OH** and **TriManαMe**), confirming the benefits of this type of presentation in low-density ligands. No synergic effect due to the presence of additional β-glucopyranosyl motifs was detected for low-density heterovalent derivatives, in full agreement with ELLA data.

In the case of the CD-scaffolded glycocluster series, SPR experiments were carried out using low and high density Con A-functionalized chips. To evaluate lectin density and stability of the lectin anchoring after a set of SPR experiments, ribonuclease B was used as standard ligand. Channel responses upon injection of 400 nM ribonuclease B (30 μL for 3 min) were in the range of 200 RU for the low-density channel and over 900 RU for the high-density chip, respectively. The results, summarized in Table 3, showed a divergence of approximately one order of magnitude in the K_a values calculated using the different chips, probably due to mass transport effects.[38,40]

As a general trend, the homo- and heteromultivalent CD-conjugates showed a much higher Con A affinity (K_a) than their low-density counterparts. In contrast to ELLA and ITC data,

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Table 3 Con A binding K_a calculated from SPR-sensorgrams

Compound	Man units	Glc units	K_a (M^{-1})[a]
TriMan-βCD	21	0	2.03×10^4 [b] 4.70×10^5 [c]
DiManGlc-βCD	14	7	5.33×10^4 [b] 6.48×10^4 [c]
DiMan-βCD	14	0	7.57×10^4 [b] 5.98×10^5 [c]
ManDiGlc-βCD	7	14	5.53×10^4 [b] 2.78×10^5 [c]
Man-βCD	7	0	2.92×10^3 [b] 6.37×10^3 [c]
TriGlc-βCD	0	21	no binding detected

[a] Average values from at least four experiments (S.D. = ±10%). [b] Low Con A-surface density sensorchip flow cell. [c] High Con A-surface density sensorchip flow cell.

valency-dependent Con A affinity increases for the highly-dense systems and so did not require the presence of the mannosyl ligands in triads to maximize it. The different nature of the phenomena measured in SPR and ELLA or ITC probably accounts for these contrasting observations. The surface-ligand interaction taking place in SPR is probably easier to saturate by densely-decorated clusters than those taking place in the bulk solution (ELLA and ITC).

In any case, SPR experiments were consistent with the *heterocluster effect* observed by ELLA and ITC. While **Man-βCD** featured moderate Con A binding affinity (K_a 6.37×10^3 M^{-1}), insertion of additional β-Glc ligands (**ManDiGlc-βCD**) increased Con A affinity to a similar level as the homogeneously decorated 21-valent **TriMan-βCD** (2.78×10^5 and 4.70×10^5 M^{-1}, respectively). Sensorgrams of the homo- and heteroclusters are rather similar (Fig. 4 and Supporting Information), supporting the reliability of the measurements and, therefore, the relevance of secondary interactions at high saccharide-surface densities also for immobilized biological receptors.

The homotetradecavalent derivative **DiMan-βCD**, featuring seven allyl groups, afforded the highest K_a value (5.98×10^5 M^{-1}), as determined by SPR, in this series. However, in this case the observed affinity increase is probably motivated on non-specific hydrophobic interactions of the clusterized allyl groups with the surface. A similar observation has been recently reported for a series of cyclopeptide-templated glycoclusters.[16] Furthermore, hydrophobic residues have been shown to beneficially contribute to enhancing lectin–glycoconjugate binding in particular systems.[41]

Conclusions

In this study, an efficient methodology to access monodisperse, well-defined homo- and heteromultivalent glycoclusters has been implemented. This diversity-oriented synthetic strategy allows an exquisite control of the molecular architecture of mixed-type glycoclusters and is very well suited to explore the effects of glycoligand density and nature on lectin binding. The binding affinity towards Con A was evaluated by a number of techniques including ELLA, ITC and SPR. The combined data showed that, while low density glycoclusters behaved according to the classical cluster effect, with a gradual increase in affinity towards Con A associated with putative ligand (α-Man) valency, highly dense mixed constructs with identical α-Man valency evidenced affinity increases due to the presence of β-Glc residues, which however are not ligands for the lectin themselves. Our results point to entropy–enthalpy compensation as the basis of this effect. We hypothesize that, once α-Man residues have been recognized by Con A, a sliding mechanism promoted by the presence of the β-Glc monomers might operate that results in improved binding. Further investigations to assess the scope and its possible biological relevance of the heterocluster effect are being developed in our laboratories.

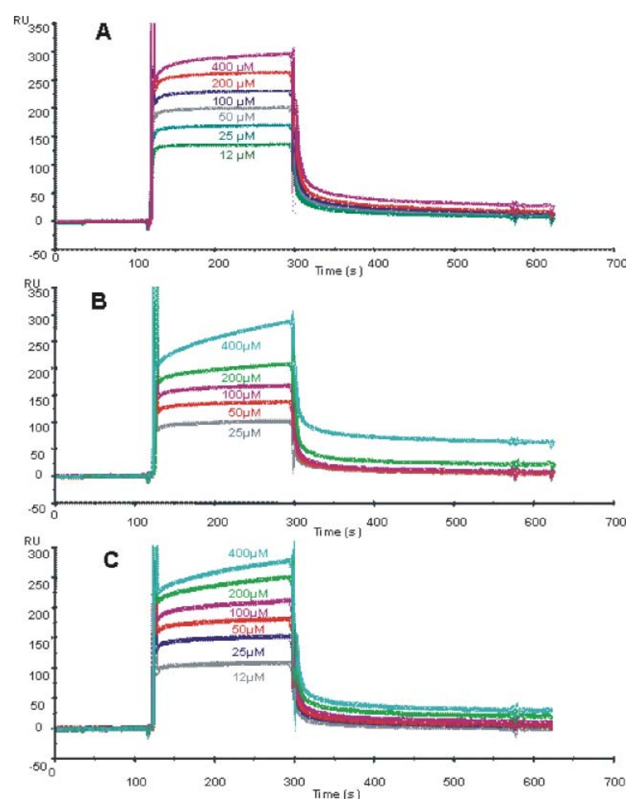

Fig. 4 SPR-Sensorgrams of **TriMan-βCD** (A), **DiManGlc-βCD** (B), and **ManDiGlc-βCD** (C) at 12.5, 25, 50, 100, 200, and 400 μM in phosphate buffer.

Experimental section

Horseradish peroxidase-labelled concanavalin A (HRP-Con A; Sigma), mannan from *Saccharomyces cerevisiae* (Sigma) and all other common reagents and materials were purchased from commercial sources. Optical rotations were measured at room temperature in 1 cm or 1 dm tubes on a Perkin-Elmer 141 MC polarimeter. Infrared (IR) spectra were recorded on a Bomem Michelson MB-120 FTIR spectrophotometer. ^{1}H (and ^{13}C NMR) spectra were recorded at 300 (75.5 for ^{13}C) and 500 (125.7 for ^{13}C) MHz with Bruker 300 and 500 DRX instruments. 1D ^{1}H TOCSY, 2D ^{1}H TOCSY, COSY, ^{1}H–^{13}C HMQC and HSQC experiments were used to assist NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with Kieselgel 60 F254 (E. Merck), with visualization by UV light and by charring with 10% H_2SO_4 or 0.2% ninhydrin. Column chromatography was carried out on Silica Gel 60 (E. Merck, 230–400 mesh).

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Gel permeation chromatography (GPC) of the fully unprotected βCD adducts was carried out on a Sephadex G-25 (eluent H_2O) column (Pharmacia Amersham) attached to a Gradifrac system using a UV detector set at 248 nm. FAB mass spectra were obtained using a Kratos MS-80 RFA instrument. The operating conditions were the following: 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 acquired on a GSG System spectrometer operating in the positive-ion mode with an accelerating voltage of 28 keV. Samples were dissolved in H_2O at millimolar concentration and mixed with a standard solution of 2,5-dihydroxybenzoic acid (DHB; 10 mg mL^{-1} in 10% aq EtOH, 2 μL) in 1 : 1 v/v relative proportions; 1 μL of the mixture was loaded onto the target plate, then allowed to air-dry at room temperature. Elemental analyses were performed at the Instituto de Investigaciones Químicas (Sevilla, Spain).

Triphenylphosphine, trifluoromethanesulfonic anhydride and *N*,*N*-dimethylformamide are indicated by the acronyms TPP, Tf_2O and DMF, respectively. 2,3,4,6-Tetra-*O*-acetyl-1-thio-α-D-mannopyranose (**2**) and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (**3**) were prepared from the corresponding per-*O*-acetates in three steps by transformation into the corresponding glycosyl halides,[42] treatment with thiourea and subsequent hydrolysis of the resulting isothiouronium salt with potassium metabisulfite ($K_2S_2O_5$).[29,43] Experimental procedures and fully assigned ^{1}H and ^{13}C NMR data for pentaerythritol glycoclusters (**TriMan-OH**, **TriGlc-OH**, **DiMan-OH**, **ManDiGlc-OH**), 4-oxahept-6-en-1-ol[44] derivatives (**21** and **Man-OH**) and monoconjugate derivatives of methyl 6-(2-aminoethylthio)-α-D-glucopyranoside[45] (**3**, **TriGlc-αMe**, **4**, **TriMan-αMe**, **10**, **DiMan-αMe**, **14**, **DiManGlc-αMe**, **17**, **ManDiGlc-αMe**, **21**, and **Man-αMe**) are described in the Supporting Information.

General procedure for the preparation of heptaconjugates of heptakis[6-(2-aminoethylthio)]cyclomaltoheptaose (TriGlc-βCD, TriMan-βCD, DiMan-βCD, DiManGlc-βCD, ManDiGlc-βCD and Man-βCD)

A solution of heptacysteaminyl βCD **25**[17] (20 mg, 11.08 μmol) in H_2O (1 mL) was adjusted to pH 8–9 with solid $NaHCO_3$ and stirred for 16 h at room temperature. A solution of the corresponding isothiocyanate (115 μmol, 1.5 eq) in acetone (1 mL) was then added and the reaction mixture was stirred at room temperature until total consumption of **25**. Acetone was evaporated under reduced pressure, the remaining aqueous suspension was freeze-dried and the solid residue was purified by column chromatography, using MeCN → 10 : 1 MeCN–H_2O as eluent, to give the corresponding hemiacetylated C_7-symmetric adducts (**32–37**). Deacetylation was effected by treatment with 1 N NaOMe in MeOH (0.1 eq per mol of acetates) at room temperature. After 5 min a white precipitate appeared, which was redissolved by addition of H_2O. The solution was stirred for 15 min, neutralised using Amberlite IR-120 (H^+) ion exchange resin, demineralised with Duolite MB-6113 (H^+, OH^-) ion exchange resin and freeze-dried to give the fully unprotected conjugates (**TriGlc-βCD**, **TriMan-βCD**, **DiMan-βCD**, **DiManGlc-βCD**, **ManDiGlc-βCD**, **Man-βCD**). Analytical samples for lectin-binding studies were obtained by gel permeation chromatography (Sephadex G-25, H_2O). For the notation of atoms in NMR assignments, see Fig. 5 and the diagrams hereinafter.

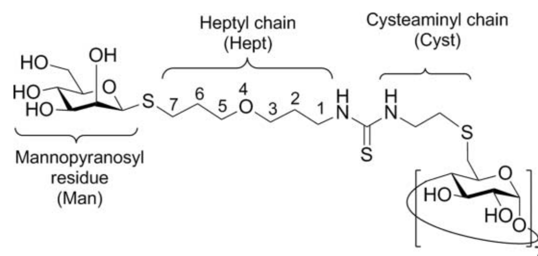

Fig. 5 Structure of compound **Man-βCD** with labels indicating the different spin systems and proton numbers.

Heptakis[6-[2-[*N*′-[2,2,2-tris[5-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]]cyclomaltoheptaose (32). Reaction time: 48 h. Yield: 47 mg (37%); $[\alpha]_D = -11.4$ (*c* 1.0, MeOH); ^{1}H NMR (500 MHz, CD_3OD, 323 K): $\delta = 5.27$ (t, 21 H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H-3$_{Glc}$), 5.04 (t, 21 H, $J_{4,5} = 10.0$ Hz, H-4$_{Glc}$), 5.03 (s, 7 H, H-1), 4.96 (t, 21 H, $J_{1,2} = 10.0$ Hz, H-2$_{Glc}$), 4.76 (d, 21 H, H-1$_{Glc}$), 4.26 (dd, 21 H, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 5.0$ Hz, H-6a$_{Glc}$), 4.16 (dd, 21 H, $J_{5,6b} = 2.5$ Hz, H-6b$_{Glc}$), 3.97 (m, 7 H, H-5), 3.92 (ddd, 21 H, H-5$_{Glc}$), 3.83 (t, 7 H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H-3), 3.72 (m, 14 H, CH_2N_{Cyst}), 3.55 (m, 14 H, H-2, H-4), 3.52 (m, 42 H, H-3$_{Pent}$), 3.42 (s, 56 H, CH_2N_{Branch}, H-1$_{Pent}$), 3.21 (m, 7 H, H-6a), 3.02 (m, 7 H, H-6b), 2.91 (bt, 14 H, CH_2S_{Cyst}), 2.82, 2.78 (2 dt, 42 H, $^2J_{H,H} = 13.0$ Hz, $^3J_{H,H} = 6.0$ Hz, H-5$_{Pent}$), 2.07, 2.05, 2.01, 1.95 (4 s, 252 H, MeCO), 1.89 (m, 42 H, H-4$_{Pent}$); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K): $\delta = 185.4$ (CS), 172.3, 171.7, 171.3, 171.2 (CO), 103.8 (C-1), 86.1 (C-4), 84.9 (C-1$_{Glc}$), 76.9 (C-5$_{Glc}$), 75.5 (C-3$_{Glc}$), 74.6 (C-3), 74.4 (C-2, C-5), 72.1 (C-1$_{Pent}$), 71.8 (C-2$_{Glc}$), 71.2 (C-3$_{Pent}$), 70.2 (C-4$_{Glc}$), 63.7 (C-6$_{Glc}$), 45.9 (C_q, CH_2N_{Branch}, CH_2N_{Cyst}), 35.1 (C-6), 34.2 (CH_2S_{Cyst}), 31.4 (C-4$_{Pent}$), 28.5 (C-5$_{Pent}$), 21.1-20.7 (*Me*CO). Anal. Calcd. for $C_{455}H_{686}N_{14}O_{238}S_{35}$: C 48.43, H 6.14, N 1.74. Found: C 48.08, H 6.02, N 1.69.

Heptakis[6-[2-[*N*′-[2,2,2-tris[5-(β-D-glucopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]]cyclomaltoheptaose (TriGlc-βCD). Yield: 32 mg (99%); $[\alpha]_D = -16.2$ (*c* 1.4 in H_2O); ^{1}H NMR (500 MHz, D_2O, 343 K): $\delta = 5.46$ (bd, 7 H, $J_{1,2} = 3.0$ Hz, H-1), 4.86 (d, 21 H, $J_{1,2} = 9.5$ Hz, H-1$_{Glc}$), 4.29 (m, 7 H, H-5), 4.28 (m, 7 H, H-3), 4.27 (d, 21 H, $J_{6a,6b} = 12.0$ Hz, H-6a$_{Glc}$), 4.13 (d, 21 H, H-6b$_{Glc}$), 4.09 (m, 14 H, CH_2N_{Cyst}), 4.07 (m, 7 H, H-2), 3.98 (t, 42 H, $^3J_{H,H} = 6.5$ Hz, H-3$_{Pent}$), 3.97 (m, 7 H, H-4), 3.89 (t, 21 H, $J_{2,3} = J_{3,4} = 9.3$ Hz, H-3$_{Glc}$), 3.84 (m, 98 H, H-4$_{Glc}$, H-5$_{Glc}$, CH_2N_{Branch}, H-1$_{Pent}$), 3.73 (t, 21 H, H-2$_{Glc}$), 3.65 (m, 7 H, H-6a), 3.38 (m, 7 H, H-6b), 3.32 (bt, 14 H, $^3J_{H,H} = 6.5$ Hz, CH_2S_{Cyst}), 3.22, 3.18 (2 dt, 42 H, $^2J_{H,H} = 13.0$ Hz, $^3J_{H,H} = 5.5$ Hz, H-5$_{Pent}$), 2.33 (m, 42 H, H-4$_{Pent}$); ^{13}C NMR (125.7 MHz, 343 K, D_2O): $\delta = 181.4$ (CS), 102.6 (C-1), 86.3 (C-1$_{Glc}$), 85.1 (C-4), 80.5 (C-5$_{Glc}$), 78.0 (C-3$_{Glc}$), 73.4 (C-3), 73.2 (C-2$_{Glc}$), 72.8 (C-2, C-5), 71.2 (C-1$_{Pent}$), 70.4 (C-3$_{Pent}$), 70.3 (C-4$_{Glc}$), 61.8 (C-6$_{Glc}$), 46.5 (C_q), 45.0 (CH_2N_{Branch}, CH_2N_{Cyst}), 34.2 (C-6), 33.1 (CH_2S_{Cyst}), 30.0 (C-4$_{Pent}$), 27.4 (C-5$_{Pent}$); MALDI-TOFMS: *m*/*z* 7745 $[M + H]^+$. Anal. Calcd. for $C_{287}H_{518}N_{14}O_{154}S_{35}$: C 44.46, H 6.75, N 2.53. Found: C 44.32, H 6.67, N 2.34.

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Heptakis[6-[2-[N'-[2,2,2-tris[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]]cyclomaltoheptaose (33). Reaction time: 48 h. Yield: 75 mg (60%); $[\alpha]_D$ = +78.1 (*c* 1.0, MeOH); R_f 0.48 (10 : 1 : 1 MeCN–H_2O–NH_4OH); ^1H NMR (500 MHz, CD_3OD, 313 K): δ = 5.36 (s, 21 H, H-1_{Man}), 5.32 (bs, 21 H, H-2_{Man}), 5.26 (t, 21 H, $J_{3,4} = J_{4,5}$ = 10.0 Hz, H-4_{Man}), 5.19 (dd, 21 H, $J_{2,3}$ = 2.5 Hz, H-3_{Man}), 5.01 (bs, 7 H, H-1), 4.37 (ddd, 21 H, $J_{5,6a}$ = 5.0 Hz, $J_{5,6b}$ = 2.0 Hz, H-5_{Man}), 4.28 (dd, 21 H, $J_{6a,6b}$ = 12.5 Hz, H-$6a_{Man}$), 4.12 (dd, 21 H, H-$6b_{Man}$), 3.97 (m, 7 H, H-5), 3.82 (m, 7 H, H-3), 3.73 (m, 14 H, CH_2N_{Cist}), 3.56 (m, 56 H, H-2, H-4, H-3_{Pent}), 3.43 (s, 56 H, CH_2N_{Branch}, H-1_{Pent}), 3.23 (m, 7 H, H-6a), 3.00 (m, 7 H, H-6b), 2.91 (m, 14 H, CH_2S_{Cyst}), 2.82, 2.80 (2 dt, 42 H, $^2J_{H,H}$ = 13.0 Hz, $^3J_{H,H}$ = 6.5 Hz, H-5_{Pent}), 2.15–1.92 (4 s, 252 H, MeCO), 1.93 (m, 42 H, H-4_{Pent}); ^{13}C NMR (125.7 MHz, CD_3OD, 313 K): δ = 183.5 (CS), 172.6–171.5 (CO), 103.9 (C-1), 86.0 (C-4), 84.0 (C-1_{Man}), 74.4 (C-3), 73.7 (C-2, C-5), 72.5 (C-2_{Man}), 72.1 (C-1_{Pent}), 71.2 (C-3_{Man}, C-3_{Pent}), 70.5 (C-5_{Man}), 67.7 (C-4_{Man}), 63.8 (C-6_{Man}), 45.9 (C_q), 45.7 (CH_2N_{Ram}, CH_2N_{Cyst}), 35.0 (C-6), 34.0 (CH_2S_{Cyst}), 30.9 (C-4_{Pent}), 29.6 (C-5_{Pent}), 21.1-20.6 (*Me*CO). Anal. Calcd. for $C_{455}H_{686}N_{14}O_{238}S_{35}$: C 48.43, H 6.14, N 1.74. Found: C 48.30, H 6.04, N 1.55.

Heptakis[6-[2-[N'-[2,2,2-tris[5-(α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]]cyclomaltoheptaose (TriMan-βCD). Yield: 52 mg (99%); $[\alpha]_D$ = +104.0 (*c* 1.0 in H_2O); ^1H NMR (500 MHz, D_2O, 353 K): δ = 5.82 (s, 21 H, H-1_{Man}), 5.61 (bs, 7 H, H-1), 4.57 (m, 21 H, H-2_{Man}), 4.46 (m, 7 H, H-5), 4.43 (m, 28 H, H-3, H-5_{Man}), 4.36 (m, 63 H, H-3_{Man}, H-$6a_{Man}$, H-$6b_{Man}$), 4.29 (m, 21 H, H-4_{Man}), 4.23 (m, 14 H, CH_2N_{Cyst}), 4.19 (m, 7 H, H-2), 4.09 (m, 49 H, H-4, H-3_{Pent}), 3.96 (m, 56 H, CH_2N_{Branch}, H-1_{Pent}), 3.82 (m, 7 H, H-6a), 3.50 (m, 7 H, H-6b), 3.47 (m, 14 H, CH_2S_{Cyst}), 3.26 (bt, 42 H, $^2J_{H,H}$ = 11.5 Hz, H-5_{Pent}), 2.46 (m, 42 H, H-4_{Pent}); ^{13}C NMR (125.7 MHz, D_2O, 343 K): δ = 181.9 (CS), 102.7 (C-1), 85.3 (C-1_{Man}), 85.1 (C-4), 73.9 (C-5_{Man}), 73.4 (C-3), 72.9 (C-2, C-5), 72.6 (C-2_{Man}), 72.1 (C-3_{Man}), 71.1 (C-1_{Pent}), 70.9 (C-3_{Pent}), 67.7 (C-4_{Man}), 61.6 (C-6_{Man}), 45.1 (CH_2N_{Branch}, CH_2N_{Cyst}), 45.0 (C_q), 34.1 (C-6), 33.2 (CH_2S_{Cyst}), 29.8 (C-4_{Pent}), 28.5 (C-5_{Pent}); MALDI-TOFMS: *m/z* 7767.50 [M + Na]$^+$. Anal. Calcd for $C_{287}H_{518}N_{14}O_{154}S_{35}$: C 44.46, H 6.75, N 2.53. Found: C 44.19, H 6.36, N 2.43.

Heptakis[6-[2-[N'-[2,2-bis[5-(2,3,4,6-tetra-*O*-acetyl-*α*-D-mannopyranosylthio)-2-oxapentyl]-2-(2-oxapent-4-enyl)ethyl]thioureido]-ethylthio]]cyclomaltoheptaose (34). Reaction time: 24 h. Yield: 81.2 mg (84%); $[\alpha]_D$ = +7.0 (*c* 1.0, MeOH); R_f 0.54 (10 : 1 : 1 MeCN–H_2O–NH_4OH); ^1H NMR (500 MHz, CD_3OD, 323 K): δ = 5.93 (ddt, 7 H, $^3J_{H,H}$ = 17.0 Hz, $^3J_{H,H}$ = 10.5 Hz, $^3J_{H,H}$ = 5.0 Hz, OCH_2CH=), 5.35 (bd, 14 H, $J_{1,2}$ = 3.3 Hz, H-1_{Man}), 5.32 (dd, 14 H, $J_{2,3}$ = 3.0 Hz, H-2_{Man}), 5.26 (t, 14 H, $J_{3,4} = J_{4,5}$ = 10.0 Hz, H-4_{Man}), 5.29 (bd, 7H, =CHa), 5.20 (dd, 14 H, H-3_{Man}), 5.18 (da, 7H, =CHb), 5.01 (bs, 7 H, H-1), 4.37 (ddd, 14 H, $J_{5,6a}$ = 5.0 Hz, $J_{5,6b}$ = 2.0 Hz, H-5_{Man}), 4.27 (dd, 14 H, $J_{6a,6b}$ = 12.5 Hz, H-$6a_{Man}$), 4.12 (dd, 14 H, H-$6b_{Man}$), 4.00 (bd, 14 H, OCH_2CH=), 3.96 (m, 7 H, H-5), 3.82 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 3.71 (m, 14 H, CH_2N_{Cyst}), 3.55 (t, 28 H, $^3J_{H,H}$ = 5.5 Hz, H-3_{Pent}), 3.51 (m, 14 H, H-2, H-4), 3.44 (m, 56 H, H-1_{Pent}, CH_2OAll, CH_2N_{Branch}), 3.20 (m, 7 H, H-6a), 2.96 (m, 7 H, H-6b), 2.91 (m, 14 H, CH_2S_{Cyst}), 2.80, 2.77 (2 dt, 28 H, $^2J_{H,H}$ = 13.0 Hz, $^3J_{H,H}$ = 6.5 Hz, H-5_{Pent}), 2.13–1.95 (4 s, 168 H, MeCO), 1.92 (m, 28 H, H-4_{Pent}); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K): δ = 183.6 (CS), 172.3–171.5 (CO), 136.4 (CH_2CH=), 117.6 (=CH_2), 103.8 (C-1), 85.9 (C-4), 84.0 (C-1_{Man}), 74.4 (C-3), 73.7 (CH_2CH=), 73.5 (C-2, C-5), 72.5 (C-2_{Man}), 72.2 (C-1_{Pent}), 71.8 (CH_2OAll), 71.2 (C-3_{Man}), 71.1 (C-3_{Pent}), 70.6 (C-5_{Man}), 67.8 (C-4_{Man}), 63.8 (C-6_{Man}), 45.8 (C_q), 45.6 (CH_2N_{Branch}, CH_2N_{Cyst}), 35.0 (CH_2S_{Cyst}), 34.0 (C-6), 30.9 (C-4_{Pent}), 29.6 (C-5_{Pent}), 21.1-20.6 (*Me*CO). Anal. Calcd for $C_{357}H_{546}N_{14}O_{175}S_{28}$: C 49.10, H 6.30, N 2.25. Found: C 49.59, H 6.51, N 2.24.

Heptakis[6-[2-[N'-[2,2-bis[5-(*α*-D-mannopyranosylthio)-2-oxapentyl]-2-(2-oxapent-4-enyl)ethyl]thioureido]ethylthio]]cyclomaltoheptaose (DiMan-βCD). Yield: 59 mg (99%); $[\alpha]_D$ = +89.0 (*c* 1.0 in H_2O); R_f 0.45 (5 : 3 : 5 MeCN–H_2O–NH_4OH); ^1H NMR (500 MHz, D_2O, 333 K): δ = 6.26 (m, 7 H, OCH_2CH=), 5.64 (m, 7 H, $^3J_{H,H}$ = 17.5 Hz, =CH_a), 5.59 (d, 14 H, $J_{1,2}$ = 1.7 Hz, H-1_{Man}), 5.57 (bd, 7 H, $^3J_{H,H}$ = 10.5 Hz, =CH_b), 5.39 (bs, 7 H, H-1), 4.34 (m, 28 H, H-2_{Man}, OCH_2CH=), 4.21 (m, 14 H, H-5_{Man}), 4.19 (m, 14 H, H-5, H-3), 4.13 (m, 14 H, H-$6a_{Man}$), 4.06 (m, 28 H, H-3_{Man}, H-$6b_{Man}$), 4.04 (m, 14 H, H-4_{Man}), 4.00 (m, 2 H, CH_2N_{Cyst}), 3.97 (bd, 7 H, $J_{2,3}$ = 9.5 Hz, H-2), 3.87 (m, 35 H, H-3_{Pent}, H-4), 3.74 (m, 44 H, H-1_{Pent}, CH_2N_{Branch}, CH_2OAll), 3.58 (m, 7 H, H-6a), 3.29 (m, 7 H, H-6b), 3.23 (m, 2 H, CH_2S_{Cyst}), 3.03 (m, 28 H, H-5_{Pent}), 2.23 (m, 28 H, H-4_{Pent}); ^{13}C NMR (125.7 MHz, D_2O, 333 K): δ = 181.5 (CS), 131.3 (CH_2CH=), 118.0 (=CH_2), 103.0 (C-1), 85.6 (C-1_{Man}), 83.6 (C-4), 73.8 (C-5_{Man}), 73.2 (C-3), 72.8 (CH_2CH=), 72.6 (C-2, C-5), 72.5 (C-2_{Man}), 71.9 (C-3_{Man}), 70.9 (C-1_{Pent}), 70.6 (C-3_{Pent}, CH_2OAll), 67.5 (C-4_{Man}), 61.4 (C-6_{Man}), 44.8, 44.2 (C_q, CH_2N_{Cyst}, CH_2N_{Branch}), 33.2 (C-6), 31.8 (CH_2S_{Cyst}), 29.6 (C-4_{Pent}), 28.4 (C-5_{Pent}); MALDI-TOFMS: *m/z* 6394.53 [M + Na]$^+$. Anal. Calcd for $C_{245}H_{434}N_{14}O_{119}S_{28}$: C 46.14, H 6.86, N 3.07. Found: C 46.62, H 6.83, N 2.87.

Heptakis[6-[2-[N'-[2-[5-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)-2-oxapentyl]-2,2-bis[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]]cyclomaltoheptaose (35). Reaction time: 48 h. Yield: 24 mg (26%); $[\alpha]_D$ = +76.8 (*c* 1.0, MeOH); R_f 0.67 (10 : 1 : 1 MeCN–H_2O–NH_4OH); ^1H NMR (500 MHz, CD_3OD, 323 K): δ = 5.36 (bs, 14 H, H-1_{Man}), 5.33 (14 H, dd, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 1.5 Hz, H-2_{Man}), 5.27 (t, 14 H, $J_{3,4} = J_{4,5}$ = 10.0 Hz, H-4_{Man}), 5.26 (t, 7 H, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, H-3_{Glc}), 5.21 (dd, 14 H, H-3_{Man}), 5.03 (t, 7 H, $J_{4,5}$ = 10.0 Hz, H-4_{Glc}), 5.02 (m, 7 H, H-1), 4.95 (t, 7 H, $J_{1,2}$ = 10.0 Hz, H-2_{Glc}), 4.74 (d, 7 H, H-1_{Glc}), 4.39 (ddd, 14 H, $J_{5,6a}$ = 5.0 Hz, $J_{5,6b}$ = 2.0 Hz, H-5_{Man}), 4.28 (dd, 14 H, $J_{6a,6b}$ = 12.5 Hz, H-$6a_{Man}$), 4.24 (dd, 7 H, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 4.0 Hz, H-$6a_{Glc}$), 4.15 (dd, 7 H, $J_{5,6b}$ = 1.5 Hz, H-$6b_{Glc}$), 4.13 (dd, 14 H, H-$6b_{Man}$), 3.96 (m, 7 H, H-5), 3.90 (ddd, 7 H, H-5_{Glc}), 3.83 (t, 7 H, $J_{2,3} = J_{3,4}$ = 8.5 Hz, H-3), 3.72 (m, 14 H, CH_2N_{Cyst}), 3.55 (m, 42 H, H-3_{Pent}), 3.54 (m, 14 H, H-2, H-4), 3.43 (s, 56 H, CH_2N_{Branch}, H-1_{Pent}), 3.22 (m, 7 H, H-6a), 3.01 (m, 7 H, H-6b), 2.91 (14 H, m, CH_2S_{Cyst}), 2.81, 2.78 (2 dt, 42 H, $^2J_{H,H}$ = 12.5 Hz, $^3J_{H,H}$ = 5.5 Hz, H-5_{Pent}), 2.14–1.96 (s, 252 H, MeCO), 1.99 (42 H, m, H-4_{Pent}); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K): δ = 183.6 (CS), 172.7-169.9 (CO), 103.8 (C-1), 85.9 (C-4), 84.9 (C-1_{Glc}), 84.0 (C-1_{Man}), 76.9 (C-5_{Glc}), 75.5 (C-3_{Glc}), 74.6 (C-3), 74.3 (C-2, C-5), 72.5 (C-2_{Man}), 72.1 (C-1_{Pent}), 71.8 (C-2_{Glc}), 71.2 (C-3_{Man}), 71.1 (C-3_{Pent}), 70.6 (C-5_{Man}), 70.1 (C-4_{Glc}), 67.8 (C-4_{Man}), 63.8 (C-6_{Man}), 63.6 (C-6_{Glc}), 45.9 (C_q), 45.7 (CH_2N_{Branch}, CH_2N_{Cyst}), 35.1 (C-6), 34.0 (CH_2S_{Cyst}), 31.4 (C-$4_{PentGlc}$), 31.0 (C-$4_{PentMan}$), 29.6 (C-$5_{PentMan}$), 28.5 (C-$5_{PentGlc}$), 21.1-20.7 (*Me*CO). Anal. Calcd. for $C_{455}H_{686}N_{14}O_{238}S_{35}$: C 48.43, H 6.14, N 1.74. Found: C 48.31, H 5.88, N 1.70.

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Heptakis[6-[2-[*N′*-[2-[5-(β-D-glucopyranosylthio)-2-oxapentyl]-2,2-bis[5-(α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]-ethylthio]]cyclomaltoheptaose (DiManGlc-βCD). Yield: 17 mg (99%); $[\alpha]_D$ = +66.0 (*c* 1.0 in H_2O); ^1H NMR (500 MHz, D_2O, 353 K): δ = 5.67 (s, 14 H, H-1_{Man}), 5.47 (bs, 7 H, H-1), 4.86 (d, 7 H, $J_{1,2}$ = 10.0 Hz, H-1_{Glc}), 4.43 (m, 14 H, H-2_{Man}), 4.29 (m, 21 H, H-5_{Man}, H-5), 4.26 (m, 7 H, H-3), 4.25 (d, 7 H, $J_{6a,6b}$ = 12.0 Hz, H-$6a_{Glc}$), 4.14 (m, 42 H, H-3_{Man}, H-$6a_{Man}$, H-$6b_{Man}$), 4.14 (m, 14 H, H-4_{Man}), 4.09 (d, 7 H, H-$6b_{Glc}$), 4.08 (m, 14 H, CH_2N_{Cyst}), 4.04 (m, 7 H, H-2), 3.95 (m, 49 H, H-3_{Pent}, H-4), 3.85 (t, 7 H, $J_{2,3}$ = $J_{3,4}$ = 8.9 Hz, H-3_{Glc}), 3.80 (m, 70 H, H-4_{Glc}, H-5_{Glc}, CH_2N_{Branch}, H-1_{Pent}), 3.71 (t, 7 H, H-2_{Glc}), 3.61 (m, 7 H, H-6a), 3.37 (m, 7 H, H-6b), 3.32 (m, 14 H, CH_2S_{Cyst}), 3.17, 3.12 (2 dt, 42 H, $^2J_{H,H}$ = 13.5 Hz, $^3J_{H,H}$ = 6.5 Hz, H-5_{Pent}), 2.30 (t, 42 H, $^3J_{H,H}$ = 6.5 Hz, H-4_{Pent}); ^{13}C NMR (125.7 MHz, D_2O, 353 K): δ = 182.0 (CS), 102.7 (C-1), 86.1 (C-1_{Glc}), 85.7 (C-1_{Man}), 85.2 (C-4), 80.5 (C-5_{Glc}), 78.0 (C-3_{Glc}), 73.9 (C-5_{Man}), 73.1 (C-3), 73.0 (C-2_{Glc}), 72.8 (C-2, C-5), 72.6 (C-2_{Man}), 72.0 (C-3_{Man}), 71.1 (C-1_{Pent}), 70.8 (C-3 $_{Pent}$), 70.3 (C-4_{Glc}), 67.7 (C-4_{Man}), 61.8 (C-6_{Glc}), 61.5 (C-6_{Man}), 45.1 (CH_2N_{Branch}, CH_2N_{Cyst}, C_q), 34.2 (C-6), 33.1 (CH_2S_{Cyst}), 30.2 (C-$4_{PentGlc}$), 29.3 (C-$4_{PentMan}$), 28.5 (C-$5_{PentMan}$), 27.5 (C-$5_{PentGlc}$); MALDI-TOFMS: *m/z* 7768 [M + Na]$^+$. Anal. Calcd. for $C_{287}H_{518}N_{14}O_{154}S_{35}$: C 44.46, H 6.75, N 2.53. Found: C 44.33, H 6.67, N 2.44.

Heptakis[6-[2-[*N′*-[2,2-bis[5-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)-2-oxapentyl]-2-[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]]cyclomaltoheptaose (36). Reaction time: 5 days. Yield: 30 mg (30%); $[\alpha]_D$ = +32.3 (*c* 1.0, MeOH); R_f 0.50 (10 : 1 : 1 MeCN–H_2O–NH_4OH); ^1H NMR (500 MHz, CD_3OD, 313 K): δ = 5.39 (s, 7 H, H-1_{Man}), 5.34 (bd, 7 H, $J_{2,3}$ = 5.0 Hz, H-2_{Man}), 5.28 (t, 14 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3_{Glc}), 5.27 (t, 7 H, $J_{3,4}$ = $J_{4,5}$ = 10.5 Hz, H-4_{Man}), 5.20 (dd, 7 H, H-3_{Man}), 5.15 (m, 7 H, H-1), 5.04 (t, 14 H, $J_{4,5}$ = 10.0 Hz, H-4_{Glc}), 4.96 (t, 14 H, $J_{1,2}$ = 10.0 Hz, H-2_{Glc}), 4.76 (d, 14 H, H-1_{Glc}), 4.39 (ddd, 7 H, $J_{5,6a}$ = 2.5 Hz, $J_{5,6b}$ = 1.5 Hz, H-5_{Man}), 4.29 (dd, 7 H, $J_{6a,6b}$ = 10.5 Hz, H-$6a_{Man}$), 4.26 (dd, 14 H, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 4.5 Hz, H-$6a_{Glc}$), 4.15 (dd, 14 H, $J_{5,6b}$ = 2.5 Hz, H-$6b_{Glc}$), 4.13 (dd, 7 H, H-$6b_{Man}$), 3.96 (m, 7 H, H-5), 3.92 (ddd, 14 H, H-5_{Glc}), 3.82 (t, 7 H, $J_{2,3}$ = $J_{3,4}$ = 10.0 Hz, H-3), 3.73 (m, 14 H, CH_2N_{Cyst}), 3.54 (m, 42 H, H-3_{Pent}), 3.52 (m, 14 H, H-2, H-4), 3.42 (s, 56 H, CH_2N_{Branch}, H-1_{Pent}), 3.22 (m, 7 H, H-6a), 3.00 (m, 7 H, H-6b), 2.92 (m, 14 H, CH_2S_{Cyst}), 2.83, 2.79 (2 dt, 42 H, $^2J_{H,H}$ = 11.5 Hz, $^3J_{H,H}$ = 6.5 Hz, H-5_{Pent}), 2.07–1.97 (s, 252 H, MeCO), 1.92 (m, 42 H, H-4_{Pent}); ^{13}C NMR (125.7 MHz, CD_3OD, 313 K): δ = 181.9 (CS), 172.2–171.1 (CO), 103.7 (C-1), 85.9 (C-4), 84.9 (C-1_{Glc}), 84.0 (C-1_{Man}), 76.9 (C-5_{Glc}), 75.4 (C-3_{Glc}), 74.5 (C-3), 74.3 (C-2, C-5), 72.5 (C-2_{Man}), 72.0 (C-1_{Pent}), 71.7 (C-2_{Glc}), 71.2 (C-3_{Man}), 71.1 (C-3_{Pent}), 70.5 (C-5_{Man}), 70.1 (C-4_{Glc}), 67.7 (C-4_{Man}), 63.8 (C-6_{Man}), 63.6 (C-6_{Glc}), 45.9 (C_q), 45.7 (CH_2N_{Branch}, CH_2N_{Cyst}), 35.0 (C-6), 34.0 (CH_2S_{Cyst}), 31.4 (C-$4_{PentGlc}$), 30.9 (C-$4_{PentMan}$), 29.6 (C-$5_{PentMan}$), 28.1 (C-$5_{PentGlc}$), 21.2-20.9 (*Me*CO). Anal. Calcd for $C_{455}H_{686}N_{14}O_{238}S_{35}$: C 48.43, H 6.14, N 1.74. Found: C 48.30, H 5.98, N 1.63.

Heptakis[6-[2-[*N′*-[2,2-bis[5-(β-D-glucopyranosylthio)-2-oxapentyl]-2-[5-(α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]]cyclomaltoheptaose (ManDiGlc-βCD). Yield: 21 mg (99%); $[\alpha]_D$ = +25.0 (*c* 1.0 in H_2O); ^1H NMR (500 MHz, D_2O, 353 K): δ = 5.87 (s, 7 H, H-1_{Man}), 5.60 (bd, 7 H, $J_{1,2}$ = 3.5 Hz, H-1), 5.00 (d, 14 H, $J_{1,2}$ = 10.0 Hz, H-1_{Glc}), 4.57 (m, 7 H, H-2_{Man}), 4.42 (m, 7 H, H-5_{Man}), 4.41 (m, 7 H, H-5), 4.39 (d, 14 H, $J_{6a,6b}$ = 12.0 Hz, H-$6a_{Glc}$), 4.38 (m, 7 H, H-3), 4.36 (m, 21 H, H-3_{Man}, H-$6a_{Man}$, H-$6b_{Man}$), 4.29 (m, 7 H, H-4_{Man}), 4.25 (dd, 14 H, $J_{5,6b}$ = 4.5 Hz, H-$6b_{Glc}$), 4.22 (m, 14 H, CH_2N_{Cyst}), 4.17 (d, 7 H, H-2), 4.09 (t, 42 H, $^3J_{H,H}$ = 5.5 Hz, H-3_{Pent}), 4.08 (m, 7 H, H-4), 4.02 (t, 14 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3_{Glc}), 3.96 (m, 84 H, H-4_{Glc}, H-5_{Glc}, CH_2N_{Branch}, H-1_{Pent}), 3.85 (t, 14 H, H-2_{Glc}), 3.78 (m, 7 H, H-6a), 3.51 (m, 7 H, H-6b), 3.46 (bt, 14 H, $^3J_{H,H}$ = 6.5 Hz, CH_2S_{Cyst}), 3.34, 3.32 (2 dt, 42 H, $^2J_{H,H}$ = 13.0 Hz, $^3J_{H,H}$ = 6.0 Hz, H-5_{Pent}), 2.45 (m, 42 H, H-4_{Pent}); ^{13}C NMR (125.7 MHz, D_2O, 353 K): δ = 181.9 (CS), 102.8 (C-1), 86.3 (C-1_{Glc}), 86.0 (C-1_{Man}), 85.3 (C-4), 80.7 (C-5_{Glc}), 78.3 (C-3_{Glc}), 74.1 (C-5_{Man}), 73.6 (C-3), 73.4 (C-2_{Glc}), 73.0 (C-2, C-5), 72.8 (C-2_{Man}), 72.3 (C-3_{Man}), 71.4 (C-1_{Pent}), 71.0 (C-3_{Pent}), 70.6 (C-4_{Glc}), 67.9 (C-4_{Man}), 62.0 (C-6_{Glc}), 61.7 (C-6_{Man}), 47.0 (C_q), 45.0 (CH_2N_{Branch}, CH_2N_{Cyst}), 34.5 (C-6), 33.3 (CH_2S_{Cyst}), 30.4 (C-$4_{PentGlc}$), 30.0 (C-$4_{PentMan}$), 28.7 (C-$5_{PentMan}$), 27.6 (C-$5_{PentGlc}$); MALDI-TOFMS: *m/z* 7768 [M + Na]$^+$. Anal. Calcd. for $C_{287}H_{518}N_{14}O_{154}S_{35}$: C 44.46, H 6.75, N 2.53. Found: C 44.29, H 6.63, N 2.34.

Heptakis[6-[2-[*N′*-[7-(α-D-mannopyranosylthio)-4-oxaheptyl]-thioureido]ethylthio]]cyclomaltoheptaose (Man-βCD). Yield: 20.4 mg (46%); R_f 0.51 (5 : 3 : 5 MeCN–H_2O–NH_4OH); $[\alpha]_D$ = +10.8 (*c* 1.0 in H_2O); ^1H NMR (500 MHz, D_2O, 343 K): δ = 5.69 (s, 7 H, H-1_{Man}), 5.49 (d, 7 H, $J_{1,2}$ = 3.0 Hz, H-1), 4.44 (m, 7 H, H-2_{Man}), 4.32 (m, 14 H, H-5_{Man}, H-5), 4.27 (t, 7 H, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, H-3), 4.23 (m, 14 H, H-$6a_{Man}$, H-$6b_{Man}$), 4.17 (dd, 7 H, $J_{3,4}$ = 9.3 Hz, $J_{2,3}$ = 2.9 Hz, H-3_{Man}), 4.12 (m, 21 H, H-4_{Man}, CH_2N_{Cyst}), 4.06 (dd, 7 H, H-2), 3.98 (t, 14 H, $^3J_{H,H}$ = 6.2 Hz, H-5_{Hept}), 3.96 (t, 14 H, $^3J_{H,H}$ = 6.0 Hz, H-3_{Hept}), 3.94 (m, 7 H, H-4), 3.89 (m, 14 H, H-1_{Hept}), 3.67 (m, 7 H, H-6a), 3.40 (m, 7 H, H-6b), 3.34 (bt, 14 H, $^3J_{H,H}$ = 6.5 Hz, CH_2S_{Cyst}), 3.15, 3.11 (2 dt, 14 H, $^2J_{H,H}$ = 13.2 Hz, $^3J_{H,H}$ = 6.4 Hz, H-7_{Hept}), 2.32 (m, 14 H, H-6_{Hept}), 2.27 (m, 14 H, H-2_{Hept}); ^{13}C NMR (125.7 MHz, D_2O, 343 K): δ = 181.0 (CS), 102.7 (C-1), 85.7 (C-1_{Man}), 85.0 (C-4), 73.9 (C-5_{Man}), 73.5 (C-3), 72.8 (C-2, C-5), 72.6 (C-2_{Man}), 72.0 (C-3_{Man}), 69.8 (C-5_{Hept}), 68.9 (C-3_{Hept}), 67.7 (C-4_{Man}), 61.6 (C-6_{Man}), 44.5 (CH_2N_{Cyst}), 42.0 (C-1_{Hept}), 34.1 (C-6), 33.1 (CH_2S_{Cyst}), 29.8 (C-2_{Hept}), 29.3 (C-6_{Hept}), 28.4 (C-7_{Hept}); MALDI-TOFMS: *m/z* 4036.74 [M + H]$^+$. Anal. Calcd for $C_{147}H_{266}N_{14}O_{70}S_{21}$: C 43.89, H 6.66, N 4.87. Found: C 44.13, H 6.51, N 4.76.

Enzyme-linked lectin assay (ELLA)

Nunc-Inmuno(tm) plates (MaxiSorp™) were coated overnight with yeast mannan at 100 μL/well diluted from a stock solution of 10 μg mL^{-1} in 0.01 M phosphate buffer saline (PBS, pH 7.3 containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}) at room temperature. The wells were then washed three times with 300 μL of washing buffer (containing 0.05% (v/v) Tween 20) (PBST). The washing procedure was repeated after each of the incubations throughout the assay. The wells were then blocked with 150 μL/well of 1% BSA/PBS for 1 h at 37 °C. After washing, the wells were filled with 100 μL of serial dilutions of horseradish peroxidase labelled concanavalin A (Con A-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/well of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (0.25 mg mL^{-1}) in citrate 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/well of 1 M

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H_2SO_4 and the absorbances were measured at 415 nm. Blank wells contained citrate-phosphate buffer. The concentration of lectin–enzyme conjugate that displayed an absorbance between 0.8 and 1.0 was used for inhibition experiments.

In order to carry out the inhibition experiments, each inhibitor was added in a serial of 2-fold dilutions (60 μL/well) in PBS with 60 μL of the desired Con A–peroxidase conjugate concentration on Nunclon(tm) (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 1 h at 37 °C. The plates were washed and the ABTS substrate was added (50 μL/well). Color development was stopped after 20 min and the absorbances were measured. The percent of inhibition was calculated as follows:

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

Results in triplicate were used for plotting the inhibition curves for each individual ELLA experiment. Typically, the IC_{50} values (concentration required for 50% inhibition of the Con A coating mannan association) obtained from several independently performed tests were in the range of ±15%. Nevertheless, the relative inhibition values calculated from independent series of data were highly reproducible.

Isothermal titration calorimetry experiments (ITC)

The lectin solutions to be used for ITC were prepared in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}, dialysed against a large volume of the same buffer and centrifuged to remove any insoluble material. The protein concentrations were determined colorimetrically following the Bradford's method,[46] measuring the absorbance at 595 nm of a solution (1 mL) containing 200 μL of the commercial reagent (BIO-RAD protein assay), distilled H_2O (780 μL) and 20 μL of protein solution containing 2–10 μg of protein. Calibration of the commercial reagent was carried out using a 10% (w/v) solution of BSA. The extinction coefficient determined for the colorimetric complex was $\varepsilon_{595} = 45$ mL mg^{-1} cm^{-1}. A multichannel thermal activity monitor (TAM) isothermal heat conduction microcalorimeter (Thermometric AB 2277/201, Järfälla, Sweden) was used. This instrument has been described in detail by Suurkuusk and Wadsö.[47] The microcalorimeter was connected to an external H_2O circulator (Hato) and the whole system was placed in a room in which the temperature was kept constant within ±0.5 °C. A 1.1 mL titration vessel was equipped with a stirring system, loaded with 0.8 mL of protein solution using a microsyringe, thermostated at 25 °C and continuously stirred at 60 rpm. The ligand was injected through a stainless steel cannula connected to a 250 μL microsyringe, which was positioned in a computer controlled syringe pump (Hamilton Microlab M). Injections were made over a period of 10 s with intervals of 6 min. The experiment was computer controlled using Digitam 4.1 software (Thermometric); the same program was used for data analysis. The microcalorimeter was electrically calibrated before each experiment. Nevertheless, in order to check the performance, the binding of Ba^{2+} (aq) to 18-crown-6 was used. The results obtained, $K_c = 5667 \pm 680$ M^{-1} and $\Delta H_m = -(31.46 \pm 0.70)$ kJ mol^{-1} are in agreement with those reported by Briggner and Wadsö:[48] $K_c = 5900 \pm 200$ M^{-1} and $\Delta H_m = -(31.42 \pm 0.20)$ kJ mol^{-1}. To minimize dilution artefacts, the ligand was dissolved in the same dialysis buffer as the protein. A separate experiment was run for each titration in order to determine the heat of dilution of the ligand in the dialysis buffer.

Surface plasmon resonance experiments (SPR)

Con A and ribonuclease B (RNase B, EC 3.1.27.5) were purchased from Sigma. Red cellulose filters were from Agilent technologies (0.22 μm × 13 mm) or from Millipore (0.22 μm × 47 mm). All SPR experiments were performed at 25 °C using a BIAcore 3000 system (BIAcore International AB, Uppsala, Sweden), using a PBS buffer (pH 7.3) consisting of 10 mM sodium phosphate, 10 mM NaCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ as running buffer. To account for the overestimation of K_a associated to mass transport limitation effects[37] at the surface of the chip, high and low density channels were used. Due to the high molecular weight of CD-glycoconjugates, mass transport effects in the flow channel on the sensor chip could not be discarded completely, even at the low density surfaces. Low and high-density chips were prepared by immobilizing Con A as follows. For the experiments a CM5 sensorchip (Pharmacia) was activated[49] and Con A (in 10 mM NaOAc, pH 4.0) was immobilised to three flow channels (FC2, FC3 and FC4, ~11600 RU). As a reference surface (FC1) an activated-deactivated carboxymethylated dextran layer was employed. Furthermore, the lectin bound to channel 3 was denatured by flowing 6 M guanidinium chloride (pH 1.0) and 0.5% SDS solution across the flow cell. The denatured lectin was used to measure the level of nonspecific binding and to serve as reference channel for analysis of the data.[40] Experiments were performed at a flow rate of 10 μL min^{-1}. For interaction experiments, typically, a solution of carbohydrate containing material (15–30 μL) was flowed across the sensor-chip for the association phase. Then, spontaneous dissociation was allowed to occur for another 3 min and subsequently the surface was regenerated, and stabilised for the next round of binding.

For calculation of the kinetics of the binding of the different compounds, samples were appropriately diluted in phosphate buffer, to cover the concentration range from 0.5 mM to 4 mM for alcohol and model glycodendrimers, and from 12.5 μM to 400 μM for βCD glycoclusters. Sample solutions at different concentrations were flown across the surfaces for 3 min, followed by a dissociation period of 6 min prior to regeneration. After a dissociation period (~ 3-6 min) remaining material was eluted from the surface using two consecutive injections of 5 mM sodium hydroxide (15 μL) and two consecutive injections of 100 mM (15 μL) methyl α-D-mannopyranoside in running buffer (regeneration A) or using two consecutive injections of 5 mM sodium hydroxide (5 μL) and two consecutive injections of 200 mM (10 μL) methyl α-D-mannopyranoside in running buffer (regeneration B). Occasionally (in between different compounds), surface regeneration was performed using 10 mM sodium acetate (pH 4.2) followed by a re-equilibration injection employing a running buffer.

Association and dissociation rate constants were calculated by nonlinear fitting of the primary sensorgram data using the BIAevaluation 3.0 software. The precision of the fit for each estimated parameter is described by the statistical χ^2. The association constant (K_a) was calculated both experimentally from the association (k_a) and dissociation (k_d) rate constants,

and by Scatchard analyses based on the equilibrium steady-state kinetics.

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