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Mannosylated G(0) Dendrimers with Nanomolar Affinities to *Escherichia coli* FimH

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Pentaerythritol and bis-pentaerythritol scaffolds were used for the preparation of first generation glycodendrimers bearing aryl α-D-mannopyranoside residues assembled using single-step Sonogashira and click chemistry. The carbohydrate precursors were built with either para-iodophenyl, propargyl, or 2-azidoethyl aglycones whereas the pentaerythritol moieties were built with terminal azide or propargyl groups, respectively. Cross-linking abilities of this series of glycodendrimers were first evaluated with the lectin from Canavalia ensiformis *(Concanavalin A). Surface plasmon resonance measurements showed these two families of mannosylated clusters as the best ligands known to date toward* Escherichia coli *K12 FimH with subnanomolar affinities. Tetramer* **4** *had a* K_d *of 0.45 nM. These clusters were 1000 times more potent than mannose for their capacity to inhibit the binding of* E. coli *to erythrocytes in vitro.*

Introduction

Rapid development of drug resistance to clinically important pathogens represents a serious public health threat. The proportion of harmful organisms resistant to methicillin, oxacillin, or nafcillin continues to rise.[1] Even more critical is the development of vancomycin-resistant *enterococci* (VRE). Vancomycin belongs to the glycopeptide drug family and has long been regarded as the last line of defense against drug-resistant strains. Unfortunately, a significant number of enterococcal infections in intensive health care units in the United States have also become resistant to vancomycin. More recently, the first case of fully vancomycin-resistant *Staphylococcus aureus* has been reported.[2] Moreover, within the last ten years, only one significant novel class of non-β-lactam antibiotics has emerged from basic research. From this family of novel oxazolidinones, the potent linezolid was approved in 2000.[3,4] However, the speed of resistance development in bacteria creates a continuous pressure toward the development of novel antibiotics. Again, oxazolidinone resistant bacteria have already appeared.[5,6]

One of the very promising groups of novel compounds for the treatment of bacterial infections appears to be within the family of glycoconjugates that can be used as vaccines[7] or inhibitors of bacterial adhesions.[8] The main advantages of glycoconjugates as antiadhesins are 1) the limited likelihood of resistance development, as resistance would require alterations of the cell surface membranes of the infected host tissues serving as receptors, and 2) their unique mode of action against multiresistant pathogens, as glycoconjugates do not affect classical antibiotic enzyme targets. A large number of bacterial toxins, viruses, and bacteria target carbohydrate derivatives on the cell surface to attach to and gain entry into the cells. Mannopyranoside-specific adhesions are among the most widely distributed type of carbohydrate-specific bacterial adhesion ligands[9] that has also been recently observed for viral infections caused by Ebola and HIV-1.[10] Taken together, these results suggest that α-D-mannopyranoside analogues might play important roles against *E. coli* or in some other viral infections. Glycoconjugates play key roles in cellular recognition, adhesion, cell-growth regulation, cancer cell metastasis, and inflammation.[11] Regrettably, monomeric carbohydrate–protein interactions often occur with low binding affinities ($K_d \approx 10^{-3}$ M).[12] However, multivalent interactions have several advantages over monomeric ones and are often used by nature to control a wide variety of cellular processes. Consequently, multivalent arrangements of ligands are generally beneficial for the accomplishment of physiologically relevant associations.[13] Structurally defined glycoconjugates, such as mannopyranoside dendrimers, should provide the opportunity to intervene in *E. coli* infection processes by blocking the initial stage of adhesion and colonization of host tissues, particularly in urinary or digestive tract infections (UTIs), therefore saturating the FimH present at the tip of the bacterial pili responsible for the adhesion.

A fundamental approach to synthetic, potent, and multivalent carbohydrate-bearing ligands is the attachment of saccharide moieties, necessary for pathogen adhesion, to structurally simple, hyper-branched molecules such as glycodendrimers.[14] Several studies[15–19] have described examples of glycodendri-

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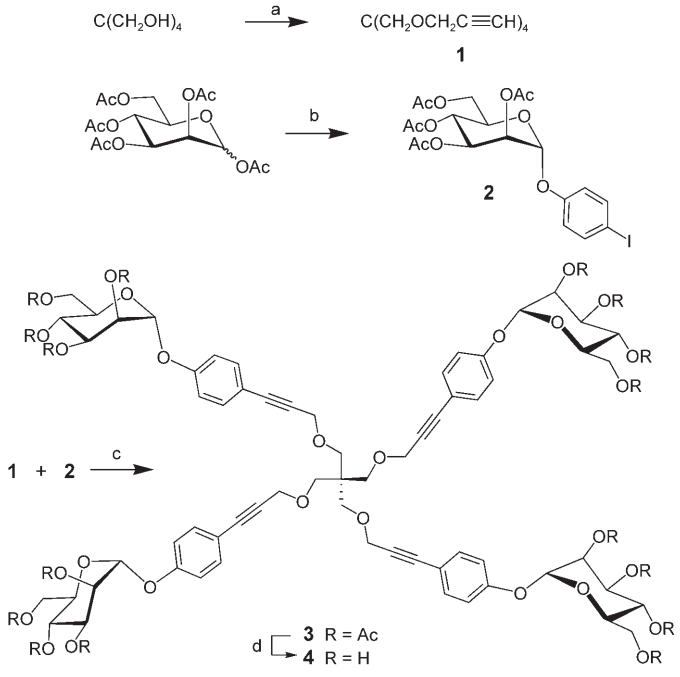
mer syntheses using PAMAM,[15] poly-L-lysine,[16] and related species as multivalent scaffolds,[17] albeit with limited success.[18] Pioneering observations by Sharon et al.[19] have also demonstrated the binding preferences of type 1 fimbriated *E. coli* to mannopyranosides bearing aromatic aglycones. These findings prompted us to evaluate the affinities of several oligomannoside clusters against *E. coli* K12 FimH. Toward this goal, single step multiple Sonogashira coupling[20] and click chemistry were used.[21] Preliminary data from this last family of compounds indicated that they were more potent inhibitors than monomeric D-mannose.[22] These results stimulated the synthesis of further pentaerythritol-based glycodendrimers having aromatic (aryl or triazole) aglycones.

Chemistry

Transition metal-catalysed cross-couplings have proven to be powerful tools for mild, highly efficient carbon–carbon bond formations. Among these processes, those involving palladium catalysis, especially Sonogashira coupling or alkynylation of aryl halides[23] are particularly useful for the synthesis of complex molecules, owing to their excellent levels of selectivity and high functional group compatibility. Consequently and on the basis of previous expertise,[20] the efficient and systematic synthesis of a family of glycodendrimers bearing mannopyranoside residues using multiple Sonogashira couplings is described herein. The target tetramer **4** was synthesised as depicted in Scheme 1. Treatment of pentaerythritol by our modified method with propargyl bromide and KOH afforded known tetrakis(2-propynyloxymethyl)methane **1**[24] in good yield. *p*-Iodophenyl α-D-mannopyranoside derivative **2**[20b] was prepared from peracetylated α,β-D-mannopyranose by glycosidation with triflic acid as a promoter. Sonogashira coupling between tetrapropargyl pentaerythritol derivative **1** and aryl mannoside **2** provided cluster **3** which after deacetylation under Zemplén conditions (NaOMe, MeOH) gave tetramer **4**.



Scheme 1. Reagents and conditions: a) Propargyl bromide, KOH, DMSO, 60 °C, 83%; b) HOPh-*p*I, TfOH, CH_2Cl_2, 0 °C to RT, 76% c) optimised conditions (Table 1, entry 8, 9), 78%; d) MeONa, MeOH, RT, 4 h, (90%).

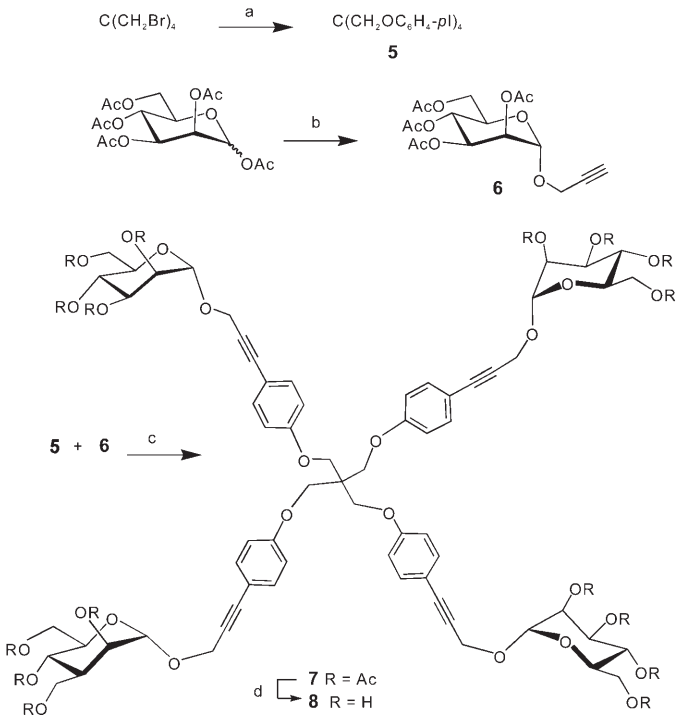
In a previous work, Roy et al.[25] have demonstrated that couplings of propargyl glycosides and aryl halides could be successfully performed at 60 °C in DMF using either Pd^0 or Pd^{II} catalysts and Et_3N in the absence of Cu^I. Initially, multiple one-step Sonogashira coupling between **1** and **2** was attempted following the above procedure. Surprisingly, these conditions did not provide **3** in any acceptable yields (Table 1, entry 1), although alkynyl **1** was consumed or even degraded. To suppress this side reaction, various Pd species and bases, Cu^I addition, and solvent effects were reinvestigated (Table 1). It was ultimately found that $Pd(PPh_3)_2Cl_2$ in the presence of Cu^I catalyst (Table 1, entries 7–10) provided the best results in comparison to $Pd(PPh_3)_4$or $Pd(OAc)_2$. Notably, bases other than piperidine (Table 1, entries 1–7: Et_3N, Et_2NH, Cs_2CO_3) known to prevent alkyne degradation also failed to provide acceptable yields. As solvents, DMF and THF were found to be equally potent (Table 1, entries 8 and 9). Interestingly, the order of addition of the alkyne and iodide played an important role. Homocoupling and degradation were prevented completely by adding tetraalkyne **1** in THF or DMF slowly, to keep its concentration in the reaction mixture at low levels. The optimised conditions were found to be 5 mol% of $Pd(PPh_3)_2Cl_2$, 10 mol% Cu^I, and piperidine.

Table 1. Optimization of the conditions for synthesis of **3**.

Entry	Conditions	**3** [%]
1	$Pd(PPh_3)_4$, Et_3N, DMF, 60 °C	trace
2	$Pd(PPh_3)_4$, CuI, Et_2NH, THF, RT	trace
3	$Pd(PPh_3)_4$, CuI, Et_3N, DMF, RT	20,[a] 25[b]
4	$Pd(PPh_3)_4$, CuI, Et_3N, THF, RT	25[a,b]
5	$Pd(OAc)_2$, Cs_2CO_3, DMF, 60 °C	trace
6	$Pd(PPh_3)_2Cl_2$, Et_3N, DMF, 60 °C	trace
7	$Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, 60 °C	33[a,b]
8	$Pd(PPh_3)_2Cl_2$, CuI, piperidine, THF, RT	44,[a] 78[b]
9	$Pd(PPh_3)_2Cl_2$, CuI, piperidine, DMF, RT	38,[a] 75[b]
10	$Pd(PPh_3)_2Cl_2$, CuI, piperidine, DMF, 60 °C	40[a,b]

[a] Tetrakis(2-propynyloxymethyl)methane **1** was added at once to the reaction mixture. [b] Dropwise addition of **1** to the reaction mixture.

Alternatively, tetramer **8**, possessing the reversed linkage functionality, that is the propargyl group installed on the mannoside residue and the aryl iodide on the pentaerythritol scaffold, was also similarly prepared to investigate the effect of the aryl pharmacophore positioning on binding. Hence, tetrakis[(4-iodophenoxy)methyl]methane **5** was considered as suitable synthon. Precursor **5** was efficiently synthesised in a single step by substitution of pentaerythritol tetrabromide with iodophenol under basic conditions (NaOH, DMF, 78%). Propargyl α-D-mannopyranoside derivative **6** was obtained from peracetylated α,β-D-mannopyranose by glycosidation with propargyl al-



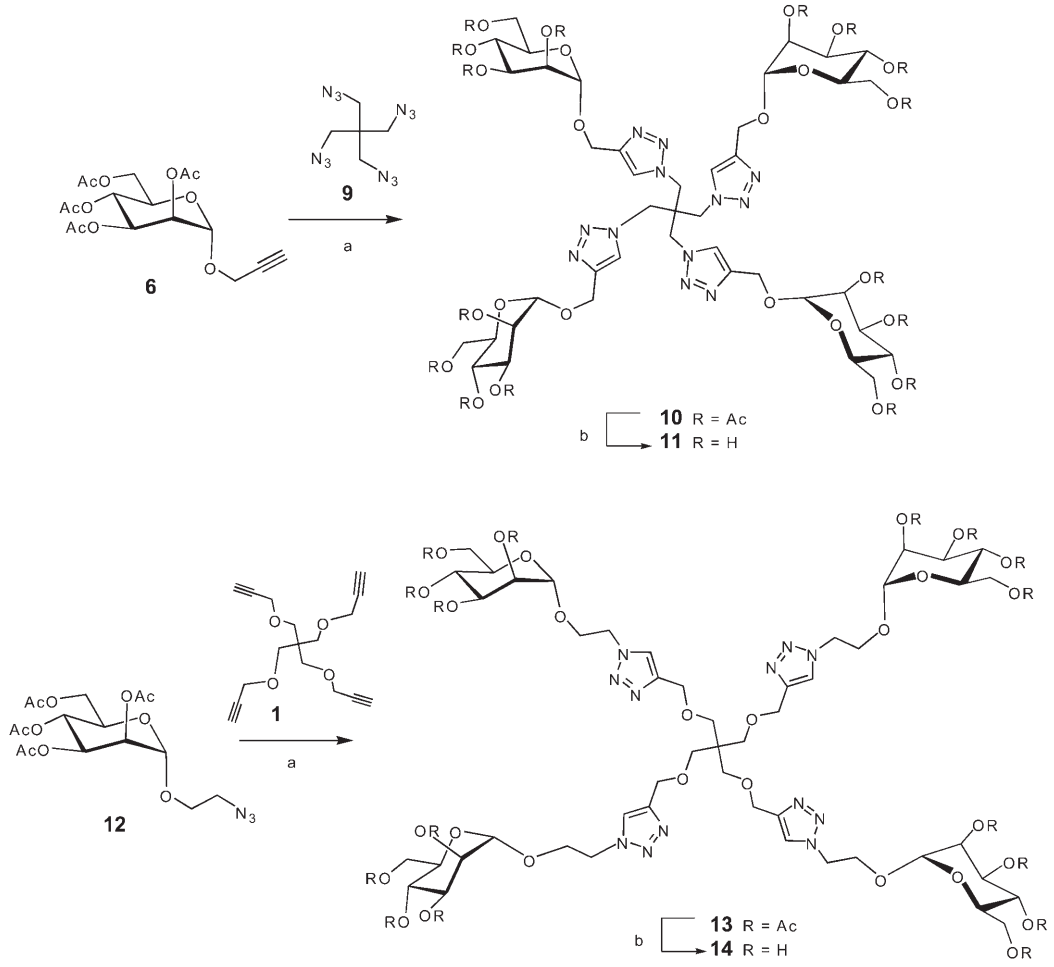
Scheme 2. Reagents and conditions: a) HOPh-*p*I, NaOH, DMF, reflux, 78%; b) propargyl alcohol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, RT, 95%; c) optimised conditions (Table 1, entry 8, 9) (80%); d) MeONa, MeOH, RT, 4 h, (92%).

cohol and $BF_3{\cdot}OEt_2$ in quantitative yield.[26] Interchanged tetramer **7** was then acquired using the optimised conditions described for **3** (Table 1, entry 8) which after deacetylation under Zemplén conditions (NaOMe, MeOH) gave unprotected tetramer **8** in 92% yield (Scheme 2).

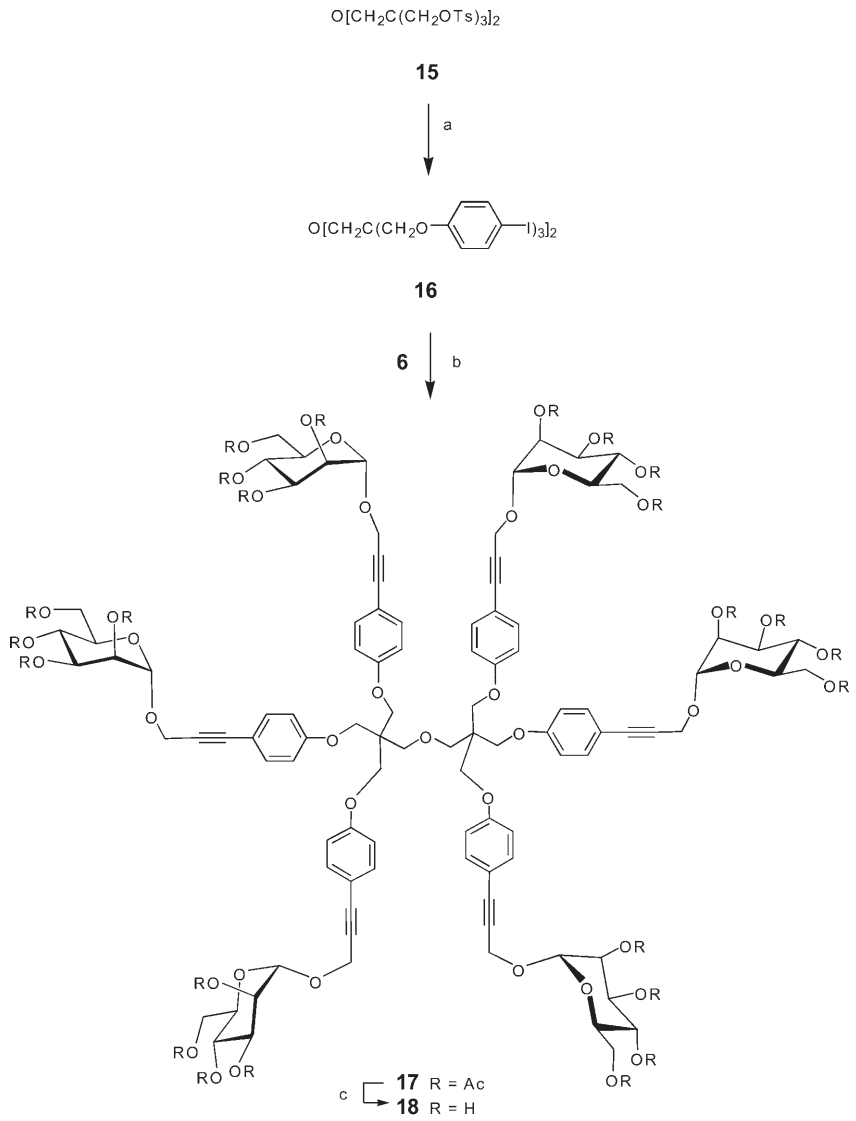
Treatment of tetra-azides **9**[27] and tetrakis(2-propynyloxymethyl)methane **1** in Cu^I catalysed click reaction conditions with prop-2-ynyl α-D-mannopyranoside **6**[26] and 2-azidoethyl-2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranoside **12**[28,29] respectively provided tetramers **11** and **14** in good yields after deacetylation (NaOMe, MeOH) (Scheme 3).

Further elaboration of these dendrimers may show much potential in the way of novel multiarmed clusters with enhanced flexibilities and various geometries. Commercially available bis-pentaerythritol was then used as scaffold. As shown in Scheme 4, hexatosylated dipentaerythritol **15**[30] was converted into the novel hexa-p-iodophenyl scaffold **16** with NaOH and iodophenol in good yield. The Sonogashira coupling reactions between **6** and **16** afforded **17**, followed by **18** after usual deprotection (Scheme 4). All attempts to prepare hexakispropargyl scaffold from bis-pentaerythritol or hexatosylated dipentaerythritol **15**[30] failed.

The novel hexameric azide **19**[21] was obtained by conversion of hexatosylated dipentaerythritol derivative **15**, which, under



Scheme 3. Reagents and conditions: a) $CuSO_4$, Na ascorbate THF/H_2O, RT, 12 h, **10** (92%), **13** (89%); b) MeONa, MeOH, RT, 4 h, **11** (90%), **14** (92%).



Scheme 4. Reagents and conditions: a) HOPh-*p*I, NaOH, DMF, reflux, 79%; b) $Pd(PPh_3)_2Cl_2$, Et_3N, DMF, 60 °C, 79%; c) MeONa, MeOH, RT, 4 h, (92%).

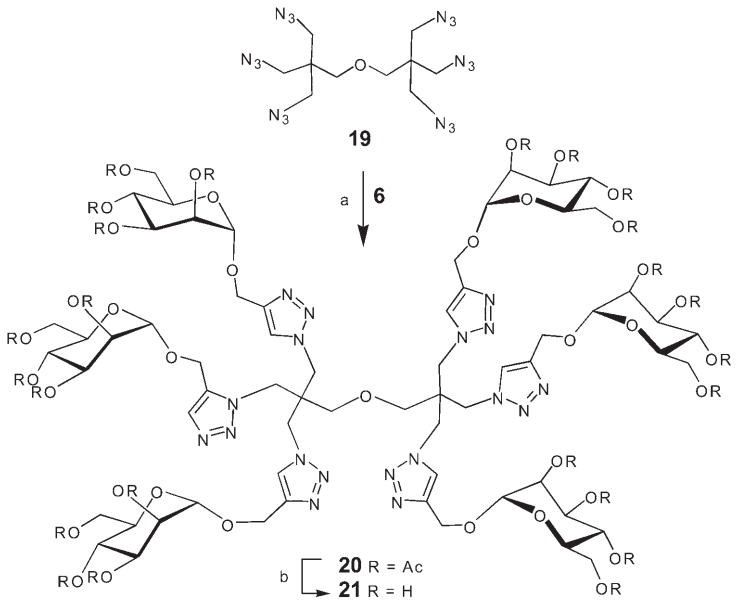
the above cycloaddition conditions afforded hexamer **21** after deacetylation (Scheme 5). The structural integrity of the carbohydrate-coated clusters (glycodendrimers of generation G0) has been fully proven by chromatographic and spectroscopic techniques, including NMR spectroscopy and mass spectrometry.

Biological Assays

Three different types of biological assays were put in place to evaluate the relative binding properties of the above tetra- and hexavalent mannosylated clusters. Initially, the cross-linking abilities of these molecules were investigated using a kinetic turbidimetric assay (nephelometry) as previously used in similar circumstances using the soluble lectin from Concanavalin A (Con A) as a model,[31] although there are also more hydrogen bonds involved at the FimH binding site than at the Con A site. To this end, tetrameric phytohaemagglutinin Concanavalin A from *Canavalia ensiformis* was treated with each of the clusters in microtiter plates. The Sonogashira-type clusters **4**, **8**, and **18** were tested separately from those of the 1,2,3-triazoles **11**, **14**, and **21**. Each of the compounds and the lectin were tested at a concentration of 1 mg mL^{-1} using the polysaccharide yeast mannan as positive control. Rapidly and within twelve minutes, insoluble cross-linked complexes were formed as judged by the cloudiness within the wells (Figure 1 and 2). The optical density (O.D.) was measured at 490 nm. The results compared favourably with previous multivalent mannosides.[31] Secondly, mannoside clusters **4**, **8**, **11**, **14**, **18**, and **21** were tested as inhibitors of the haemagglutination of guinea pig or rabbit erythrocytes by type-1 piliated clinical isolate *E. coli* UTI89. Their inhibitory potential was compared with those of D-mannose and HeptαMan.[32] Inhibition of mannose-sensitive haemagglutination of red blood cells by type-1 piliated *E. coli* by the multivalent mannoside clusters is a fast way to compare their relative inhibitory capacities.[19] Finally, each compound was evaluated for their relative binding affinity by surface plasmon resonance (SPR) against FimH bound to a monoclonal antibody. The results are also reported in thermodynamic terms (Table 2).

Results and Discussion

A critical step in host tissue colonisation is achieved through bacterial adhesion commonly mediated by carbohydrate-binding lectin-like proteins expressed on or shed from bacterial surfaces. Type 1 pili are the most common type of adhesive appendages in *E. coli* and several other enterobacteria that can mediate mannose-specific adhesion via the 30 kDa lectin-like subunit FimH.[24] Initially, evaluation of binding abilities of D-mannose-coated clusters towards Concanavalin A, by turbidimetric measurements in microtiter plates at 490 nm, demonstrated a significant activity of glycoclusters when they were used as ligands in interactions with protein receptors. The time course of formation of insoluble precipitin complexes between Con A and yeast mannan or clusters is illustrated in Figure 1. In



Scheme 5. Reagents and conditions: a) $CuSO_4$, Na ascorbate THF/H_2O, RT, 12 h, 72%; b) MeONa, MeOH, RT, 4 h, 85%.

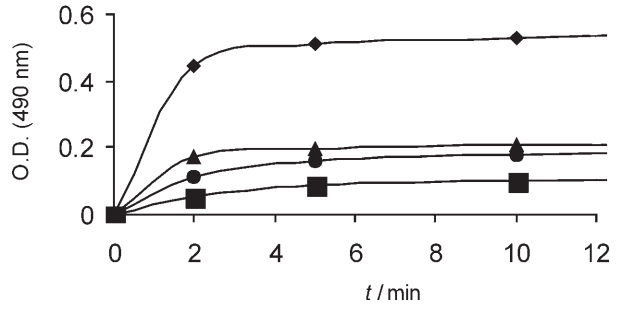


Figure 1. Turbidimetric analysis (micro-precipitation) of Con A with tetramer **4** (◆), tetramer **8** (■), hexamer **18** (●) and yeast mannan (▲) as positive control. Measurements were done in PBS at 1 mg mL^{-1} using an ELISA plate reader at 25 °C and are the average of triplicate values.

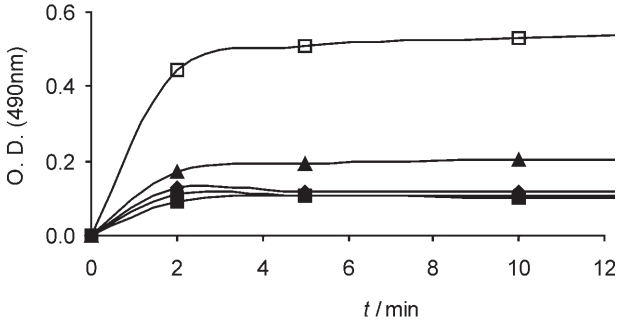


Figure 2. Turbidimetric analysis (micro-precipitation) of Con A with tetramer **4** (□), tetramer **11** (●), tetramer **14** (◆) hexamer **21** (■), and yeast mannan (▲) taken as positive control. Measurements were done in PBS at 1 mg mL^{-1} using an ELISA plate reader at 25 °C and are the average of triplicate values.

both cases, maximum turbidity occurred after approximately 12 min. As shown in Figure 1 and 2, the results clearly illustrate the rapid preference of tetraaryl mannoside **4**, obtained by Sonogashira coupling, to form a cross-linked lattice toward Concanavalin A, over the alternate functional isomer **8** or hexamer **18**. The latter two were significantly less potent than the reference yeast mannan.

Figure 2 shows the relative cross-linking abilities of tetrazole-based G0 glycodendrimers **11**, **14**, and **21** in comparison to **4**. However, as opposed to the above series, there was no marked variation in the cross-linking behaviours arising from tetramers **11** and **14** and the hexamer **21**. Again tetramer **4** was the best candidate with an almost quantitative precipitation of the tetrameric lectin occurring after only 2 min. Obviously, clusters having the triazole heterocycle or the extended series from the Sonogashira coupling were less efficient.

The above results can be rationalised on the basis of the relative stability of the resulting insoluble complexes.[31] Molecular modelling of tetramer **4** in one of its low energy extended conformations is illustrated in Figure 3. Each mannopyranoside residue is at the apex of a tetrahedron which are 18.6 and 16.0 Å apart. This distance can easily accommodate the clustering of four different tetrameric lectins. Figure 4a illustrates such complexes (only one of the possible tetrameric lectin is shown using Connolly surface). By analogy, it is also expected that tetramer **4** would form such strong complexes with FimH (bottom Figure 4b)

Table 2. *E. coli* K12 FimH relative affinities of mannopyranosylated clusters as determined by surface plasmon resonance.

Compd	K_d [nM][a]	K_d [nM] per Man[b]	R K_d[c]	ΔG° [kcal mol^{-1}]
Mannose	2300	2300	0.96	−7.6
MeαMan	2200	2200	1	−7.7
HeptαMan	5	5	440	−11.3
PNPαMan	44	44	50	−10.0
4	0.45	1.8	4889	−12.5
8	273	1092	8.1	−8.9
11	14	56	157	−10.7
14	53.8	215.2	40.9	−9.9
18	267	1602	8.2	−9.0
21	3	18	733	−11.6

[a] $K_d = k_d/k_a$. [b] Relative affinity on a per mannoside residue basis. [c] Relative K_d values based on methyl α-D-mannopyranoside as standard, that is, K_d (MeαMan)/K_d (test compd).

The relative binding ability of the mannoside clusters was then evaluated by SPR measurements on a Biacore3000 as described before for other synthetic monomeric mannosides.[32] The affinity of the lectin domain of isolated FimH of *E. coli* K12 toward our clusters was obtained by competition between an immobilised anti-FimH antibody(1C10) and free mannosylated clusters. Table 2 shows the thermodynamic results from the SPR measurements. The second column gives the K_d values in the nanomolar range, the third column contains the values corrected per mannoside residue, the fourth one the relative affinities (R K_d), and finally, the ΔG° of the synthetic mannoside

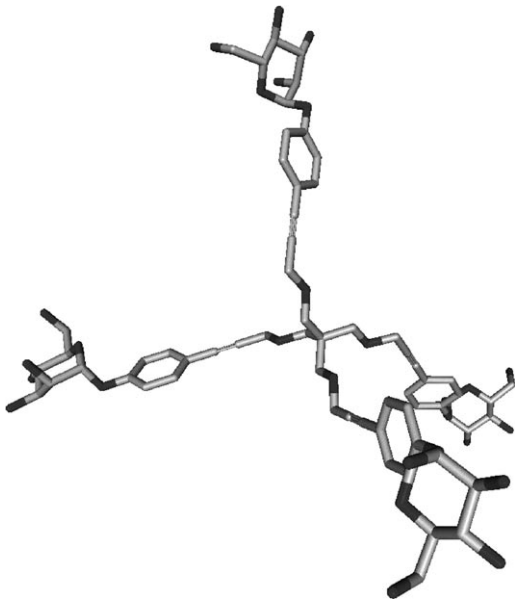


Figure 3. Extended conformation of tetramer **4** showing the relative distances between the α-D-mannopyranoside residues.

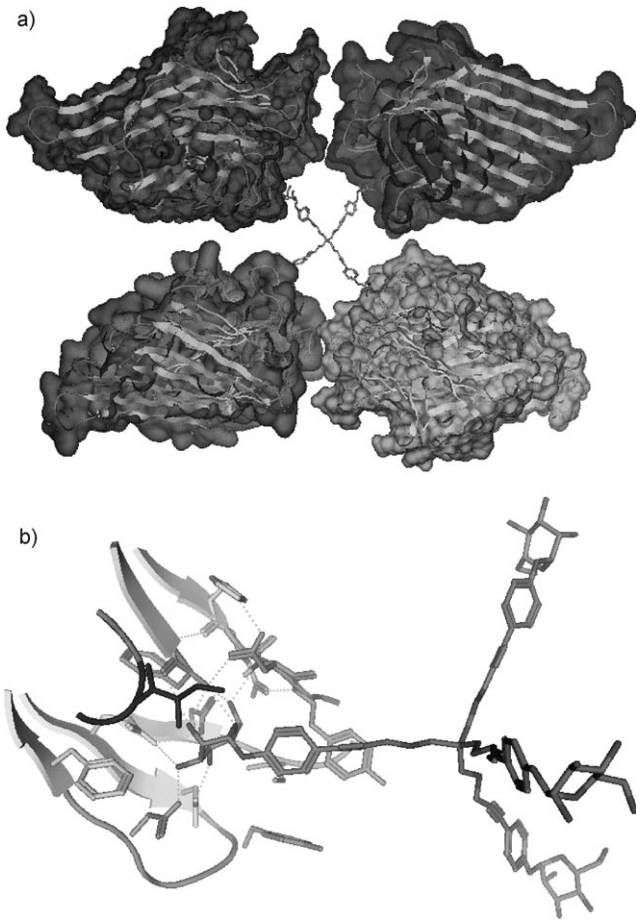


Figure 4. a) Modelled extended tetramer **4** cross-linking four individual Concanavalin A lectin monomers illustrating the capability of compound **4** to form insoluble cross-linked lattices. b) Modelled *E. coli* FimH bound to tetramer **4**.

clusters in comparison to the best ligands known to date.[32] The strongest monovalent inhibitor known to date for FimH is heptyl α-D-mannopyranoside (HeptαMan, $K_d = 5$ nM), which is eight times better than the known *p*-nitrophenyl α-D-mannopyranoside (PNPαMan, $K_d = 44$ nM).[32] As shown in Table 2, the SPR measurements designated tetramer **4** as the best ligand with a K_d in the subnanomolar range ($K_d = 0.45$ nM).

The affinity of all mannosylated glycodendrimers were compared based on the number of mannoside moieties they contained (K_d per Man in Table 2). On the basis of corrected values on a per mannoside residue, tetramer **4**, with a K_d of 0.45 nM ($K_d = 1.8$ nM/Man), readily surpassed the affinity of mannose by 1277-fold and methyl α-D-mannopyranoside by 1222. The strongest monosaccharide ligand known to date, heptyl α-D-mannopyranoside ($K_d = 5$ nM) and *p*-nitrophenyl α-D-mannopyranoside ($K_d = 44$ nM) did not reach the high affinity of FimH toward **4** (K_d 1.8 nM/Man), which is still three times better than HeptαMan and 25 times better than PNPαMan. On the other hand, the position of the phenyl ring appeared to be rather important with regard to modulating the activity of **8** ($K_d =$ 273 nM) which differs from **4** only by the relative positioning of the phenyl ring.

Compounds **11**, **14**, and **21** to which a triazole ring was introduced by click chemistry showed significant affinity (Table 2). The distance between the anomeric oxygen and the triazole ring is important for affinity, as compound **11**, having a single methylene and mannoside moiety attached to the C4 carbon of the triazole ring, showed better affinity (**11**, $K_d =$ 14 nM) than extended **14** ($K_d = 53.8$ nM). Tetramer **11**, directly prepared from tetraazide **9** is thus four times better than tetramer **14** obtained from tetrakis(2-propynyloxymethyl)methane **1**, and surpassed the affinity potency of mannose by 164 and methyl α-D-mannopyranoside by 157. In addition, hexamers **18** and **21** obtained by Sonogashira coupling and cycloaddition, respectively have also been evaluated toward *E. coli* FimH. The spatial arrangement of the hexamer having the triazole rings appeared to be a determinant for affinity as **21** ($K_d = 3$ nM) was nearly five times better than **11** ($K_d = 14$ nM), thus illustrating again the influence of multivalency on this scaffold. The introduction of four or six mannopyranoside moieties using extended precursors and Sonogashira coupling had a minor effect on the relative affinity, as compounds **8** and **18** having four and six mannoside residues, respectively, were almost equipotent (**8**, $K_d = 273$ nM; **18**, $K_d = 267$ nM).

Finally, the mannoside clusters were tested as inhibitors of haemagglutination of erythrocytes by type 1 piliated UTI89 clinical isolate *E. coli* by inhibition of haemagglutination. Guinea pig and rabbit erythrocytes gave similar titers for all compounds. The inhibition titre (IT) is the lowest concentration of the inhibitor at which no agglutination occurs. Tetramer **4** was the best inhibitor of haemagglutination, with an inhibition titer of about 3 μM, or a factor 6000 compared to its affinity (Table 2). It was a 1000-fold better inhibitor than D-mannose, that had an inhibition titer of 3 mM, or a factor 1000 compared to its affinity (Table 2). HeptαMan, **11** and **21** were 500-fold better than mannose. Nevertheless, no multivalency effect was obvious for tetramer **4**, when compared to monomeric Heptα

Man. Compound **18** inhibited 250 times better than mannose. Compounds **8** and **14** showed no inhibition at the minimal concentrations used (200 μM), which may be too low compared with their affinities for FimH (Table 2). Possibly, compound **18** can overcome the lack of inhibition by compound **8** due to a multivalency effect. Hexamer **18** and tetramer **8** carry identical mannoside substituents, however the longer distances in **18** linking the aglycons on the monovalent arms could possibly enable inhibition of haemagglutination by compound **18** in contrast to **8**.

Cautiously however, these results have to be placed in proper perspective when compared to previous data using a binding assay that measures the binding of ^{125}I-labeled highly mannosylated neoglycoprotein to the same type 1 fimbriated *E. coli* K12 in suspension.[16] The inhibitory values (IC_{50}) obtained by glycodendrimers in the labelled method were much lower than those obtained from the inhibition of haemagglutination above, presumably because of the better dynamic equilibrium process allowed in the previous assay. In all fairness, the inhibitors had a better opportunity to displace a freely soluble protein than erythrocytes extensively covered by numerous mannoside residues.

Conclusions

In this report, the synthesis of six mannosylated G(0) glycodendrimers was described using pentaerythritol and bis-pentaerythritol scaffolds, and Pd^0-catalysed Sonogashira and Cu^I-catalysed click chemistry. The Sonogashira coupling conditions had to be optimised with these scaffolds and it was found that $Pd(PPh_3)Cl_2$, piperidine in the presence of Cu^I species gave the best results. The cross-linking ability against Con A, the relative binding affinity against type 1 fimbrial lectin of *E. coli* K12, and the inhibition of haemagglutination of pig and rabbit erythrocytes were determined. The results demonstrated that clusters **4**, **11**, and **21** possessing an aryl moiety in the vicinity of the anomeric oxygen showed the best overall qualifications. Overall, tetramer **4** was the best noncovalent cross-linker of Con A and the best ligand known to *E. coli* K12 FimH. It is clear that the above compounds represent potential therapeutic candidates for the inhibition of adhesion of *E. coli* toward urothelial infections and work is in progress toward this goal. A small library of monosaccharidic mannoside ligands based on the above observations has been synthesised and the report will be presented in due course together with thermodynamic data.

Experimental Section

Flash chromatography was performed using Merck silica gel 60 (40–63 μm). TLC was performed on Kieselgel 60 F254 plates from Merck. Detection was carried out under UV light or by spraying with 20% ethanolic sulfuric acid or molybdate solution followed by heating. Tetrahydrofuran was distilled prior to use. Infrared spectra were recorded on a Perkin–Elmer 1600 FTIR instrument. NMR spectra were measured with a Varian 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C NMR) spectrometer. Chemical shifts are in ppm, relative to internal TMS ($\delta = 0.00$ for ^{1}H and ^{13}C NMR) or solvent peaks. Where necessary, DEPT, APT, and two-dimensional ^{1}H–^{1}H COSY experiments were performed for complete signal assignments. Optical rotations were obtained using a JASCO P-1000 Polarimeter (Na-D line, 589 nm, cell length 5 cm). ESI-MS analyses were carried out on a MICROMASS Quattro LC instrument.

Tetrakis(2-propynyloxymethyl)methane (1). A round-bottomed flask, equipped with a magnetic stir bar, was charged with pentaerythritol (2 g, 0.014 mmol) and KOH (12.5 g, 0.22 mmol). Anhydrous DMF (25 mL) was added by a syringe and the reaction mixture was stirred at 5 °C for 30 min. Propargyl bromide (20 g, 0.17 mmol) was slowly added over a 30 min period. The reaction mixture was then heated at 40 °C overnight. Water (100 mL) was added after cooling, and the mixture was extracted with ether (3×50 mL). The organic layers were combined, washed with water (3×50 mL) and then with brine (3×50 mL), and dried over Na_2SO_4. Removal of solvent by evaporation under reduced pressure left a residue that was purified by flash chromatography on silica gel eluting with ethyl acetate/hexanes (2:8 *v/v*) to give tetrakis(2-propynyloxymethyl)methane **1** (3.5 g, 83%) as an orange solid; mp: 52–54 °C; $R_f = 0.23$ (ethyl acetate/hexanes 30:70); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta =$ 4.12 (d, ^3J (H,H) = 2.4 Hz, 8H, HCCCH_2), 3.54 (s, 8H, C(CH_2)$_4$), 2.4 ppm (t, ^3J (H,H) = 2.4 Hz, 4H, *H*CCCH$_2$); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 80.0$ (HCCCH$_2$), 74.0 (HCCCH$_2$), 69.0 (C(CH$_2$)$_4$), 58.7 (HCCCH$_2$), 44.7 ppm (*C*(CH$_2$)$_4$); ESI-MS calcd for $C_{17}H_{20}O_4$ + (Na^+) 311.13; found: 311.13.

4-Iodophenyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (2). Triflic acid (19 μL, 0.21 mmol) was added to a solution of penta-*O*-acetyl-α,β-D-mannopyranose (0.54 g, 1.38 mmol) and 4-iodophenol (0.61 g, 2.79 mmol) in dry CH_2Cl_2 (20 mL). The reaction mixture was kept at 0 °C and the course of the reaction was monitored by TLC (EtOAc/hexanes 1:1) until complete disappearance of the starting material (12 h). Triflic acid was neutralised by addition of Et_3N (20 μL) and after evaporation of the solvent, the resulting crude product was purified by flash chromatography (EtOAc/hexanes 1:4) to give 4-iodophenyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **2** (577 mg, 76%) as a white solid; mp: 127–129 °C (Ref. [20b] mp: 127–129 °C); $R_f = 0.61$ (ethyl acetate/hexanes 1:1); $[\alpha]_D^{20} = +65$ ($c =$ 1.0 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.57$ (d, ^3J (H,H) = 9.0 Hz, 2H, H$_{ar}$-*meta*), 6.85 (d, ^3J (H,H) = 9.0 Hz, 2H, H$_{ar}$-*ortho*), 5.50 (dd, ^3J (H,H) = 3.5, 10.1 Hz, 1H, H-3), 5.46 (d, ^3J (H,H) = 1.9 Hz, 1H, H-1), 5.40 (dd, ^2J (H,H) = 1.9, 3.5 Hz, 1H, H-2), 5.33 (t, ^3J (H,H) = 10.0 Hz, 1H, H-4), 4.24 (dd, ^2J (H,H) = 5.5, 12.4 Hz, 1H, H-6a), 4.06–3.99 (m, 2H, H-5, H-6b), 2.17, 2.03, 2.00 ppm (3×s, 12H, 4×COCH_3); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 170.5$, 170.0, 169.7 (CO), 155.4 (OC$_{ar}$), 138.5 (CH$_{ar}$), 118.8 (CH$_{ar}$), 85.5 (IC$_{ar}$), 95.8 (C-1), 69.4 (C-2), 69.3 (C-4), 68.8 (C-6), 65.8 (C-3), 62.1 (C-5), 20.9–20.7 ppm (CH$_3$CO).

Tetravalent cluster 3. $PdCl_2(PPh_3)_2$ (13 mg, 0.018 mmol (5 mol%)), Cu^I (7 mg, 0.036 mmol (10 mol%)), and piperidine (1 mL) was added to a solution of 4-iodophenyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **2** (198.5 mg, 0.36 mmol) in 3 mL THF. The mixture was stirred for 30 min and then tetrakis(2-propynyloxymethyl)methane **1** (20 mg, 0.069 mmol) in 1.5 mL of THF was added dropwise over a 30 min period. TLC (eluent: ethyl acetate/hexanes 8:2) indicated the reaction was complete after 3 h. Water (10 mL) and dichloromethane (30 mL) were added to the reaction mixture and the phases separated. The aqueous phase was extracted with dichloromethane (2×30 mL) and the combined organic phases were washed with saturated aqueous NH_4Cl (2×25 mL) and brine (2× 25 mL). The organic layers were dried over Na_2SO_4 and were evaporated under vacuum, leaving a yellow oil. Purification by

flash chromatography (eluent: ethyl acetate/hexanes 30:70) produced **3** as a pale solid (107 mg, 80% yield); mp: 94–96 °C; R_f = 0.19 (ethyl acetate/hexanes 40:60); $[\alpha]_D^{20} = +46.1$ (c = 1.0 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.40–7.37 (d, ^3J (H,H) = 8.8 Hz, 8H, H_{ar}), 7.03–7.00 (d, ^3J (H,H) = 8.8 Hz, 8H, H_{ar}), 5.56–5.51 (m, 8H, H-1, H-3), 5.44–5.42 (m, 4H, H-2), 5.38–5.32 (t, ^3J (H,H) = 9.9 Hz, 4H, H-4), 4.34 (s, 8H, OCH_2CC), 4.30–4.23 (dd, ^2J (H,H) = 5.5, 12.6 Hz, 8H, H-6a), 4.07–4.03 (m, 8H, H-6b, H-5), 3.65 (s, 8H, C(CH_2)$_4$), 2.05–2.02 ppm (4×s, 48H, COCH_3); ^{13}C NMR (75 MHz; $CDCl_3$, 25 °C): δ = 170.4, 169.9, 169.7 (COCH_3), 155.4 (OC_{ar}), 133.2 (CH_{ar}), 117.4 (CH_{ar}), 116.3 (CC_{ar}), 95.6 (C-1), 85.1 (C$_{ar}$CC), 85.0 (C$_{ar}$CC), 69.25 (C-2), 69.21 (C-6), 69.1 (C(CH_2)$_4$), 68.7 (C-4), 65.8 (C-3), 62.0 (C-5), 59.5 (CH_2CC), 45.2 (C(CH$_2$)$_4$), 20.8 (COCH_3), 20.64 (COCH_3), 20.61 ppm (COCH_3); ESI-MS calcd for $C_{97}H_{108}O_{44}$ + (Na^+) 1999.61; found: 1999.5.

Tetravalent cluster 4. Acetylated tetravalent cluster **3** (50 mg, 0.025 mmol) was dissolved in dry MeOH (3 mL), a solution of sodium methoxide (5.4 μL, 1 M in MeOH, 0.5 equiv) was added and the reaction mixture was stirred at room temperature until disappearance of the starting material. The solution was neutralised by addition of ion-exchange resin (Amberlite IR 120, H^+), filtered, washed with MeOH, and the solvent was removed in vacuo. The residue was then lyophilised to yield the fully deprotected glycocluster **4** in a quantitative yield. R_f = 0.23 (MeOH/CH_2Cl_2 5:95); $[\alpha]_D^{20} = +43.2$ (c = 1.0 in MeOH); ^{1}H NMR (300 MHz, [D$_6$]DMSO, 25 °C): δ = 7.36–7.34 (d, ^3J (H,H) = 8.24 Hz, 8H, H_{ar}) 7.05–7.02 (d, ^3J (H,H) = 8.24 Hz, 8H, H_{ar}), 5.38 (s, 4H, H-1), 5.04–5.03 (d, ^3J (H,H) = 4.1 Hz, 4H, H-2), 4.84–4.82 (d, ^3J (H,H) = 5.49 Hz, 4H, H-6a), 4.77–4.76 (d, ^3J (H,H) = 5.8 Hz, 4H, H-5), 4.45–4.41 (t, ^3J (H,H) = 5.8 Hz, 4H, H-6b), 4.35 (s, 8H, CH_2CC), 3.81 (s, 4H, H-3), 3.65 (br s, 4H, H-4), 3.53 ppm (s, 8H, C(CH_2)$_4$); ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 156.5 (OC_{ar}), 132.9 (CH_{ar}), 116.8 (CH_{ar}), 115.2 (CC_{ar}), 98.6 (C-1), 85.4 (C$_{ar}$CC), 84.9 (C$_{ar}$CC), 75.0 (C-2), 70.6 (C-5), 69.9 (C-3), 68.7 (C(CH$_2$)$_4$), 66.5 (C-6), 60.9 (C-4), 58.8 (CH_2CC), 44.6 ppm (C(CH$_2$)$_4$); HRMS calcd for $C_{65}H_{76}O_{28}$ + (H^+) 1305.4595; found: 1305.4586.

Tetrakis[(4-iodophenoxy)methyl]methane (5). A solution of pentaerythrityl tetrabromide (1.5 g, 3.86 mmol), iodophenol (4.25 g, 19.3 mmol), and NaOH (0.77 g, 20 mmol) in DMF (30 mL) was heated at reflux overnight. The mixture was then cooled, water (100 mL) was added, and the mixture was extracted with ether (3× 50 mL). The organic layers were combined, washed with water (3× 50 mL) and then with brine (3×50 mL), and dried over Na_2SO_4. Removal of solvent by evaporation under reduced pressure left a residue that was purified by flash chromatography on silica gel, ethyl acetate/hexanes (5:95) to give tetrakis[(4-iodophenoxy)methyl]methane **5** (2.84 g, 78%) as a white solid; mp: 122–124 °C; R_f = 0.15 (ethyl acetate/hexanes 10:90); ^{1}H NMR (300 MHz, [D$_6$]DMSO, 25 °C): δ = 7.53 (d, ^3J (H,H) = 8.9 Hz, 8H, H_{ar}), 6.67 (d, ^3J (H,H) = 8.9 Hz, 8H, H_{ar}), 4.26 ppm (s, 8H, CH_2); ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 158.5 (C_{ar}), 138.2 (CH_{ar}), 117 (CH_{ar}), 83.4 (C_{ar}), 66.5 (CH_2), 44.7 ppm (CCH_2); ESI-MS calcd for $C_{29}H_{24}I_4O_4$ + (H^+) 943.78; found: 944.79.

Prop-2-ynyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (6). BF_3·etherate (1.77 mL, 14.1 mmol) was added to a solution of penta-*O*-acetyl-α,β-D-mannopyranose (1.1 g, 2.82 mmol) and propargyl alcohol (683 μL, 11.28 mmol) in dry CH_2Cl_2 (30 mL). The reaction mixture was kept at 0 °C and the course of the reaction was monitored by TLC (EtOAc/hexanes 1:1) until complete disappearance of the starting material (48 h). CH_2Cl_2 (50 mL) was added and the solution was washed with a 20% aqueous Na_2CO_3 solution (2× 50 mL) and water (2×50 mL). After drying (Na_2SO_4) and evaporation of the solvent, the resulting crude product was purified by flash chromatography (EtOAc/hexanes 1:2) to give prop-2-ynyl 2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranoside **6** (828 mg, 76%) as a white solid; mp: 99–100 °C; (Ref. [26] mp: 100 °C) R_f = 0.57 (ethyl acetate/hexanes 1:1); $[\alpha]_D^{20} = +56$ (c = 2.0 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 5.31 (dd, ^3J (H,H) = 3.4, 10.0 Hz, 1H, H-3), 5.26 (t, ^3J (H,H) = 10.0 Hz, 1H, H-4), 5.23 (dd, ^3J (H,H) = 3.4, 1.7 Hz, 1H, H-2), 4.99 (d, ^3J (H,H) = 1.7 Hz, 1H, H-1), 4.24 (dd, ^2J (H,H) = 5.2, 12.2 Hz, 1H, H-6b), 4.24 (d, ^4J (H,H) = 2.4 Hz, 2H, H-1′), 4.07 (dd, ^2J (H,H) = 2.5 Hz, 1H, H-6a), 4.00 (m, 1H, H-5), 2.44 (t, ^4J (H,H) = 2.4 Hz, 1H, H-2′), 2.12, 2.07, 2.01, 1.96 ppm (4×s, 12H, $COCH_3$); ^{13}C NMR (75 MHz; $CDCl_3$, 25 °C): δ = 170.5, 169.8, 169.7, 169.6 (CO), 96.2 (C-1), 77.9 (C-2′), 75.0 (C-3′), 70.6, 69.3, 68.9, 67.9 (C-2, 3, 4, 5), 62.3 (C-6), 54.9 (C-1′), 20.8, 20.7, 20.6, 20.6 ppm (CH_3CO).

Tetramer 7. Compound **7** was obtained using the same procedure described for **3** to give tetrakis[(4-iodophenoxy)methyl]methane **5** (100 mg, 0.1 mmol) and prop-2-ynyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **6** (213 mg, 0.55 mmol), $PdCl_2(PPh_3)_2$ (19.3 mg, 0.027 mmol (5 mol%)), and Cu^I (10.5 mg, 0.055 mmol (10 mol%)), tetravalent cluster, **7**, (166 mg, 80%) was obtained after purification by flash chromatography (eluent: ethyl acetate/hexanes 3:7) as a pale solid in 80% (107 mg) yield; mp: 112–114 °C; R_f = 0.48 (ethyl acetate/hexanes 8:2); $[\alpha]_D^{20} = +41.2$ (c = 1.0 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.38–7.35 (d, ^3J (H,H) = 9.0 Hz, 8H, H_{ar}), 6.87–6.84 (d, ^3J (H,H) = 9.0 Hz, 8H, H_{ar}), 5.40–5.28 (m, H-2, H-3, 12H, H-4), 5.11–5.10 (d, ^3J (H,H) = 1.37 Hz, 4H, H-1), 4.47 (s, 8H, OCH_2CC), 4.33 (s, 8H, C(CH_2)$_4$), 4.32–4.27 (m, 4H, H-6a), 4.13–4.04 (m, 8H, H-6b, H-5), 2.16–1.99 ppm (4×s, 48H, COCH_3); ^{13}C NMR (75 MHz; $CDCl_3$, 25 °C): δ = 170.6, 169.9, 169.8, 169.6 (COCH_3), 158.8 (OC_{ar}), 133.3 (CH_{ar}), 114.7 (CC_{ar}), 114.6 (CH_{ar}), 96.1 (C-1), 86.9 (C$_{ar}$CC), 81.9 (C$_{ar}$CC), 69.4 (C-2), 68.9 (C-6), 68.8 (C(CH_2)$_4$), 66.4 (C-4), 65.9 (C-3), 62.2 (C-5), 55.75 (CH_2CC), 44.4 (C(CH$_2$)$_4$), 20.8 (COCH_3), 20.68 (COCH_3), 20.64 (COCH_3), 20.61 ppm (COCH_3); ESI-MS calcd for $C_{97}H_{108}O_{44}$ + (Na^+) 1999.61; found: 1999.54.

Tetramer 8. Compound **8** was prepared from tetramer **7** (50 mg, 0.025 mmol) and a solution of sodium methoxide (12 μL, 1 M in MeOH, 0.5 equiv) using the procedure described above for the synthesis of **4**. Lyophilisation gave tetravalent cluster **8** (92% 30.3 mg) as a white foam; R_f = 0.4 (CH_3CN/H_2O 7:3); $[\alpha]_D^{20} = +39.2$ (c = 1.0 in MeOH); ^{1}H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 7.37–7.34 (d, ^3J (H,H) = 8.6 Hz, 8H, H_{ar}), 6.99–6.96 (d, ^3J (H,H) = 8.6 Hz, 8H, H_{ar}), 4.86–4.79 (m, 12H, H-1, CH_2CC), 4.64 (br s, 4H, H-2), 4.54 (br s, 4H, H-6a), 4.46–4.37 (m, 8H, H-5, H-6b), 4.28 (s, 8H, C(CH_2)$_4$), 3.66–3.60 ppm (br s, 8H, H-3, H-4); ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 158.7 (OC_{ar}), 133.1 (CH_{ar}), 115.1(CC_{ar}), 114.2 (CH_{ar}), 98.2 (C-1), 85.6 (C$_{ar}$CC), 84.2(C$_{ar}$CC), 74.4 (C-2), 70.9 (C-5), 70.1 (C-3), 66.9 (C-(CH_2)$_4$), 66.1 (C-4), 61.1 (C-6), 53.7 (CH_2CC), 44.2 ppm (C(CH$_2$)$_4$); HRMS calcd for $C_{65}H_{76}O_{28}$ + (Na^+) 1327.4415; found: 1327.4445.

General procedure for the click reaction catalysed by $CuSO_4$: A solution of azide cluster (0.1 mmol), prop-2-ynyl α-D-mannopyranoside (0.12 mmol per azide), $CuSO_4$ (1% per azide), and sodium ascorbate (5% per azide) were dissolved in a 1:1 mixture of water and tetrahydrofuran (3 mL) and after 12 h of reaction time, the general workup procedure described above was followed. The residue was then purified by flash column chromatography eluting with dichloromethane and gradually increasing the polarity to MeOH/CH_2Cl_2 (3:97).

Tetramer 10. Tetraazido pentaerythritol **9** (50 mg, 0.21 mmol), prop-2-ynyl α-D-mannopyranoside **6** (389.4 mg, 1 mmol), *N*,*N* diisopropylethylamine (293.4 μL, 1.68 mmol), and Cu^I (1.60 mg, 0.008 mmol) were dissolved in THF (5 mL) and after 12 h of reaction time following the general procedure described above, compound **10** (301 mg, 80%) was obtained as a white foam. R_f = 0.21

(MeOH/CH_2Cl_2 5:95); $[\alpha]_D^{20} = +46.1$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (300 MHz. $CDCl_3$, 25 °C): $\delta = 8.29$ (s, 4H, *CH*=C), 5.30–5.28 (m, 8H, H-3, H-4), 5.16 (brs, 4H, H-2), 4.98 (d, ^3J (H,H) = 1.6 Hz, 4H, H-1), 4.88 (d, ^3J (H,H) = 12.2 Hz, 4H, CH_2O), 4.70 (d, ^3J (H,H) = 12.2 Hz, 4H, CH_2O), 4.46 (s, 8H, CH_2N), 4.30 (dd, ^2J (H,H) = 5.4, 12.6 Hz, 4H, H-6a), 4.15–4.11 (m, 8H, H-5, H-6b), 2.11, 2.08, 2.06, 1.94 ppm (4s, 48H, COCH_3); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 170.6$, 169.98, 169.8, 169.6 (*C*OCH_3), 142.8 (4 *C*=CH), 127.6 (4 C=*CH*), 96.7 (4 C-1), 69.4 (4 C-2), 68.9 (4 C-5), 68.7 (4 C-3), 66.0 (4 C-4), 62.3 (3 C-6), 60.3 (4 CH_2O), 49.1 (4 CH_2N), 46.8 (*C*CH_2), 20.8, 20.7, 20.6, 20.6 ppm (CO*C*H_3); ESI-MS calcd for $C_{73}H_{96}O_{40}N_{12}$ + (H^+): 1781.59; found: 1781.60.

Tetramer 11. Compound **10** (100 mg, 0.001 mmol) and sodium methoxide (28 μL from 1 M solution in MeOH) in 3 mL of methanol were stirred for 4 h at RT and treated following the procedure described for the synthesis of **4** above, deprotected glycocluster **11** (56 mg, 90%) was obtained as a white solid; mp: 165 °C; $R_f = 0.3$ (H_2O/CH_3CN 3:7); $[\alpha]_D^{20} = +28.3$ ($c = 1.0$ in CH_3OH); ^{1}H NMR (300 MHz, D_2O, 25 °C): $\delta = 7.91$ (s, 4H, *CH*=C), 4.67 4.99 (brs, 4H, H-1), 4.49–4.37 (m, 16H, CH_2O, CH_2N), 3.62–3.34 ppm (m, 24H, H-2, H-4, H-3, H-5, H-6a, H-6b); ^{13}C NMR (75 MHz, D_2O, 25 °C): $\delta = 142.9$ (4 *C*=CH), 127.5 (4 C=*CH*), 99.2 (4 C-1), 72.8 (4 C-2), 70.2 (4 C-5), 69.7 (4 C-3), 66.5 (4 C-4), 60.7 (4 C-6), 59.3 (4 CH_2O), 50.2 (4 CH_2N), 44.5 ppm (*C*CH_2O); ESI-MS calcd for $C_{41}H_{64}O_{24}N_{12}$ + (H^+): 1109.3; found: 1109.4.

Tetramer 13. Compound **1** (20 mg, 0.07 mmol), 2′-azidoethyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **12** (138 mg, 0.33 mmol), $CuSO_4 \cdot 5H_2O$ (3.4 mg, 0.01 mmol), and sodium ascorbate (2.73 mg, 0.01 mmol) were dissolved in THF/H_2O (1:1) (5 mL), and after 12 h of reaction time, the mixture was treated following the general procedure described above, glycocluster **13** (125 mg, 92%) was obtained as a white foam; $R_f = 0.16$ (MeOH/CH_2Cl_2 5:95); $[\alpha]_D^{20} = +45.6$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta =$ 7.72 (s, 4H, *CH*=C), 5.29–5.19 (m, 12H, H-2, H-3, H-4), 4.81 (d, ^3J (H,H) = 1.4 Hz, 4H, H-1), 4.61 (brs, 8H, CH_2-N), 4.56 (brs, 8H, CH=CCH_2), 4.20 (dd, ^2J (H,H) = 4.9, 12.3 Hz, 4H, H-6a), 4.14–4.09 (m, 4H, OCH_2CH_2), 4.02 (dd, ^2J (H,H) = 2.4, 12.3 Hz, 4H, H-6b), 3.92–3.89 (m, 8H, OCH_2CH_2), 3.61 (brs, 4H, H-5), 3.47 (s, 8H, CH_2C); 2.12, 2.08, 2.02, 1.97 ppm (s, 48H, COCH_3); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta =$ 170.5, 169.9, 169.8, 169.6 (*C*OCH_3), 145.5 (4*C*=CH), 123.7 (4C=*CH*), 97.5 (4 C-1), 69.1 (4 C-2), 68.4 (4C-5), 68.8 (8C-3, C-4), 66.3 (4CH=CCH_2), 65.6 (4OCH_2), 64.7 (4CH_2C), 62.1 (4 C-6), 49.5 (4 CH_2N), 45.2 (*C*CH_2), 20.8, 20.7, 20.63, 20.60 ppm (16COCH_3); ESI-MS calcd for $C_{81}H_{112}O_{44}N_{12}$ + (Na^+): 1979.6; found: 1980.2.

Tetramer 14. Compound **13** (110 mg, 0.05 mmol) and sodium methoxide (30 μL from 1 M solution in MeOH) in 3 mL of methanol were stirred for 4 h and the mixture was treated following the procedure for the synthesis of **4** described above. Deprotected glycocluster **14** (66 mg, 92%) was obtained as a white foam. $R_f = 0.18$ (H_2O/CH_3CN 3:7); $[\alpha]_D^{20} = +39.3$ ($c = 1.0$ in CH_3OH); ^{1}H NMR (300 MHz, CD_3OD_3, 25 °C): $\delta = 7.88$ (s, 4H, *CH*=C), 4.60 (brs, 4H, H-1), 4.52–4.50 (m, 8H, OCH_2CH_2N), 4.38 (s, 8H, CH=CCH_2O), 3.97–3.94 (m, 4H, OCH_2CH_2N), 3.79–3.75 (m, 4H, OCH_2CH_2N), 3.72–3.71 (m, 4H, H-2), 3.63–3.42 (m, 16H, H-3, H-4, H-6a, H-6b), 3.26 (s, 8H, OCH_2C), 2.95–2.91 ppm (m, 4H, H-5); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 143.8$ (4CH=*C*), 124.9 (4*CH*=C), 99.1 (4C-1), 72.3 (4C-2), 70.0 (4C-3), 69.5 (4C-5), 67. 6 (4CH=CCH_2O), 65.9 (4C-4), 65.0 (4OCH_2CH_2N), 63.0 (4CCH_2O), 60.2 (4C-6), 49.6 (4OCH_2CH_2N), 44.2 ppm (*C*CH_2); HRMS calcd for $C_{49}H_{80}O_{28}N_{12}$ + (H^+): 1285.5277; found: 1285.5279.

1,1′-Oxybis-[3-(4-iodophenoxy)-2,2-bis[(4-iodophenoxy)methyl]]-propane (16). A mixture of dipentaerythrityl hexatosylate **15**[30] (1.5 g, 1.2 mmol), 4-iodophenol (2.09 g, 9.5 mmol), and NaOH (0.4 g, 10 mmol) in DMF (15 mL) was heated at reflux overnight. The mixture was then cooled, water (100 mL) was added, and the mixture was extracted with ether (3×50 mL). The organic layers were combined, washed with water (3×50 mL) and then with brine (3×50 mL), and dried over Na_2SO_4. Removal of solvent by evaporation under reduced pressure left a residue that was purified by flash chromatography on silica gel, CH_2Cl_2-hexanes (2:8) to give 1,1′-oxybis-[3-(4-iodophenoxy)-2,2-bis[(4-iodophenoxy)methyl]]propane **16** (1.47 g, 79%) as a white solid; mp: 78–80 °C; $R_f =$ 0.21 (MeOH/CH_2Cl_2 5:95); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 7.51$–7.48 (d, ^3J (H,H) = 8.3 Hz, 6H, H_{ar}), 6.56–6.53 (d, ^3J (H,H) = 8.3 Hz, 6H, H_{ar}), 4.03 (s, 6H, ArO(CH_2)$_3$), 3.75 ppm (s, 2H, C(CH_2)O); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 158.5$ (OC_{ar}), 138.3 (CH_{ar}), 116.8 (CH_{ar}), 83.3 (IC_{ar}), 69.7 (ArO(CH_2)$_3$), 66.6 (C(CH_2)O), 44.8 ppm (*C*(CH_2)$_4$); ESI-MS calcd for $C_{46}H_{40}I_6O_7$ + (Na^+) 1488.70; found: 1488.69.

Hexamer 17. $Pd(PPh_3)_2Cl_2$ (10 mg, 0.014 mmol (3.5 mol%)), Cu^I (4 mg, 0.021 mmol (5 mol%)), prop-2-ynyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **6** (238 mg, 0.61 mmol) and triethyl amine (1 mL) were added to a solution of compound **16** (100 mg, 0.068 mmol) in 3 mL of DMF. The solution was stirred under nitrogen at 60 °C for 6 h. The solvent and TEA were evaporated under reduced pressure. Water (10 mL) and dichloromethane (30 mL) were added to the reaction mixture and the phases separated. The aqueous phase was extracted with dichloromethane (2×30 mL) and the combined organic phases were washed with saturated aqueous NH_4Cl (2×25 mL) and brine (2×25 mL). The organic layers were dried over Na_2SO_4 and evaporated under reduced pressure, leaving a yellow oil. Purification by flash chromatography (eluent: ethyl acetate/hexanes 40:60) produced **17** as a pale solid in 79% (162.5 mg) yield; mp: 94–96 °C; $R_f = 0.21$ (MeOH/CH_2Cl_2 5:95); $[\alpha]_D^{20} = +49$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta =$ 7.33–7.30 (d, ^3J (H,H) = 8.4 Hz, 12H, H_{ar}), 6.73–6.70 (d, ^3J (H,H) = 8.4 Hz, 12H, H_{ar}), 5.39–5.28 (m, 18H, H-2, H-3, H-4), 5.11 (s, 6H, H-1), 4.49 (s, 12H, CH_2CC), 4.33–4.27 (dd, ^2J (H,H) = 4.9, 12.3 Hz, 6H, H-6a), 4.12–4.08 (m, 24H, (CH_2)$_3$$CH_2$C, H-5, H-6b), 3.78 (s, 4H, (CH_2)$_3$$CH_2$C), 2.15–1.98 ppm (4×s, 72H, COCH_3); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 170.6$, 169.9, 169.8, 169.6 (*C*OCH_3), 158.9 (OC_{ar}), 133.3 (CH_{ar}), 114.5 (CC_{ar}), 114.4 (CH_{ar}), 96.1 (C-1), 86.9 (C_{ar}CC), 82.0 (C_{ar}*C*C), 69.4 (C-2), 69.0 (C-4, (CH_2)$_3$$CH_2$C), 68.8 (C-6), 66.5 (($CH_2$)$_3$$CH_2$C), 66.0 (C-3), 62.2 (C-5), 55.7 (CH_2CC), 44.7 ((CH_2)$_3$$CH_2$*C*), 20.8, 20.67, 20.63, 20.5 ppm (COCH_3); ESI-MS calcd for $C_{148}H_{166}O_{67}$ + (Na^+) 3037.95; found: 3037.95.

Hexamer 18. Compound **18** was prepared from hexamer **17** (70 mg, 0.023 mmol) and a solution of sodium methoxide (11 μL, 1 M in MeOH, 0.5 equiv) using the procedure described for the synthesis of **4**. Lyophilisation gave hexavalent cluster **18** (93% 42.7 mg) as a white foam. $R_f = 0.38$ (CH_3CN/H_2O 7:3); $[\alpha]_D^{20} = +42$ ($c = 1.0$ in MeOH); ^{1}H NMR (300 MHz, [D_6]DMSO, 25 °C): $\delta = 7.33$–7.30 (d, ^3J (H,H) = 8.5 Hz, 12H, H_{ar}), 6.86–6.83 (d, ^3J (H,H) = 8.6 Hz, 12H, H_{ar}), 4.84 (s, 12H, CH_2CC), 4.78–4.76 (d, ^3J (H,H) = 4.6 Hz, 6H, H-1), 4.63–4.61 (d, ^3J (H,H) = 5.5 Hz, 6H, CH), 4.55–4.34 (m, 18H, CH), 4.09 (s, 12H, (CH_2)$_3$$CH_2$C), 3.69–3.62 (m, 12H, CH), 3.40 ppm (s, 4H, (CH_2)$_3$$CH_2$C); ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): $\delta = 158.8$ (OC_{ar}), 133.1 (CH_{ar}), 114.9 (CC_{ar}), 114.13 (CH_{ar}), 98.3 (C-1), 85.5 (C_{ar}*C*C), 84.2 (C_{ar}C*C*), 74.4 (C-2), 70.9 (C-5), 70.1 (C-3), 66.9 (C-4), 66.5 (C-6), 61.1 ((CH_2)$_4$C), 53.8 (CH_2CC), 44.5 ppm ((CH_2)$_4$*C*); HRMS calcd for $C_{100}H_{118}O_{43}$ + (2Na^+) 1026.3415; found: 1026.34312.

Dipentaerythrityl hexaazide (19). NaN_3 (1.35 g, 0.02 mol) was added to a solution of **15**[30] (1 g, 0.84 mol) in DMF (20 mL) under

N_2 and the resulting mixture was warmed to 80 °C. After 24 h, the solution was cooled, poured into water (250 mL), and extracted with Et_2O (100 mL then 75 mL×4). Because of the potential explosiveness of azides, the organic fractions were combined, dried (Na_2SO_4), and concentrated under vacuum at <40 °C. Purification by column chromatography (eluent: EtOAc/hexanes 2:98) gave **19** (250 mg, 73%) as a colourless oil: $R_f = 0.32$ (EtOAc/hexanes 1:9); **IR** (NaCl): $\tilde{\nu} = 2099\ cm^{-1}$ (N_3); ^1H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 3.35$ (s, 12H, CH_2N_3), 3.33 ppm (s, 4H, OCH_2); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 70.0$ ($2OCH_2$), 51.5 ($6CH_2N_3$), 44.6 ppm ($2CH_2CCH_2$); ESI-MS calcd for $C_{10}H_{16}ON_{18} + (Na^+)$: 427.1; found: 427.2.

Hexamer 20. Compound **19** (51 mg, 0.12 mmol), prop-2-ynyl α-D-mannopyranoside **6** (333.8 mg, 0.86 mmol), $CuSO_4{\cdot}5H_2O$ (21.5 mg, 0.08 mmol), and sodium ascorbate (8.5 mg, 0.04 mmol) were dissolved in THF/H_2O (1:1) (5 mL). After 12 h of reaction time following the general procedure described above, glycocluster **20** (245 mg, 72%) was obtained as a white foam. $R_f = 0.35$ (MeOH/CH_2Cl_2 5:95); $[\alpha]_D^{20} = +32.8$ ($c = 1.0$ in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta = 8.39$ (s, 6H, *CH*=C), 5.28 (m, 12H, H-3, H-4), 5.16 (m, 6H, H-2), 4.98 (d, ^3J (H,H) = 1.4 Hz, 6H, H-1), 4.86 (d, ^3J (H,H) = 12.1 Hz, 6H, OCH_2), 4.69 (d, ^3J (H,H) = 12.4 Hz, 6H, OCH_2), 4.54 (s, 12H, CH_2N), 4.29 (dd, ^2J (H,H) = 4.9, 12.4 Hz, 6H, H-6b), 4.14–4.10 (m, 12H, H-5, H-6a), 3.33 (d, ^3J (H,H) = 10.5 Hz, 2H, O*CH*$_2$C), 3.21 (d, ^3J (H,H) = 10.5 Hz, 2H, O*CH*$_2$C), 1.94, 2.01, 2.09, 2.10 (4 s, 72H, CO*CH*$_3$); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 170.6$, 169.9, 169.8, 169.6 ($COCH_3$), 143.2 (6*C*=CH), 127.0 (6C=*CH*), 96.8 (C-1), 69.4 (C-2), 68.9 (C-5), 68.7 (C-3), 65.9 (C-4), 62.3 (C-6), 60.6 (O*CH*$_2$N), 49.0 (*CH*$_2$O*CH*$_2$), 46.3 (CH_2CCH_2), 20.6, 20.61, 20.7, 20. 8 ppm (CO*CH*$_3$); ESI-MS calcd for $C_{112}H_{148}O_{61}N_{18} + (Na^+)$: 2721.9; found: 2722.5.

Hexamer 21. Compound **20** (55 mg, 0.02 mmol) and sodium methoxide (17 μL from 1 M solution in MeOH) were stirred at room temperature in 3 mL of methanol. After 4 h of reaction time, the mixture was treated following the procedure described above for the synthesis of **4**. Deprotected glycocluster **21** (48 mg, 85%) was obtained as a white foam. $R_f = 0.15$ (H_2O/CH_3CN 3:7); $[\alpha]_D^{20} = +49.2$ ($c = 1.0$ in CH_3OH); ^1H NMR (300 MHz, D_2O, 25 °C): $\delta = 8.1$ (s, 6H, *CH*=C), 4.82 (m, 6H, H-1), 4.58–4.54 (m, 12H, O*CH*$_2$), 4.48 (br s, 12H, CH_2N), 3.76–3.70 (m, 12H, CH), 3.61–3.59 (m, 12H, CH), 3.51–3.49 (m, 12H, CH), 3.26 ppm (br s, 4H, O*CH*$_2$C); ^{13}C NMR (75 MHz, D_2O, 25 °C): $\delta = 142.9$ (6 *C*=CH), 126. 9 (6*CH*=C), 99.0 (6C-1), 72.5 (6C-2), 70.0 (6C-5), 69.5 (6C-3), 66.2 (6C-4), 60.4 (6C-6), 59.2 (6O*CH*$_2$N), 49.9 (*CH*$_2$O*CH*$_2$), 44.3 ppm (CH_2CCH_2); HRMS calcd for $C_{64}H_{100}O_{37}N_{18} + (H^+)$: 1713.6569; found: 1713.6562.

Turbidimetric analysis: Turbidimetry experiments were performed in microtitration plates where 100 μL/well of stock Con A solution prepared from (1 mg mL^{-1} PBS) were mixed with 100 μL/well of mannosylated clusters solution and incubated at room temperature for 15 min (Figures 1 and 2). The turbidity of the solutions was monitored by reading the optical density (O.D.) at 490 nm at regular time intervals until no noticeable changes could be observed. Each test was done in triplicate.

Surface plasmon resonance measurements on a Biacore3000

Expression and purification. Bacteria were grown in minimal medium containing 40 μg mL^{-1} of all the amino acids, 0.4% glucose, 2 μg mL^{-1} biotin, 2 μg mL^{-1} thiamine, 2 mM $MgCl_2$, and 25 μg mL^{-1} kanamycin at 37 °C. At $OD_{600nm} = 0.6$ the C43 (DE3) cells were induced with 1 mM IPTG. After overnight incubation at 37 °C, cells were collected and the periplasmic content was extracted. The lectin domain of FimH was purified by dialysing it 4 h at 4 °C against 20 mM Na formate pH 4 and loading it on a Mono S column (Pharmacia). The protein was eluted with 20 mM Na formate, 1 M NaCl pH4. Fractions containing FimH lectin domain were pooled and dialysed overnight at 4 °C against 20 mM HEPES, 150 mM NaCl pH 8.

Immobilization of Fab fragments of the monoclonal antibody 1C10. Affinity measurements using SPR was performed as described in materials and methods in Bouckaert et al. (2005).[1] All SPR measurements were performed on a Biacore3000. A monoclonal antibody 1C10 against the mannose-binding pocket of FimH was produced by a mouse hybridoma cell line at Medimmune and its Fab fragments were purified. We used these Fab fragments to determine the solution affinity of the lectin domain of FimH for different mannose derivatives. The surface of flow cell 2 (Fc2) was activated with 35 μL of EDC/NHS (mixture of EDC (200 mM) and NHS (50 mM)). 1C10 Fabs dissolved at 100 μg mL^{-1} in 10 mM sodium acetate buffer pH 5 have been subsequently covalently coupled as ligands onto a CM5 biosensor chip (BIAapplications Handbook, Biacore AB, Uppsala, Sweden) at 530 RU (resonance units = pg ligand per mm^2) in Fc2 via free amine groups. The excess of succinimide esters on the surface of the chip have been deactivated by the injection of 35 μL of 1 M ethanolamine at pH 8.5. Fc1 was activated and deactivated the same way as Fc2, but without Fab immobilisation, and was used as the reference cell.

Determination of the affinity of FimH for the immobilised Fab fragments. The kinetic constants for binding of the lectin domain of FimH to the immobilised 1C10 antibody have been measured by flowing a twofold serial dilution of FimH, ranging from 2000 nM to 1.95 nM in running buffer [20 mM HEPES pH 7.4, 150 mM NaCl, 0.005% surfactant P20, and 3 mM EDTA], sequentially over Fc1 and Fc2, at 298 K. The evolution of the optical signal from Fc2 minus Fc1, measured in RUs, was followed in time. The flow rate was 20 μL min^{-1}, the association time was 4.5 min. The dissociation was allowed to proceed for 25 min, by injecting only running buffer, to completely dissociate FimH from the 1C10 antibody before starting another binding cycle. A zero concentration data was obtained by injecting only running buffer over the sensor chip. The binding of FimH to 1C10 has been evaluated using the BIAevaluation software version 4.1. A Langmuir binding isotherm with a 1:1 stoichiometry has been fitted simultaneously to the association and dissociation phases, to obtain in global reaction rate constants k_a and k_d, and the maximum analyte binding response R_{max}.

Affinity measurements and fittings. The affinity of the lectin domain of FimH for different mannose derivatives was obtained by competition between antibody and sugar for the FimH lectin domain. Each mannose derivative has been diluted minimal 11 times in a twofold serial dilution. To each of the sugar concentrations, including a zero concentration, a concentration of the lectin domain of FimH was added that was close to the dissociation constant at equilibrium K_d of the FimH-antibody interaction, calculated from the foregoing experiment. Analyses of all binding cycles were again performed using the BIAevaluation software version 4.1. The concentrations of FimH lectin domain free from sugar and thus able to interact with the immobilised 1C10 antibody were obtained by fitting a Langmuir binding isotherm with 1:1 stoichiometry to the data, using the global parameters k_a, k_d, and R_{max} from the former experiment. These FimH concentrations were plotted in function of the sugar concentrations. Fitting using the solution affinity interaction model $(B\text{-}A\text{-}K_d)/2 + (0.25\,(A+B+K_d)^2\,A\,B)^{0.5}$, where A is the concentration sugar, B is the initial fixed concentration of the lectin domain of FimH added to each sugar concentration delivered K_d, the dissociation constant at equilibrium or the affinity of FimH for the sugar.

Inhibition of haemagglutination: The bacteria were grown statically for 48 h in LB at 37 °C.

1) Haemagglutination. The haemagglutination titer of the type-1 piliated UTI89 clinical isolate was determined in U-shaped 96-well microtiter plates (Greiner). In a first well of row only 25 μL ice-cold PBS was used as a negative control. From the second well on there is a twofold serial dilution of the UTI89 bacteria, starting at $OD_{600nm} = 11$. Finally, 25 μL of 5% rabbit or guinea pig red blood cells in PBS were added. All agglutinations were performed on ice.

2) Inhibition of haemagglutination. Eight sugars have been tested: HeptαMan, mannose, and dendrimers **4**, **8**, **11**, **14**, and **21** (20 mM stock solutions in 50% DMSO). A twofold serial dilution of the sugar is made per row in 25 μL PBS, starting at 200 μM (200 mM for mannose). Next 25 μL of UTI89 at $OD_{600nm} = 1$ is added to all wells. Finally, 25 μL of 5% rabbit or guinea pig red blood cells at about 5% in PBS were added.

Computational methods: In silico modelling and analysis of tetramer **4** and its interaction with Concanavalin A (PDB code: 1VAM) was performed with MOE software (Molecular Operating environment).[33] The extended conformation of tetramer **4** was designed from inside out by keeping a tetrahedral symmetry throughout. Docking of tetramer **4** into Con A was performed by the following procedure: α-D-Mannopyranoside atoms from tetramer **4** were superposed onto those of the α-D-mannopyranoside atoms from the ligand 4-nitrophenyl α-D-mannopyranoside that was already complexed with one of the Concanavalin A lectin monomer (PDB code: 1VAM). Then, 4-nitrophenyl α-D-mannopyranoside was removed from the complex and a new in silico complex was created. This step was accomplished four times at each monomer of the tetramer structure. Finally, to avoid clashes between the four monomers the entire system was minimised with AMBER 94 molecular force fields. To visualise the volume of each monomer, analytic Connolly molecular surface were generated. The best quaternary structure indicated that a tetrahedral shape was preferred for multivalent interaction involving four Con A monomers. An analogous procedure was used to generate the structure of the FimH (PDB code: 1UWF) complexed with ligand **4** in Figure 4b. The ligand (butyl α-D-mannopyranoside from 1UWF was superimposed with compound **4** and the latter was then removed.

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