Synthesis of Lactose Dendrimers and Multivalency Effects in Binding to the Cholera Toxin B Subunit

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The synthesis of lactose-containing dendrimers is described; the dendrimers used were based on the 3,5-di(2-aminoethoxy)benzoic acid repeating unit. Dendrimers of generation 1, 2, and 3 – containing 2, 4, and 8 endgroups, respectively – were used. These were coupled to lactose isothiocyanate, resulting in thiourea-linked glycodendrimers, characterized by ^{13}C NMR and mass spectrometry. The lactose-functionalized dendrimers were evaluated by fluorescence assay for their ability to bind to the cholera toxin B subunit. Binding affinities determined in the fluorescence assay (K_d's) ranged from 18 mM for monovalent lactose to 33 μM for an octavalent lactose dendrimer.

Multivalency in ligand-receptor interactions is an important principle used in nature to increase weak interactions to biologically relevant levels.[1] The principle is especially prevalent in carbohydrate recognition events, in which monovalent affinities are generally quite low and such an enhancement is required.[2] It can be used in the design of biologically active compounds to endow them with binding power greater than that of their monovalent counterparts.[3] The generation of multivalent carbohydrate ligands has several potential areas of application in glycobiology.[4] In this respect, glycodendrimers are growing in importance as a class of dendrimers[5] and glycoconjugates.[6] They have potential as antiviral agents,[7] antiadhesion agents of bacteria,[8] and bacterial toxins,[9] as drug targeting agents[10] and possibly also as in the development of synthetic vaccines.[11] The true nature of multivalency is still poorly understood and requires further study. One effect that can result in multivalent binding enhancement is the 'chelate effect', commonly explained in terms of entropy.[12,13] After the first ligand binds, the entropic barriers for a linked second ligand can be much lower if the spacer is designed properly. Another mechanism is based on ligand-induced receptor clustering in solution, which explains why multivalent systems with spacers too short for chelation can still exhibit enhanced affinity, possibly due to favorable protein-protein interactions.[14] Various types of clusters or aggregates, both soluble or insoluble, exist and so precipitation can be a cause of incorrect data interpretation in multivalent systems. Aggregation occurs especially when the receptor sites and/or the ligands are divergently oriented. This may result in large supramolecular assemblies.[15] Such aggregates can be responsible for biological effects, as has been documented for several lectin-glycoprotein aggregates.[15] This type of association may or may not be accompanied by tighter overall binding. Enhanced binding has been explained by reduced off-rates from such an assembly.[16] Finally, enhanced affinities in very flexible multivalent systems have been observed and explained in terms of an increased local concentration of a second ligand after the first one binds; it was found to be optimal when the effective length of the spacer matches the spacing of the binding sites.[9b,17]

One thing that the various mechanisms of multivalency have in common is that the magnitude of the enhancement they cause is highly dependent on the structure of the linking moiety between the ligands. The effectiveness of the chelate effect depends on the spatial match between the receptors and the ligands and on the flexibility of the spacer arms.[3] This has also been found to be the case in systems that exhibit receptor aggregation.[18] Dendrimers are well suited to address the fundamental issues of the origin of multivalency effects in a given system, provided that enough structural diversity can be created. This can be achieved by variation of the number and/or the nature of the branching building blocks. Recent synthetic activities in this area have already resulted in a significant number of glycodendrimers.[19] Affinity studies of some of these systems for lectins − concanavalin A for the mannose-functionalized dendrimers,[18,19e−19g,19j] for example − have shown that significant binding enhancements are possible with relatively little backbone design. It has also been shown that this lectin is especially prone to the receptor clustering mechanism of affinity enhancement.[14,18,20]

We are involved in a program of glycodendrimer synthesis and the study of their biological application. We have shown that lactose dendrimers may exhibit large multivalency effects with galectins and related galactose-binding lectins but that these effects are highly dependent both on the receptor and on the matrix used in solid-phase inhibition assays.[21] Here we report on the synthesis of a series of lactose-functionalized dendrimers and their binding to the cholera toxin B subunit (CTB), investigated by use of a direct fluorescence binding assay. The preparation of these

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dendrimers, besides offering the hope of uncovering some of the fundamental aspects of multivalency, is further motivated by their potential application as a cholera therapeutic.

Cholera toxin is produced by the *Vibrio cholerae* bacterium and has an AB_5 hexameric architecture. The B-subunits of the toxin bind to GM1 gangliosides present on the intestinal cell surface, which is the first step towards the disease cholera. For this reason, an effective ligand for the toxin may prevent the disease. It is not feasible to make multigram quantities of a multivalent GM1, due to the limited availability of this compound,[22] although a simplified version of the compound has been made without loss of activity.[23] We chose to take the widely available disaccharide lactose as our monovalent starting point. Lactose has been shown to bind to CTB in the millimolar range,[24] and an X-ray structure with 5 units of this compound bound to the closely related heat-labile enterotoxin from *E. coli*[25] (LT, 80% sequence identity with CT) shows a binding mode of the terminal galactose in this structure similar to that of this residue in the structure of the CTB-GM1 pentasaccharide complex.[26] In CTB, all binding sites are on the same face of the molecule, which creates the potential for simultaneous binding of several or even all the binding sites to a single multivalent ligand and suggests that enhanced affinity through the chelate effect should be possible. Recently, highly flexible pentavalent galactose molecules have been shown to be capable of inhibiting LT in an ELISA assay.[9b] Reductions in IC_{50} of four orders of magnitude relative to that of a monovalent galactose derivative were observed. No studies that provide a direct measure of affinity under equilibrium conditions have been reported either with LT or with CT. This is significant since indirect assays may reflect linked aggregation phenomena, which may be biologically relevant but are not a direct measure of protein-carbohydrate interaction.[20] Evidence that binding to pentameric toxins can be quite complicated was recently obtained by Toone et al., who showed that binding to the Shiga-like toxin by divalent ligands was enhanced by about one order of magnitude when assayed by microcalorimetry and by two orders by a competitive ELISA assay. These provided evidence for different binding mechanisms, including bridging between toxin pentamers, depending on the nature of the spacer.[27] An interesting mix of chelation of binding sites within a single toxin and bridging between toxin molecules has been observed for the Shiga-like toxin with a multivalent molecule containing ten ligands.[9a] In a competitive ELISA assay, this dodecavalent system was seven orders of magnitude more effective than monovalent reference compounds.

Results and Discussion

Synthesis

The dendrimer backbone structures used here have been prepared previously[28] and are members of an easily variable class with significant rigidity and considerable distances between the endgroups: features that might be favorable for multivalent binding of biological receptors. The dendrimers used in our work are based on the 3,5-bis(2-aminoethoxy)benzoic acid repeating unit. These building blocks can easily be synthesized and their coupling can be performed by peptide chemistry methods. They are synthesized by a convergent approach[29] that ensures the absence of deletions. Dendrimers of the first, second, and third generation, the poly-HCl and TFA salts of compounds **1**−**3**, were used (Figure 1). These molecules contain various amino groups for functionalization with carbohydrates and also one methyl ester group, which plays no role in this study but might be used to functionalize the system further, depending on its biological application. For the linkage of the carbohydrates, β-lactose isothiocyanate **4** was used (Scheme 1). This compound was initially prepared from acetylated lactose bromide by use of KSCN as described by Lindhorst and Kieburg;[30] for larger scale preparations, however, it proved more convenient to use treatment of acetylated lactose amine with thiophosgene.[31] The isothiocyanate was coupled to the dendrimer amino groups (Scheme 1) in CH_2Cl_2 in the presence of iPr$_2$NEt. The coupling products (**5**−**7**) were purified by column chromatography and obtained in good yields (60−80%). The carbon NMR spectra and elemental analyses of these compounds provided evidence for the structures shown. Deprotection of the hydroxyl groups of **5** was possible under standard Zemplén conditions. However, this was not the case for the higher generations **6** and **7**, because of the insolubility of these compounds in MeOH. Because of the large differences in the solubility properties of the protected and the deprotected glycodendrimers, a two-step procedure was used. Firstly, addition of aqueous NaOH to a homogeneous solution of the protected glycodendrimer in a MeOH/dioxane mixture resulted in rapid formation of a precipitate, which redissolved after addition of some water. Deprotection was complete within 15 min. The resulting deprotected dendrimers were characterized by ^{13}C NMR spectroscopy and MS. Their ^{1}H NMR spectra were very broad and not instructive and even the ^{13}C NMR of **9** and (especially) **10** showed signal broadening, although all the signals of the dendrimer framework, the lactose moiety, and the C=S of the thiourea linkage could still be identified. The ESI-MS of the divalent system **8** showed a clean spectrum with essentially one peak $[M + H]^+$ (100%). For the tetravalent system **9**, the peaks of highest intensity were the doubly charged ions ($[M + 2\,H]^{2+}$, $[M + H + Na]^{2+}$, $[M + 2\,Na]^{2+}$). Smaller singly charged peaks were also seen ($[M + Na]^+$, 20%). Furthermore, peaks corresponding to the loss of a single sugar unit, presumably the galactose, were seen (approx. 20%), as well as peaks due to minor ester hydrolysis (approx. 10%) resulting from the presence of water during the deprotection. For the octavalent system **10**, the trends observed for **8** and **9** continued. Here, the triply charged ions showed the highest intensity over the doubly charged. Multiple peaks due to the various numbers of sodium ions and protons attached were observed, and significant intensities due to the loss of one and even two

terminal sugar moieties were seen. Ester hydrolysis did not seem to play a significant role in this case. In short, the carbon NMR could and indeed did show that all the expected parts of the molecules were present whereas the complementary MS techniques were able to show whether a system was monodisperse or not. In our system we detected some methyl ester hydrolysis in **9** by mass spectrometry, as well as some cleavage of a terminal carbohydrate moiety. The ester hydrolysis occurred because of the water needed in the deprotection, but was only minimal (approx. 10%). Future versions of the molecules will be further functionalized (as amides) at the carboxyl site, so ester hydrolysis will no longer play a role. The sugar cleavages occurred despite the mild conditions used during the deprotection of the acetylated hydroxyls; there is a distinct possibility that these cleavages occurred inside the mass spectrometer. As monovalent comparison compounds we used, besides lactose, the lactose thiourea-linked compounds **11** and **12** (Figure 2), which were prepared by the same methods.

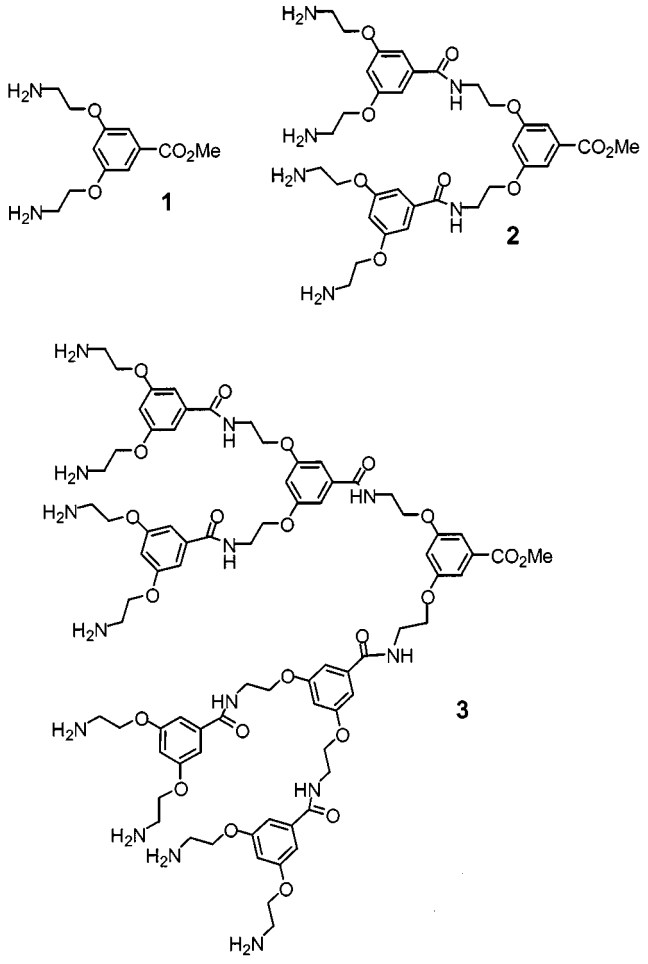
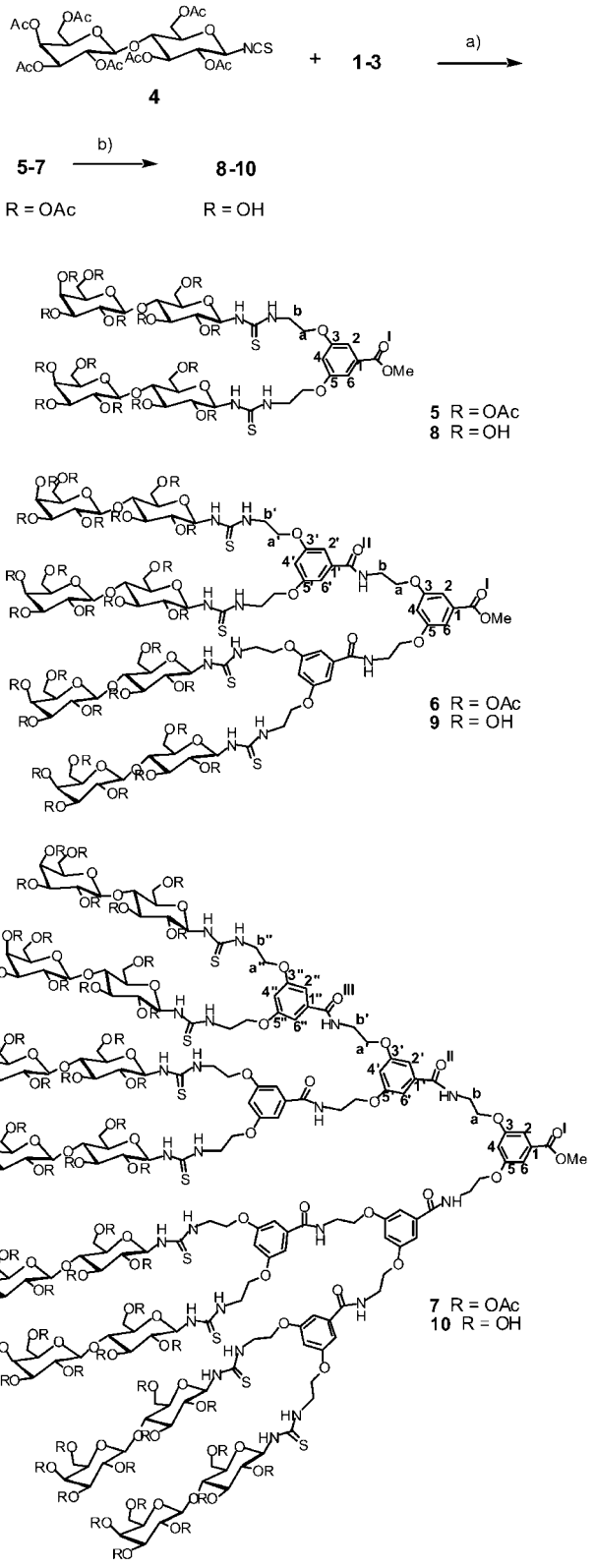


Figure 1. Structures of the dendrimers used for carbohydrate attachment



Scheme 1.Reaction conditions: a) *i*Pr_2NEt, CH_2Cl_2, room temp.; b) for **8**: NaOMe, MeOH, 2 h, room temp.; for **9** and **10**: NaOH, MeOH, dioxane, H_2O, 15 min, room temp.

Binding Studies

The assay used to determine the affinities of the glycodendrimers and the reference compounds for the cholera toxin B subunit was fluorescence spectroscopy, as described for monovalent ligands.[24] CTB contains one tryptophan residue (Trp 88), the fluorescence of which is affected by

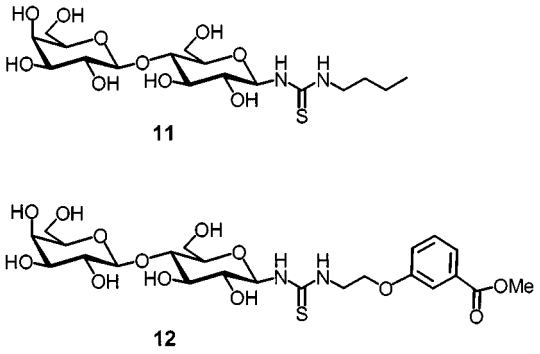


Figure 2. Structures of the monovalent reference compounds **11** and **12**

the binding of carbohydrate ligands. The degree to which this happens is greatly dependent on the nature of the ligand. For example, a much larger drop in fluorescence was observed with **11**, compared to that seen with lactose (Figure 3). Interestingly, we noted that the fluorescence quenching was complete − i.e., the fluorescence of the occupied receptor at saturation was zero − for all ligands containing the thiourea group and an aromatic ring. For lactose, which has neither of these features, the quenching was minor, while **11**, which contains only the thiourea moiety, represented an in-between case. For lactose and **11**, the change in fluorescence at 346 nm could be fitted to a simple one binding site model (**a** in Figure 4, for example). The apparent K_d values obtained here were 18 mM for lactose and 2.7 mM for compound **11** (Table 1). The enhanced binding of **11** as compared to lactose may be caused by hydrogen bonding involving the thiourea group, or otherwise the butyl tail may make hydrophobic contacts to the toxin. For the compounds **8**, **9**, **10**, and **12**, the simple binding model was unsatisfactory. To describe the fluorescence data better, we explored several binding models including a two-site binding model and a cooperative model. In these cases, no contribution of the occupied receptor to the fluorescence had to be included, since its fluorescence was quenched completely. It appeared that a Hill model[32] including cooperativity described the fluorescence well; the improved fit on using the Hill model can be seen in Figure 4 (**b**). For the divalent **8**, an apparent K_D of 235 μM was determined with a Hill coefficient of $n = 1.4$. The involvement of cooperativity in CTB binding is a well known phenomenon, observed previously with the GM1 oligosaccharide but not with mono- or disaccharides.[24,33] By use of the same analysis, the K_d for the tetravalent **9** was determined to be 99 μM ($n = 1.4$), while the octavalent **10** exhibited the strongest binding, with a K_d of 33 μM ($n = 1.4$) and a relative potency of 545. The binding studies indicate a modest multivalent enhancement on going from **8** to **10**. The relative potency on a per lactose basis increases from 38 to 68. However, it is also clear that either the thiourea linkage or the hydrophobic spacer contribute to the affinity, as can be concluded from the enhanced affinity of **11** over lactose. Additionally, the substituted aryl group contributes, as can be concluded from the exceptional affinity of the monovalent **12**. The K_d of 248 μM for this compound is 73 times lower than that for lactose and surprisingly rivals the affinity of other designed monovalent CT or LT binders.[34,35]

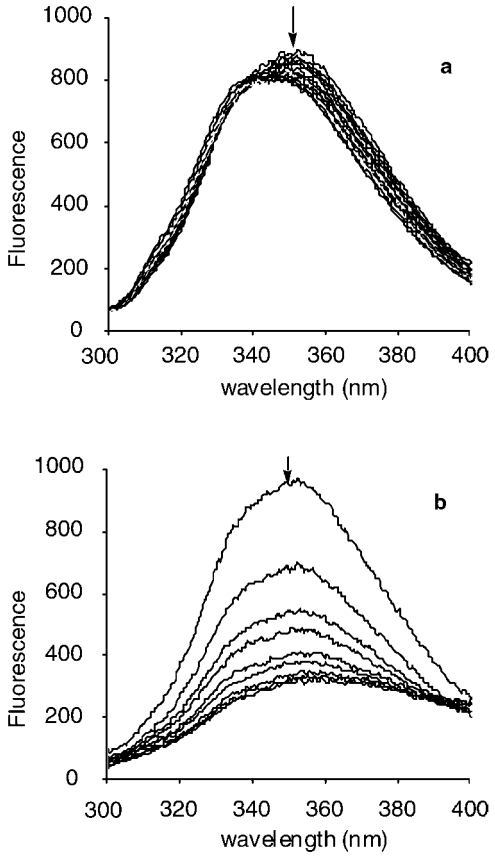


Figure 3. Fluorescence titrations of CTB (0.5 μM) with: a) lactose (K_d = 18 mM) and b) **11** (K_d = 2.7 mM). Conditions: buffer: Tris·HCl (50 mM), pH 7.5, 0.15 M NaCl, T = 23 °C, excitation wavelength 282 nm

Table 1. Apparent dissociation constants of the binding of various lactose derivatives to CTB. Fluorescence titration conditions: [CTB] = 0.5 μM, buffer: Tris·HCl (50 mM), pH 7.5, 0.15 M NaCl, T = 23 °C, excitation wavelength 282 nm

Entry	Compound	K_d [μM]	Rel. potency (per lactose.)	Hill coefficient n
1	lactose	18000	1 (1)	1.0
2	**11**	2700	7 (7)	1.0
3	**12**	248	73 (73)	1.5
4	**8**	235	77 (38)	1.5
5	**9**	99	182 (46)	1.4
6	**10**	33	545 (68)	1.4

To explain the observed data further, a closer examination of the glycodendrimer structures provided some insight. The distance between two ligands in the divalent **8**, measured between the anomeric oxygens of the terminal galactoses, was about 28 Å in an extended conformation. For comparison, the distances between the anomeric oxygens of the terminal galactoses of GM1 molecules in the crystal

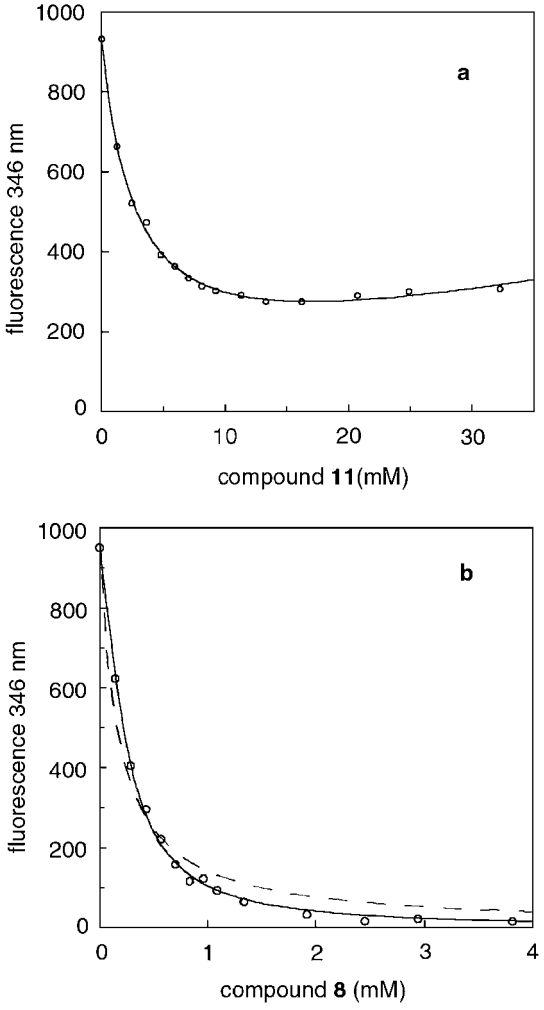


Figure 4. Fluorescence signal at 346 nm upon ligand binding to 0.5 μM CTB in Tris HCl buffer (50 mM), pH 7.5, 0.15 M NaCl. a) Compound **11**. The line is the fit according to Equation (1) (see Experim. Section), yielding a K_d of 2.7±0.1 mM. b) Compound **8**. The lines represent the fit using the Hill cooperativity model Equation (2), (solid line) and a noncooperative one-site binding model (broken line). The Hill model yielded a K_d of 235±6 μM and n = 1.4±0.1.

structure of the complex with CTB is 31 Å. Tetravalent **9** and octavalent **10** can also display larger distances and certainly should be able to bridge binding sites, which may explain the modest multivalency effects observed. Whether the divalent **8** can bridge two binding sites seems less likely. The stretched conformation required to do so would take away any thermodynamic advantages this might have. The similar efficiencies of **8** and **9** suggest that a chelation model is not the major binding mechanism. Furthermore, the striking similarity of the Hill cooperativity coefficients for compounds **8**−**10** and **12** suggest very similar modes of binding for these compounds. For CTB, cooperativity is known for binding of the monovalent GM1, binding sites next to occupied sites displaying higher affinity.[33] In fact, the cholera toxin is one of only a few systems known to exhibit such enthalpically enhanced binding.[1] These effects are not the entropically based effects targeted with the multivalent systems; rather they might occur simultaneously. Our results indicate that cooperativity exists in the binding of monovalent **12** (n = 1.5). The binding results of **8**−**10** argue in favor of a model in which the multivalent sugars bridge between toxin molecules, rather than bind to a single one. For the larger compounds such as **10**, binding to a single toxin would not result in cooperativity because this single molecule could satisfy all the binding sites of one molecule of CTB, thus no sites of higher affinity would be available, and a Hill coefficient of close to 1 would be expected. Although the formation of soluble aggregates would be consistent with this model, no aggregation was observed in the binding of a pentavalent galactose system to the related LT system, as concluded from dynamic light scattering measurements.[9b] However, a bridging model has recently been deduced from studies with the pentameric Shiga-like toxin and certain divalent ligands.[27] Aggregation often results in precipitation, and it is therefore relevant to note that in none of the titration experiments did we observe any sign of precipitation. Further affinity studies with longer, shorter, more rigid, and more flexible systems, as well as complementary assay methods, to optimize affinities and to decipher the mechanism further, are currently in progress.

Experimental Section

General Remarks: Chemicals were obtained from commercial sources and used without further purification unless stated otherwise. The solvents CH_2Cl_2, MeOH, and dioxane were purchased from Biosolve, the Netherlands. The solvents CH_2Cl_2 and MeOH were stored on molecular sieves (4 Å and 3 Å, respectively). The base iPr$_2$NEt was distilled from ninhydrin and KOH. Column chromatography was performed on Merck Kieselgel 60 (40−63 μm). For neutralization, Dowex 50 × 8 (H^+-form; 20−50 mesh) purchased from Fluka, was used. CTB_5 was obtained from Sigma. ^{13}C NMR spectra were recorded on a Varian G-300 spectrometer at 75.4 MHz. in 7% $CD_3OD/CDCl_3$ (referenced to $CDCl_3$ at δ = 77.0) and in 5% CD_3OD/D_2O (referenced to CD_3OD at δ = 49.0). Electrospray ionisation (ESI) mass spectrometry was carried out with a Shimadzu LCMS QP-8000 single quadrupole benchtop mass spectrometer (m/z range < 2000), coupled with a QP-8000 data system, for the low molecular weight glycodendrimers **8** and **9**. For all deprotected glycodendrimers (compounds **8**−**10**), electrospray MS (nano ES-TOF-MS) was run on a Micromass Q-tof hybrid tandem mass spectrometer by spraying a CH_3CN/H_2O solution from a gold coated glass capillary in a Z-spray nanospray ionisation source (cone voltage 30−40). Elemental analyses were measured at H. Kolbe, Mikroanalytisches Laboratorium, Mülheim an der Ruhr, Germany. For the fluorescence measurements a Perkin−Elmer LS50 luminescence spectrometer was used, connected to a PC with the program FL Winlab 1.1.

$MeO_2C[G_1](LacAc)_2$ (5): iPr$_2$NEt (44 μL, 250 μmol) was added to a stirred suspension of compounds **1**·2HCl (33 mg, 100 μmol) and **4** (136 mg, 200 μmol) in CH_2Cl_2 (3 mL) at room temp. The reaction mixture was stirred until total consumption of **1** (1 h), as monitored by TLC (10% MeOH/CH_2Cl_2). The solvent was evaporated in vacuo and the residue was redissolved in EtOAc (5 mL). The organic layer was washed with $KHSO_4$ (1 N, 2 × 5 mL) and brine (2 ×

5 mL), and dried (Na_2SO_4). After evaporation of the solvent, the residue was purified chromatographically (silica gel, 3% MeOH/CH_2Cl_2) to give 99 mg (62 μmol, 61%) of **5**; m.p. > 133 °C (decomp.). ^{13}C NMR (7% $CD_3OD/CDCl_3$, 75.4 MHz): δ = 184.3 (C=S), 171.3, 170.7, 170.5, 170.3, 170.2, 169.6, 169.1 (7 × $C{=}O^{OAc}$), 166.7 ($C{=}O^{I}$), 159.4 ($C^{3/5}$), 131.9 (C^{1}), 108.0, 106.3 ($C^{2/6}$ + C^{4}), 100.6 ($C^{Gal}_{anom.}$), 82.0 ($C^{Glc}_{anom.}$), 75.9, 74.0, 72.8, 70.9, 70.5, 68.9, 66.6 (all CH^{Lac}), 66.5 (CH^{a}_{2}), 62.0, 60.8 (2 × CH^{Lac}_{2}), 52.3 (CH_3O), 43.7 (CH^{b}_{2}), 20.7, 20.6, 20.5, 20.4, 20.3 (all CH^{OAc}_{3}). $C_{66}H_{88}N_4O_{38}S_2$ (1609.54) calcd. C 49.25, H 5.51, N 3.48; found C 49.09, H 5.43, N 3.40.

$MeO_2C[G_2](LacAc)_4$ (6): This synthesis used **2**·4TFA as starting material and was essentially identical to the preparation of **5** but was complete after 2 days (monitored by TLC, 5% MeOH/CH_2Cl_2). Identical workup followed by column chromatography (silica, 5% MeOH/CH_2Cl_2) gave 158 mg (46 μmol, 68%) of **6**; m.p. > 161 °C (decomp.). ^{13}C NMR (7% $CD_3OD/CDCl_3$, 75.4 MHz): δ = 184.1 (C=S), 170.7, 170.4, 170.3, 170.1, 169.6, 169.1 (all × $C{=}O^{OAc}$), 168.2 ($C{=}O^{II}$), 166.7 ($C{=}O^{I}$), 159.6, 159.3 ($C^{3/5}$ + $C^{3'/5'}$), 135.6 ($C^{1'}$), 132.1 (C^{1}), 108.6, 107.5, 106.1 ($C^{2/6}$ + $C^{2'/6'}$ + C^{4} + $C^{4'}$), 100.6 ($C^{Gal}_{anom.}$), 82.0 ($C^{Glc}_{anom.}$), 75.8, 74.0, 73.0, 70.9, 70.7, 70.4, 68.9, 66.8 (8 × CH^{Lac}), 66.5 (CH^{a}_{2} + $CH^{a'}_{2}$), 62.0 (CH^{Glc}_{2}), 60.7 (CH^{Gal}_{2}), 52.4 (CH_3O), 43.7 ($CH^{b'}_{2}$), 39.7 (CH^{b}_{2}), 20.6, 20.5, 20.4, 20.3 (all CH^{OAc}_{3}). $C_{142}H_{186}N_{10}O_{78}S_4$ (3409.28) calcd. C 50.03, H 5.50, N 4.11; found C 49.79, H 5.57, N 3.97.

$MeO_2C[G_3](LacAc)_8$ (7): This synthesis used **3**·8TFA as starting material and was essentially identical to the preparation of **5** but was complete after 4 days (monitored by TLC, 5% MeOH/CH_2Cl_2). Identical workup followed by column chromatography (silica, 5% MeOH/CH_2Cl_2) gave 206 mg (29 μmol, 74%) of **7**; m.p. > 163 °C (decomp.). ^{13}C NMR (7% $CD_3OD/CDCl_3$, 75.4 MHz): δ = 184.2 (C=S), 170.7, 170.5, 170.3, 170.2, 169.6, 169.2 (all × $C{=}O^{OAc}$), 168.0 ($CO^{II,III}$), 166.7 (CO^{I}), 159.7−159.3 ($C^{3/5}$ + $C^{3'/5'}$ + $C^{3''/5''}$), 135.9, 135.5 ($C^{1'}$ + $C^{1''}$), 131.8 (C^{1}), 108.5−102.7, ($C^{2/6}$ + $C^{2'/6'}$+ $C^{2''/6''}$ C^{4} + $C^{4'}$ + $C^{4''}$), 100.6 ($C^{Gal}_{anom.}$), 82.0 ($C^{Glc}_{anom.}$), 75.9, 74.1, 73.1, 70.9, 70.7, 70.4, 68.9, 66.6 (8 × CH^{Lac}), 66.2 (CH^{a}_{2} + $CH^{a'}_{2}$ + $CH^{a''}_{2}$), 62.0 (CH^{Glc}_{2}), 60.7 (CH^{Gal}_{2}), 52.4 (CH_3O), 43.8 ($CH^{b''}_{2}$), 39.7 (CH^{b}_{2} + $CH^{b'}_{2}$), 20.6, 20.5, 20.3 (all CH^{OAc}_{3}). $C_{294}H_{382}N_{22}O_{158}S_8$ (7008.76) calcd. C 50.38, H 5.49, N 4.40; found C 50.42, H 5.59, N 4.29.

$MeO_2C[G_1](Lac)_2$ (8): NaOMe (30 wt% solution in MeOH, 200 μL, 1.05 mmol) was added to a solution of **5** (268 mg, 167 μmol) in dry MeOH (10 mL). The resulting suspension was stirred at room temp. for 2 h. The reaction mixture was neutralized with Dowex resin. After addition of water (3 mL) and removal of the resin by filtration, the solvents were evaporated in vacuo. The material was dissolved and lyophilized from H_2O to yield 160 mg (157 μmol, 94%) of **8** as a white solid: ^{13}C NMR (5% CD_3OD/D_2O, 75.4 MHz): δ = 184.2 (C=S), 169.0 ($C{=}O^{I}$), 160.0 ($C^{3/5}$), 132.2 (C^{1}), 109.3, 107.6 ($C^{2/6}$ + C^{4}), 104.0 ($C^{Gal}_{anom.}$), 84.2 ($C^{Glc}_{anom.}$), 79.0, 77.0, 76.3, 76.1, 73.5, 72.8, 71.9, 69.6 (8 × CH^{Lac}), 67.3 (CH^{a}_{2}), 62.0 (CH^{Glc}_{2}), 61.0 (CH^{Gal}_{2}), 53.8 (CH_3O), 44.8 (CH^{b}_{2}). ESI MS: *m*/*z* = 1021.3 [*M* + H]$^+$.

$MeO_2C[G_2](Lac)_4$ (9): Aqueous NaOH (1 N, 0.3 mL) was added to a solution of **6** (338 mg, 99 μmol) in dioxane (2.1 mL) and MeOH (0.75 mL). The resulting white suspension redissolved on addition of H_2O (2 mL) and the solution was stirred at room temp. for 15 min. The reaction mixture was brought to pH 4−5 with Dowex resin and the solvents were evaporated in vacuo. The material was dissolved and lyophilized from H_2O to yield **9** as a white solid: (210 mg, 94 μmol, 95%): ^{13}C NMR (5% CD_3OD/D_2O, 75.4 MHz): δ = 184.3 (C=S), 169.7, ($C{=}O^{II}$), 168.5 ($C{=}O^{I}$), 160.1 ($C^{3/5}$ + $C^{3'/5'}$), 136.4 ($C^{1'}$), 132.0 (C^{1}), 107.2, 106.0 ($C^{2/6}$ + $C^{2'/6'}$+ C^{4} + $C^{4'}$), 104.0 ($C^{Gal}_{anom.}$), 84.3 ($C^{Glc}_{anom.}$), 79.1, 76.9, 76.3, 73.5, 72.8, 71.9, 69.5 (all CH^{Lac}), 67.2 (CH^{a}_{2} + $CH^{a'}_{2}$), 62.0 (CH^{Glc}_{2}), 61.0 (CH^{Gal}_{2}), 53.7 (CH_3O), 44.7 (CH^{b}_{2}), 40.4 ($CH^{b'}_{2}$). ESI MS: *m*/*z* = 1057.4 [*M* − Glc + 2 Na]$^{2+}$, 1116.9 [*M* + 2H]$^{2+}$, 1127.4 [*M* + H + Na]$^{2+}$, 1138.9 [*M* + 2 Na]$^{2+}$, 2091.7 [*M* − Glc + Na]$^+$, 2253.8 [*M* + Na]$^+$.

$MeO_2C[G_3](Lac)_8$ (10): Compound **7** (396 mg, 57 μmol) was dissolved in a mixture of dioxane (4.2 mL) and MeOH (1.5 mL). The solution was treated with aqueous NaOH (0.8 N, 0.6 mL), after which H_2O (4 mL) was added and the mixture was stirred for 15 min. The reaction mixture was brought to pH 4−5 with Dowex resin and the solvents were evaporated in vacuo. The material was dissolved and lyophilized from H_2O to yield **10** as a white solid (238 mg, 51 μmol, 90%): ^{13}C NMR (5% CD_3OD/D_2O, 75.4 MHz): δ = 184.3 (C=S), 169.8 (br, all C=O), 160.2 ($C^{3/5}$ + $C^{3''/5''}$ + $C^{3'''/5'''}$), 136.8 (br, C^{1} + $C^{1'}$ + $C^{1''}$), 107.3 (br, $C^{2/6}$ + $C^{2''/6''}$ + $C^{2'''/6'''}$ + C^{4} + $C^{4'}$ + $C^{4''}$), 104.0 ($C^{Gal}_{anom.}$), 84.3 ($C^{Glc}_{anom.}$), 79.1, 76.3, 73.5, 72.8, 71.9, 69.5 (all CH^{Lac}), 67.2 (br, CH^{a}_{2} + $CH^{a'}_{2}$ + $CH^{a''}_{2}$), 62.0, 61.1 (2 × CH^{Lac}_{2}), 54.0 (CH_3O), 44.9, 40.4 (br, CH^{b}_{2}, $CH^{b'}_{2}$, $CH^{b''}_{2}$). ESI MS: *m*/*z* = 1465 [*M* − 2 Glc + 3 Na]$^{3+}$, 1472 [*M* − 2 Glc + 4 Na − H]$^{3+}$, 1480 [*M* − 2 Glc + 5 Na − 2 H]$^{3+}$, 1519 [*M* − Glc + 3 Na]$^{3+}$, 1526 [*M* − Glc + 4 Na − H]$^{3+}$, 1534 [*M* − Glc + 5 Na − 2 H]$^{3+}$, 1574 [*M* + 3 Na]$^{3+}$, 1581 [*M* + 4 Na − H]$^{3+}$, 1588 [*M* + 5 Na − 2 H]$^{3+}$, 2207 [*M* − 2 Glc + 4 Na − 2 H]$^{2+}$, 2289 [*M* − Glc + 4 Na − 2 H]$^{2+}$, 2371 [*M* + 4 Na − 2 H]$^{2+}$.

Monovalent Lactose Derivative 11: This compound was prepared by methods very similar to the preparation of **8** via **5**, starting in this case from *n*-butylamine instead of the HCl salt of $G1(NH_2)_2$ ^{13}C NMR (5% CD_3OD/D_2O, 75.4 MHz): δ = 183.0 (C=S), 103.9 ($C^{Gal}_{anom.}$), 84.0 ($C^{Glc}_{anom.}$), 78.9, 76.9, 76.3, 76.1, 73.5, 72.7, 71.9, 69.5 (8 × CH^{Lac}), 62.0 (CH^{Glc}_{2}), 60.9 (CH^{Gal}_{2}), 45.6, 31.2, 20.4 (3 × CH^{tBuyl}_{2}) 13.9 CH^{tBuyl}_{3}. ESI MS: *m*/*z* = 457.2 [M + H]$^+$ (100%). $C_{17}H_{32}N_2O_{10}S$ (456.2) calcd. C 44.73, H 7.07, N 6.14; found C 44.52, H 7.16, N 6.02.

Monovalent Lactose Derivative 12: This compound was prepared by methods very similar to the preparation of **8** via **5**, starting in this case from the HCl salt of methyl 3(2-aminoethoxy)benzoate instead of $G1(NH_2)_2$. ^{13}C NMR (5% CD_3OD/D_2O, 75.4 MHz): δ = 184.4 (C=S), 169.6 (C=O), 159.2 (C^{3}), 131.8 (C^{1}), 131.0, 123.4, 121.2, 115.9 ($C^{2/4/5/6}$), 104.0 ($C^{Gal}_{anom.}$), 84.3 ($C^{Glc}_{anom.}$), 79.0, 77.1, 76.4, 76.3, 73.7, 72.8, 72.0, 69.7 (8 × CH^{Lac}), 67.4 (CH^{a}_{2}), 62.1 (CH^{Glc}_{2}), 61.0 (CH^{Gal}_{2}), 53.5 (CH_3O), 44.8 (CH^{b}_{2}). ESI MS: *m*/*z* = 579.3 [M + H, 100%]$^+$. $C_{23}H_{34}N_2O_{13}S$ (578.2) calcd. C 47.75, H 5.92, N 4.84; found C 47.58, H 6.10, N 4.86.

Fluoresence Titrations: A solution (1000 μL) of CTB_5 (0.5 μM) in buffer (Tris·HCl 50 mM, pH 7.5, 0.15 M NaCl) was placed in a cuvette (1 × 1 cm). The fluorescence spectrum was recorded between 300 nm and 400 nm, using an irradiation wavelength of 282 nm, and the temperature was maintained at 23 °C. Incremental additions (> 10 datapoints) of a solution containing the lactose (derivative) and CTB_5 (0.5 μM) were made until at least 75% saturation was achieved. The fluorescence spectra were recorded by averaging four scans of 50 nm/min and the data were stored. In the case of the aryl-containing lactose derivatives an additional spectrum of a reference cuvette with the same amount of the ligand but no CTB_5 was taken for each datapoint. These spectra were subtracted from those in which CTB_5 was present to correct for some inherent ligand fluorescence. All titrations were run in duplicate from freshly

prepared solutions. The samples were left to incubate for about 10 min after each addition before the measurements were started. It was shown that an incubation time of 2 hours (using **10**) had no significant effect on the spectrum. Fluorescence data at 346 nm were used to determine the apparent dissociation constant by fitting them to Equation (1), (for lactose and **11**). Equation (1) represents a one-site binding model, taking into account fluorescence of free CTB (flu_R), occupied CTB (flu_{RL}), and free ligand (flu_L):

$$flu_{tot} = flu_R \cdot \{[R]_{tot} / (K_a \cdot [L] + 1)\} + flu_L \cdot \{[L] - [R]_{tot} \cdot (K_a \cdot [L] / \{K_a \cdot [L] + 1\})\} + flu_{RL} \cdot [R]_{tot} \cdot \{K_a \cdot [L] / (K_a \cdot [L] + 1)\} \quad (1)$$

in which $[R]_{tot}$ is the total concentration of CTB, [L] the ligand concentration, and K_a is the association binding constant.

The binding of **8**−**10** and **12** could be described well by a cooperativity model according to Hill,[32] taking into account the fluorescence of free CTB only. The fluorescence of the ligands was subtracted for each datapoint as mentioned above, and occupied CTB exhibits no fluorescence in these cases, as is demonstrated by the complete quenching.

$$flu_{tot} = flu_R \cdot [R]_{tot} \cdot \{K_d^n / (K_d^n + [L]^n)\} \quad (2)$$

Non-linear curve-fitting according to the specified models was performed with SlideWrite Plus for Windows, version 4 (Advanced Graphics Software Inc., Carlsbad, CA).

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