ARTICLE

OBC
www.rsc.org/obc

Synthesis and cholera toxin binding properties of multivalent GM1 mimics †

Daniela Arosio,[a,b] Ioannis Vrasidas,[a,b] Paola Valentini,[b] Rob M. J. Liskamp,[b] Roland J. Pieters*[b] and Anna Bernardi*[a]

[a] *Dipartmento di Chimica Organica e Industriale, via Venezian 21, 20133 Milano, Italy; Fax: +39-02-50314092; Tel: anna.bernardi@unimi.it*

[b] *Department of Medicinal Chemistry, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, NL-3508 TB Utrecht, The Netherlands. E-mail: r.j.pieters@pharm.uu.nl; Fax: +31-30-253 6655*

Received 13th April 2004, Accepted 26th May 2004
First published as an Advance Article on the web 30th June 2004

Dendrimers based on the 3,5-di-(2-aminoethoxy)-benzoic acid branching unit were used to attach multiple copies of a GM1 mimic for inhibition of cholera toxin binding. Systems up to octavalent were synthesized along with relevant reference compounds that contained in one case the ligand in a monovalent format and in another case the scaffold but not the ligand. Using a surface plasmon resonance inhibition assay the prepared inhibitors showed good inhibition. While the monovalent GM1 mimic showed the expected inhibition in the 200 μM range the multivalent scaffolds led to increased binding. The tetravalent compound was shown to be 440-fold more potent than its monovalent counterpart. The octavalent analog, however, was the most potent compound as determined using an ELISA assay.

Introduction

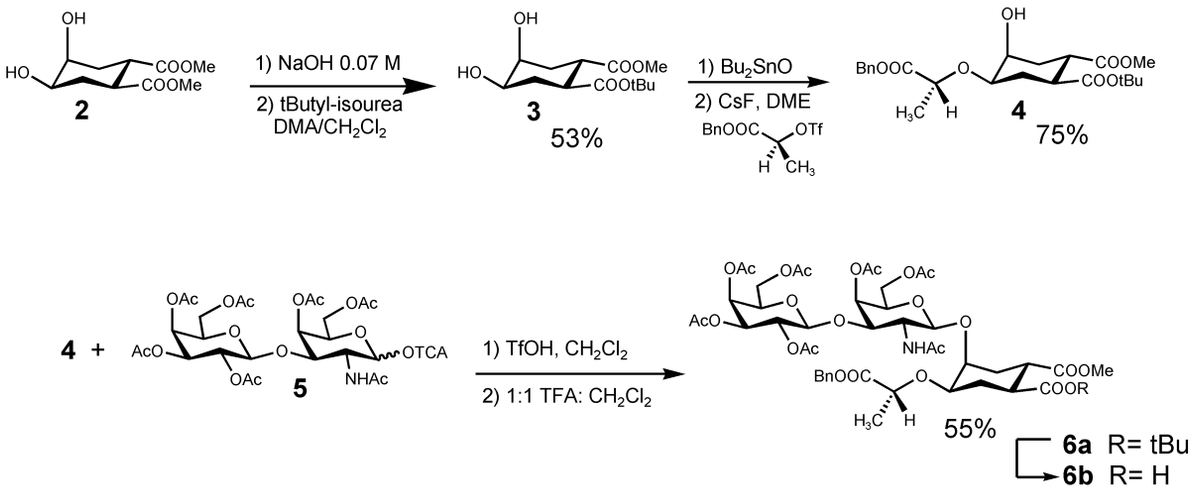
Toxins of the AB_5 type are a major source of disease and death.[1] As the causative agent of cholera, the cholera toxin (CT) is an important member of this family. Its mechanism of action begins with the binding of the B subunit to ganglioside GM1 molecules on the cell surface of the intestinal tract. This interaction is crucial for cell-uptake of the toxin and development of the disease. Interference with this process is therefore an attractive strategy with therapeutic potential. The protein–carbohydrate interactions are multivalent, since the five B subunit binding sites present in the multi-protein complex are well positioned for simultaneous interaction with the surface-bound GM1 molecules. The oligosaccharide part of ganglioside GM1 binds strongly to the B subunits with a K_d of 43 nM at 25 °C,[2] and the multivalent display of the GM1 molecules makes the affinity of the toxin to the cell surface even higher. Effective interference is therefore a tremendous challenge. We describe here our strategy of interference that is based on the combined effects of structure optimization at the monovalent and at the multivalent level. Simplified mimics of the complicated GM1 oligosaccharide were used and multivalency enhancement was approached by the use of dendritic multivalent scaffolds. The compounds were evaluated by an SPR competition assay and an ELISA type assay.

The carbohydrate mimics were developed by a process of structure-based design and experimental verification. This has led to a group of pseudo-saccharides designed to retain the specific orientation of the GM1 binding determinants (the terminal galactose residue and the sialic acid carboxy group) while progressively simplifying the molecular structure (and hence the synthetic complexity) of the ligand. These compounds bind to CT with dissociation constants that vary from 1 mM up to the potency of GM1.[3] Among them, for the present project we selected **1b**, based on the previously made **1a**, a 190 μM ligand of CT. Compound **1a** retains the galactose epitope of GM1 and uses an (*R*)-lactic acid as a substitute for the NeuAc residue. The interaction of **1a** with CT has been studied in detail using NMR spectroscopy and molecular modeling.[4] Unlike GM1, **1b** can be synthesized on a gram scale, and therefore has a higher potential of being used for prevention of cell intoxication. However, its low affinity is a serious drawback, which could be overcome by exploiting the power of multivalent presentation.

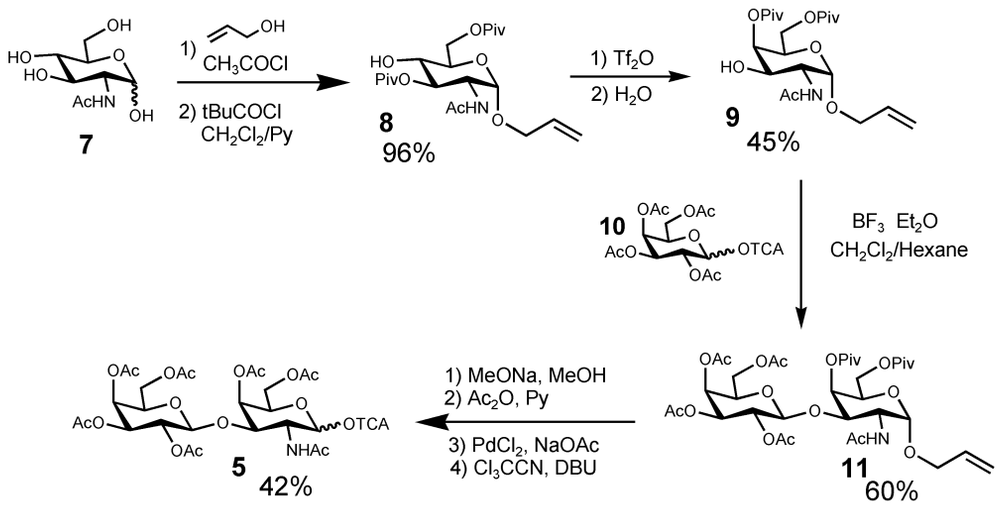
Multivalency is a common phenomenon of interacting biomolecules and also an emerging strategy for affinity optimization.[5] While the origin of the affinity enhancements by synthetic multivalent ligands is not always clear, mechanisms involving chelation *versus* aggregation can be distinguished. In chelation the goal is to bridge binding sites by use of a suitable spacer, thus reducing the entropic barriers of the association of the second and higher (sub)ligands after binding of the first.[6] Aggregation is another mechanism of soluble systems where a spacer cannot bridge binding sites, yet multivalency effects are observed nonetheless.[7] In this case aggregates can form whose ligands dissociate with reduced off-rates, thus enhancing overall affinity.[8] The AB_5 toxins represent an ideal case for harnessing multivalency effects based on chelation of binding sites, due to their positioning on the same face of the multimeric protein complex. Indeed, the multivalency aspects of AB_5 toxin binding have been actively studied over the last years. Highly active compounds based on a pentavalent scaffold have been developed for both the Shiga-like toxin[9] and the heat-labile enterotoxin of *E. coli* (LT)[10] and also theoretical models have been developed to help future design efforts.[11] Our approach to multivalency has involved the use of glycodendrimers as scaffolds for multivalent display of carbohydrate ligands, which in specific cases gave strong multivalency effects of over three orders of magnitude.[12] Dendrimers or dendritic scaffolds are easily synthesized and also easily modified with respect to valency and geometry. Therefore they are useful tools in the study of multivalency aspects of protein–carbohydrate interactions.[13] In contrast to the previously mentioned pentameric inhibitors, a dendrimer-based system does not have the exact complementarity to the target in terms of the number of attached ligands, either four or eight ligands for five binding sites. However, a recent demonstration of an octavalent glycodendrimer ligand that outperformed a pentavalent core-derived

† Electronic supplementary information (ESI) available: characterization of the polyvalent compounds – imide by-products. See http://www.rsc.org/suppdata/ob/b4/b405344c/

DOI: 10.1039/b405344c



Scheme 1 Synthesis of the protected monovalent ligand **6**.



Scheme 2 Synthesis of the Galβ1,3GalNAc donor **5**.

system in the inhibition of the Shiga-like toxin,[11b] further supports our approach. The dendrimeric cores used in this project are based on the 3,5-di-(2-aminoethoxy)-benzoic acid branching unit.[12,14] They have already been used to synthesize polyvalent CT ligands using lactose as the monovalent inhibitor.[12a] The present study incorporates two improvements on that system. Firstly, here a designed GM1 mimic is used, a monovalent ligand of much higher affinity than lactose. Secondly, the scaffold was outfitted with elongated arms, as long spacer arms were shown to be beneficial in other systems.[10]

Synthesis

The synthesis of **1a** (Chart 1) was previously reported.[4] For the synthesis of **1b**, the reported sequence was slightly modified to achieve differentiation of the carboxy group in the cyclohexanediol moiety, and to improve the overall yield, also reducing the number of steps (Scheme 1). In brief, the known diol **2**[15] was treated with NaOH, and the resulting mono acid[15] was transformed into the t-butyl ester using *N*,*N*-diisopropyl-*O*-*tert*-butyl isourea[16] in DMA/CH_2Cl_2. The resulting diol **3**, now with two orthogonally protected carboxy groups, was transformed into the mono ether **4** using a published procedure.[4] Finally **4** was glycosylated in 55% yield with the Galβ1,3GalNAc donor **5**, whose synthesis, improved over reported procedures,[4] is depicted in Scheme 2. Quantitative removal of the t-butyl ester yielded the protected monovalent ligand **6b** ready for conjugation.

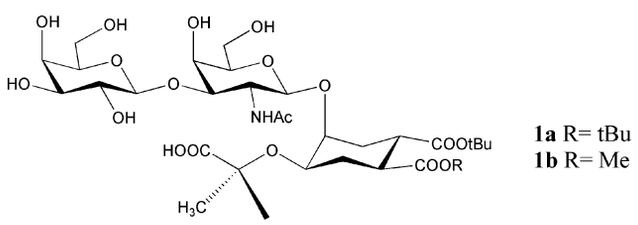
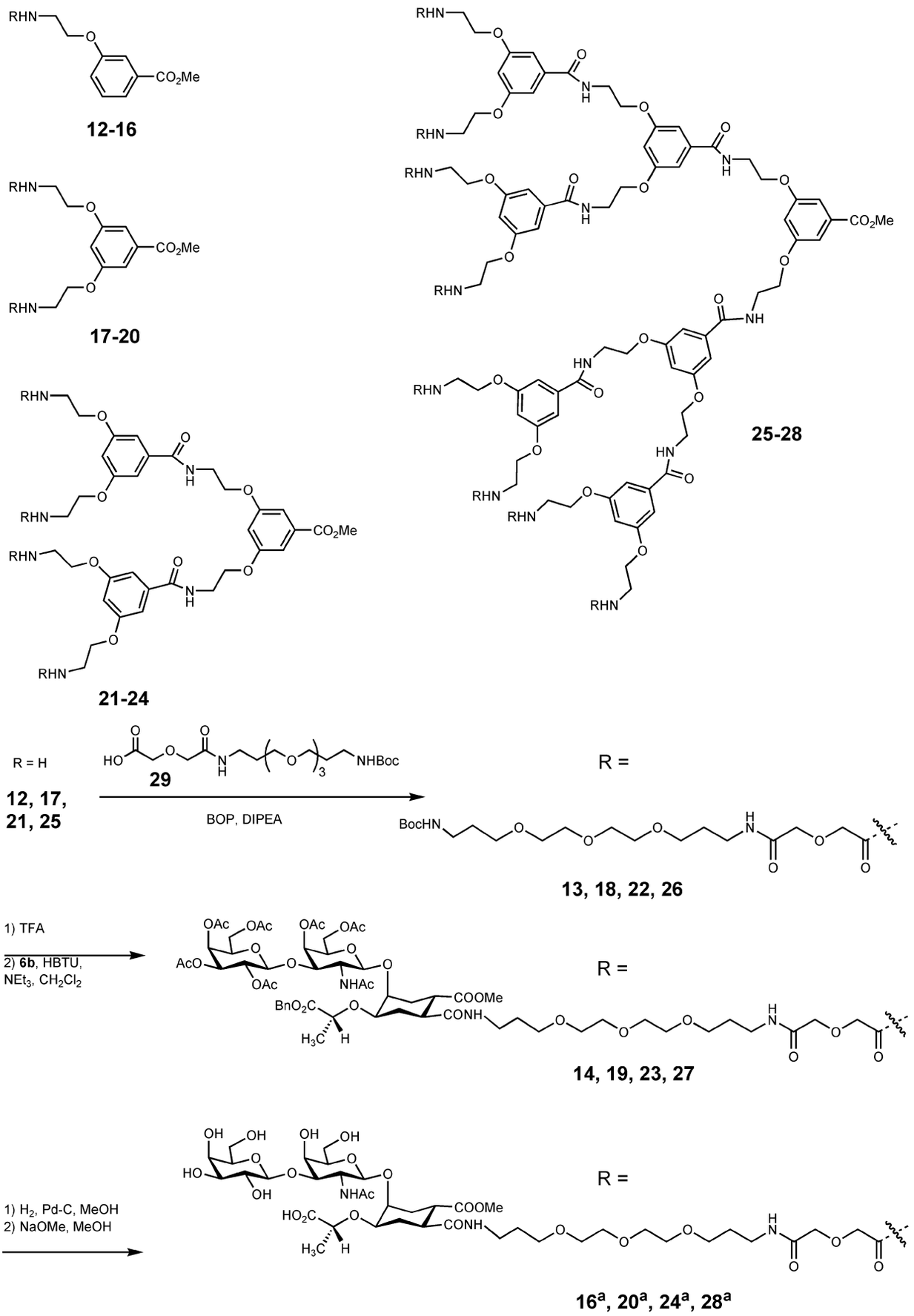


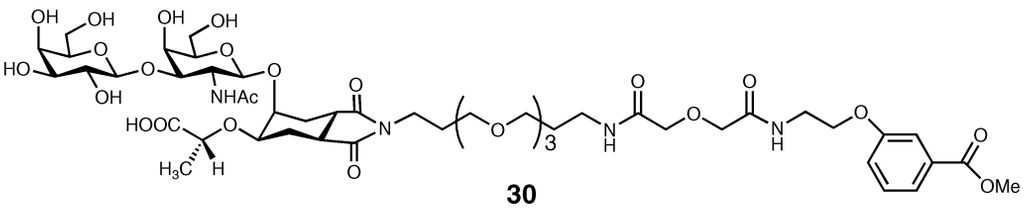
Chart 1 The monovalent ligand **1**.

The synthesis of the polyvalent ligands started from the dendritic cores **12**, **17**, **21** and **25**,[12a] and followed a common protocol. Thus the monovalent core **12** (Scheme 3) was elongated (BOP, DIPEA) to give **13**, using the amino acid spacer **29**.[12d,17] Deprotection of **13**, followed by condensation (HBTU) with an excess of **6b** yielded the fully protected monovalent reference compound **14** in 73% overall yield. Its benzyl ester was removed by standard hydrogenolysis on Pd–C. This reaction went to completion in 1 hour yielding the free carboxylic acid **15** (not shown), which was purified by short-path flash column chromatography (60 : 35 : 5 $CHCl_3$/MeOH/H_2O). Zemplén's hydrolysis of the acetates (MeONa in MeOH) proceeded within a few minutes yielding **16**. However, a by-product of mass M-32 was also observed in the ESI-MS spectrum of the final product. The by-product was isolated by careful chromatography using 60 : 35 : 5 $CHCl_3$/MeOH/H_2O and was characterized as imide **30** (Chart 2). This assignment was confirmed by the ^{1}H-NMR data that showed significant shifts for the protons adjacent to the carboxy groups in the cyclohexanediol fragment. Furthermore, an analog of **30** with OH groups instead of the disaccharide and lactic acid moieties also showed formation of the imide under the same reaction conditions (not shown).



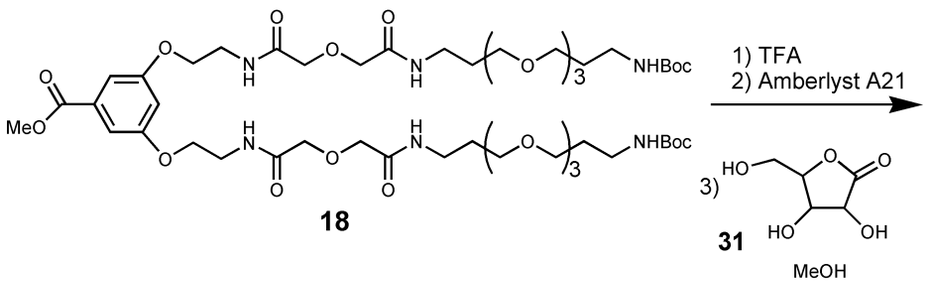
Scheme 3 Synthesis of the multivalent CTB ligands. [a]Compounds **16**, **20**, **24** and **28** also include cyclic imide containing spacer arms as in **30**, see text and electronic supplementary information. †

Starting from the appropriate dendritic cores **17**, **21**, and **25** (Scheme 3), the same process afforded the divalent **19**, the tetravalent **23** and the octavalent **27**, in 65%, 40% and 14% yields, respectively. Characterisation of these compounds was performed by NMR spectroscopy and ESI-MS analysis. Despite the high molecular weight, at this stage all compounds

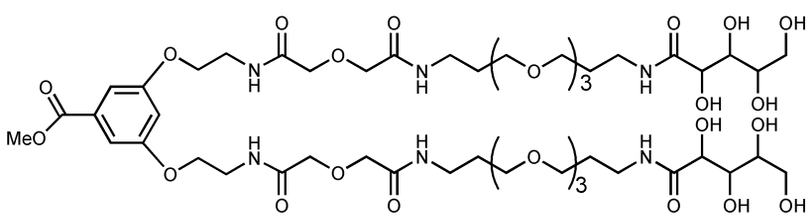


30

Chart 2 Obtained side product **30**.



18 **31**



32

Scheme 4 Synthesis of the water soluble divalent reference compound **32**.

were well-behaved in terms of solubility in common organic solvents, they could be chromatographed on silica gel with $CHCl_3$/MeOH and gave reasonably well-defined signals in ^{1}H-NMR. ESI-MS revealed the presence of multiple-charge peaks of the derivatives while underglycosylated by-products were not detected. Deprotection was performed following the procedure discussed for the monovalent compound **14** to yield the polyvalent **20**, **24** and **28**. Depending on the substrate and on the reaction conditions, variable amounts (20–35%) of inseparable imide by-products were obtained upon deacetylation, as judged by ^{1}H-NMR integration of the reaction crudes. The final polyvalent pseudo-glycoconjugates were purified by precipitation with acetone from water solution. All the products **16**, **20**, **24** and **28** were obtained as white solids, water soluble up to 7 mg ml^{-1}. They were all fully characterized by ^{1}H-NMR and ^{13}C-NMR and by mass spectrometry. ESI-MS revealed multiple charge peaks of the compounds and also of the corresponding [M-32] by-product. The [M-64] by-product was detected only in the case of the tetravalent ligand **24**, while the [M-96] by-product was never detected. Nevertheless we cannot exclude the presence of multiple imides in the higher generation polyvalent ligands because the presence of small quantities of these by-products was hard to identify in the complex mass spectra characteristic of these polyvalent compounds. The compounds were also analyzed by MALDI-MS, which allowed the detection of singly charged peaks for the monovalent and divalent compounds **16** and **20**.

With the aim of addressing the issue of non-specific interactions that could occur between the scaffolds and the cholera toxin, we also synthesized the divalent reference compound **32** (Scheme 4) by coupling **18** to a polyhydroxylated system that does not interact with CT. This compound, which represents a water soluble version of the divalent aglycon, was obtained by deprotecting the elongated divalent core **18** with TFA and treating the resulting diamine with excess D-(+)-ribonic acid-γ-lactone **31** in MeOH. The diamide **32** was not easily separated from unreacted ribonic lactone, but the reaction crude was acetylated and the functionalized dendrimer was isolated by flash chromatography. Finally, classical deacetylation reaction yielded pure **32**. All the compounds synthesized were tested as CT binders using SPR and an ELISA-type assay.

Results of the SPR analysis

The prepared compounds were analyzed with a surface plasmon resonance (SPR) competition assay, using the cholera toxin B pentamer CTB as the target. The glycoprotein ASF was covalently attached to a dextran functionalized gold SPR chip *via* NHS active ester intermediacy. ASF is a protein obtained after desialylation of the major plasma protein fetuin. It contains three *N*-glycosylation sites with complex type triantennary chains either with *N*-acetyllactosamine terminus (74%) or with the Galβ1,3GlcNAc isomer in the outer Manα1,3-arm (9%) or a biantennary chain (17%). Furthermore three *O*-linked chains, primarily displaying Galβ1,3GalNAcα– are also present.[18] Despite the fact that the glycoprotein does not display the GM1-oligosaccharide as the optimal ligand, CTB exhibited good affinity for the chip. A series of measurements with increasing CTB concentrations yielded a binding isotherm and a K_{chip}[19] of 3 μM. Inhibition studies were then performed which showed that the prepared compounds were able to inhibit the CTB (at 3 μM) binding to the chip. First, lactose was measured and an IC_{50} of 9.4 mM (Table 1) was determined for this disaccharide. The monovalent GM1 mimic **1b** showed much better inhibition with an IC_{50} of 97 μM. Both values are in the range that is close to their K_d as measured by other methods such as fluorescence titration.[12a,3e] As a monovalent reference, compound **16** was measured (Fig. 1). This compound exhibited an IC_{50} of 221 μM. The slightly reduced inhibitory power in comparison with **1b** clearly indicates that the attached spacer does not benefit the binding, rather it reduces it roughly two-fold. It is also possible that the reduced affinity is an expression of the presence of the imide side product **30**, whose affinity was not individually assessed. The divalent **20** showed an IC_{50} of 13 μM which indicates almost a 17-fold enhanced binding in comparison to the monovalent

Table 1 Surface plasmon resonance (SPR) inhibition studies using CTB (3 μM), an ASF-coated SPR sensorchip, and varying concentrations of inhibitors

Compound	Valency	IC_{50} [μM]	Relative potency (per sugar)
Lactose	1	9400	0.02 (0.02)
1b	1	97	2.3 (2.3)
16	1	221	1
20	2	13	17 (8.5)
24	4	0.5	442 (111)
28	8	0.5[a]	442 (55)
GM1-OS	1	10[a]	22 (22)

[a] Value an underestimation of the affinity since the assay limit was reached, see text for details.

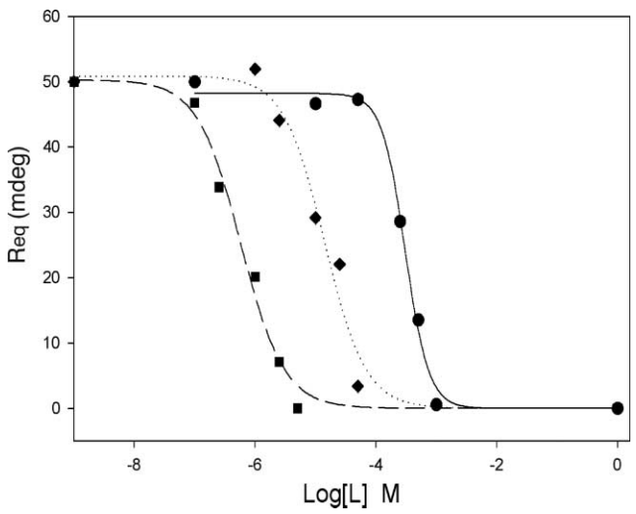


Fig. 1 Inhibition curves of CTB binding to an ASF functionalized SPR sensorchip. Normalised inhibition curves are shown for the monovalent ligand **16** (circles, solid line), the divalent ligand **20** (diamonds, dotted line), the tetravalent ligand **24** (squares, dashed line).

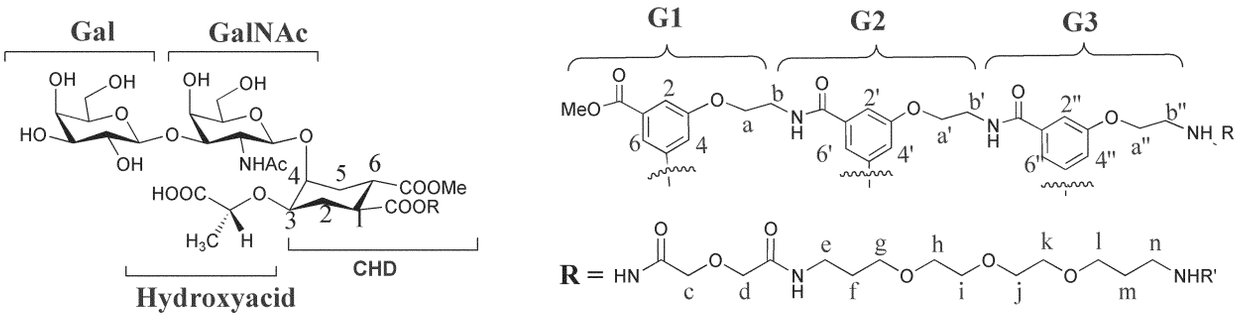
reference compound. The tetravalent **24** showed a large multivalency effect with an IC_{50} of 0.5 μM or a 442-fold enhanced binding (111-fold per sugar mimic). Moving to the octavalent compound **28** did not lead to a further enhancement in this assay, and its potency on a per sugar basis is actually lower than that of tetravalent **24**. The GM1-oligosaccharide (GM1-OS) showed an IC_{50} of 10 μM in this assay. The last two experiments indicated that these high-affinity compounds reached the limit of the assay (see discussion), due to the relatively moderate inherent affinity of CTB for the ASF chip. The divalent reference scaffold compound **32** was measured up to 500 μM and showed no effect.

ELISA

In order to better evaluate the affinity of the octavalent **28** a different assay was employed. For this reason an ELISA-like assay was used. In this assay the ganglioside GM1 was coated onto the ELISA wells (0.1 μg of GM1 per well) and a CTB–horseradish peroxidase conjugate (CT-HRP) was used as the binding protein.[20] The binding could clearly be visualized by the HRP catalyzed conversion of *o*-phenylene diamine (OPD) and measured by a multiwell plate spectrophotomer. GM1-OS was capable of inhibition, although concentrations of over 100 μM were required for full inhibition, reflecting the multivalent GM1 display on the ELISA plate. With the multivalent systems inhibition was observed only with the octavalent **28**, but not with its analogs of lower valency. With the concentrations employed (up to 400 μM) only 20% inhibition was observed with **28**. Thus, contrary to the SPR results on ASF chips, GM1-OS appears to be a stronger binder of CTB than **28** when their relative potency is estimated by ELISA on GM1-coated plates. On the other hand, both methods imply that the affinity of the multivalent ligands increases with the valency of the compound.

Discussion

Multivalent GM-1 mimics were prepared based on their activity as monovalent entities. In the synthesis a cyclic imide product was formed, which could theoretically complicate our binding studies. However, the formation of the imide is not expected to create a major distortion of the DCCHD ring. The first non-chair conformation of the parent ring system (*i.e.* the imide with two methyl ethers on the diol) is still 12 kJ mol^{-1} above the global minimum by MM3*, compared with 17 kJ mol^{-1} for the "diester" compound. Hence, the imide formation should have little effect on the relative orientation of the binding determinants (galactose and carboxylate) which define the affinity of the ligand for CT.[2] Experimentally we observed similar behavior of **16** and the shown **16/30** mixture (see Electronic Supplementary Information †) in a fluorescence assay.[21,25] The fact that a multivalency effect was observed in the SPR assay when moving from mono- to tetravalent compounds is consistent with the geometry of the compounds, *i.e.* their expected ability to bridge adjacent binding sites. In the crystal structure of the complex of five GM1 molecules and CTB[22] the distance between neighboring ligands was 31 Å, as measured between the anomeric oxygens of the terminal galactoses. The two GM1 mimics of the divalent **20** are separated by about 70 Å in a mostly extended conformation. The fact that the average distance or the so-called effective length between ligands separated by flexible spacers is significantly shorter than its extended conformation was previously shown.[10,23] The observed 17-fold enhancement (nine-fold per sugar mimic) indicates that chelation is indeed taking place. Such an enhancement is sizeable considering the large distance of 31 Å between the binding sites and the flexibility of the spacer. For the Shiga-like toxin a series of divalent ligands was prepared to bridge sites separated by only 11 Å, and enhancements per sugar between 3.8 and 23.7 were measured.[23] The effect observed for tetravalent **24** was larger with an enhancement of 442-fold (111-fold per sugar mimic). Due to the higher valency more binding sites can simultaneously be reached. In the recently reported thermodynamic model of Kitov and Bundle[11b] avidity entropy, a factor expressing the probability of association, was coined as a determining factor in chelation-based multivalency effects. For a tetravalent ligand binding to a pentavalent receptor this probability should be significantly larger than for the divalent system, but the octavalent **28** should yield the highest probability. Therefore it seemed surprising that **28** did not show a lower IC_{50} in the SPR assay. However, it can be understood when the stoichiometry of the components in the assay is considered. A CTB concentration of 3 μM was used, while the IC_{50} of the tetravalent **24** was 0.5 μM. It seems as if each individual GM1 mimic is blocking more than one CT binding site. Besides unlikely experimental error this suggests that aggregation may also be taking place in which more than one toxin binds to the arms of a single glycodendrimer. In such an aggregate[24] some of the free binding sites may be sterically blocked for binding to the chip surface. Although the existence of aggregates in this study was not experimentally confirmed it is clear that the tetravalent compound **24** reached the boundaries of the assay. For the octavalent **28**, which based on the ELISA results appears to be a better binder than **24**, this was also the case. More support for this interpretation came from the measurement of the GM_1–OS, a ligand with a K_d of 43 nM,[2] which in our SPR assay inhibited the CTB binding, only with an IC_{50} of 10 μM, a number close to the number of available carbohydrate binding sites under the assay conditions. This is again an indication that the assay can only differentiate



affinities up to the low micromolar range. The ELISA assay on GM1-coated plates clearly showed GM_1–OS to be the best ligand, and consistent with expectations it also showed the octavalent **28** to be the best of the multivalent ligands. Unfortunately, the ligands could not all be compared in the same assay.[25]

Conclusions

Our approach to high-affinity ligands for cholera toxin represents a combination of structure-based design of monovalent ligands with further enhancement by multivalent presentation using dendrimers. Our experiments showed that multivalent compounds were accessible which included an advanced GM1 mimic attached to dendritic scaffolds with elongated spacer arms. To some extent the synthetic strategy led to additional cyclic imide formation in the linking unit, with possible effects on the conformation of the GM1 mimic. The SPR inhibition assay showed that the tetravalent **24** in comparison to lactose was almost 19000-fold more active than lactose. The multivalency enhancement of 442 relative to the monovalent **16**, was of the same order of magnitude (263-fold) as the enhancement observed by Hol *et al.* with their pentavalent version of the *m*-nitrophenyl-α-D-galactoside ligand.[26] In contrast to their hypothesis, our results clearly show that a pentavalent ligand design is not a crucial factor for strong multivalency binding enhancement to AB_5 toxins. Further support for this notion also comes from the success of a functionalized PAMAM dendrimer for interference with binding of the Shiga-like toxin.[11b] Precisely how the most effective compound, octavalent **28**, compared to the tetravalent **24** could not be quantified with our assay methods, however its affinity was clearly higher. In general, our results showed that multivalent presentation of designed ligands can indeed bring their affinity closer to that required for practical application against AB_5 toxins. In order to do so the compounds need to be considerably larger than monovalent ligands to be effective. Considering the toxins reside in the intestinal tract this is not a problem, in fact it is an added bonus if the structure turns out to be too large and too polar for absorption. We are currently still in the phase where the optimal multivalent geometry for toxin neutralization, be it cyclic, radial, dendritic or polymeric, is not yet known, although several promising ones are now available. For the longer term, concise syntheses of both simple but effective ligands and similarly straightforward multivalent scaffolds may bring practical intervention within reach.

Experimental section

General

Chemicals were obtained from commercial sources and used without further purification, unless stated otherwise. Pyridine, DMF, dimethylacetamide (DMA), DME, and allyl alcohol were dried over 4 Å molecular sieves. All other dry solvents were distilled under nitrogen shortly before use. THF, hexane, dioxane and benzene were distilled from Na; CH_2Cl_2, MeOH, Et_3N, iPr_2NEt, were distilled from CaH_2.

Flash chromatography was performed using Macherey-Nagel Kieselgel 60, 230–400 mesh. Optical rotation values were measured using a Perkin-Elmer 241 polarimeter, at 589 nm, in 1 ml cells.

Electrospray Ionisation (ESI) mass spectrometry was carried out with a Shimadzu LCMS QP-8000 single quadrupole benchtop mass spectrometer (m/z < 2000), or a ThermoQuest Finnigan LCQDeca mass spectrometer (FINNIGAN MAT, San Jose, CA, USA) (m/z < 2000), or an Apex II ICR FTMS (for HRMS).

MALDI mass spectrometry was performed with a Bruker OMNIFLEX spectrometer.

FAB^+ mass spectrometry was performed with a VG 7070 EQ-HF spectrometer.

^{1}H-NMR and ^{13}C-NMR were recorded using Bruker Ac-200, Ac-300, and Avance-400. TMS was used as internal standard. For ^{13}C-NMR spectra, only selected values are reported.

Product numbering for spectral assignment

The unconventional numbering of the DCCHD moiety in the GM1 mimic was used in analogy with the Gal-II residue of the GM1 ganglioside. To avoid misunderstandings, the same numbering was adopted for non glycosylated dicarboxycyclohexanediols. Protons belonging to the (*R*)-lactic acid chain were identified as CH_3-L and H-L.

Dendrimers are numbered as reported in the picture. The methylenic protons of the linker are identified by the letter assigned to the corresponding carbon atom. The amide protons are numbered progressively starting from the scaffold.

Synthesis of 3

To dimethyl ester **2** (2 g, 8.6 mmol, 1 mol eq.) a 0.07 M solution of NaOH (182 ml, 12.75 mmol, 1.5 mol eq.) was added. The reaction mixture was stirred at rt for 1.5 h monitoring by TLC. After reaction completion HCl 6 M was added to pH 1, and the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel (8 : 2 $CHCl_3$/MeOH + 1% AcOH) to yield the monoacid[16] (80%).

To a suspension of the monoacid (602 mg, 2.76 mmol, 1 mol eq.) in dry CH_2Cl_2 (12 ml) and under N_2, DMA (2 ml) was added dropwise, then *t*-butyl isourea[16] (1.32 ml, 5.52 mmol, 2 mol eq.) was added. The reaction mixture was stirred at 40 °C for 6 h adding 2 equivalents of isourea every two hours (8 eq. in total) and monitoring by TLC. Two hours after the last addition, the solvent was evaporated under reduced pressure and the crude was taken up with Et_2O. The suspension was filtered over a pad of Celite until the urea was completely removed. The organic phase was evaporated and the crude was purified by flash chromatography on silica gel ($CHCl_3$/MeOH 95 : 5) to yield **3** as a white solid (66%). $[\alpha]^{20}_D$: +19.8 (c = 1.11, $CHCl_3$). MS (FAB+): 275 [M + H^+]; 297 [M + Na^+]. **^{1}H-NMR ($CDCl_3$, 200 MHz):** 1.45 (s, 9H, OC(CH_3)$_3$); 1.6 (ddd, 1H, **H-5ax**, $J_{gem} \cong J_{6ax\text{-}1}$ = 2 Hz; $J_{6ax\text{-}4}$ = 2.5 Hz); 1.8 (ddd, 1H, **H-2ax**, $J_{gem} \cong J_{3ax\text{-}2} \cong J_{3ax\text{-}4}$ = 12 Hz); 2.05 (ddd, 1H, **H-2eq**, J_{gem} = 12 Hz; $J_{3eq\text{-}2} \cong J_{3ax\text{-}4}$ = 12 Hz); 2.21 (ddd, 1H, **H-5eq**, J_{gem} = 12 Hz; $J_{6eq\text{-}1} \cong J_{6eq\text{-}4}$ = 3.5 Hz); 2.45 (s, 2H,O**H**); 2.62 (ddd, 1H, **H-1**, $J_{2\text{-}1} \cong J_{2\text{-}3ax}$ = 12 Hz; $J_{2\text{-}3eq}$ = 3.5 Hz); 2.98 (ddd, 1H, **H-6**, $J_{1\text{-}2} \cong J_{1\text{-}6ax}$ =

12 Hz; $J_{1\text{-}6eq}$ = 3.5 Hz); 3.68 (s, 3H, OC**H**$_3$), 3.7–3.75 (m, 1H, **H-3**); 3.85–4.05 (br s, 1H, **H-4**). ^{13}C-NMR ($CDCl_3$, 50.3 MHz): 27.8; 30.1; 33.0; 38.3; 44.1; 51.7; 67.7; 70.3; 80.8; 173; 175.5.

Synthesis of the triflate of *(S)*-lactic acid benzyl ester

To a solution of *(S)*-lactic acid benzyl ester (260 mg, 1.443 mmol, 1 mol eq.) in dry CH_2Cl_2 (5 ml), under N_2 and at 0 °C, trifluoromethanesulfonic anhydride (1.6 ml, 5.94 mmol, 1.1 mol eq.) was added. After 5 min 2,6-lutidine (195 µl, 1.682 mmol, 1.16 mol eq.) was added. The reaction mixture was stirred at room temperature for 20 min monitoring by TLC. At reaction completion the solvent was evaporated and the crude purified by short path flash chromatography (hexane/EtOAc 9 : 1. 90% yield). **^{1}H-NMR ($CDCl_3$, 200 MHz):** 1.7 (d, 3H, C**H**$_3$, *J* = 7 Hz); 5.25 (m, 3H, C**H**$_2$, C**H**); 7.38 (s, 5H, **aromatic protons**).

Synthesis of the monoether 4

A solution of diol **3** (146 mg, 0.53 mmol, 1 mol eq.) and Bu_2SnO (132 mg, 0.53 mmol, 1eq.), in dry benzene (4 ml) was refluxed under N_2, while continuously removing water (4 Å molecular sieves were inserted between the flask and the reflux condenser). After 8 h the solvent was evaporated under reduced pressure. The residue was taken up in dry DME (1.5 ml) and, under N_2, the triflate of (*S*)-benzyllactate (248 mg, 0.79 mmol, 1.5 mol eq.) and CsF (120 mg, 0.79 mmol, 1.5 mol eq.) were added. The reaction mixture was stirred at room temperature for 30 min, monitoring by TLC. After completion, Et_2O was added and the organic phase was washed with H_2O. The organic phase, dried with Na_2SO_4, was evaporated and the crude purified by flash chromatography on silica gel (hexane/EtOAc 65 : 35) to afford **4** in 75% yield, as a white solid. $[\alpha]^{20}_D$: +22 (*c* = 1.55, $CHCl_3$). ^{1}H-NMR ($CDCl_3$, 300 MHz): 1.55–1.52 (m, 11H, COOC(C**H**$_3$)$_3$, C**H**$_3$-**L**, **H-5ax**); 1.76 (ddd, 1H, **H-2ax**, $J_{gem} \cong J_{3ax\text{-}2} \cong J_{3ax\text{-}4}$ = 12 Hz); 2.05 (ddd, 1H, **H-2eq**, J_{gem} = 12 Hz, $J_{3eq\text{-}2} \cong J_{3ax\text{-}4}$ = 12 Hz); 2.25 (ddd, 1H, **H-5eq**, J_{gem} = 2 Hz; $J_{6eq\text{-}1} \cong J_{6eq\text{-}4}$ = 3.5 Hz); 2.41 (ddd, 1H, **H-1**, $J_{2\text{-}1} \cong J_{2\text{-}3ax}$ = 12 Hz, $J_{2\text{-}3eq}$ = 3.5 Hz); 2.85 (ddd, 1H, **H-6**, $J_{1\text{-}2} \cong J_{1\text{-}6ax}$ = 12 Hz, $J_{1\text{-}6eq}$ = 3.5 Hz); 3.37 (m, 1H, **H-3**); 3.65 (s, 3H, OC**H**$_3$), 4.05 (br s, 1H, **H-4**); 5.19 (m, 2H, OC**H**$_2$Ph); 7.35 (s, 5H, **aromatic protons**). ^{13}C-NMR ($CDCl_3$, 50.3 MHz): 18.7; 27.8; 27.9; 32.5; 38.0; 44.2; 51.5; 65.0; 66.8; 72.3; 76.4; 77.0; 77.6; 80.3; 128.1; 128.5; 172.6; 174.1.

Synthesis of 1-allyl-2-*N*-acetyl-2-deoxy-3,6-pivaloylglucose 8

Acetyl chloride (27.6 ml, 388 mmol, 3.4 mol eq.) was added dropwise to allylic alcohol (208 ml), under N_2 and at 0 °C. At room temperature, *N*-acetyl glucosamine **7** (25 g, 113 mmol, 1 mol eq.) was added. The reaction mixture was stirred at 70 °C for 3 h, monitoring by TLC ($CHCl_3$/MeOH 8 : 2). After reaction completion, solid $NaHCO_3$ was added to pH 7 and the suspension was filtered through a Celite pad, washing several times with MeOH. The solvent was removed under reduced pressure and the residue was digested with Et_2O. Finally the solvent was decanted and the α-1-allyl-2-*N*-acetyl glucosamine was recovered as a white solid. (99%). **Mp** 145 °C **^{1}H-NMR (D_2O, 400 MHz):** 2.0 (s, 3H, NHCOC**H**$_3$); 3.45 (m, 1H, **H-4**); 3.65–3.79 (m, 3H, **H-3**, **H-5**, **H-6**); 3.8–3.9 (m, 2H, **H-2, H-6′**); 3.95–4.23 (m, 2H, OC**H**$_2$CH=CH$_2$); 4.89 (d, 1H, **H-1**, $J_{1\text{-}2}$ = 3.6 Hz); 5.2–5.32 (m, 2H, OCH$_2$CH=C**H**$_2$); 5.85–5.99 (m, 1H, OCH$_2$C**H**=CH$_2$). To a suspension of α-1-allyl-2-*N*-acetyl glucosamine (16 g, 61.2 mmol, 1 mol eq.) in dry CH_2Cl_2/pyridine 1 : 2 (242 ml), under N_2 and at 0 °C, *t*BuCOCl (20.7 ml, 171.0 mmol, 2.8 mol eq.) was added dropwise. The reaction mixture was stirred for 2 h, monitoring by TLC (hexane/EtOAc 3 : 7). After completion, CH_2Cl_2 was added and the organic phase was washed with HCl 5%, $NaHCO_3$ 5% and H_2O. The organic phase was dried with Na_2SO_4 and the solvent was evaporated to yield **7** (98%), as an oil. **^{1}H-NMR ($CDCl_3$, 200 MHz):** 1.2 (s, 9H, COC(C**H**$_3$)$_3$); 1.25 (s, 9H, COC(C**H**$_3$)$_3$); 1.92 (s, 3H, NHCOC**H**$_3$); 2.95 (br s, 1H, O**H**); 3.45–3.61 (m, 1H, **H-4**); 3.8–3.89 (m, 1H, **H-5**); 3.98–4.25 (m, 2H, OC**H**$_2$CH=CH$_2$); 4.35–4.42 (m, 2H, **H-2**, **H-6**); 4.4–4.5 (m, 1H, **H-6′**); 4.85 (d, 1H, **H-1**, $J_{1\text{-}2}$ = 3.5 Hz); 5.04–5.15 (m, 1H, **H-3**); 5.21–5.37 (m, 2H, OCH$_2$CH=C**H**$_2$); 5.75 (d, 1H, N**H**COCH$_3$, *J* = 12 Hz); 5.82–5.95 (m, 1H, OCH$_2$C**H**=CH$_2$).

Synthesis of 1-allyl-2-*N*-acetyl-2-desoxy-4,6-pivaloylgalactose 9

To a solution of **8** (22 g, 51.2 mmol, 1 mol eq.) in dry CH_2Cl_2/pyridine 20 : 1 (489 ml), under N_2 and at −35 °C, Tf_2O (10.53 ml, 62.6 mmol, 1.2 mol eq.) was added. The reaction mixture was stirred at 0 °C for 2 h, monitoring by TLC (hexane/EtOAc 4 : 6), then H_2O (22 ml, 122.2 mmol, 2.4 mol eq.) was added and the reaction was refluxed for 13 h. After completion, CH_2Cl_2 was added and the organic phase was washed with HCl 5%, $NaHCO_3$ 5% and H_2O. The organic phase was dried with Na_2SO_4 and the solvent evaporated. The crude was purified by flash chromatography on silica gel (hexane/EtOAc 2 : 8) yielding **9** (45%). **^{1}H-NMR ($CDCl_3$, 400 MHz):** 1.2 (s, 9H, COC(C**H**$_3$)$_3$); 1.28 (s, 9H, COC(C**H**$_3$)$_3$); 2.1 (s, 3H, NHCOC**H**$_3$); 3.95–4.1 (m, 2H, **H-3**, **Ha**); 4.1–4.2 (m, 3H, **H-5**, **H-6**, **H-6′**); 4.25 (m, 1H, **Hb**); 4.95 (d, 1H, **H-1**, *J* = 6 Hz); 5.26–5.32 (m, 3H, **H-4**, OCH$_2$CH=C**H**$_2$); 5.85–5.95 (m, 2H, OCH$_2$C**H**=CH$_2$, N**H**COCH$_3$).

Synthesis of *O*-allyl-Gal(β1–3)GalNAc 11

To a solution of **9** (11.9 g, 27.7 mmol, 1 mol eq.) in dry hexane/CH_2Cl_2 1 : 1 (234 ml), under N_2 and at 0 °C, $BF_3{\cdot}Et_2O$ (4.17 ml, 33.24 mmol, 1.2 mol eq.) was added. The solution was warmed to room temperature and a solution of tetra-acetyl-galactose trichloroacetimidate (13.6 g, 27.7 mmol, 1 mol eq.) in dry CH_2Cl_2 (117 ml) was added. The reaction mixture was stirred at room temperature for 2 h then a second equivalent of trichloroacetimidate dissolved in the same amount of CH_2Cl_2 was added. The reaction was stirred for 12 h monitoring by TLC (hexane/EtOAc 2 : 8). After completion, TEA was added to pH 7 and the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel (toluene/acetone 8 : 2) to afford **11** (60%). **^{1}H-NMR (C_6D_6, 200 MHz):** 1.2 (s, 9H, OCOC(C**H**$_3$)$_3$); 1.25 (s, 9H, OCOC(C**H**$_3$)$_3$); 1.98 (s, 3H, OCOC**H**$_3$); 2.0 (s, 3H, OCOC**H**$_3$); 2.07 (s, 3H, OCOC**H**$_3$); 2.1 (s, 3H, OCOC**H**$_3$); 2.19 (s, 3H, NHCOC**H**$_3$); 3.8–4.3 (m, 9H, OC**H**$_2$CH=CH$_2$, GalNAc**H-6**, GalNAc**H-6′**, Gal**H-6**, Gal**H-6′**, GalNAc**H-1**, GalNAc**H-5**, Gal**H-5**); 4.53 (m, 1H, GalNAc**H-2**); 4.6 (m, 1H, Gal**H-1**); 4.9–5 (m, 2H, GalNac**H-3**); 5.1 (m, 1H, Gal**H-2**); 5.2–5.4 (m, 4H, OCH$_2$CH=C**H**$_2$, GalNAc**H-4**, Gal**H-4**), 5.65 (d, 1H, N**H**COCH$_3$); 5.75–6.1 (m, 1H, OCH$_2$C**H**=CH$_2$).

Synthesis of the trichloroacetimidate 5

To a solution of the disaccharide **11** (5.7 mg, 7.5 mmol, 1 mol eq.) in dry MeOH (500 ml), under N_2, 1 M MeONa in MeOH (13 ml, 13 mmol) was added. The reaction mixture was stirred at room temperature for 40 h, monitoring by TLC. After reaction completion, Amberlite IR-120 (H^+ form) was added to pH 6. The organic phase was filtered and evaporated under reduced pressure. The crude was redissolved in dry pyridine (80 ml) and, at 0 °C, Ac_2O (11 ml, 114 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 24 h, monitoring by TLC (hexane/EtOAc 2 : 8). After completion, the solvent was evaporated under reduced pressure and the residue was taken up with EtOAc. The organic phase was washed with HCl 5%, $NaHCO_3$ 5% and H_2O, then dried with Na_2SO_4 and the solvent was evaporated. The crude was purified by flash chromatography on silica gel (hexane/EtOAc 2 : 8) yielding the polyacetylated disaccharide (70% over the two steps). $[\alpha]^{20}_D$ = +66.7 (*c* = 1.39, $CHCl_3$). **^{1}H-NMR ($CDCl_3$, 400**

MHz): 1.94 (s, 3H, OCOC**H**$_3$); 1.96 (s, 3H, OCOC**H**$_3$); 1.99 (s, 3H, OCOC**H**$_3$); 2.09 (s, 3H, OCOC**H**$_3$); 2.11 (s, 3H, OCOC**H**$_3$); 2.16 (s, 3H, OCOC**H**$_3$); 2.19 (s, 3H, NHCOC**H**$_3$); 3.90 (m, 1H, Gal**H-5**); 3.99–4.20 (m, 8H, OC**H**$_2$CH=CH$_2$, GalNAc**H-6**, GalNAc**H-6′**, Gal**H-6**, Gal**H-6′**, GalNAc**H-1**, GalNAc**H-5**); 4.51 (m, 1H, GalNAc**H-2**); 4.62 (m, 1H, Gal**H-1**); 4.90–5.01 (m, 2H, GalNac**H-3**, Gal**H-3**, Gal**H-2**); 5.11 (d, 1H, GalN**H-1**, J_{1-2} = 3.7 Hz); 5.28–5.40 (m, 4H, OCH$_2$C**H**=C**H**$_2$, GalNAc**H-4**, Gal**H-4**), 5.66 (d, 1H, N**H**OCH$_3$, J_{NH-H2} = 8.83 Hz); 5.88 (m, 1H, OCH$_2$C**H**=CH$_2$). ^{13}C-NMR-HETCOR (CDCl$_3$, 400 MHz): 20.8; 21.0; 21.1; 21.1; 23.7; 49.3; 61.5; 63.0; 67.1; 67.9; 68.9; 69.1; 69.2; 71.2; 71.3; 73.2; 97.3; 100.9; 118.7.

To a solution of the acetylated disaccharide (3.56 g, 5.30 mmol, 1 mol eq.) in AcOH/H$_2$O 20 : 1 (11 ml), NaOAc (1.03 g, 12.6 mmol, 2.4 mol eq.) and PdCl$_2$ (1.03 g, 5.83 mmol, 1.1 mol eq.) were added. The reaction mixture was stirred at room temperature for *ca.* 20 h monitoring by TLC. After completion the mixture was filtered through a Celite pad washing several times with EtOAc. The organic phase was washed with a saturated solution of NaHCO$_3$, dried with Na$_2$SO$_4$ and finally the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel (EtOAc/CHCl$_3$ 9 : 1 + 2% MeOH) yielding the anomerically deprotected disaccharide (70%). $[\alpha]^{20}_D$ = +54.8 (*c* = 1.035, CHCl$_3$). **^{1}H-NMR (CDCl$_3$, 400 MHz):** 2.00 (s, 3H, OCOC**H**$_3$); 2.03 (s, 3H, OCOC**H**$_3$); 2.07 (s, 3H, OCOC**H**$_3$); 2.09 (s, 3H, OCOC**H**$_3$); 2.11 (s, 3H, OCOC**H**$_3$); 2.15 (s, 3H, OCOC**H**$_3$); 2.19 (s, 3H, NHCOC**H**$_3$); 3.58 (br s, 1H, O**H**); 3.91 (dt, 1H, Gal**H-5**, J_{H5-H6}= 7.2 Hz); 4.01–4.21 (m, 5H, GalNAc**H-6**, GalNAc**H-6′**, Gal**H-6**, Gal**H-6′**, GalNAc**H-3**); 4.36 (t, 1H, GalNAc**H-5**, J_{5-6} = 6 Hz); 4.44 (m, 1H, GalNAc**H-2**); 4.74 (d, 1H, Gal**H-1**, J_{1-2} = 8 Hz); 4.99 (dd, 1H, Gal**H-3**, J_{3-2} = 10.4 Hz, J_{3-4} = 3.4 Hz); 5.17 (dd, 1H, Gal**H-2**, J_{2-1} = 8 Hz); 5.27 (dd, 1H, Gal**H-4**); 5.38–5.42 (m, 2H, GalNAc**H-1**, GalNAc**H-4**); 5.87 (d, 1H, N**H**COCH$_3$, J_{NH-H2} = 8.1 Hz). ^{13}C-NMR-HETCOR (CDCl$_3$, 400 MHz): 20.6; 21.2; 23.8; 27.4; 27.7; 47.6; 50.2; 62.0; 63.3; 63.5; 67.6; 68.7; 71.4; 72.1; 92.3; 92.5; 101.2.

To a solution of the anomerically deprotected disaccharide (1.56 g, 2.45 mmol, 1 mol eq.) in dry CH$_2$Cl$_2$ (20 ml), under N$_2$, Cl$_3$CCN (1.32 ml, 13.2 mmol, 5.5 mol eq.) and DBU (63 µl, 0.43 mmol, 0.175 mol eq.) were added. The reaction mixture was stirred at room temperature for *ca.* 2 h, then the solvent was evaporated under reduced pressure and the crude was purified by short pattern flash chromatography on silica gel (hexane/EtOAc 1 : 1) yielding **5** in 86% yield, as a foam. **^{1}H-NMR (CDCl$_3$, 200 MHz):** 1.98 (s, 6H, OCOC**H**$_3$); 2.07 (s, 3H, OCOC**H**$_3$); 2.10 (s, 3H, OCOC**H**$_3$); 2.15 (s, 3H, OCOC**H**$_3$); 2.18 (s, 3H, OCOC**H**$_3$); 2.20 (s, 3H, NHCOC**H**$_3$); 3.9–4.4 (m, 7H, Gal**H-5**, GalNAc**H-6**, GalNAc**H-6′**, Gal**H-6**, Gal**H-6′**, GalNAc**H-3**, GalNAc**H-5**); 4.6 (m, 1H, GalNAc**H-2**); 4.72 (d, 1H, Gal**H-1**, J_{1-2} = 7.4 Hz); 5.0 (dd, 1H, Gal**H-3**, J_{3-4} = 3.3 Hz, J_{3-2} = 10.4 Hz); 5.2 (dd, 1H, Gal**H-2**, J_{2-1} = 7.4 Hz, J_{2-3} = 10.4 Hz); 5.4 (d, 1H, Gal**H-4**, J_{4-3} = 3.3 Hz); 5.47 (d, 1H, GalNAc**H-4**, J_{4-3} = 2.2 Hz); 5.72 (d, 1H, N**H**COCH$_3$, J_{NH-H2} = 7.4 Hz); 6.55 (d, 1H, GalNAc**H-1**, J_{1-2} = 3 Hz); 8.74 (s, 1H, N**H**=C–CCl$_3$).

Synthesis of the pseudotrisaccharide 6

A solution of trichloroacetimidate **5** (495 mg, 0.57 mmol, 0.5 mol eq.) and monoether **4** (500 mg, 1.14 mmol, 1 mol eq.) in dry CH$_2$Cl$_2$ (8 ml), under N$_2$ and in the presence of 4 Å molecular sieves was stirred for 15 min at rt before adding TfOH (1.3 µl, 0.015 mmol, 0.05 mol eq.). The reaction mixture was stirred at room temperature for 1 h then was heated at 40 °C and another 0.05 eq. of TfOH were added. After reaction completion (24 h, monitoring by TLC hexane/EtOAc 3 : 7), TEA was added to pH 7 and the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel (toluene/acetone 7 : 3) to afford the pseudotrisaccharide **6a**, as a white solid, in 55% yield. $[\alpha]^{20}_D$ = + 0.34 (*c* = 0.77, CHCl$_3$). MS (FAB+): 1055 [M + H]$^+$; 1077 [M + Na]$^+$. **HRMS (ESI$^+$):** [C$_{49}$H$_{67}$NO$_{24}$Na]$^+$ requires 1076.39452, found 1076.39722. **Mp** 75–78 °C. **^{1}H-NMR (C$_6$D$_6$, 300 MHz):** 1.26 (m, 1H, CHD**H-5ax**); 1.43 (d, 3H, C**H**$_{3L}$, J_{CH3-HL} = 6.7 Hz); 1.49 (s, 9H, OCOC(C**H**$_3$)$_3$); 1.69 (s, 3H, OCOC**H**$_3$); 1.82 (s, 3H, OCOC**H**$_3$); 1.83 (s, 3H, OCOC**H**$_3$); 1.84 (s, 3H, OCOC**H**$_3$); 1.89 (s, 3H, OCOC**H**$_3$); 1.96 (s, 3H, OCOC**H**$_3$); 2.05 (s, 3H, NHCOC**H**$_3$); 2.22 (m, 1H, CHD**H-2ax**); 2.30 (m, 1H, CHD**H-2eq**); 2.45 (td, 1H, CHD**H-5eq**, $J_{5eq-5ax}$ = 10 Hz, $J_{5eq-6} \cong J_{5eq-4} \cong 4$ Hz); 2.78 (dt, 1H, CHD**H-1**, $J_{1-6} \cong J_{1-2ax} \cong$ 12 Hz, J_{1-2eq} = 3.8 Hz); 3.02 (m, 1H, CHD**H-3**); 3.25 (m, 1H, CHD**H-6**); 3.3 (s, 3H, OC**H**$_3$); 3.35 (m, 1H, Gal**H-5**); 3.65 (t, 1H, GalNAc**H-5**, J_{5-6} = 7 Hz); 3.8–3.9 (m, 2H, **H**$_L$, GalNAc**H-2**); 3.92 (br s, 1H, CHD**H-4**); 4.12–4.30 (m, 4H, Gal**H-6**, Gal**H-6′**, GalNAc**H-6**, GalNAc**H-6′**); 4.33 (d, 1H, Gal**H-1**, J_{1-2} = 6 Hz); 4.54 (dd, 1H, GalNAc**H-3**, J_{3-2} = 11 Hz, J_{3-4} = 3.4 Hz); 5.0 (dd, 1H, OC**H**$_2$Ph, J_{gem} = 12 Hz); 5.16 (m, 1H, Gal**H-3**); 5.45–5.60 (m, 3H, Gal**H-2**, Gal**H-4**, GalNAc**H-4**); 5.80 (d, 1H, GalNAc**H-1**, J_{1-2} = 6.3 Hz); 7.25–7.31 (m, 5H, aromatic protons) ^{13}C-NMR (C$_6$D$_6$, 75 MHz): 18.5; 20.6; 23.5; 27.7; 29.2; 33.2; 38.6; 39.0; 44.5; 51.0; 54.2; 56.2; 67.0; 68.7; 70.6; 71.2; 72.4; 78.1; 100.6; 101.2; 127.8; 128.7; 135.8; 168.9; 170.0; 172.4; 173.2; 174.6; 174.8.

To a solution of **6a** (310 mg, 0.294 mmol, 1 mol eq.) in CH$_2$Cl$_2$ (3 ml), TFA (3 ml) was added. The reaction mixture was stirred at room temperature for 1 h, monitoring by TLC (CHCl$_3$/MeOH 9 : 1). After completion, the solvent was evaporated and the residue taken up with toluene, which was evaporated several times to completely remove the TFA. Crude **6b** (99%) was dried under vacuum and used for the following reactions without any further purification.

Synthesis of 1b

To a solution of pseudo trisaccharide **6a** (22 mg, 0.021 mmol, 1 mol eq.) in MeOH (1 ml), a catalytic amount of Pd/C 10% was added. The mixture was stirred under H$_2$ atmosphere at room temperature for 1 h. After reaction completion, the mixture was filtered through a Celite pad, washing with MeOH. The collected organic phase was evaporated under reduced pressure and the crude (19 mg, 0.02 mmol, 1 mol eq.) redissolved in dry MeOH (1 ml). Under N$_2$, a solution of 1 M MeONa in MeOH (0.03 ml, 0.03 mmol, 1.5 mol eq.) was added. The reaction was stirred at room temperature for 45 min monitoring by TLC (CHCl$_3$/MeOH/H$_2$O 60 : 35 : 5). After completion, the reaction was quenched adding Amberlite IR 120 (H$^+$ form) to pH 5–6. Finally the resin was filtered and washed with MeOH. The organic phase was evaporated under reduced pressure and the crude was purified by flash chromatography on silica gel (CHCl$_3$/MeOH/H$_2$O 60 : 35 : 5) to yield **1b** (60%), as a white solid. $[\alpha]^{20}_D$ = −8.1 (*c* = 0.63, MeOH). MS (FAB+): 712 [M + H]$^+$; 734 [M + Na]$^+$. **^{1}H-NMR (D$_2$O, 300 MHz):** 1.42 (d, 3H, C**H**$_{3L}$, $J_{CH3L-HL}$ = 6.7 Hz); 1.54 (s, 9H, COOC(C**H**$_3$)$_3$); 1.63–1.85 (m, 2H, CHD**H-5ax**, CHD**H-2ax**); 2.11 (s, 3H, NHCOC**H**$_3$); 2.25 (d, 1H, CHD**H-2eq**, $J_{2eq-2ax}$ = 13.6 Hz); 2.36 (d, 1H, CHD**H-5eq**, $J_{5eq-5ax}$ = 13.6 Hz); 2.74 (t, 1H, CHD**H-1**, $J_{1-6} \cong J_{1-2ax} \cong$ 12.9 Hz); 3.01 (t, 1H, CHD**H-6**, $J_{6-1} \cong J_{6-5ax} \cong$ 12.9 Hz); 3.58–3.72 (m, 3H, Gal**H-2**, CHD**H-3**, Gal**H-3**); 3.72–3.91 (m, 9H, Gal**H-5**, GalNAc**H-5**, COOC**H**$_3$, Gal**H-6**, Gal**H-6′**, GalNAc**H-6**, GalNAc**H-6′**); 3.91–4.06 (m, 2H, GalNAc**H-3**, Gal**H-4**); 4.12–4.23 (m, 2H, GalNAc**H-2, H**$_L$); 4.28 (m, 1H, GalNAc**H-4**); 4.48 (br s, 1H, CHD**H-4**); 4.57 (d, 1H, Gal**H-1**, J_{1-2} = 6.8 Hz); 5.06 (d, 1H, GalNAc**H-1**, J_{1-2} = 8.2 Hz). ^{13}C-NMR-HETCOR (D$_2$O, 300 MHz): 19.2; 23.5; 28.5; 29.3; 33.0; 40.1; 45.5; 52.2; 53.4; 62.0; 69.1; 69.2; 71.3; 72.6; 73.0; 74.4; 75.6; 77.6; 81.1; 102.0; 105.3.

Synthesis of 13

To a solution of **12** (35 mg, 0.18 mmol, 1 mol eq.) in dry CH$_2$Cl$_2$ (1 ml), under N$_2$, *i*Pr$_2$NEt (94 µl, 0.54 mmol, 3 mol eq.) was

added. The mixture was stirred at room temperature for 10 min and a solution of the linker **29** (95 mg, 0.22 mmol, 1.2 mol eq.) in dry CH_2Cl_2 (1 ml) and BOP (97 mg, 0.22 mmol, 1.2 mol eq.) was added. The resulting homogeneous solution was stirred at room temperature for 24 h monitoring by TLC ($CHCl_3$/MeOH 9 : 1 + 3% TEA). After completion the solvent was evaporated under reduced pressure and the residue was taken up with CH_2Cl_2. The organic phase was washed with 0.5 M NaOH, (2 × 10 ml), 1 M $KHSO_4$ (1 × 10 ml) and brine (1 × 10 ml), was finally dried with Na_2SO_4 and evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel ($CHCl_3$/MeOH 95 : 5) to yield pure **13** product as a colourless oil (98%). MS (ESI): 614.2 $[M + H]^+$. **^{1}H-NMR ($CDCl_3$, 300 MHz):** 1.39 (s, 9H, OCOC(C**H**$_3$)$_3$); 1.6–1.8 (m, 4H, C**H**$_2$**-m**, C**H**$_2$**-f**); 3.12 (m, 2H, C**H**$_2$**-n**); 3.35 (m, 2H, C**H**$_2$**-e**); 3.4–3.62 (m, 12H, C**H**$_2$**-g**, C**H**$_2$**-h**, C**H**$_2$**-i**, C**H**$_2$**-j**, C**H**$_2$**-k**, C**H**$_2$**-l**); 3.68 (m, 2H, C**H**$_2$**-b**); 3.85 (s, 3H, OC**H**$_3$); 3.99 (s, 2H, C**H**$_2$**c**); 4.02 (s, 2H, C**H**$_2$**-d**); 4.05 (m, 2 H, C**H**$_2$**-a**); 5.01 (br s, 1H, N**H**Boc); 7.03 (m, 1H, C**H-4**); 7.2 (s, 1H, N**H-II**); 7.28 (t, 1H, C**H-5**, $J_{5-4} \cong J_{5-6}$ = 7.9 Hz); 7.38 (br s, 1H, N**H-I**); 7.48 (s, 1H, C**H-2**); 7.58 (d, 1H, C**H-6**, J_{6-5} = 7.8 Hz). ^{13}C-NMR ($CDCl_3$, 75.0 MHz): 28.4; 28.9; 29.6; 37.4; 38.4; 38.5; 52.2; 66.7; 69.4; 69.7; 70.0; 70.2; 70.3; 71.1; 71.2; 78.8; 114.8; 119.6; 122.4; 129.5; 131.5; 156.2; 158.4; 166.8; 168.4; 169.1.

The same procedure as for the monovalent ligand was adopted to functionalise the divalent, tetravalent and octavalent dendrimeric cores using equivalents of reagents in proportion to the number of free amine groups. The solvents used in the purification and the characterization of each product are reported.

MeO$_2$C-[G1](LinkerNHBoc)$_2$ 18. The pure product was obtained after flash chromatography on silica gel ($CHCl_3$/MeOH 9 : 1) as a colourless oil (98%). MS (ESI): 1091.8 $[M + H]^+$. **^{1}H-NMR ($CDCl_3$, 200 MHz):** 1.42 (s, 18 H, OCOC(C**H**$_3$)$_3$); 1.65–1.86 (m, 8H, C**H**$_2$**-m**, C**H**$_2$**-f**); 3.12–3.28 (m, 4H, C**H**$_2$**-n**); 3.38–3.48 (m, 4H, C**H**$_2$**-e**); 3.48–3.64 (m, 24H, C**H**$_2$**-g**, C**H**$_2$**-h**, C**H**$_2$**-i**, C**H**$_2$**-j**, C**H**$_2$**-k**, C**H**$_2$**-l**); 3.64–3.78 (m, 4H, C**H**$_2$**-b**); 3.92 (s, 3H, OC**H**$_3$); 4.02 (s, 4H, C**H**$_2$**c**); 4.03–4.18 (m, 8 H, C**H**$_2$**-d**, C**H**$_2$**-a**); 5.03 (br s, 2H, N**H**Boc); 6.68 (s, 1H, C**H-4**); 7.2 (s, 2H, C**H-2**, C**H-6**); 7.22 (br s, 2H, N**H-II**), 7.4 (br s, 2H, N**H-I**). ^{13}C-NMR ($CDCl_3$, 50.3 MHz): 28.5; 29.0; 29.8; 37.4; 38.6; 43.3; 52.4; 66.8; 69.4; 69.7; 70.1; 70.2; 70.4; 71.1; 71.2; 77.9; 106.7; 108.4; 132.2; 156.4; 159.7; 166.7; 169.0; 169.8.

MeO$_2$C-[G2](LinkerNHBoc)$_4$ 22. The pure product was obtained after flash chromatography on silica gel ($CHCl_3$/MeOH 95 : 5⟶8 : 2) as a colourless oil (50%). MS (ESI): 1187.3 $[M + 2H]^{2+}$; 1197.7 $[M + H + Na]^{2+}$; 1209.5 $[M + 2Na]^{2+}$. **^{1}H-NMR ($CDCl_3$, 200 MHz):** 1.42 (s, 36H, OCOC(C**H**$_3$)$_3$); 1.64–1.86 (m, 16H, C**H**$_2$**-m**, C**H**$_2$**-f**); 3.10–3.22 (m, 8H, C**H**$_2$**-n**); 3.30–3.43 (m, 8H, C**H**$_2$**-e**); 3.43–3.78 (m, 56H, C**H**$_2$**-g**, C**H**$_2$**-h**, C**H**$_2$**-i**, C**H**$_2$**-j**, C**H**$_2$**-k**, C**H**$_2$**-l**, C**H**$_2$**-b′**); 3.78–3.96 (m, 7H, C**H**$_2$**-b**, OC**H**$_3$); 3.99–4.18 (s, 24H, C**H**$_2$**c**, C**H**$_2$**-d**, C**H**$_2$**-a′**); 4.18–4.26 (m, 4H, C**H**$_2$**-a**); 5.06 (br s, 4H, N**H**Boc); 6.58 (s, 2H, C**H-4′**); 6.72 (s, 1H, C**H-4**); 7.0 (d, 4H, C**H-2′**, C**H-6′**, $J_{2'-4'} \cong J_{6'-4'}$ = 2.2 Hz); 7.2 (d, 2H, C**H-2**, C**H-6**, $J_{2-4} \cong J_{6-4}$ = 2.2 Hz); 7.34 (br s, 4H, N**H-III**); 7.46 (br s, 4H, N**H-II**); 7.63 (br s, 2H, N**H-I**). ^{13}C-NMR ($CDCl_3$, 50.3 MHz): 28.6; 29.1; 29.8; 37.5; 38.5; 39.5; 52.4; 66.6; 66.9; 69.5; 69.9; 70.0; 70.2; 70.3; 70.5; 70.6; 71.1; 79.1; 104.6; 106.5; 106.8; 108.4; 132.2; 136.8; 156.3; 159.9; 166.7; 167.5; 168.8; 169.5.

MeO$_2$C-[G3](LinkerNHBoc)$_8$ 26. The pure product was obtained after flash chromatography on silica gel ($CHCl_3$/MeOH 9 : 1⟶8 : 2) as a colourless oil (40%). MS (ESI): 1256.8 $[M + 4H]^{4+}$; 1627.1 [M-Boc + 2Na + H]$^{3+}$; 1634.5 [M-Boc + 3Na]$^{3+}$; 1668.0 $[M + 3Na]^{2+}$; 1673.2 [M + 2Na + K]$^{3+}$. **^{1}H-NMR ($CDCl_3$, 200 MHz):** 1.41 (s, 72H, OCOC(C**H**$_3$)$_3$); 1.64–1.86 (m, 32H, C**H**$_2$**-m**, C**H**$_2$**-f**); 3.18 (m, 16H, C**H**$_2$**-n**); 3.35 (m, 16H, C**H**$_2$**-e**); 3.43–3.67 (m, 108H, C**H**$_2$**-g**, C**H**$_2$**-h**, C**H**$_2$**-i**, C**H**$_2$**-j**, C**H**$_2$**-k**, C**H**$_2$**-l**, C**H**$_2$**-b**, C**H**$_2$**-b′**); 3.68–3.8 (m, 16H, C**H**$_2$**-b″**); 3.85 (s, 3H, OC**H**$_3$); 3.93–4.18 (s, 60H, C**H**$_2$**c**, C**H**$_2$**-d**, C**H**$_2$**-a**, C**H**$_2$**-a′**, C**H**$_2$**-a″**); 5.1 (br s, 8H, N**H**Boc); 6.42 (s, 4H, C**H-4″**); 6.52 (s, 2H, C**H-4′**); 6.68 (s, 1H, C**H-4**); 6.92 (s, 8H, C**H-2″**, C**H-6″**); 6.98 (s, 4H, C**H-2′**, C**H-6′**); 7.16 (s, 2H, C**H-2**, C**H-6**); 7.56 (br s, 8H, N**H-IV**); 7.76 (br s, 8H, N**H-III**); 7.8–7.98 (bm, 6H, N**H-I**, N**H-II**).

^{13}C-NMR ($CDCl_3$, 50.3 MHz): 28.6; 29.2; 29.8; 37.4; 38.6; 39.9; 52.5; 66.6; 69.5; 69.7; 70.2; 70.3; 70.5; 71.1; 79.2; 102.4; 104.6; 106.4; 132.2; 136.6; 156.3; 159.7; 159.8; 166.7; 167.8; 168.9; 169.7.

Synthesis of 14

To a solution of **13**, (26 mg, 0.042 mmol, 1 mol eq.) in dry CH_2Cl_2 (0.25 ml), TFA (0.25 ml) was added. The reaction mixture was stirred at rt for 1 h then the solvent was evaporated and the residue was taken up with toluene and evaporated several times to completely remove the TFA. The crude was dried under vacuum for *ca.* 18 h and redissolved in dry CH_2Cl_2 (1.5 ml). Under N_2, Et_3N (17 µl, 0.126 mmol, 3 mol eq.) was added and after 10 min, HBTU (24 mg, 0.063 mmol, 1.5 mol eq.) and the pseudosaccharide **6b** (50 mg, 0.05 mmol, 1.2 mol eq.) were added. The reaction mixture was stirred at rt for 18 h, monitoring by TLC (CH_2Cl_2/MeOH 9 : 1). After reaction completion the solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2. The organic phase was washed with 0.5 M NaOH (2 times), 1 M $KHSO_4$ (1 time) and brine (1 time), dried with Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel ($CHCl_3$/MeOH 95 : 5) to yield **14** (76%). MS (ESI): 1493.8 $[M + H]^+$; 1516.0 $[M + Na]^+$. **^{1}H-NMR (acetone-D6, 400 MHz):** 1.41 (d, 3H, C**H**$_{3L}$, J_{CH3-HL} = 7 Hz); 1.49 (dd, 2H, CHD**H-5ax**, $J_{5ax-5eq} \cong J_{5ax-6}$ = 13 Hz); 1.72 (m, 2H, C**H**$_2$**-m**); 1.76 (m, 2H, C**H**$_2$**-f**); 1.80–1.98 (m, 8H, OCOC**H**$_3$, NHCOC**H**$_3$, CHD**H-2ax**, CHD**H-2eq**); 1.99–2.02 (m, 6H, 2 OCOC**H**$_3$); 2.06 (m, 3H, OCOC**H**$_3$); 2.10 (m, 3H, OCOC**H**$_3$); 2.11 (m, 3H, OCOC**H**$_3$); 2.18 (m, 1H, CHD**H-5eq**); 2.49 (m, 1H, CHD**H-1**); 3.02 (m, 1H, CHD**H-6**); 3.25 (m, 2H, C**H**$_2$**-n**); 3.32 (m, 2H, C**H**$_2$**-e**); 3.5 (m, 2H, C**H**$_2$**-l**); 3.52–3.63 (m, 14H, C**H**$_2$**-g**, C**H**$_2$**-h**, C**H**$_2$**-i**, C**H**$_2$**-j**, C**H**$_2$**-k**, CHD**H-3**, OC**H**$_3$); 3.69 (m, 2H, C**H**$_2$**-b**); 3.89–3.96 (m, 5H, OC**H**$_3$, GalNAc**H-5**, GalNAc**H-2**); 4.0 (m, 1H, GalNAc**H-6** or GalNAc**H-6′**); 4.04 (s, 2H, C**H**$_2$**-d**); 4.07–4.17 (s, 6H, GalNAc**H-6** or GalNAc**H-6′**, C**H**$_2$**c**, Gal**H-6**, Gal**H-6′**, Gal**H-5**); 4.19 (m, 2 H, C**H**$_2$**-a**); 4.27 (br s, 1H, CHD**H-4**); 4.37 (m, 1H, **H**$_L$); 4.40 (m, 1H, GalNAc**H-3**); 4.90 (d, 1H, Gal**H-1**, J_{1-2} = 10.8 Hz); 5.04–5.1 (m, 3H, Gal**H-2**, Gal**H-3**, GalNAc**H-1**); 5.22 (m, 2H, OC**H**$_2$Ph); 5.36 (br s, 1H, Gal**H-4**); 5.42 (d, 1H, GalNAc**H-4**); 7.0 (br s, 1H, N**H-III**); 7.2–7.28 (m, 2H, NHCOCH$_3$, C**H-4**); 7.35–7.48 (m, 6H, C**H-5** aromatic protons); 7.54–7.6 (m, 2H, N**H-II**, C**H-2**), 7.03 (d, 1H, C**H-6**); 7.88 (br s, 1H, N**H-I**).^{13}C-NMR-HETCOR (acetone-D6, 400 MHz): 18.8; 20.0; 20.1; 23.1; 29.9; 30.0; 33.9; 37.0 (2 carbons); 38.8; 39.2; 45.3; 51.2; 52.0; 53.5; 61.4, 63.0; 66.9; 67.3; 67.8; 69.0; 69.1; 69.9; 70.7; 71.0; 71.5 (3 carbons); 72.0; 72.3; 73.0; 76.2; 79.0; 101.9; 102.0; 106.0; 120.4; 129.0; 130.7.

The same procedure as for the monovalent ligand was adopted to functionalise the divalent, tetravalent and octavalent dendrimers with the sugar. Reagents were used in proportion to the number of free amine groups. The solvents used for the purification and the characterization of each product are reported.

Divalent compound 19

The pure product was obtained after flash chromatography on silica gel ($CHCl_3$/MeOH 95 : 5⟶9 : 1) as a colourless oil (66%). MS (ESI): 951.2 $[M + 3H]^{3+}$; 1117.2 [M-GalGalNAc + 2H]$^{2+}$; 1226.2 [M-Gal + 2H]$^{2+}$; 1426.6 $[M + 2H]^{2+}$; 1437.9 [M + Na + H]$^{2+}$. **^{1}H-NMR (acetone-D6, 400 MHz):** 1.41 (d, 6H, C**H**$_{3L}$, J_{CH3-HL} = 6.7 Hz); 1.5 (dd, 2H, CHD**H-5ax**, $J_{5ax-5eq} \cong J_{5ax-6}$ = 13

Hz); 1.66–1.80 (m, 8H, C**H**$_2$-**m**, C**H**$_2$-**f**); 1.86–2.02 (m, 28H, 3 OCOC**H**$_3$, NHCOC**H**$_3$, CHD**H**-**2ax**, CHD**H**-**2eq**); 2.02–2.15 (m, 18H, 3 OCOC**H**$_3$); 2.18 (m, 2H, CHD**H**-**5eq**); 2.50 (m, 2H, CHD**H**-**1**); 3.02 (m, 2H, CHD**H**-**6**); 3.25 (m, 4H, C**H**$_2$-**n**); 3.32 (m, 4H, C**H**$_2$-**e**); 3.47–3.63 (m, 32H, C**H**$_2$-**g**, C**H**$_2$-**h**, C**H**$_2$-**i**, C**H**$_2$-**j**, C**H**$_2$-**k**, C**H**$_2$-**l**, CHD**H**-**3**, OC**H**$_3$); 3.68 (m, 4H, C**H**$_2$-**b**); 3.85–3.92 (m, 7H, OC**H**$_3$, GalNAc**H**-**5**, GalNAc**H**-**2**); 3.92–4.18 (s, 18H, GalNAc**H**-**6**, GalNAc**H**-**6′**, C**H**$_2$-**d**, C**H**$_2$**c**, Gal**H**-**6**, Gal**H**-**6′**, Gal**H**-**5**); 4.18 (m, 4 H, C**H**$_2$-**a**); 4.26 (br s, 2H, CHD**H**-**4**); 4.32–4.41 (m, 4H, **H**$_L$, GalNAc**H**-**3**); 4.89 (d, 2H, Gal**H**-**1**, J_{1-2} = 6 Hz); 5.02–5.1 (m, 6H, Gal**H**-**2**, Gal**H**-**3**, GalNAc**H**-**1**); 5.2 (m, 4H, OC**H**$_2$Ph); 5.36 (br s, 2H, Gal**H**-**4**); 5.42 (d, 2H, GalNAc**H**-**4**); 6.73 (s, 1H, C**H**-**4**); 7.06 (br s, 2H, N**H**-**III**); 7.18 (d, 2H, C**H**-**2**, C**H**-**6**, $J_{2'-4'} \cong J_{6'-4'}$ = 2.2 Hz); 7.26 (d, 2H, N**H**COCH$_3$, J_{NH-H2} = 7.8 Hz); 7.42 (s, 10H, aromatic protons); 7.65 (br s, 2H, N**H**-**II**); 7.92 (br s, 2H, N**H**-**I**). ^{13}C-NMR-HETCOR (acetone-D6, 400 MHz): 18.5; 20.1 (6 carbons); 23.1; 29.6; 29.8; 29.9; 33.9; 36.8 (2 carbons); 36.9; 38.6; 45.4; 51.5; 51.8; 53.4; 61.1; 62.8; 66.6; 67.3; 67.9; 69.2; 69.8; 70.8; 71.4; 71.5 (2 carbons); 72.0 (2 carbons); 72.3; 72.7; 76.4; 78.8; 101.7; 102.0; 106.8; 108.7; 129.6.

Tetravalent compound 23

The pure product was obtained after flash chromatography on silica gel (CHCl$_3$/MeOH 95 : 5⟶8 : 2) as a colourless oil (55%). MS (ESI): 1474.1 $[M + 4H]^{4+}$; 1495.1 $[M + 4Na]^{4+}$. **^{1}H-NMR (acetone-D6, 400 MHz):** 1.4 (d, 12H, C**H**$_{3L}$, J_{CH3-HL} = 7 Hz); 1.5 (dd, 4H, CHD**H**-**5ax**, $J_{5ax-5eq} \cong J_{5ax-6}$ = 13 Hz); 1.70 (m, 8H, C**H**$_2$-**m**); 1.76 (m, 8H, C**H**$_2$-**f**); 1.90–2.02 (m, 56H, 3 OCOC**H**$_3$, NHCOC**H**$_3$, CHD**H**-**2ax**, CHD**H**-**2eq**); 2.06 (s, 12H, OCOC**H**$_3$); 2.11 (s, 12H, OCOC**H**$_3$); 2.13 (s, 12H, OCOC**H**$_3$); 2.19 (m, 4H, CHD**H**-**5eq**); 2.5 (ddd, 4H, CHD**H**-**1**, $J_{1-6} \cong J_{1-2ax} \cong$ 10.8 Hz, J_{1-2eq} = 3.8 Hz); 3.03 (ddd, 4H, CHD**H**-**6**, $J_{6-1} \cong J_{6-5ax} \cong$ 11 Hz); 3.24 (m, 8H, C**H**$_2$-**n**); 3.33 (m, 8H, C**H**$_2$-**e**); 3.49 (m, 8H, C**H**$_2$-**l**); 3.51–3.63 (m, 56H, C**H**$_2$-**g**, C**H**$_2$-**h**, C**H**$_2$-**i**, C**H**$_2$-**j**, C**H**$_2$-**k**, CHD**H**-**3**, OC**H**$_3$); 3.66 (m, 8H, C**H**$_2$-**b′**); 3.83 (m, 4H, C**H**$_2$-**b**); 3.88 (s, 3H, OC**H**$_3$); 3.89–4.03 (m, 12H, GalNAc**H**-**5**, GalNAc**H**-**2**, GalNAc**H**-**6** or GalNAc**H**-**6′**); 4.05 (s, 8H, C**H**$_2$-**d**); 4.07–4.18 (s, 32H, GalNAc**H**-**6** or GalNAc**H**-**6′**, C**H**$_2$**c**, Gal**H**-**6**, Gal**H**-**6′**, Gal**H**-**5**, C**H**$_2$-**a′**); 4.23–4.30 (m, 8H, CHD**H**-**4**, C**H**$_2$-**a**); 4.33–4.40 (m, 8H, **H**$_L$, GalNAc**H**-**3**); 4.90 (d, 4H, Gal**H**-**1**, J_{1-2} = 11.5 Hz); 5.03–5.1 (m, 12H, Gal**H**-**2**, Gal**H**-**3**, GalNAc**H**-**1**); 5.22 (m, 8H, OC**H**$_2$Ph); 5.36 (br s, 4H, Gal**H**-**4**); 5.42 (d, 4H, GalNAc**H**-**4**, J_{4-3} = 3.4 Hz); 6.7 (s, 2H, C**H**-**4′**); 6.88 (s, 1H, C**H**-**4**), 7.08 (br s, 4H, N**H**-**IV**); 7.1 (d, 4H, C**H**-**2′**, C**H**-**6′**); 7.16 (d, 2H, C**H**-**2**, C**H**-**6**); 7.25 (d, 4H, N**H**COCH$_3$, J_{NH-H2} = 7.8 Hz); 7.42 (s, 20H, aromatic protons); 7.68 (br s, 4H, N**H**-**III**); 7.93 (br s, 4H, N**H**-**II**); 8.02 (br s, 2H, N**H**-**I**). ^{13}C-NMR-HETCOR (acetone-D6, 400 MHz): 18.3; 19.9; 20.1; 23.0; 29.7; 29.9; 33.7; 36.8 (2 carbons); 38.4; 39.2; 39.7; 45.1; 51.3; 52.1; 53.1; 61.1; 62.9; 66.6; 67.0; 67.1; 67.6; 69.1 (2 carbons); 69.8; 70.4; 70.8; 71.0; 71.1; 71.6; 72.2; 72.6; 76.2; 78.8; 101.6; 101.7; 104.9; 106.8; 107.0; 108.9; 129.1.

Octavalent compound 27

The pure product was obtained after flash chromatography on silica gel (CHCl$_3$/MeOH 9 : 1⟶8 : 2) as a colourless oil (35%). MS (ESI): 1433.4 $[M + 7Na]^{7+}$; 2018.7 $[M + 6Na]^{6+}$; 2417.6 $[M + 5Na]^{5+}$; 3015.5 $[M + 4Na]^{4+}$. **^{1}H-NMR (acetone-D6, 400 MHz):** 1.41 (d, 24H, C**H**$_{3L}$, J_{CH3-HL} = 7 Hz); 1.5 (dd, 8H, CHD**H**-**5ax**, $J_{5ax-5eq} \cong J_{5ax-6}$ = 11.4 Hz); 1.70 (m, 16H, C**H**$_2$-**m**); 1.76 (m, 16H, C**H**$_2$-**f**); 1.90–2.02 (m, 112H, 3 OCOC**H**$_3$, NHCOC**H**$_3$, CHD**H**-**2ax**, CHD**H**-**2eq**); 2.06 (s, 24H, OCOC**H**$_3$); 2.11 (s, 24H, OCOC**H**$_3$); 2.13 (s, 24H, OCOC**H**$_3$); 2.19 (m, 8H, CHD**H**-**5eq**); 2.51 (ddd, 8H, CHD**H**-**1**, $J_{1-6} \cong J_{1-2ax} \cong$ 10.8 Hz, J_{1-2eq} = 3.8 Hz); 3.03 (ddd, 8H, CHD**H**-**6**, $J_{6-1} \cong J_{6-5ax} \cong$ 11.4 Hz); 3.24 (m, 16H, C**H**$_2$-**n**); 3.33 (m, 16H, C**H**$_2$-**e**); 3.45–3.63 (m, 128H, C**H**$_2$-**g**, C**H**$_2$-**h**, C**H**$_2$-**i**, C**H**$_2$-**j**, C**H**$_2$-**k**, C**H**$_2$-**l**, CHD**H**-**3**, OC**H**$_3$); 3.63–3.68 (m, 16H, C**H**$_2$-**b″**); 3.75–3.83 (m, 12H, C**H**$_2$-**b′**, C**H**$_2$-**b**); 3.86 (s, 3H, OC**H**$_3$); 3.89–4.04 (m, 24H, GalNAc**H**-**5**, GalNAc**H**-**2**, GalNAc**H**-**6** or GalNAc**H**-**6′**); 4.06 (s, 16H, C**H**$_2$-**d**); 4.07–4.18 (m, 64H, GalNAc**H**-**6** or GalNAc**H**-**6′**, C**H**$_2$**c**, Gal**H**-**6**, Gal**H**-**6′**, Gal**H**-**5**, C**H**$_2$-**a″**); 4.20–4.28 (m, 20H, CHD**H**-**4**, C**H**$_2$-**a′**, C**H**$_2$-**a**); 4.33–4.40 (m, 16H, **H**$_L$, GalNAc**H**-**3**); 4.90 (d, 8H, Gal**H**-**1**, J_{1-2} = 11.5 Hz); 5.03–5.08 (m, 24H, Gal**H**-**2**, Gal**H**-**3**, GalNAc**H**-**1**); 5.21 (m, 16H, OC**H**$_2$Ph); 5.35 (br s, 8H, Gal**H**-**4**); 5.42 (d, 8H, GalNAc**H**-**4**, J_{4-3} = 3.4 Hz); 6.65 (s, 4H, C**H**-**4″**); 6.70 (s, 2H, C**H**-**4′**); 6.86 (s, 1H, C**H**-**4**), 7.08–7.18 (m, 22H, N**H**-**V**, C**H**-**2″**, C**H**-**6″**, C**H**-**2′**, C**H**-**6′**, C**H**-**2**, C**H**-**6**); 7.25 (d, 8H, N**H**COCH$_3$, J_{NH-H2} = 7.8 Hz); 7.42 (s, 40H, aromatic protons); 7.78 (br s, 8H, N**H**-**IV**); 8.02 (br s, 8H, N**H**-**III**); 8.35 (br s, 6H, N**H**-**II**, N**H**-**I**). ^{13}C-NMR (acetone-D6, 75.0 MHz): 19.2; 20.7; 20.9; 23.8; 29.5; 29.8; 30.03; 34.3; 37.2; 39.1; 39.7; 45.7; 51.5; 51.8; 53.6; 61.7, 63.5; 67.1; 67.4;68.0; 69.4; 69.6; 69.7; 70.1; 70.8; 71.1; 71.2; 71.7; 72.1; 72.7; 73.0; 76.9; 79.0; 101.9 (2 anomeric carbons); 107.1; 108.2; 128.9; 129.1; 129.5; 137.6; 160.7; 167.7; 169.8; 170.4; 170.7; 173.7; 174.0; 176.0.

Synthesis of 16

To a solution of **14** (42 mg, 0.028 mmol, 1 mol eq.) in MeOH (1.5 ml), a catalytic amount of Pd/C 10% was added. The mixture was stirred under H_2 atmosphere at room temperature for *ca.* 1 h. After reaction completion, the mixture was filtered through a Celite pad, washing several times with MeOH and the collected organic phase was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel (CHCl$_3$/MeOH/H$_2$O 60 : 35 : 5) and the pure product (35 mg, 0.025 mmol, 1 mol eq.) was dissolved in dry MeOH (2 ml). Under N_2, a solution of 1 M MeONa in MeOH (45 µl, 0.045 mmol, 1.8 mol eq.) was added and the reaction was stirred at room temperature for *ca.* 45 min monitoring by TLC (CHCl$_3$/MeOH/H$_2$O 60 : 35 : 5 or EtOAc/AcOH/MeOH/H$_2$O 4 : 3 : 3 : 2). Just after 5 min a white solid precipitated. After completion, the reaction was quenched adding Amberlite IR 120 (H^+ form) to pH 5–6. The suspension was stirred for some minutes till the white solid completely dissolved, finally the resin was filtered and washed several times with MeOH. The organic phase was evaporated under reduced pressure. The final product was obtained as a white solid and results contaminated by 33% of imide by-product (79% over two steps). MS (ESI): 754.7 $[M\text{-}GalGalNAc\text{-}32 + H]^+$; 786.8 $[M\text{-}GalGalNAc + H]^+$; 1119.8 $[M\text{-}32 + H]^+$; 1151.7 $[M + H]^+$; 1174.6 $[M + Na]^+$. **^{1}H-NMR (D$_2$O, 400 MHz):** 1.32 (d, 3H, C**H**$_{3L}$, J_{CH3-HL} = 7 Hz); 1.52 (dd, 1H, CHD**H**-**5ax**, J_{5ax-6} = 13 Hz); 1.62–1.88 (m, 5H, C**H**$_2$-**m**, C**H**$_2$-**f**, CHD**H**-**2ax**); 1.9 (m, 1H, CHD**H**-**2eq**); 2.0 (s, 3H, NHCOC**H**$_3$); 2.26 (m, 1H, CHD**H**-**5eq**); 2.5 (dd, 1H, CHD**H**-**1**, $J_{1-6} \cong J_{1-2ax}$ = 12 Hz); 2.98 (dd, 1H, CHD**H**-**6**, $J_{6-1} \cong J_{6-5ax}$ = 13 Hz); 3.18 (m, 2H, C**H**$_2$-**n**); 3.24 (m, 2H, C**H**$_2$-**e**); 3.34–3.54 (m, 6H, C**H**$_2$-**g**, C**H**$_2$-**l**, CHD**H**-**3**, Gal**H**-**2**); 3.54–3.68 (m, 16H, C**H**$_2$-**h**, C**H**$_2$-**i**, C**H**$_2$-**j**, C**H**$_2$-**k**, OC**H**$_3$, Gal**H**-**3**, Gal**H**-**5**, GalNAc**H**-**5**, C**H**$_2$-**b**); 3.68–3.80 (m, 4H, Gal**H**-**6**, Gal**H**-**6′**, GalNAc**H**-**6**, GalNAc**H**-**6′**); 3.82 (m, 1H, GalNAc**H**-**3**); 3.85 (d, 1H, Gal**H**-**4**, J_{4-3} = 3.3 Hz); 3.87 (m, 3H, OC**H**$_3$); 3.92–4.01 (m, 3H, GalNAc**H**-**2**, C**H**$_2$-**d**); 4.40 (s, 2H, C**H**$_2$**c**); 4.13 (d, 1H, GalNAc**H**-**4**, J_{4-3} = 3.1 Hz); 4.2 (m, 2H, C**H**$_2$-**a**); 4.25–4.35 (m, 2H, **H**$_L$, CHD**H**-**4**); 4.42 (d, 1H, Gal**H**-**1**, J_{1-2} = 7.6 Hz); 4.73 (m, 1H, GalNAc**H**-**1**); 7.21 (d, 1H, C**H**-**4**, J_{4-5} = 8.2 Hz); 7.42 (dd, 1H, C**H**-**5**, $J_{5-4} \cong J_{5-6}$ = 8.2 Hz); 7.53 (br s, 1H, C**H**-**2**); 7.60 (d, 1H, C**H**-**6**, J_{6-5} = 8.2 Hz). ^{13}C-NMR-HETCOR (D$_2$O, 400 MHz): 18.9; 23.0; 28.9; 29.7; 32.9; 36.5; 36.8 (2 carbons); 38.9; 39.1; 45.2; 52.1; 53.0; 53.1; 61.7; 67.7; 68.8; 69.1; 70.0; 70.4; 71.2; 72.6; 73.0; 73.1; 75.5; 78.1; 80.3; 102.0; 105.7.

The same procedure as for the monovalent ligand **14** was adopted to deprotect the divalent, tetravalent and octavalent ligands. Reagents were used in proportion to the number of sugar moieties to be deprotected. The yield and the characterization of each product are reported.

Divalent compound 20

The pure product was obtained as a white solid and results contaminated by 20% of imide by-product (83% over two steps). MS (ESI): 1068.5 $[M-32 + 2H]^{2+}$; 1083.9 $[M + 2H]^{2+}$. **^{1}H-NMR (D_2O, 400 MHz):** 1.39 (d, 6H, **CH_{3L}**, $J_{CH3\text{-}HL}$ = 7 Hz); 1.54 (dd, 2H, CHD**H-5ax**, $J_{5ax\text{-}5eq} \cong J_{5ax\text{-}6}$ = 11.7 Hz); 1.66–1.83 (m, 10H, **CH_2-m**, **CH_2-f**, CHD**H-2ax**); 1.92 (m, 2H, CHD**H-2eq**); 2.0 (s, 6H, NHCO**CH_3**); 2.29 (m, 2H, CHD**H-5eq**); 2.55 (dd, 2H, CHD**H-1**, $J_{1\text{–}6} \cong J_{1\text{–}2ax}$ = 12 Hz); 3.0 (dd, 2H, CHD**H-6**, $J_{6\text{–}1} \cong J_{6\text{–}5ax}$ = 11.7 Hz); 3.18 (m, 4H, **CH_2-n**); 3.26 (m, 4H, **CH_2-e**); 3.34–3.54 (m, 12H, **CH_2-g**, **CH_2-l**, CHD**H-3**, Gal**H-2**); 3.54–3.71 (m, 32H, **CH_2-h**, **CH_2-i**, **CH_2-j**, **CH_2-k**, O**CH_3**, Gal**H-3**, Gal**H-5**, GalNAc**H-5**, **CH_2-b**); 3.71–3.85 (m, 8H, Gal**H-6**, Gal**H-6′**, GalNAc**H-6**, GalNAc**H-6′**); 3.85–3.93 (m, 7H, GalNAc**H-3**, Gal**H-4**, O**CH_3**); 4.01 (m, 2H, GalNAc**H-2**); 4.03 (s, 4H, **CH_2-d**); 4.10 (s, 4H, **CH_2_c**); 4.13–4.22 (m, 6H, **CH_2-a**, GalNAc**H-4**); 4.22–4.36 (m, 4H, **H_L**, CHD**H-4**); 4.44 (d, 2H, Gal**H-1**, $J_{1\text{–}2}$ = 7.8 Hz); 4.79 (d, 2H, GalNAc**H-1**, $J_{1\text{–}2}$ = 9.4 Hz); 6.80 (s, 1H, C**H-4**); 7.18 (s, 2H, C**H-2**, C**H-6**).

^{13}C-NMR-HETCOR (D_2O, 400 MHz): 18.6; 22.9; 28.7; 29.4; 32.8; 36.8 (2 carbons); 39.0; 39.3; 45.1; 52.1; 53.0; 53.2; 61.3; 67.8; 68.9; 69.0; 70.0; 70.4; 72.9; 75.3; 78.0; 80.3; 101.8; 105.2; 107.9; 109.1.

Tetravalent ligand 24

The pure product was obtained as a white solid and contained 30% of the imide by-product (79% over two steps). MS (ESI): 1115.7 $[M-64 + 4H]^{4+}$; 1132.0 $[M + 4H]^{4+}$; 1508.8 $[M + 3H]^{3+}$; 1536.7 $[M + 2Na + K]^{3+}$. **^{1}H-NMR (D_2O, 400 MHz):** 1.40 (d, 12H, **CH_{3L}**, $J_{CH3\text{-}HL}$ = 7 Hz); 1.54 (dd, 4H, CHD**H-5ax**, $J_{5ax\text{-}5eq} \cong J_{5ax\text{-}6}$ = 11.7 Hz); 1.69–1.86 (m, 20H, **CH_2-m**, **CH_2-f**, CHD**H-2ax**); 1.95 (m, 4H, CHD**H-2eq**); 2.03 (s, 12H, NHCO**CH_3**); 2.32 (m, 4H, CHD**H-5eq**); 2.57 (dd, 4H, CHD**H-1**, $J_{1\text{–}6} \cong J_{1\text{–}2ax}$ = 12 Hz); 3.02 (dd, 4H, CHD**H-6**, $J_{6\text{–}1} \cong J_{6\text{–}5ax}$ = 11.7 Hz); 3.12–3.30 (m, 16H, **CH_2-n**, **CH_2-e**); 3.43–3.54 (m, 24H, **CH_2-g**, **CH_2-l**, CHD**H-3**, Gal**H-2**); 3.54–3.72 (m, 64H, **CH_2-h**, **CH_2-i**, **CH_2-j**, **CH_2-k**, O**CH_3**, Gal**H-3**, Gal**H-5**, GalNAc**H-5**, **CH_2-b′**); 3.72–3.85 (m, 20H, Gal**H-6**, Gal**H-6′**, GalNAc**H-6**, GalNAc**H-6′**, **CH_2-b**); 3.87 (s, 3H, O**CH_3**); 3.89–3.98 (m, 8H, GalNAc**H-3**, Gal**H-4**); 3.98–4.09 (m, 12H, GalNAc**H-2**, **CH_2-d**); 4.09–4.18 (m, 16H, **CH_2c**, **CH_2-a′**); 4.18–4.30 (m, 12H, GalNAc**H-4**, **CH_2-a**, **H_L**); 4.33 (br s, 4H, CHD**H-4**); 4.48 (d, 4H, Gal**H-1**, $J_{1\text{–}2}$ = 7.8 Hz); 4.82 (d, 4H, GalNAc**H-1**, $J_{1\text{–}2}$ = 9.4 Hz); 6.62 (s, 2H, C**H-4′**); 6.70 (s, 1H, C**H-4**); 7.78 (s, 4H, C**H-2′**, C**H-6′**); 7.05 (s, 2H, C**H-2**, C**H-6**). ^{13}C-NMR-HETCOR (D_2O, 400 MHz): 18.9; 23.0; 29.0; 29.8; 33.0; 37.0; 39.1; 39.5; 40.5; 45.6; 52.5; 53.1; 53.4; 61.9, 67.6; 67.8; 69.0; 69.2; 69.6; 70.8; 71.0; 71.9; 73.1; 73.4; 74.9; 76.0; 78.2; 80.9; 102.3; 105.9; 106.9; 107.0; 108.5; 110.0.

Octavalent ligand 28

The pure product was obtained as a white solid and contained 20% of imide by-product (86% over two steps). MS (ESI): 1264.6 [M-32-GalGalNac + 7H]$^{7+}$; 1270.9 [M-GalGalNac + 7H]$^{7+}$; 1475.7 [M-32-GalGalNac + 6H]$^{6+}$; 1481.4 [M-GalGalNac + 6H]$^{6+}$; 1565.9 [M + 6Na]$^{6+}$; 1579.6 [M + 6K]$^{6+}$; 1868.3 [M-32 + 5Na]$^{5+}$; 1874.8 [M + 5Na]$^{5+}$. **^{1}H-NMR (D_2O, 400 MHz):** 1.32 (d, 24H, **CH_{3L}**, $J_{CH3\text{-}HL}$ = 7 Hz); 1.52 (dd, 8H, CHD**H-5ax**, $J_{5ax\text{-}5eq} \cong J_{5ax\text{-}6}$ = 12.4 Hz); 1.59–1.68 (m, 40H, **CH_2-m**, **CH_2-f**, CHD**H-2ax**); 1.93 (m, 8H, CHD**H-2eq**); 2.0 (s, 24H, NHCO**CH_3**); 2.28 (m, 8H, CHD**H-5eq**); 2.53 (dd, 8H, CHD**H-1**, $J_{1\text{–}6} \cong J_{1\text{–}2ax}$ = 13.6 Hz); 3.02 (dd, 8H, CHD**H-6**, $J_{6\text{–}1} \cong J_{6\text{–}5ax}$ = 12.4 Hz); 3.08–3.37 (m, 32H, **CH_2-n**, **CH_2-e**); 3.35–3.46 (m, 48H, **CH_2-g**, **CH_2-l**, CHD**H-3**, Gal**H-2**); 3.46–3.62 (m, 128H, **CH_2-h**, **CH_2-i**, **CH_2-j**, **CH_2-k**, O**CH_3**, Gal**H-3**, Gal**H-5**, GalNAc**H-5**, **CH_2-b″**); 3.62–3.77 (m, 44H, Gal**H-6**, Gal**H-6′**, GalNAc**H-6**, GalNAc**H-6′**, **CH_2-b′**, **CH_2-b**); 3.77–3.88 (m, 19H, O**CH_3**, GalNAc**H-3**, Gal**H-4**); 3.88–4.07 (m, 68H, GalNAc**H-2**, **CH_2-d**, **CH_2c**, **CH_2-a″**, **CH_2-a′**, **CH_2-a**); 4.12 (m, 8H, GalNAc**H-4**); 4.17–4.30 (m, 16H, **H_L**, CHD**H-4**); 4.49 (d, 8H, Gal**H-1**, $J_{1\text{–}2}$ = 7.8 Hz); 4.80 (d, 8H, GalNAc**H-1**, $J_{1\text{–}2}$ = 9.4 Hz); 6.40–6.55 (m, 7H, C**H-4″**, C**H-4′**, C**H-4**); 6.67–6.82 (m, 14H, C**H-2″**, C**H-6″**, C**H-2′**, C**H-6′**, C**H-2**, C**H-6**). ^{13}C-NMR-HETCOR (D_2O, 400 MHz): 19.0; 23.0; 29.0; 29.8; 33.0; 37.0; 39.1; 39.5; 40.5; 45.6; 52.5; 53.1; 53.4; 61.9, 67.6; 67.8; 69.0; 69.2; 69.6; 70.8; 71.0; 71.9; 73.1; 73.4; 74.9; 76.0; 78.2; 80.9; 102.3; 105.9; 106.0; 107.0; 108.5; 110.0.

Synthesis of 32

To a solution of MeO_2C-[G1](LinkerNHBoc)$_2$ **18**, (62.5 mg, 0.057 mmol, 1 mol eq.) in dry CH_2Cl_2 (0.1 ml), TFA (0.1 ml) was added. The reaction mixture was stirred for 1 h, then the solvent was evaporated and the residue taken up with toluene, which was evaporated several times to completely remove the TFA. Then, the crude was dissolved in CH_2Cl_2 and Amberlyst A-21 (-N$(CH_3)_2$ form) was added to pH 8. The suspension was stirred for 45 min. Finally the resin was filtered and washed several times with MeOH. The organic phase was evaporated under reduced pressure. The crude was dissolved in dry MeOH (0.5 ml) and, under N_2, D-(+)-ribonic-γ-lactone **31** (34 mg, 0.228 mmol, 4 mol eq.) was added. The reaction mixture was stirred at 45 °C for 18 h, monitoring by TLC ($CHCl_3$/MeOH 9 : 1 + 1% H_2O). After reaction completion the solvent was evaporated and the crude redissolved in Ac_2O (0.4 ml). Under N_2, at 0 °C, pyridine (0.45 ml) was added. The reaction mixture was stirred at room temperature for 18 h monitoring by TLC ($CHCl_3$/MeOH 8 : 2 + 1% H_2O). After reaction completion the solvent was evaporated. The residue was dissolved in CH_2Cl_2 and washed with brine (2 times). The organic phase was dried with Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel ($CHCl_3$/MeOH 8 : 2 + 1% H_2O) yielding 26.4 mg of fully acetylated compound (30%). This compound (23 mg, 1.5 μmol, 1 mol eq.) was dissolved in dry MeOH (1 ml), under N_2, and a solution of 1 M MeONa in MeOH (0.05 ml, 0.05 mmol, 3.5 mol eq.) was added. The reaction was stirred at room temperature for 1 h monitoring by TLC ($CHCl_3$/MeOH/H_2O 60 : 35 : 5). After completion, the reaction was quenched adding Amberlite IR 120 (H^+ form) to pH 5–6. Finally the resin was filtered, washing several times with MeOH, and the organic phase was evaporated under reduced pressure. The final product **32** was obtained as a white solid (60% yield). **^{1}H-NMR (D_2O, 400 MHz):** 1.67–1.78 (m, 8H, **CH_2-m**, **CH_2-f**); 3.2–3.27 (m, 8H, **CH_2-n**, **CH_2-e**); 3.45–3.52 (m, 8H, **CH_2-g**, **CH_2-l**); 3.52–3.61 (m, 18H, **CH_2-h**, **CH_2-i**, **CH_2-j**, **CH_2-k**, Rib-**H4′**); 3.61–3.65 (m, 4H, **CH_2-b**); 3.72–3.79 (m, 4H, Rib**H-3**, Rib**H-4**); 3.84–3.90 (m, 5H, Rib**H-2**, O**CH_3**); 4.0 (s, 4H, **CH_2-d**); 4.50 (s, 4H, **CH_2_c**); 4.16 (m, 4H, **CH_2-a**); 4.27 (d, 2H, Rib**H-1**, $J_{1\text{–}2}$ = 3.4 Hz), 6.76 (s, 1H, C**H-4**); 7.15 (s, 2H, C**H-2**, C**H-6**). ^{13}C-NMR-HETCOR (D_2O, 400 MHz): 28.6; 36.9; 39.2; 53.0; 63.9 (2 carbons); 67.8; 69.3; 70.6; 71.0; 71.9; 73.8; 74.0; 108.2; 109.5.

Surface plasmon resonance

The SPR experiments were performed on a double channel surface plasmon Autolab ESPRIT instrument (Ecochemie, Utrecht, The Netherlands) at 25°C in HBS buffer (10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl and 0.005% Tween-20, pH 7.4). The cuvet-based instrument was mounted with a CMD6 biosensor chip (XanTec bioanalytics GmbH, Münster, Germany) containing a carboxymethylated dextran layer with a molecular weight of 6000. Onto this surface the glycoprotein ASF was immobilized according to a standard amine coupling procedure. Prior to activation of the carboxy functional group the sensor was presoaked in buffer for a few hours. Activation was achieved in 7 min by a mixture of *N*-ethyl-*N′*-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, 0.2 M) and *N*-hydroxysuccinimide (NHS, 0.05 M). The protein, ASF at

a concentration of 400 μg ml^{-1} in 10 mM sodium acetate buffer, pH 4, was coupled to the activated surface for 15 min, after which the remaining NHS esters were blocked by the addition of ethanolamine (1 M, pH 8.5) for 10 min. The double channel system consisted of a sample and reference cell (volume 35 μl) that were treated identically, however, the reference biosensor surface did not contain immobilized protein. The collected sample signals were corrected for the reference values and analyzed further using the SPR instrument software. After each binding experiment the surface was regenerated with a mixture of 10 mM NaOH and 0.2% SDS for 1 min. Prior to the start of a competition experiment a mixture of CTB was made with a variable concentration of the synthetic inhibitors, and the mixture was subsequently left to stand at room temperature for a specific period of time. The equilibrated sample was then introduced to the sensor-immobilized protein. Binding constants (K_D or K_{chip}) were obtained from equilibrium signals that were fitted with a non-linear binding isotherm.[19] Binding in solution was measured using competition experiments and was expressed as inhibitory concentration (IC_{50}). The concentration of CTB used in inhibition experiments was 3 μM.

ELISA

50 μl of a 0.002 μg μl^{-1} solution of GM1 in EtOH (0.1 μg of GM1) was transferred to the wells and allowed to air dry overnight. Unattached ganglioside was removed by washing the wells twice with PBS (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4). Additional binding sites on the plate surface were blocked by incubating the wells with 150 μl of a 2% (w/v) bovine serum albumin (BSA)-PBS solution for 2.5 h at rt and then washing them with 200 μl of PBS solution three times.

The test samples were dissolved in 50 μl of a 0.025 μg ml^{-1} solution of CT-HRP conjugate in 0.1% BSA-PBS and incubated for 4 h at r. t.. Toxin and inhibitor solutions were added in 50 μl volumes per well and incubated for 2 h at room temperature. Unbound toxin was removed by washing four times with 200 μl of PBS. Toxin bound to GM1 was then revealed by the following sequence: (I) incubation with 50 μl of freshly made *o*-phenylenediamine (OPD) solution (OPD solution: 21.6 mg *o*-phenylenediamine in 50 ml of 0.05 M citrate buffer at pH 5 and 40 μl of 30% hydrogen peroxide) for 15 min at room temperature; (II) quenching with 150 μl of 2 M H_2SO_4 and (III) recording the data on a multiwell plate spectrophotometer. All experiments were carried out in triplicate.

Acknowledgements

We thank Dr M. J. E. Fischer (Utrecht University) for help with the SPR measurements, Dr S. André and Professor H.-J. Gabius (Ludwig-Maximilians-Universität München) for providing the ASF and Professor S. Sonnino (Universita′ di Milano) for providing the GM1-OS. This research has been supported by Marie Curie Training Site Fellowships (to I. V., under contract HPMT-CT-2001–00293 and to D. A. under contract QLK2-CT-2000–60047) of the European Union. The Royal Netherlands Academy of Arts and Sciences (KNAW) is gratefully acknowledged for a fellowship to R. J. P.

Notes and references

1 E. Fan, E. A. Merritt, C. L. M. J. Verlinde and W. G. J. Hol, *Curr. Opin. Struct. Biol.*, 2000, **10**, 680–686.
2 W. B. Turnbull, B. L. Precious and S. W. Homans, *J. Am. Chem. Soc.*, 2004, **126**, 1047–1054.
3 (*a*) A. Bernardi, D. Arosio and S. Sonnino, *Neurochem. Res.*, 2002, **27**, 539–545 and references therein; (*b*) A. Bernardi, D. Arosio, L. Manzoni, D. Monti, H. Posteri, D. Potenza, S. Mari and J. Jimenez-Barbero, *Org. Biomol. Chem.*, 2003, **1**, 785–792; (*c*) D. Arosio, S. Baretti, S. Cattaldo, D. Potenza and A. Bernardi, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3831–3834; (*d*) A. Bernardi, A. Checchia, P. Brocca, S. Sonnino and F. Zuccotto, *J. Am. Chem. Soc.*, 1999, **121**, 2032–2036; (*e*) A. Bernardi, L. Carrettoni, A. Grosso Ciponte, D. Monti and S. Sonnino, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2197–2200.
4 A. Bernardi, D. Potenza, A. M. Capelli, A. García-Herrero, F. J. Cañada and J. Jiménez-Barbero, *Chem. Eur. J.*, 2002, **8**, 4597–4612.
5 (*a*) M. Mammen, S.-K. Chio and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794; (*b*) L. L. Kiessling, L. E. Strong and J. E. Gestwicki, *Ann. Rep. Med. Chem.*, 2000, **35**, 321–330; (*c*) J. J. Lundquist and E. J. Toone, *Chem. Rev.*, 2002, **102**, 555–578; (*d*) T. K. Lindhorst, *Top. Curr. Chem.*, 2002, **218**, 201–235; (*e*) R. Roy, *Curr. Opin. Struct. Biol.*, 1996, **6**, 692–702; (*f*) R. T. Lee and Y. C. Lee, *Glycoconjugate J.*, 2002, **17**, 543–551; (*g*) W. B. Turnbull and J. F. Stoddart, *Rev. Mol. Biotechnol.*, 2002, **90**, 231–255; (*h*) M. J. Cloninger, *Curr. Opin. Chem. Biol.*, 2002, **6**, 742–748; (*i*) R. Roy, *Trends Glycosci. Glycotechnol.*, 2003, **15**, 291–310.
6 J. Rao, J. Lahiri, R. M. Weis and G. M. Whitesides, *J. Am. Chem. Soc.*, 2000, **122**, 2698–2710.
7 (*a*) J. J. Lundquist and E. J. Toone, *Chem. Rev.*, 2002, **102**, 555–578; (*b*) S. D. Burke, Q. Zhao, M. C. Schuster and L. L. Kiessling, *J. Am. Chem. Soc.*, 2000, **122**, 4518–4519.
8 R. T. Lee and Y. C. Lee, *Glycoconjugate J.*, 2000, **17**, 543–551.
9 P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Arnstrong, H. Ling, N. S. Pannu, R. J. Read and D. R. Bundle, *Nature*, 2000, **403**, 669–672.
10 (*a*) E. Fan, Z. Zhang, W. E. Minke, Z. Hou, C. L. M. J. Verlinde and W. G. J. Hol, *J. Am. Chem. Soc.*, 2000, **122**, 2663–2664; (*b*) Z. Zhang, E. A. Merritt, M. Ahn, C. Roach, Z. Hou, C. L. M. J. Verlinde, W. G. J. Hol and E. Fan, *J. Am. Chem. Soc.*, 2002, **124**, 12991–12998.
11 (*a*) J. M. Gargano, T. Ngo, J. Y. Kim, D. W. K. Acheson and W. J. Lees, *J. Am. Chem. Soc.*, 2001, **123**, 12909–12910; (*b*) P. I. Kitov and D. R. Bundle, *J. Am. Chem. Soc.*, 2003, **125**, 16271–16284.
12 (*a*) I. Vrasidas, N. J. de Mol, R. M. J. Liskamp and R. J. Pieters, *Eur. J. Org. Chem.*, 2001, 4685–4692; (*b*) S. André, R. J. Pieters, I. Vrasidas, H. Kaltner, I. Kuwabara, F.-T. Liu, R. M. J. Liskamp and H.-J. Gabius, *ChemBioChem*, 2001, **2**, 822–830; (*c*) I. Vrasidas, S. André, P. Valentini, C. Böck, M. Lensch, H. Kaltner R. M. J. Liskamp, H.-J. Gabius and R. J. Pieters, *Org. Biomol. Chem.*, 2003, **1**, 803–810; (*d*) R. Autar, A. S. Khan, M. Schad J. Hacker, R. M. J. Liskamp and R. J. Pieters, *ChemBioChem*, 2003, **4**, 1317–1325.
13 (*a*) R. Roy, *Trends Glycosci. Glycotechnol.*, 2003, **15**, 291–310; (*b*) T. K. Lindhorst, *Top. Curr. Chem.*, 2002, **218**, 201–235.
14 A. J. Brouwer, S. J. E. Mulders and R. M. J. Liskamp, *Eur. J. Org. Chem.*, 2001, 1903–1915.
15 A. Bernardi, D. Arosio, L. Manzoni, F. Micheli, A. Pasquarello and P. Seneci, *J. Org. Chem.*, 2001, **66**, 6209–6216.
16 C. Santini, R. G. Ball and G. D. Berger, *J. Org. Chem.*, 1994, **59**, 2261–2266.
17 I. Vrasidas, PhD thesis, Utrecht University, 2003.
18 D. Gupta, H. Kaltner, X. Dong, H.-J. Gabius and C. F. Brewer, *Glycobiology*, 1996, **6**, 843–849.
19 N. J. de Mol, E. Plomp, M. J. E. Fischer and R. Ruijtenbeek, *Anal. Biochem.*, 2000, **279**, 61–70.
20 M. Carpo, E. Nobile-Orario, V. Chigorno and S. Sonnino, *Glycoconjugate J.*, 1995, **12**, 729–773.
21 J. A. Mertz, J. A McCann and W. D. Picking, *Biochem. Biophys. Res. Commun.*, 1996, **226**, 140–144.
22 E. A. Merrit, S. Sarfaty, F. van den Akker, C. L'hoir, J. A. Martial and W. G. J. Hol, *Protein Sci.*, 1994, **3**, 166–175.
23 P. I. Kitov, H. Shimizu, S. W. Homans and D. R. Bundle, *J. Am. Chem. Soc.*, 2003, **125**, 3284–3294.
24 J. J. Lundquist, S. D. Debenham and E. J. Toone, *J. Org. Chem.*, 2000, **65**, 8245–8250.
25 Fluorescence spectroscopy can be used for this purpose but competing filter effects of the dendritic backbone limited its reliability at higher concentrations.
26 E. A. Merritt, Z. Zhang, J. C. Pickens, M. Ahn, W. G. J. Hol and E. Fan, *J. Am. Chem. Soc.*, 2002, **124**, 8818–8824.