Design, Synthesis and Evaluation of Glycodendrimers as Multivalent Ligands

Huibert Marinus Branderhorst

Cover picture by Esther Branderhorst: “Multivalent presentation of purple berries by *Callicarpa bodineri”*

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Design, Synthesis and Evaluation of Glycodendrimers as Multivalent Ligands

Ontwerp, synthese en evaluatie van glycodendrimeren als multivalente liganden

(met een samenvatting in het Nederlands)

Proefschrift

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Huibert Marinus Branderhorst

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Promotor: Prof. dr. R. M. J. Liskamp

Co-promotor: Dr. R. J. Pieters

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Chapter 1

General Introduction

Synthesis of glycosides and glycodendrimers and their binding properties

General Introduction

Carbohydrates are increasingly gaining interest in the fields of medicinal chemistry and chemical biology. Their biological role is more and more recognized and thus a new field in biochemistry has emerged: glycomics.[1] This field is fueled by the enormous boost in synthetic methods and the developments in the analysis of carbohydrate recognition by proteins. Synthetic methods have been developed that range from the classic solution phase synthesis to solid phase strategies,[2] one-pot multiple glycosylation procedures[3] and regio- and stereoselective enzymatic glycosylations.[3] The biological properties of these newly created compounds can be investigated by well-established methods like Heamagglutination Inhibition Assays (HIA),[4] Enzyme-Linked Lectin Assays (ELLA),[5] Surface Plasmon Resonance (SPR)[6] and Isothermal Titration Calorimetry (ITC).[7] Despite the reliability of these techniques, there is a need for microarray technology as a high throughput method to speed up the binding analysis of compound libraries or peptide libraries. An important fact of carbohydrate-protein interactions, is the weak affinity, often in the millimolar range.[8] Multivalency is used by nature itself to overcome this low affinity,[9,10] the so called cluster effect.[11] For this reason multivalency is a possible method to increase the affinity of ligands. Many multivalent ligands have been prepared to identify carbohydrate binding proteins that favor a multivalent ligand presentation. This multiple binding principle was extensively studied for certain groups of carbohydrate binding proteins, like bacterial adhesion proteins (Adhesins),[12,13] lectins,[12] and members of the disease causing AB_5 toxin family.[14]

In this introducing chapter an overview is given including recent and important developments in the field of glycomics. First, recent developments in carbohydrate solid phase synthesis will be discussed. Subsequently, an overview of the observed multivalency effects for binding towards bacteria, AB_5 toxins and lectins will be given. Finally, the developments in microarray technology as a high throughput screening method for the detection of multivalent interactions will be described.

Solid Phase synthesis of complex carbohydrates

The principle of solid phase chemistry was first demonstrated by Merrifield in 1963 with the synthesis of peptides.[15] Major advantages of this strategy were the easy purification

after each coupling step and the high conversion rate because of the excess of reagents that were used. The solid phase methodology was extended towards the synthesis of DNA and RNA biomolecules and this set the stage for the enormous boost in both genomics and proteomics. Already since the first publication by Merrifield, scientists have been working on the extension of this strategy from peptides and oligonucleotides to oligosaccharides. The first procedure was published by Frechet and Schuerch in 1971.[16] They used an allyl alcohol functionalized polystyrene resin. Glycosyl building block **1** was used for the preparation of trisaccharide **2** which was isolated after oxidative cleavage (Figure 1). Based on this principle novel strategies were developed, concerning new resins, linkers, protecting groups and glycosylating reagents. Most strategies are based on an *on-bead* glycosylation, followed by *on-bead* selective deprotection. Reiteration of this process can lead to an oligomer. A major achievement was the development of an automated synthesizer by Seeberger *et al.* in 2001.[17] Without taking the time-consuming building block synthesis into consideration, an oligosaccharide can nowadays be constructed within 24 hours with the synthesizer. In the next section the important and most relevant developments of solid phase glycoside synthesis will be discussed.

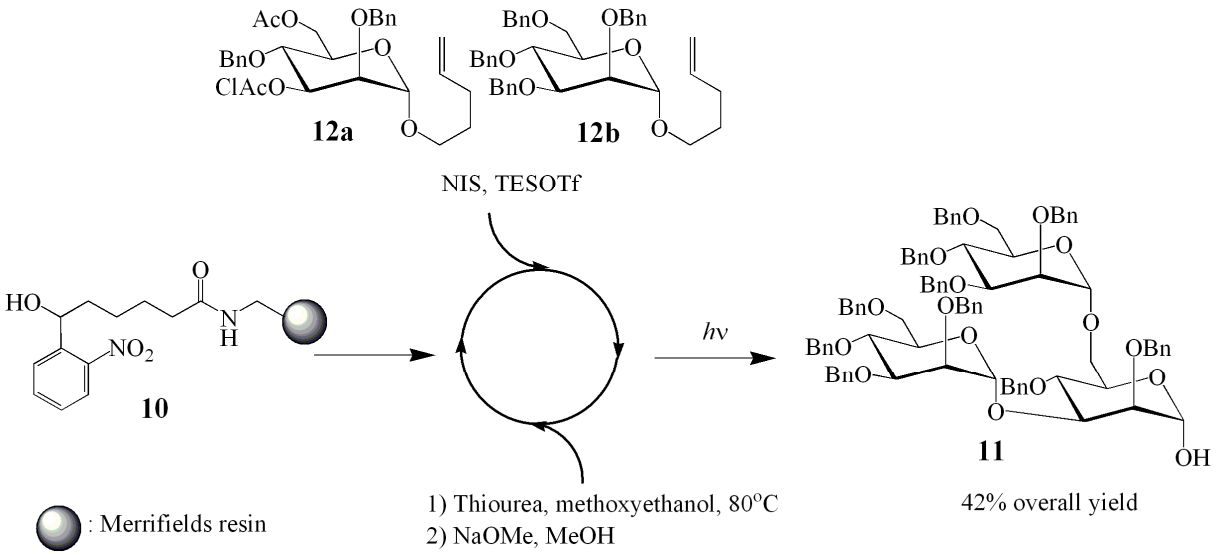


Figure 1 *The first Solid Phase carbohydrate synthesis applied by Frechet and Schuerch in 1971*[16]

Solid Phase Strategies

The goal in the synthesis of oligomers is the regioselective introduction of a chemical linkage between monomers. Oligonucleotide chemistry is based on the introduction of

phosphate esters while peptide chemistry relies on the formation of amide bonds. For carbohydrate oligomers both the regio- and stereoselective introduction of glycosidic bonds are the goals. As carbohydrates have multiple functional groups, only one should be accessible for chemical bond formation while the others have to be protected. Temporary protecting groups need to be cleavable under mild conditions without affecting the linker. Orthogonal protecting groups are necessary for branching glycosides and need to be cleavable on demand. Hydroxyl groups can be permanently blocked by, for example, benzyl groups until the final deprotection. Chemical bond formation involves the reaction of a glycosyl donor with a glycosyl acceptor. The glycosyl donor is a sugar with a good leaving group at the anomeric center. Upon activation this electrophile will be attacked by a nucleophilic hydroxyl group from the acceptor, yielding the desired glycosidic bond. The design of a solid phase route starts with the choice of a donor-bound or an acceptor-bound strategy. The acceptor-bound strategy was used by Frechet and Schuerch (Figure 1) and has several major advantages.[16] The glycosyl donor is the least stable reaction partner and can undergo several side reactions, including decomposition. With an acceptor-bound strategy these side reactions will occur in solution, so the side products will easily be washed away. In the alternative approach a resin-bound donor will suffer from side reactions and will give resin-bound impurities, hence a drop in overall yield will result. A consequence of the donor-bound strategy is that the carbohydrate can not have an anomeric connection to the resin, instead it must be linked via the remaining hydroxyl functions. Acceptor-bound strategies do not have this limitation and in general the anomeric linkage is used. Because of the donor instability, solution phase synthesis is based on an excess of glycosyl donor to force the reaction to completion. In a donor-bound strategy the nucleophilic acceptor is used in excess but still unwanted side reactions will occur. For these reasons the acceptor-linked strategy is by far the most used and this overview will be mainly focused on this strategy. Important enough to mention is the extensively studied donor-bound strategy by Danishefsky *et al.*, using the glycal assembly method (Figure 2).[18] Resin-bound galactal residue **3** was activated upon epoxidation; coupling to acceptor **4** was achieved by activation of the epoxide with $ZnCl_2$. Despite the limitations of the donor-bound strategy an impressive array of oligosaccharide structures was synthesized via this method. A synthesis in which both acceptor-bound and donor-bound strategy were used is shown in Figure 2.

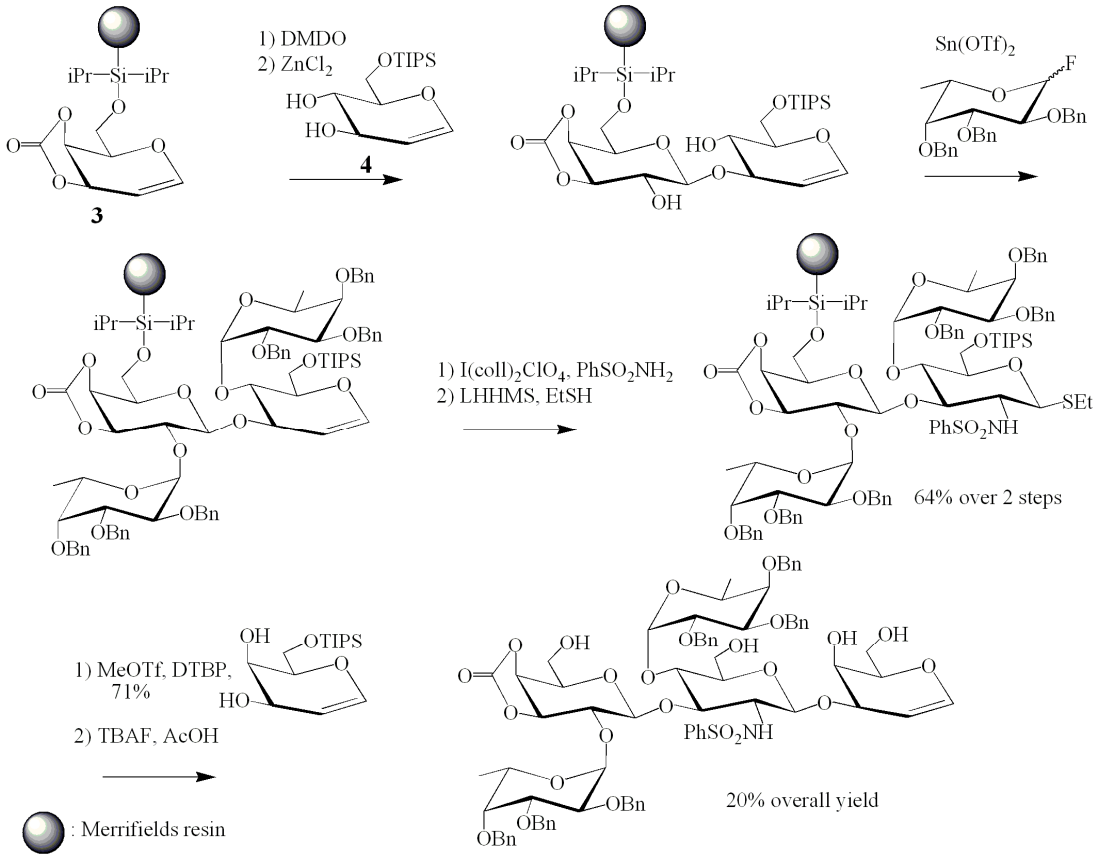


Figure 2 *Synthesis of a branched pentasaccharide by the use of a donor- and a subsequent acceptor-bound strategy*[18]

A bidirectional strategy was published by Zhu and Boons (Figure 3).[19] Resin-bound monosaccharide **5** was first used as an acceptor and subsequently used as a glycosyl donor. Crucial is the use of an orthogonal set of glycosyl donors.

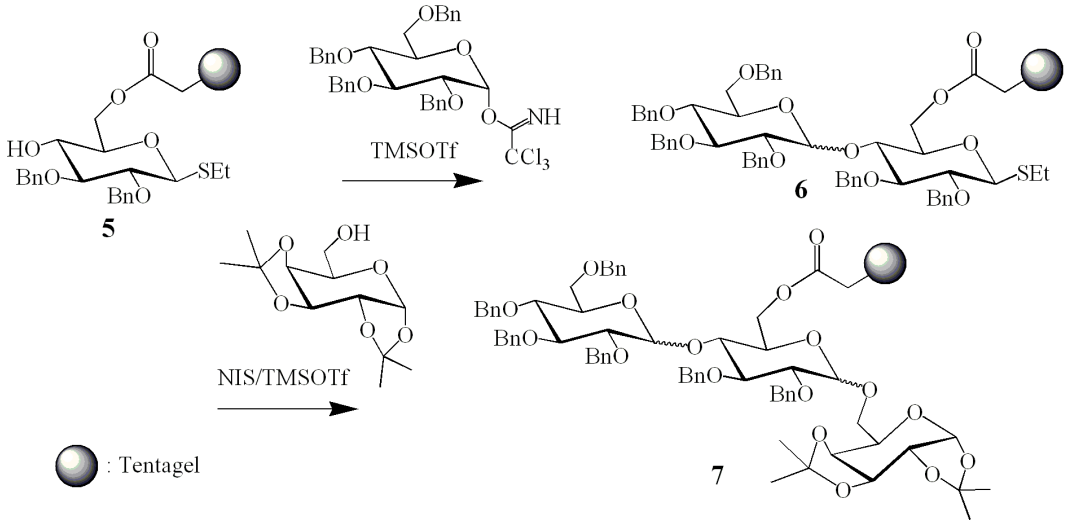


Figure 3 *The bidirectional strategy by Boons*[19]

Resin bound acceptor **5** was first glycosylated with a trichloroacetimidate donor. Subsequently, the obtained S-ethyl donor **6** was activated with NIS/TMSOTf and treated with a glycosyl acceptor to give resin-bound trisaccharide **7**.

Resins

The development of a synthetic strategy starts with the choice of the resin which influences the overall strategy, the choice of reagents and the reaction conditions. Most solid phase oligosaccharide syntheses relied on polystyrene resin **8** (Figure 4). This polystyrene is based on vinylbenzene cross-linked with 1% of divinylbenzene. It is available with a wide range of functionalities, like amines, carboxylic acids, chloromethyl groups (**8**), hydroxyl groups and thiols. In general these resins have a high loading capacity (around 1 mmol/g), are stable against a broad range of chemical conditions and show high swelling properties in apolar organic solvents. Economically they are also attractive because of the low price. The polarity of a polystyrene resin can easily be increased by introduction of polyethylene glycol chains onto the backbone (**9**) (Figure 4). Commercially available resins like Tentagel and Argogel that are based on this principle show a higher swelling capacity in polar solvents but suffer from a lower loading capacity (0.25 – 0.40 mmol/g). Other PEG based resins were developed but unlike the Argogel and Tentagel resins, they have not found widespread use in the field of carbohydrate synthesis.

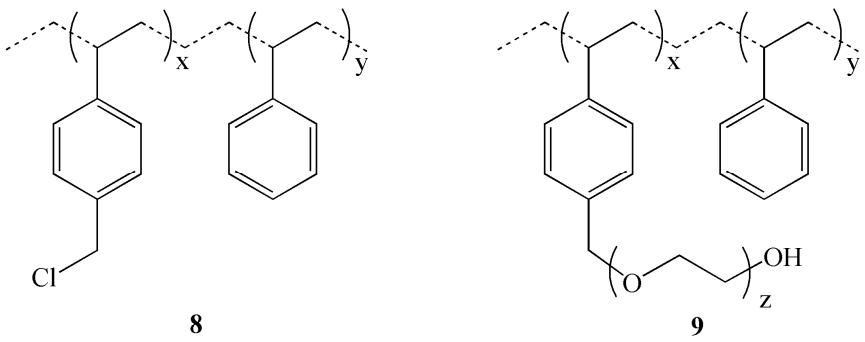


Figure 4 *The most commonly used resins in solid phase oligosaccharide synthesis*

Non-swelling controlled pore glass supports are commonly used in automated DNA synthesis. Because of the structure of the particles the loading is low and the resin is incompatible with silyl ether protecting groups. Therefore the use of this support is very limited in carbohydrate synthesis. Next to the insoluble supports there are literature reports

based on soluble organic supports. While insoluble supports often require extensive optimization of the reaction conditions, the advantage of using soluble resins is the homogeneous reaction mixture during the chemistry. After each reaction the support must be precipitated by the addition of an apolar solvent. Serious disadvantages are the limitations in solvent polarity that can be used and the loss of resin during work up which significantly lowers the yield after several couplings steps.

Linkers

The choice of the linker is of critical importance for the solid phase protocol. The linker needs to be stable under the synthesis conditions and quantitatively cleavable on demand. Several methods for sugar attachment have been developed. Most strategies are based on anomeric attachment of the carbohydrate. This overview will only discuss the most common anomeric linkers. Photo-cleavable linkers contain a light-sensitive *o*-nitrobenzyl moiety. Nicolaou *et al.* used the primary *o*-nitrobenzyl linker but cleavage was slow and incomplete.[20] A more efficient photo-cleavable linker was introduced by Geysen *et al.* (Figure 5).[21] Using the secondary *o*-nitrobenzyl linker **10**, branched trimannoside **11** was synthesized from mannosyl *n*-pentenyl donors **12a** and **12b** in 42% overall yield. A chloroacetyl and an acetyl group were used to discriminate between the branching positions.

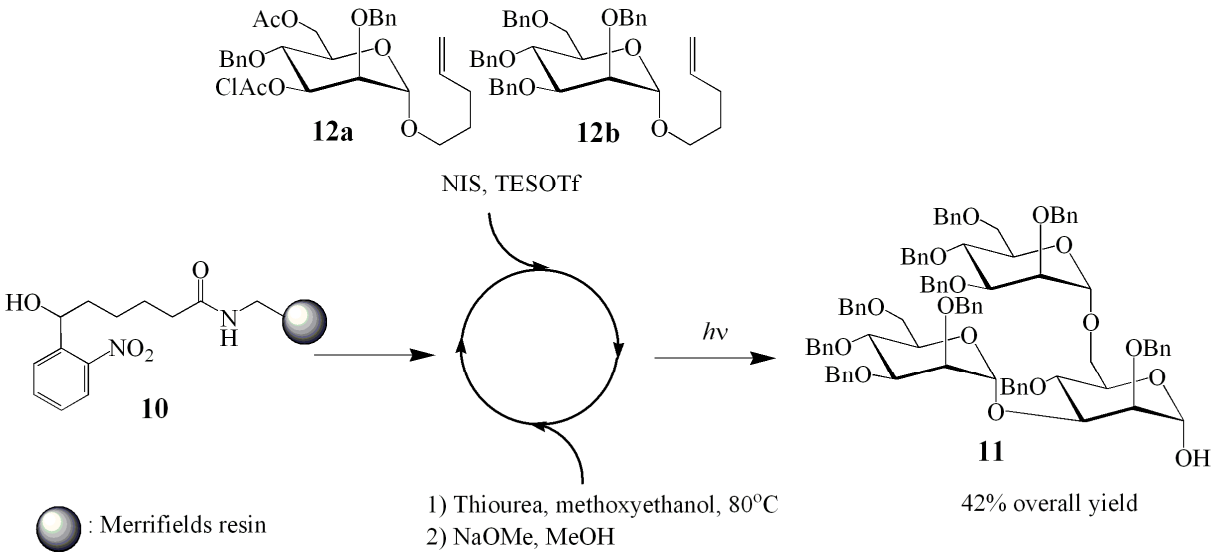


Figure 5 *Synthetic cycle for the preparation of a branched trimannoside using a photo-cleavable linker*[21]

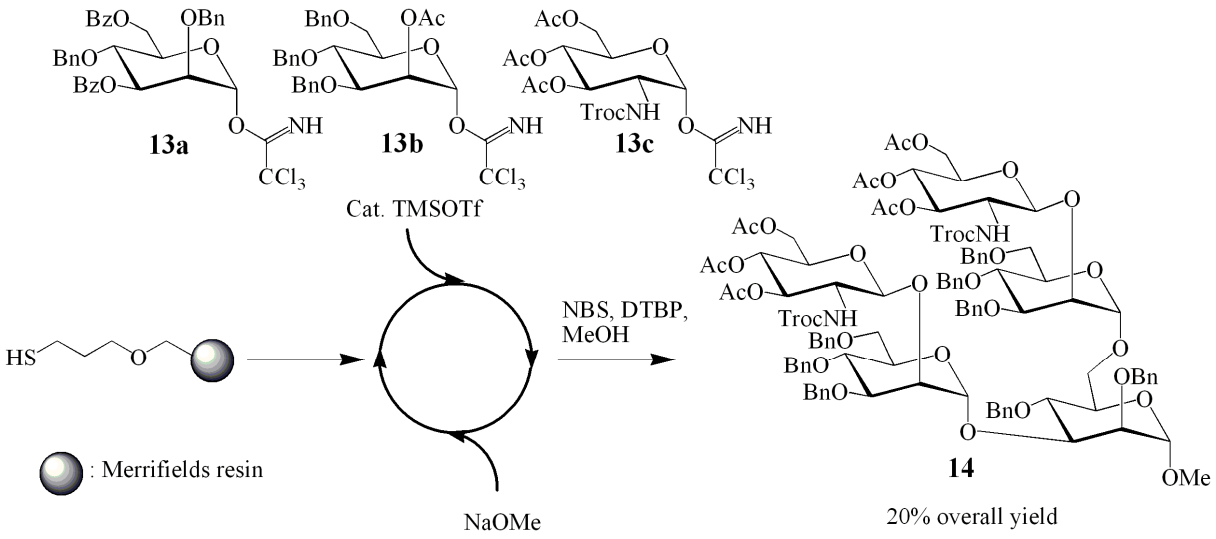


Figure 6 *Construction of a branched oligomer by Rademann and Schmidt*[22]

Thioglycosidic linkers were introduced in 1972 and frequently used by Rademann and Schmidt.[22] Cleavage relies on the nucleophilicity of the sulphur atom. It reacts with several electrophiles like MeI and N-bromosuccinimide (NBS) to give a resin bound sulphonium ion which is susceptible to nucleophilic attack by H_2O and MeOH. Reactivity of the linker can be fine-tuned by the choice of thio substituents. A thiopropyl linker on a Merrifield resin was used together with trichloroacetimidate donors **13a** - **c** for the preparation of branched pentasaccharide **14**. Cleavage was affected with NBS, MeOH and di-*tert*butyl pyridine (Figure 6).

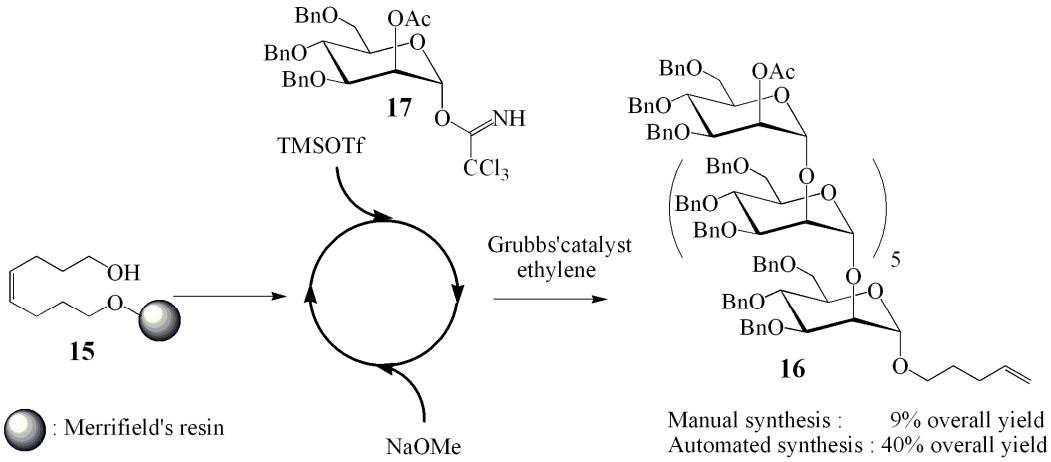


Figure 7 *Solid phase carbohydrate synthesis using an octenediol linker and glycosyl trichloroacetimidate donors*[24]

Knerr and Schmidt were the first to report the use of ring-closing metathesis for the cleavage of a linker containing a δ,ε-double bond.[23] Recent publications by Seeberger *et al.* focused on the use of octenediol linker **15** (Figure 7).[24] This linker can quantitatively be cleaved by olefin cross metathesis with Grubbs' catalyst under an ethylene atmosphere. The obtained n-pentenyl glycoside **16** can be used as a glycosyl donor and for ligation to scaffolds. The octenediol linker is nicely compatible with trichloroacetimidate donors like **17** and this combination has proven to be extremely useful in solid phase carbohydrate synthesis, even in an automated fashion.[17]

Glycosyl Donors

The lack of powerful glycosylation methods has hampered the developments in solid phase carbohydrate chemistry for years. With the development of novel, strong glycosylating agents, the area gained a renewed interest, and nowadays trichloroacetimidate donors are most commonly used.[25] These donors are activated under mild conditions by a catalytic amount of TMSOTf.

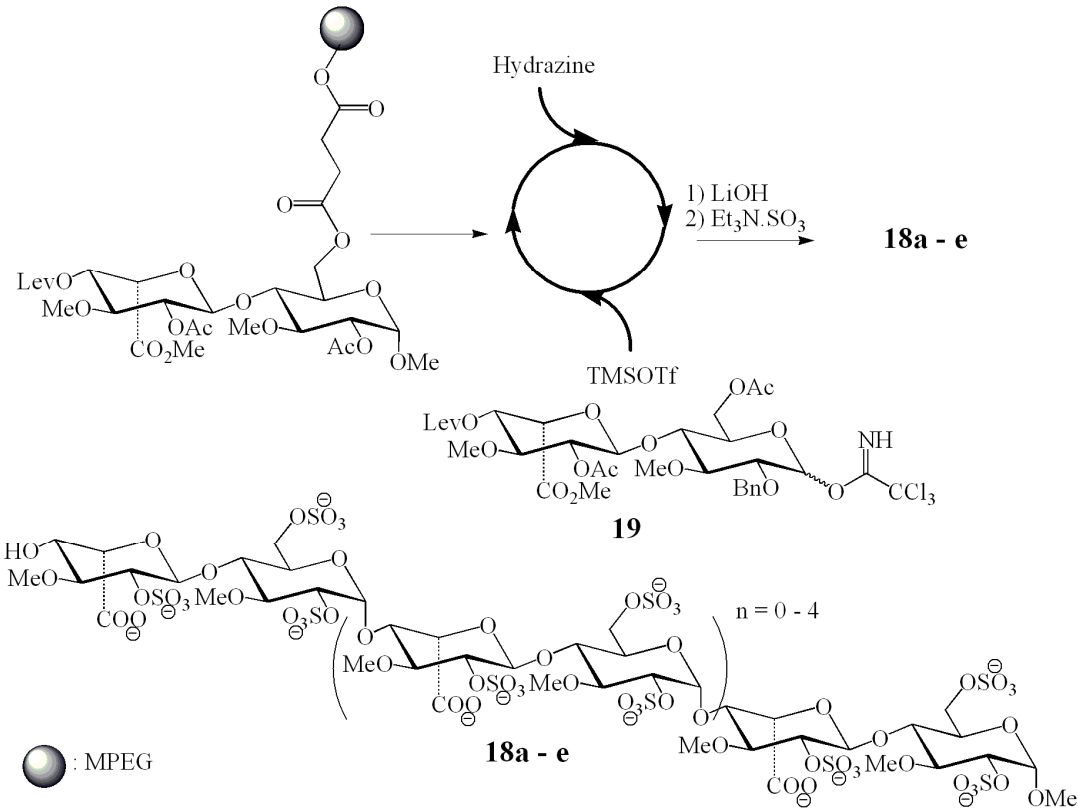


Figure 8 *Construction of heparin sulfate oligomers using an orthogonal protected trichloroacetimidate disaccharide building block*[26]

Trichloroacetimidate donors are versatile, give high coupling yields and high stereoselectivity, therefore they are valuable building blocks for solid phase oligosaccharide assembly. As mentioned above, Rademann and Schmidt used trichloroacetimidate donors for the synthesis of branched pentasaccharides (Figure 6).[22] Seeberger *et al.* used glycosyl trichloroacetimidates for the construction of a linear α(1-2) mannose heptasaccharide.[24] More complex heparin sulfate-like oligomers **18a** – **e** were prepared from trichloro-acetimidate disaccharide building block **19** by van Boeckel *et al.* (Figure 8).[26]

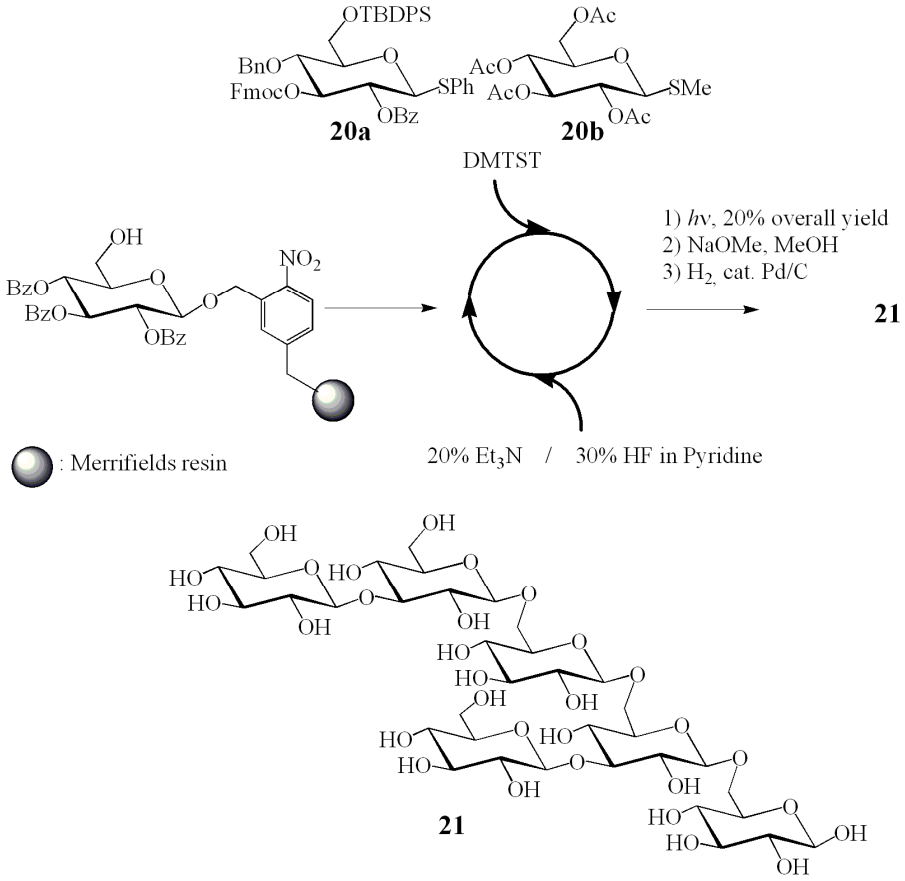


Figure 9 *Thioglycoside building blocks for the construction of a hexasaccharide. Continuation of the cycle afforded the corresponding dodecasaccharide*[20]

Thioglycosides[27] are useful glycosyl donors because of their easy availability and chemical stability. They are selectively activated by thiophilic reagents like dimethylthiosulfonium triflate, methyl triflate and NIS/triflic acid. Important to mention is the high α- or β-selectivity that can be achieved with them. Thioglycosides **20a** and **20b** were used by Nicolaou *et al.* for the construction of branched hexasaccharide **21** on a resin (Figure 9)[20] and they have found widespread use.

Finally, glycosyl phosphate donors as **22** have recently been explored in the Seeberger laboratory for the construction of complex glycan structures (Figure 10).[24] Activation proceeds by stoichiometric amounts of TMSOTf and excellent coupling yields and stereoselectivities were obtained. Linear trisaccharide **23** was obtained in 53% overall yield after cleavage. Also glycosylation of sterically hindered acceptors was achieved, and because of the high reliability, glycosyl phosphates are excellent building blocks for future solid phase developments.

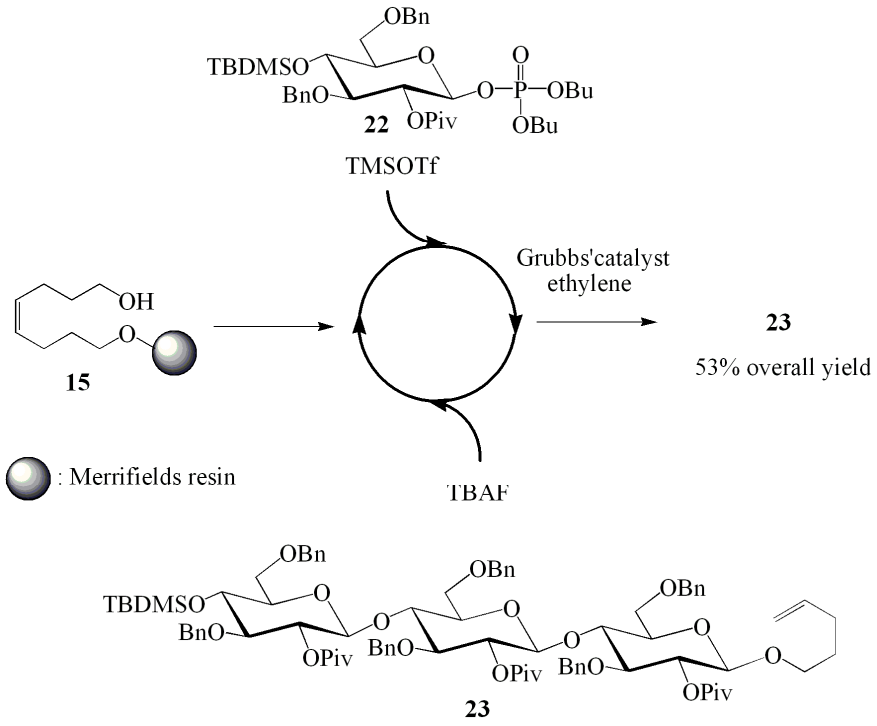


Figure 10 *Synthesis of a linear trisaccharide*[24]

Future strategies

A milestone was reached with the development of an automated synthesizer by Seeberger *et al.* in 2001. The successful octenediol linker was used in combination with powerful glycosylating agents like the trichloroacetimidate donors (Figure 7) and phosphate donors (Figure 10). A previously manually synthesized heptamannoside was now prepared in a fully automated fashion within 19 hours. The yield was superior to the yield of the manual protocol, i.e. 40% versus 9% (Figure 7).[24] Initially, only linear and simple branched oligosaccharides were assembled. Much effort was put into the optimization of glycosylation reactions, for example the conditions for the introduction of challenging α-galactoside linkages were optimized.[28]

The synthesizer can nowadays be used for the preparation of complex oligosaccharide sequences like the tumor-associated carbohydrate antigen Globo-H (**24**) (Figure 11).[28] It is likely that carbohydrate synthesizers will take over the chemical construction of oligosaccharides. Large scale synthesis of glycosyl building blocks will be optimized and within a few years simple building blocks will probably become commercial available. Despite these recent developments, the synthesis of complex carbohydrates will never be as straightforward as is the case with peptides and oligonucleotides.

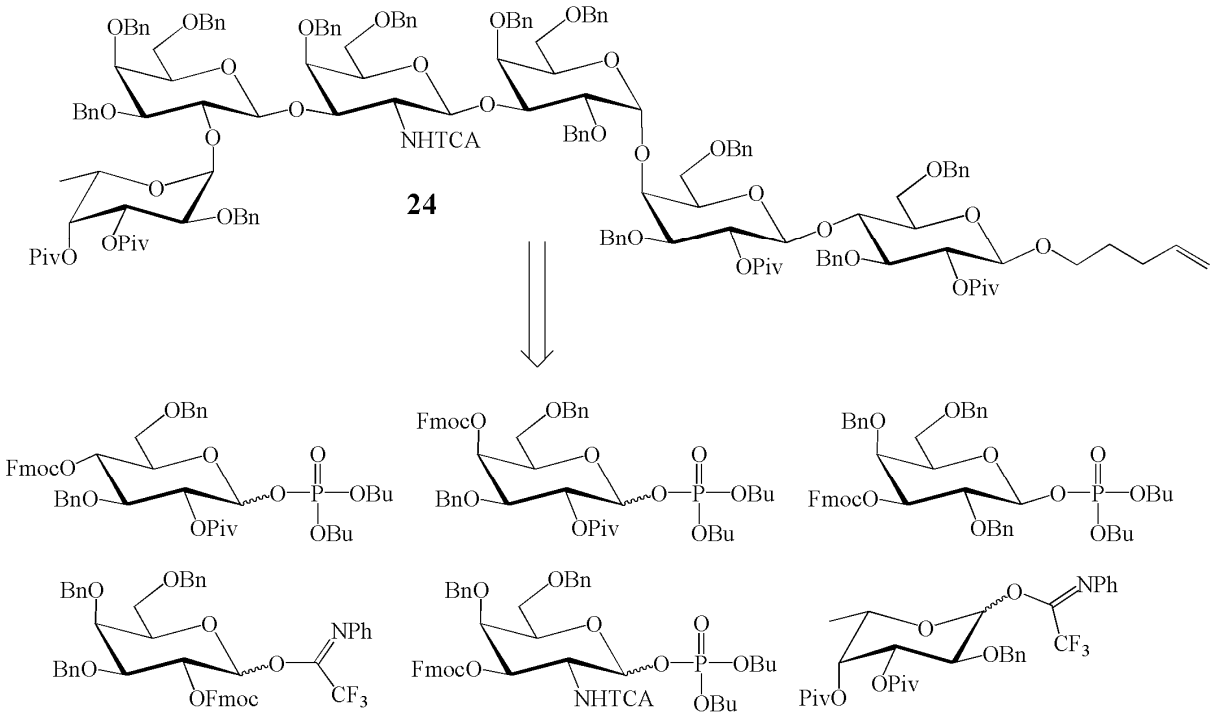


Figure 11 *The tumor-associated carbohydrate antigen Globo-H was build from monosaccharide building blocks in an automated fashion*[28]

As method for the building block synthesis, recently novel one-pot synthetic methodology was published by Hung *et al.* for the regio-selective protection of carbohydrates (Figure 12).[29] These reactions are based on silicon chemistry and can be performed on a large scale. Both acceptors and thioglycoside donors were synthesized and showed excellent yields and selectivities in glycosylation reactions. This method will set the stage for a new boost in synthetic oligosaccharide synthesis, both in solution phase and solid phase strategies.

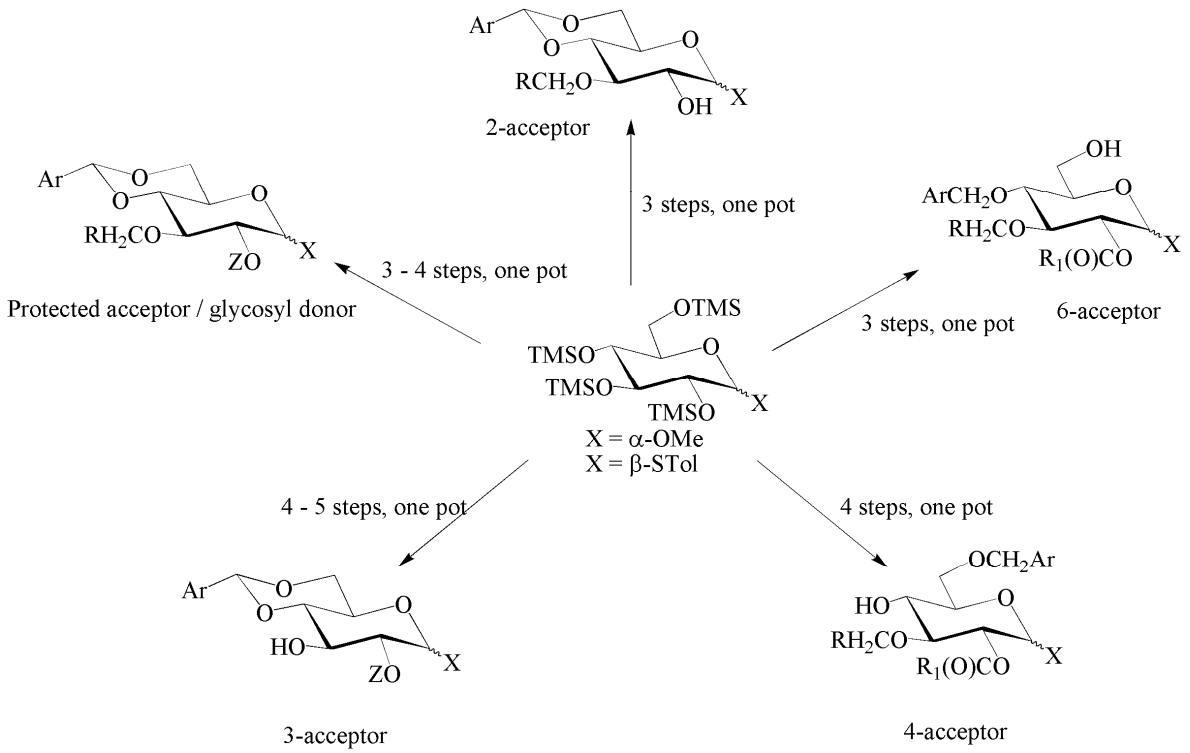


Figure 12 *One pot synthesis of glycosyl acceptors and glycosyl donors*[29]

Binding properties of multivalent carbohydrate ligands

Carbohydrate-protein interactions are essential for all living creatures. These interactions play a crucial role in many biological processes. Carbohydrate binding proteins other than enzymes and antibodies are also called lectins and they exist in many living organisms, like viruses, bacteria, plants and mammalians. Lectins are involved in diverse biological processes, such as the clearance of glycoproteins,[30] the adhesion of infectious agents to host cells,[31] the recruitment of leukocytes to inflammatory sites,[32] cell-cell communication in the immune system, malignancy and metastasis in cancer.[33]A better understanding of the exact role of carbohydrates and lectins is needed for the development of new diagnostics and therapeutics. Isolated saccharides from natural sources can be used but more and more synthetic derivatives become available by recent progress in synthetic organic chemistry.

Despite the weak affinity, typical dissociation constants are in the millimolar range for monosaccharides, lectins have a high specificity towards carbohydrate ligands.[34] Lectins can be divided into three major groupes, based on their structure. The first group are the "simple" lectins. Simple lectins consist of a small number of subunits. The subunits have a low molecular weight (< 40 kD) and are not necessarily identical. Each subunit contains at

least one carbohydrate binding site. The legume lectins represent the largest and most thoroughly studied subgroup of the simple lectins. Concanavalin A (ConA) is a lectin that is isolated from the jack bean (*Canavalia ensiformis*) and was used in many multivalency studies. The group of β-galactoside-recognizing animal lectins, better known as galectins, also belong to the group of simple lectins. Galectins are rapidly gaining interest in the field of biomedical sciences. Galectins are found to act in biological processes like apoptosis,[35] metastasis[36] and angiogenesis.[37] Galectin-3 for example, is overexpressed in many types of cancer cells and therefore an interesting target in the development of therapeutics and diagnostics. The second group of lectins are the mosaic lectins. They are composed of several protein domains, only one domain has a carbohydrate binding site. Mosaic lectins include viral hemagglutinins and the animal type C-, P- and I-lectins. The most studied examples are the siglecs (sialic acid-binding immunoglobulin superfamily lectins)[38] and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule 3 Grabbing Nonintegrin).[39] The final group of lectins are the macromolecular lectins, commonly present on bacteria. These assemblies are filamentous organelles and consist of helically arranged subunits (pilins) assembled in a well-defined order. As the group of legume lectins is by far the best studied, they will be discussed in more detail.

Lectin monosaccharide binding is relatively weak, with typical dissociation constants in the millimolar range.[34] The origin of this weakness lies in the fact that carbohydrate binding pockets are shallow and solvent exposed. The interactions can be divided into hydrogen bonding interactions and hydrophobic interactions. Nevertheless in biological systems, lectin-carbohydrate recognition is characterized by both a high-affinity and specificity. The reason for this strong affinity is the multimeric protein architecture allowing simultaneous multiple carbohydrate-receptor interactions. These multivalent association processes are widespread in nature to achieve the necessarily high affinity for biological effects. The so-called “cluster glycoside effect” is previously defined as an “affinity enhancement achieved by multivalent ligands over monovalent ones that is greater than would be expected from a simple effect of concentration”[40] and is generally accepted by scientists. Therefore, as a consequence both the location and arrangement of the carbohydrate binding sites are important for the design of multivalent compounds. For several “simple” lectins crystal structures are available and they give insight in the orientation of the carbohydrate binding sites. Multivalent association can be divided into two mechanisms; chelation and

aggregation. Chelation only occurs with proteins having closely separated carbohydrate binding sites; the multimeric ligand must be capable of bridging the distance between the receptor sites. If this is not possible and still multivalency effects are observed, the aggregation mechanism is more likely to occur. A network of multimeric ligands and proteins is formed and this network can sometimes be visualized by turbidity measurements.

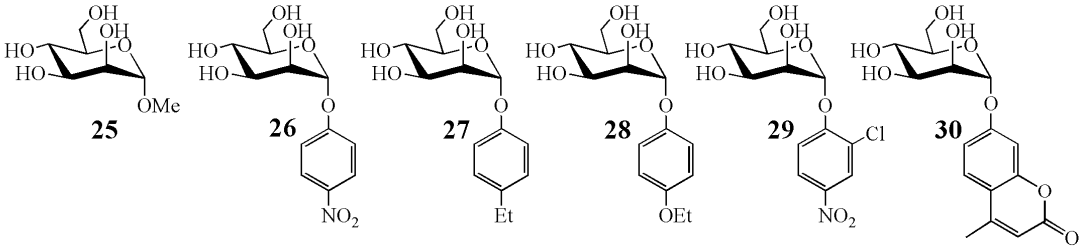
In this overview three important biological targets, that favor a multivalent ligand presentation, will be described: pathogenic bacteria (via adhesin proteins located on the cell surface or fimbriae), members of the AB_5 toxin family (Cholera Toxin, Shiga Toxin) and the legume lectins.

Prevention of Bacterial Adhesion

Bacterial infections often start with bacterial binding to mammalian cell surfaces. This binding is mediated by receptor proteins, called adhesins. These receptors are located on the bacterial surface and often bind specifically to carbohydrates located on a cell surface. Inhibition of this adhesion process is believed to be suitable strategy towards a novel generation of antibacterial agents.[13,41] Commonly used antibiotics suffer from increasing resistance[42] and for the future of mankind new antibiotics are needed. Unlike general antibiotics, prevention of bacterial binding does not stimulate the bacteria to develop resistance because the bacteria survive the treatment. Non-adhering bacteria will be excreted from the human body by regular cleansing mechanisms. Bacteria might be able to mutate the adhesin binding site so the anti-adhesion compounds will not be effective anymore, but by this mutation bacterial binding to the cell surface is reduced to the same extent, as the same ligand is presented by both antibiotic and human cell surface. To overcome the low affinity of monovalent carbohydrates for adhesin proteins, research was performed towards the development of multivalent carbohydrates. These systems present multiple carbohydrate residues within a single molecule. Since bacteria bind in a multivalent manner to cell surfaces, this approach seems a logical step to overcome the low affinity problem. Over the years several mono- and multivalent anti-adhesion compounds have been synthesized and the most impressive ones will be discussed.

Monovalent Inhibition

Initial studies were performed with type 1 fimbriated *E. coli* which is able to bind mannose. The bacterial agglutination of yeast was inhibited by mannose derivatives; methyl α-D-mannoside **25** was used as a reference compound with a relative potency of 1. The affinity was improved by di- and trimannosides (rel. pot. 26 and 11) but the best ligands were obtained from monovalent aryl mannosides. The *p*-nitrophenyl α-D-mannoside **26** showed a 70 times higher affinity then **25**. The affinity increase was thus gained by the introduction of an aromatic moiety and this research was extended to the introduction of several aromatic substituents **26** - **30** (Figure 13).[43]



Yeast agglutination inhibition (rel. pot.)	1	69	77	154	717	600
Inhibition of E. coli binding to epithelial cells (rel. pot.)	1	70	150	240	470	1010

Figure 13 *Monovalent mannose inhibitors used to prevent bacterial adhesion*[43]

Relative affinity over **25** could finally be improved to 717 by a *p*-nitro-*o*-chlorophenyl substituent (**29**). Examination of the adhesin crystal structure showed a hydrophobic pocket just outside the mannose binding site which explained the preference for aromatic substituents. The IC_{50} values observed depend on the assays performed, nevertheless a trend in relatively potency can be deduced (Figure 13).

P-fimbriated *E. coli* recognizes the galabiose sequence (**31**) and a range of deoxy and deoxyfluoro derivatives were prepared for the identification of contributing hydroxyl functions.[44] It was found which hydroxyl groups were important for binding and at which positions substituents would enhance binding. Introduction of a *o*-thiobenzoic acid substituent and a cysteine-like anomeric spacer led to **32** as a more potent inhibitor for the PapGII adhesin while *o*-nitrobenzyl derivative **33** has improved the affinity for the PapGI adhesin, both with a relative potency of 7 over reference compound **31** (Figure 14).

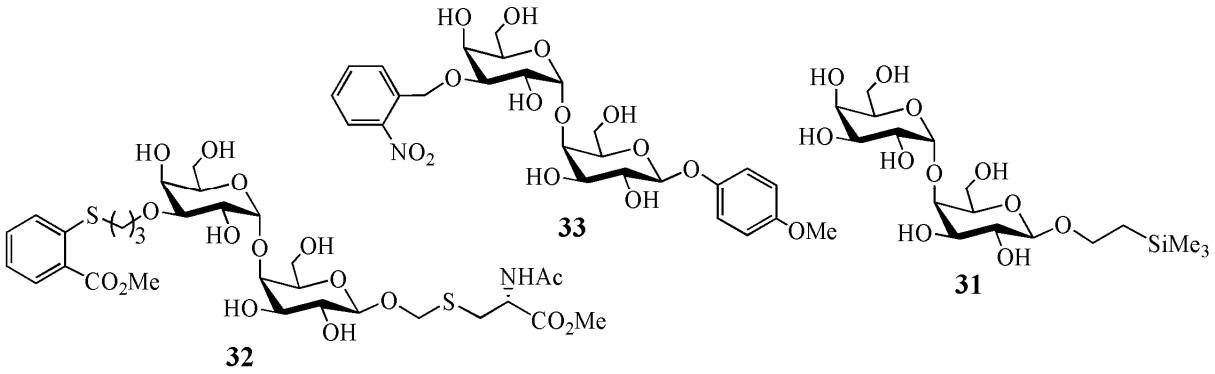


Figure 14 *Inhibitors of E. coli's PapG adhesins*[44]

Streptococcus suis also recognizes the galabiose epitope but a different substitution pattern was preferred. Evaluation of a range of galabiose derivatives showed enhanced binding upon introduction of hydrophobic substituents at the 2' and 3' positions. Both 2' and 3' substituted galabiose derivatives **34** and **35** showed a gain in relative potency of 16 for subtype P_N and 5 for subtype P_O compared to reference compound **36** (Figure 15).

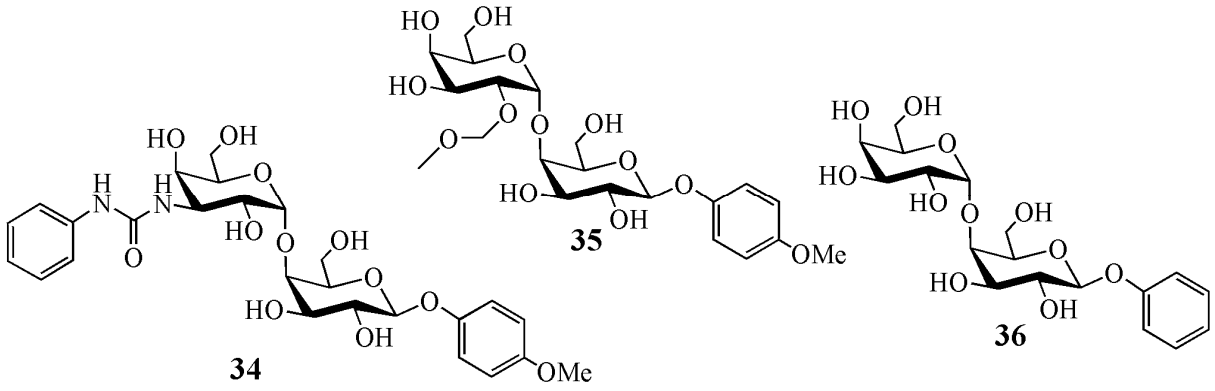
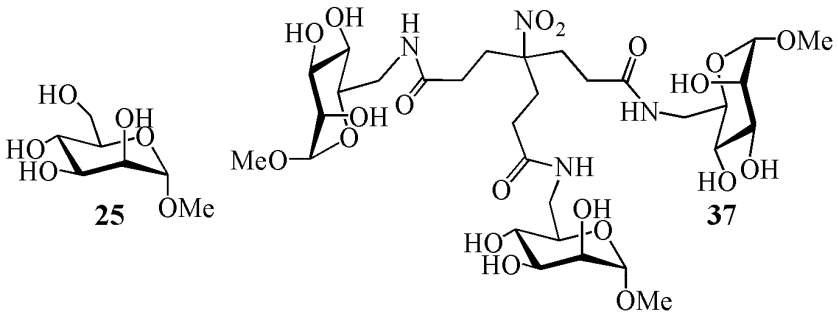


Figure 15 *Galabiose-based inhibitors of Streptococcus suis adhesion*[44]

Multivalent Inhibition

All the compounds described above were able to prevent *in vitro* bacterial binding and demonstrate the power of the anti-adhesion strategy. A major drawback is the concentration necessary for inhibition and therefore a multivalent display of ligands may overcome this hurdle. Bacteria even use this multivalency principle for the strong adhesion to cell surfaces. Until now it is uncertain how the bacterial adhesins are spatially arranged on the various pathogens and therefore a diverse choice of multivalent scaffolds is desirable.

Adhesion of uropathogenic type 1 fimbriated *E. coli* was extensively studied with monovalent inhibitors as was discussed in the previous section and these bacteria were also the first subject in a study with multivalent ligands. Lindhorst *et al.* has created several multivalent systems and it was shown that trivalent **37** performs the best (Figure 16) with an affinity higher then **26** (13 fold in a heamagglutination assay, however only 1.5- fold in an ELISA).[45]



	MIC (μM)	Rel. Pot. (per ligand)
Monovalent **25**	3900	1 (1)
Trivalent **37**	1.1	3545 (1182)
PNPαMan **26**	42	93 (93)

Figure 16 *Multivalent mannosides prepared by Lindhorst*[45]

A different trivalent system with aryl glycosides showed surprisingly less affinity over **26**. Other research groups synthesized larger glycodendrimers but these showed affinity enhancements of only 10-fold. In our research group mannose- containing dendrimers and polymers have been prepared and both series of compounds showed a relative potency of 20 - 40 fold over free mannose.[46] The observed binding enhancement was not caused by multivalency but by the aglycon part of the ligand.

Binding prevention of the lung pathogen *Pseudomonas aeruginosa* was also investigated in our group.[47] The sugar sequence GalNAcβ(1,4)Gal was synthesized and conjugated to dendritic scaffolds. The resulting dendritic compounds **38a** - **c** were tested for their ability to prevent bacterial binding to an asialoGM1 coated surface (Figure 17). Two *P. aeruginosa* strains (PAK and PAO) were tested in this inhibition assay. The results showed a 15-fold increase in affinity for tetravalent compound **38c** over the monovalent ligand **38a**. We concluded that binding mainly benefits from a lipophilic aglycon part besides a modest multivalency effect.

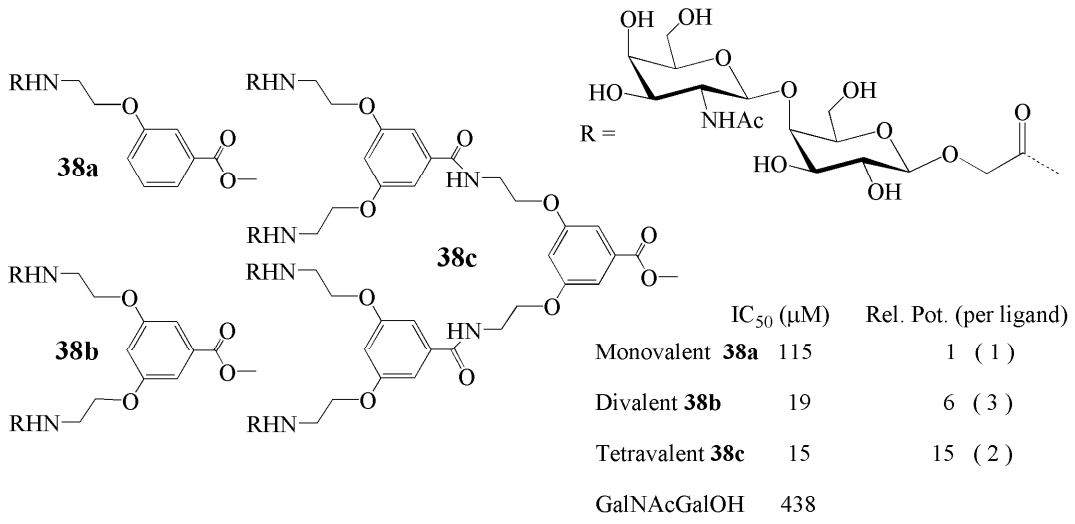


Figure 17 *Inhibitors for binding of P. aeruginosa towards an asialoGM1surface*[47]

Two important multivalency studies involving adhesion inhibition of the pathogenic *Streptococcus suis* were published. Magnusson *et al.* published in 1997 the synthesis and evaluation of mono-, di- and tetravalent galabiose ligands.[48] Galabiose (**31**) is the natural ligand for *S. suis* and it was found that tetravalent ligand presentation (**39c**) enhanced binding 25-fold (Figure 18).

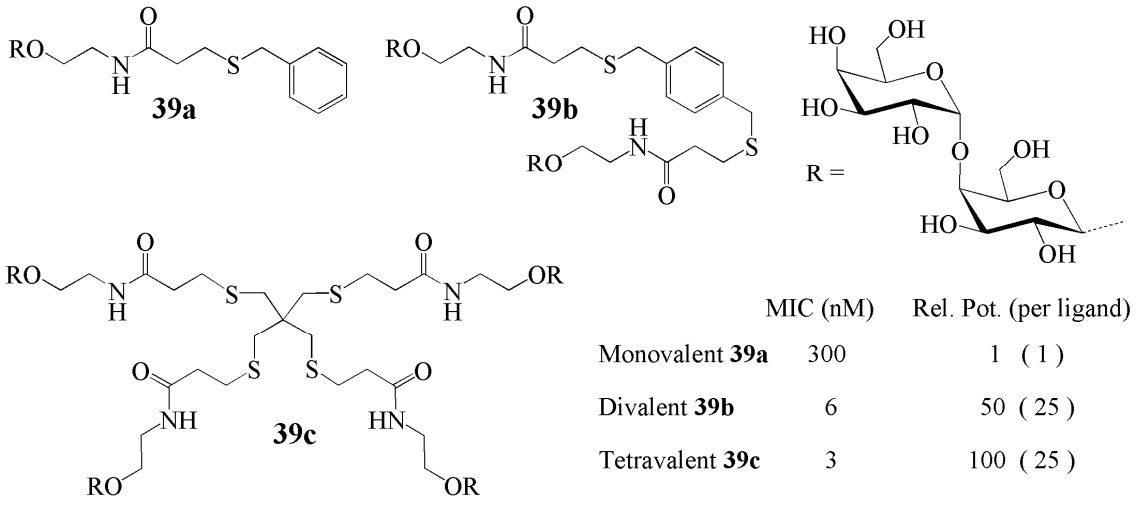
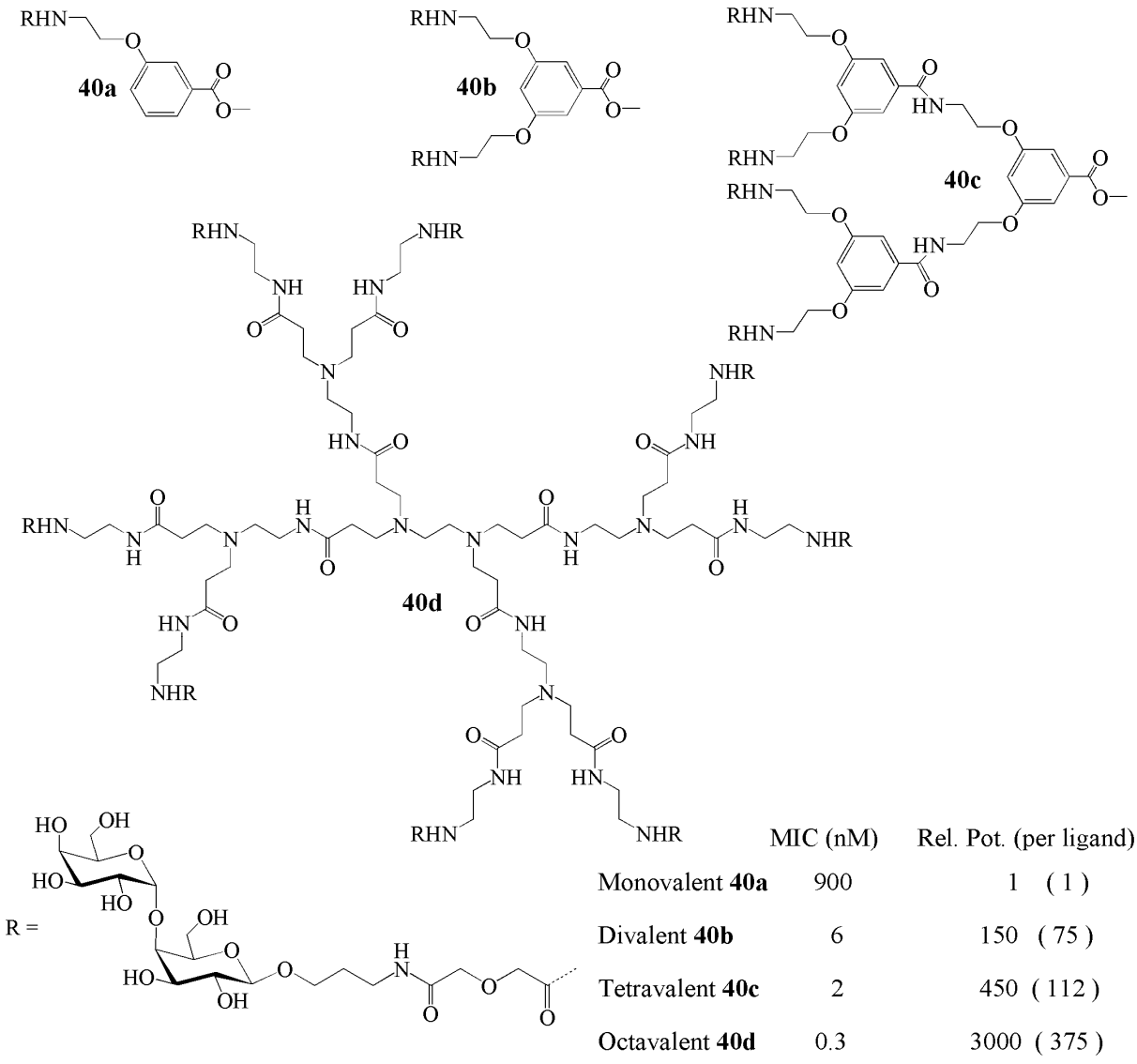


Figure 18 *Galabiose dendrimers by Magnusson*[48]

A higher multivalency effect was observed in our group.[49] Mono- to octavalent glycodendrimers **40a – 40d** were synthesized and although the monovalent compound **40a** showed a low MIC, the observed multivalency effect was clear (Figure 19). The octavalent

PAMAM glycodendrimer **40d** showed an MIC of 0.3 nM and a relative potency per sugar of 375.



	MIC (nM)	Rel. Pot. (per ligand)
Monovalent **40a**	900	1 (1)
Divalent **40b**	6	150 (75)
Tetravalent **40c**	2	450 (112)
Octavalent **40d**	0.3	3000 (375)

Figure 19 *High-affinity ligands to prevent S. suis adhesion*[49]

Despite these successful approaches towards novel antibiotics, there is still room for improvement. Monovalent inhibitors can be decorated with functionalities to improve the binding properties. Improved multivalent inhibitors can be based on both optimized ligands and an optimized multivalent presentation. For the future there is a need for high-throughput screening methods which can be used for detection of bacteria to speed up the identification of dangerous pathogens and to ensure treatment with appropriate antibiotics.

Inhibition of AB_5 toxins

The AB_5 toxins are named after their protein composition: they consist of an A-subunit surrounded by five identical B-subunits. The B-pentamer is responsible for binding to cell surface carbohydrates in a multivalent manner, subsequently followed by internalization of the A-subunit. This A-subunit is the disease-causing part of the protein complex. The AB_5 toxins can be divided into three subgroups on the basis of sequence homology and catalytic activity of the A-subunit. The first subgroup is formed by the Cholera Toxins, including Cholera Toxin (CT) and the *E. coli* heat-labile Enterotoxins (LT-I and LT-II). The Shiga Toxin (SHT) subgroup includes toxins from *Shigella dysenteriae* and the Shiga-like Toxins (SLT-I and SLT-II) excreted by certain *E. coli* strains. The third subgroup includes the Pertussis Toxin (PT) produced by *Bordetella pertussis*. PT has a very low sequence homology with the CT- and SHT-subgroups, although the structural homology is preserved. AB_5 toxins have a wide range of toxic effects on humans. Most important is the life-threatening diarrhea caused by *Vibrio cholerae* as well as the hemolytic uremic syndrome caused by members of the SHT family. Because of these toxic effects and the annually estimated million deaths,[50] there is a need to understand the mode of binding and inhibit the internalization of the toxin. A major difference between the subgroups is the number of binding sites per B-subunit. Despite the relative small size, a SLT-I and SLT-II-B subunit has three distinct binding sites (I, II and III) for the globotriose ligand **54** (Galα(1,4)Galβ(1,4)Glc). Binding sites I and II contribute most to the cytotoxicity while site III is hardly involved in binding. A CT B-subunit contains only one binding site for the GM1 ganglioside ligand which is located on the cell membranes of the human intestinal cell surface.

Important research has been performed in the design of carbohydrate derivatives that inhibit the binding of bacterial toxins to the human intestinal cell surface. GM1os analogues were synthesized and tested for the ability to prevent CT binding. These mimics ranged from close mimics of the natural ganglioside to monosaccharide derivatives of the non-reducing galactose residue. Turnbull *et al.* showed by ITC experiments the importance of this terminal galactose residue.[51] GM1os **41** was dissected into fragments and these fragments were each individually tested for their inhibition capacity (Figure 20). GM1os **41** showed a good affinity while GM2os **42**, which lacks the terminal galactose residue, showed a

dramatic decrease in affinity. Fragments **43** – **46** showed also weak affinity (Figure 20). Therefore they conclude that both the terminal galactose and the sialic acid residue are necessary for a good affinity towards CT.

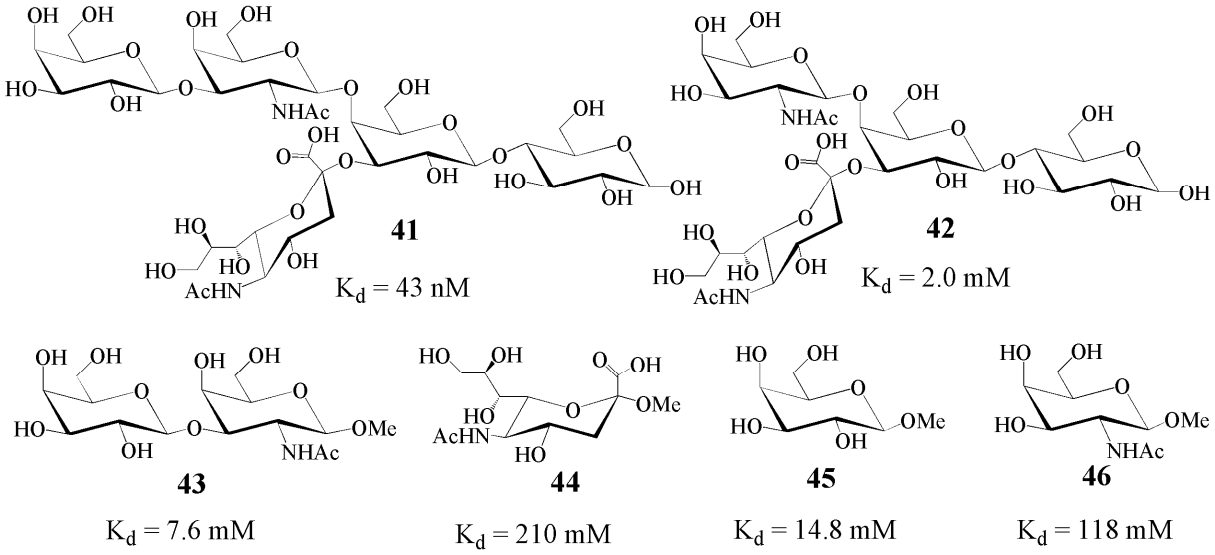


Figure 20 *Fragments of GM1os showed poor binding to CT compared to GM1os*

Bernardi *et al.* focused on GM1os mimic **47** in which the branching lactose disaccharide was replaced by a cyclohexane scaffold for correct positioning of the Gal-β(1-4)-GalNAc sequence and sialic acid residue (Figure 21).[52] The designed mimic **47** showed a comparable affinity as **41**. Despite this successful design, synthetic introduction of sialic acid remains a challenge and this mimic is therefore not suited for large scale manufacturing processes. Hol *et al.* aimed at the development of small inhibitors based on galactose.[53] A series of galactosides was prepared and *m*-nitrophenyl α-galactoside **48** (Figure 24) was found to give the highest inhibition of CT binding.

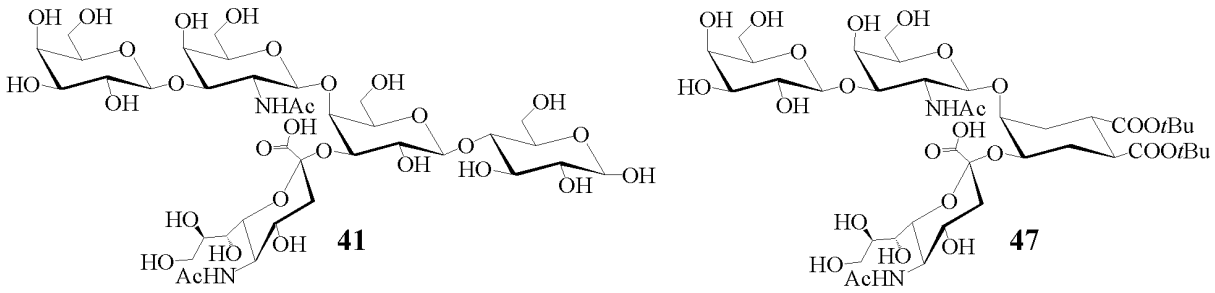
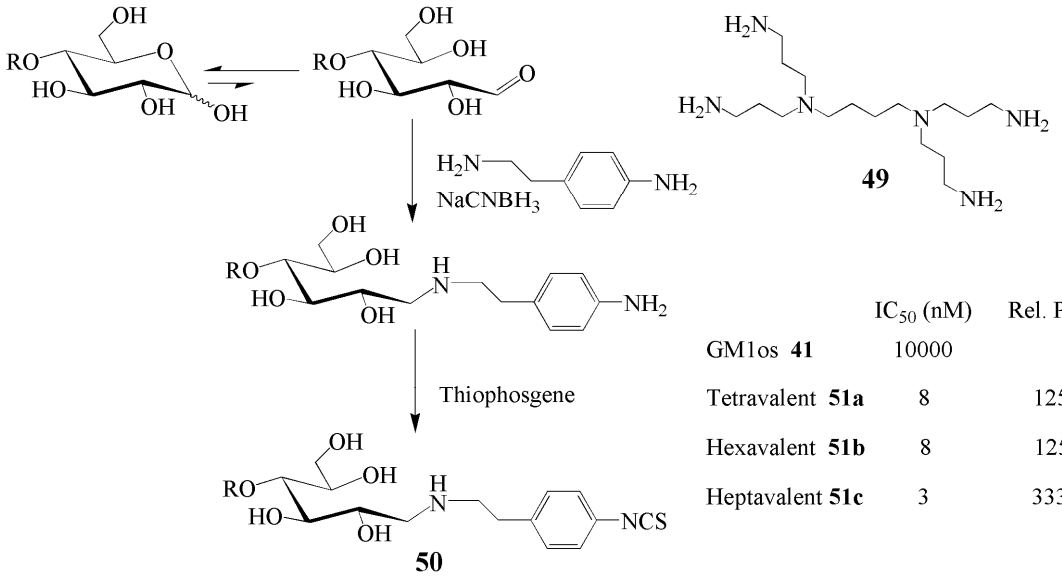


Figure 21 *GM1os compared with Bernardi's mimic*

Because of the toxin's pentameric architecture, scientists aimed at the development of multivalent inhibitors to improve the affinity. The first example of multivalent CT inhibition was published by Thompson and Schengrund (Figure 22).[54] GM1os **41** was isolated from natural sources, converted to derivative **50** and subsequently conjugated to polyamino dendrimers, such as **49**. The just prepared GM1-dendrimers **51a** - **c** were only obtained on a microscale and could therefore not be properly characterized by analytical methods like NMR and mass analysis. The affinity for CT and LT of the thus obtained dendrimers was tested by an ELISA-like assay and the IC_{50} values were compared with **41**. A clear increase in affinity was observed.

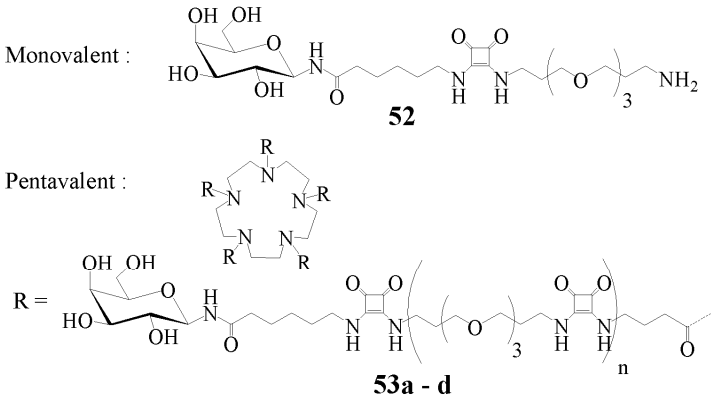


	IC_{50} (nM)	Rel. Pot. (per ligand)	
GM1os **41**	10000	1	(1)
Tetravalent **51a**	8	1250	(312)
Hexavalent **51b**	8	1250	(208)
Heptavalent **51c**	3	3333	(476)

Figure 22 *Micro scale preparation of multivalent GM1os ligands*

Hol *et al.* have developed a pentameric scaffold which was expected to match perfectly with the pentameric CTB protein.[55] The scaffold was decorated with galactose ligand **52** via a range of spacers with different length (**53a** – **d**). An inhibition study with LT showed a decrease in IC_{50} values with increasing spacer length (Figure 23). Because of solubility problems, compounds having longer spacers could not be synthesized. Improved water solubility was achieved with guanidinium-containing derivatives **54** (n = 2, 4, 6, 8) and then indeed longer spacers could be introduced (Figure 24). Compound **54** (n = 4) was found to be the most effective inhibitor (IC_{50} = 1.4 μM). A major drawback of this system was the instability of the inhibitors. The pentavalent scaffold was also used for conjugation with the

optimized *m*-nitrophenyl α-galactoside derivative **48** using this optimized spacer length to give pentavalent dendrimer **55** (Figure 24).[56]



		IC_{50} (μM)	Rel. Pot. (per ligand)	
Mono	**52**	5000	1	(1)
n = 1	**53a**	242	20	(4)
n = 2	**53b**	16	313	(63)
n = 3	**53c**	6	833	(167)
n = 4	**53d**	0.56	8929	(1786)
Galactose		58000		
GM1os	**41**	0.01		

Figure 23 *Flexible pentavalent galactose dendrimers*

The thus performed ELLA with horseradisch peroxidase conjugated CTB_5 showed a dramatic decrease in IC_{50} for the *m*-nitro-phenyl galactose derivative **55** compared to the pentavalent galactose derivative **54** (n = 4), which is 1.4 μM vs 6 nM respectively (Figure 24). This approach was the first to show a synthetic derivative with a higher inhibitory potency for CT than GM1os **41** (17 nM).

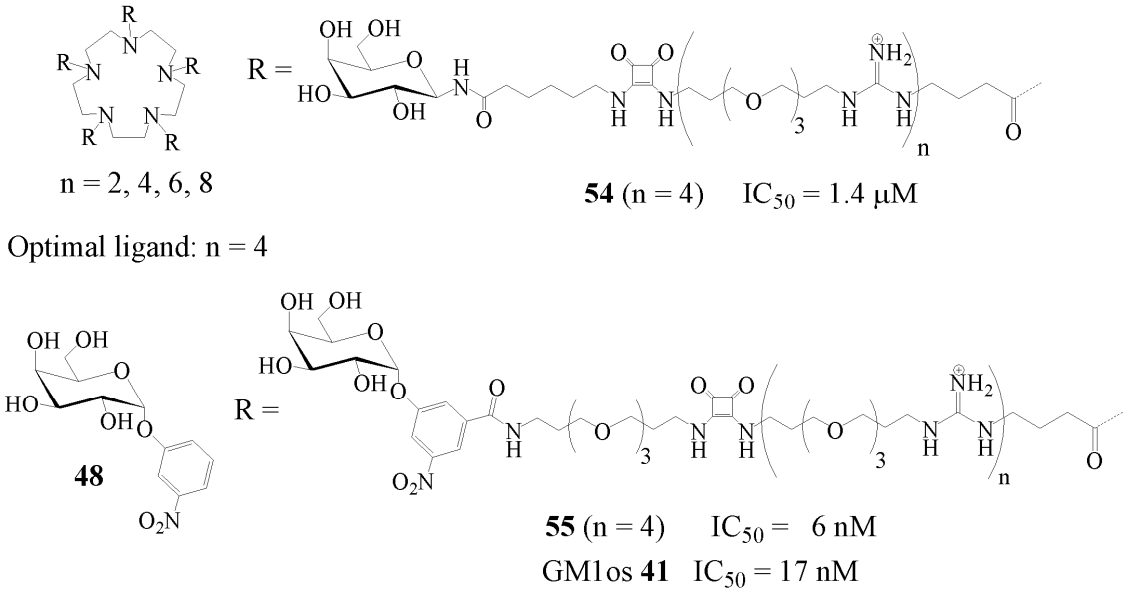
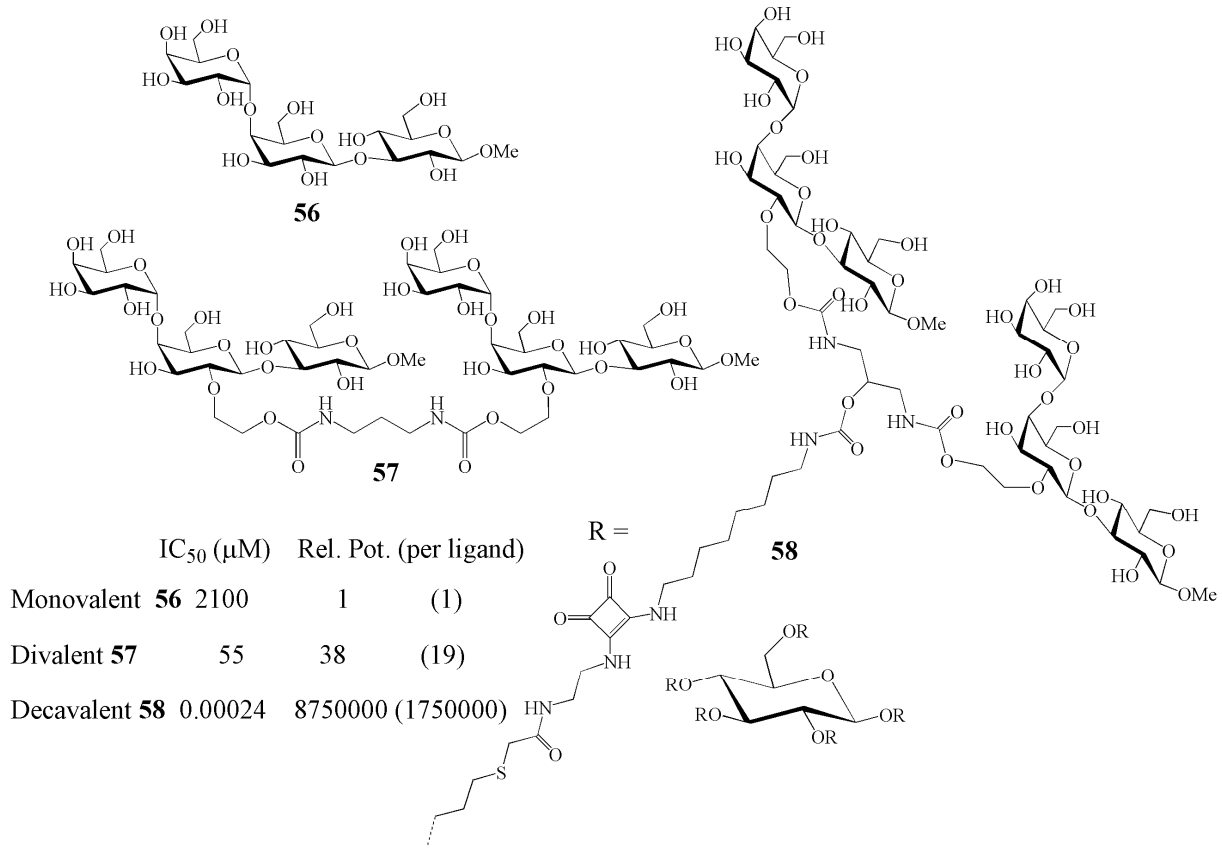


Figure 24 *Pentavalent dendrimers with improved solubility and affinity*

In 2000 Bundle *et al.* published a multivalent inhibition study with globotriose dendrimers towards SLT.[57] Globotriose **56** is a natural substrate for SLT and has a less complex structure than GM1. Monovalent compound **56** was compared to a divalent (**57**) and decavalent (**58**) system (Figure 25). Originally the ligands were expected to bind two of the three available binding sites per B subunit. Ligand **58** showed a huge multivalency effect of 8750.000 compared to monovalent **56** (Figure 25). Interestingly, crystallographic analysis revealed that one dendrimer was complexed with two SLT-proteins in a hamburger-like shape.[58] The origin of these observation is the difference in affinity for binding sites I, II and III. Earlier it was already observed that binding site II exhibits a much higher affinity for globotriose than site I. The formation of a dimer with occupation of 10 type-II binding sites was favored over complexation of a single pentamer with occupation of 5 type-I and 5 type-II binding sites.



	IC_{50} (μM)	Rel. Pot. (per ligand)	
Monovalent **56**	2100	1	(1)
Divalent **57**	55	38	(19)
Decavalent **58**	0.00024	8750000	(1750000)

Figure 25 *Decavalent ligand presentation greatly enhances the affinity towards SLT*

The just mentioned experiments by Hol *et al.* and Bundle *et al.* have been performed with pentavalent ligands because of the pentavalent protein architecture. Surprisingly, Bundle *et*

al. performed experiments that showed that octavalent inhibitors can be better inhibitors than pentavalent dendrimers.[59] The octavalent ligand **60** showed an IC_{50} of 1.26 nM which is considerably better than 410 nM for the pentavalent **59** (Figure 26).

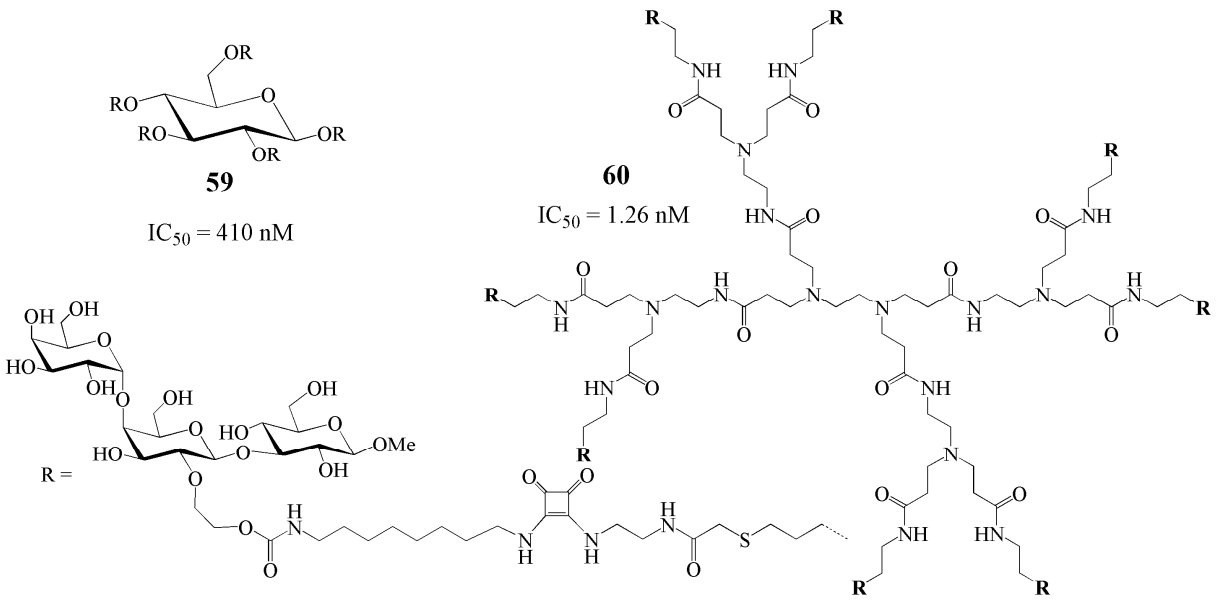


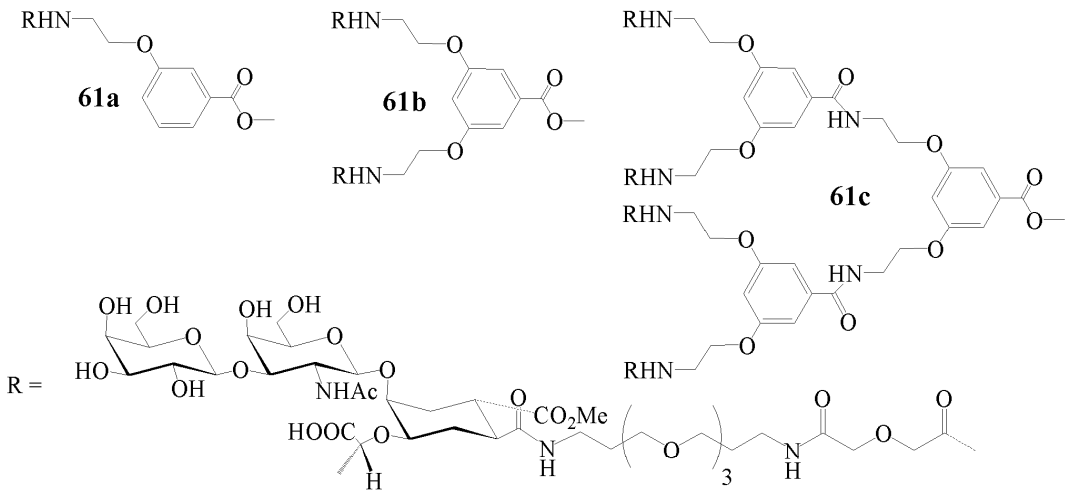
Figure 26 *Octavalent dendrimer showed enhanced affinity over a pentavalent scaffold*

From these experiments they introduced a calculation method with a statistical term called the probability of binding which is considerably higher for the octavalent dendrimer. Another explanation could be the possible aggregation that can occur with dendrimers that showed a higher valency compared to the receptor protein. More research to study this aggregation phenomena is necessary.

Bernardi *et al.* simplified the monovalent GM1os mimic **47** (Scheme 21) by the replacement of the sialic acid residue by lactic acid and mono- to tetravalent compounds **61a** - **c** were prepared to increase the affinity (Figure 27).[60] The compounds were studied by SPR and a 10 fold enhancement was observed for the divalent ligand while the tetravalent system showed a ca. 100 fold binding enhancement per ligand.

To conclude, AB_5 toxins are extremely useful proteins for the study of multivalency phenomena and the inhibitory potency of ligands can be enormously increased. Despite this increase in potency, for therapeutic use a ligand is needed with a high affinity, which can conveniently be prepared on a large scale. Chemists are still searching for this simple ligand

that fulfills both requirements. From the research reviewed above, it became clear that a multivalent ligand presentation is necessary to overcome the weak affinity of simple ligands.



	IC_{50} (μM)	Rel. Pot. (per ligand)
Monovalent **61a**	221	1 (1)
Divalent **61b**	13	17 (8.5)
Tetravalent **61c**	0.5	442 (111)

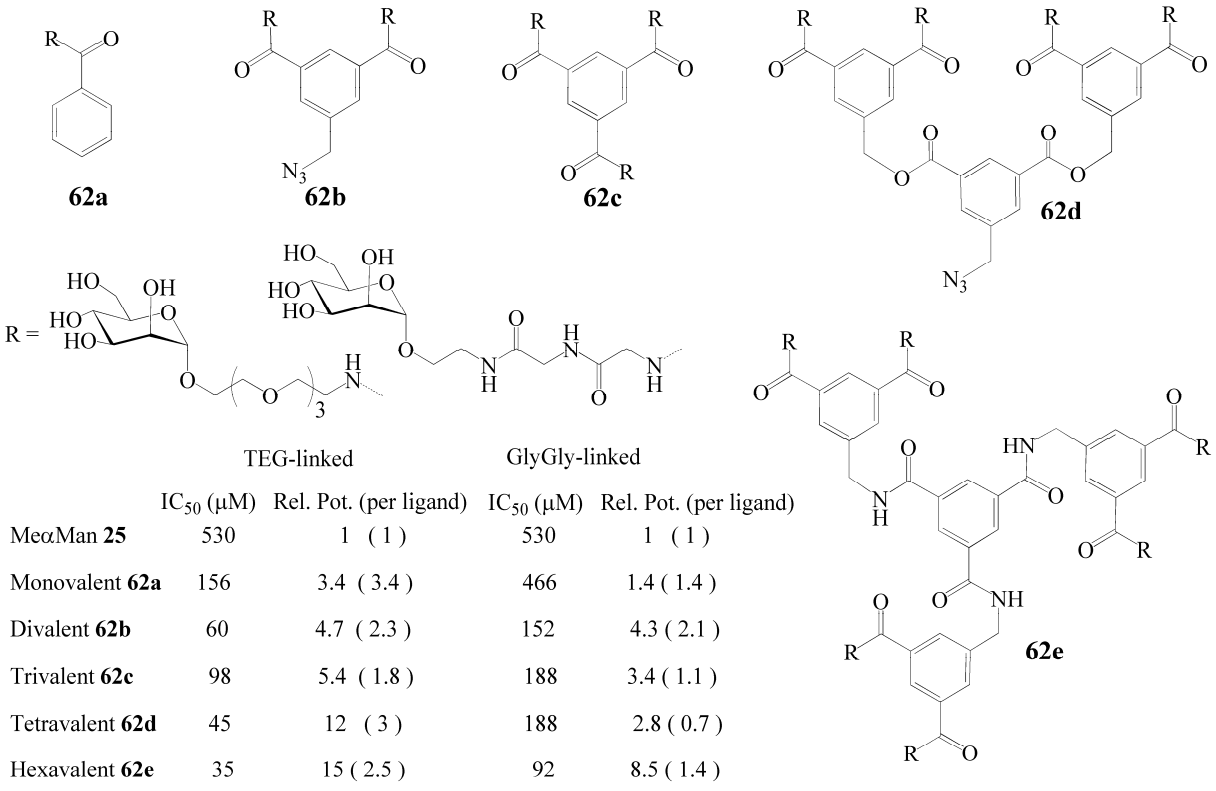
Figure 27 *Dendrimers decorated with GM1 mimics*

Multivalency and Simple Lectins

The largest most thoroughly studied subgroup of the simple lectins, are the legume lectins. They are often isolated from the seeds of the plants in which they are present. Multivalency studies were mainly performed with the lectin isolated from *Canavalia ensiformis* (ConA) as it is the prototype member of this subgroup. ConA recognizes both mannose and glucose and at neutral pH it exists as a homo-tetramer of which all four binding sites are available. Thus, in principle it can favor a multivalent ligand presentation although the binding sites are divergently oriented and spaced ca. 70Å apart.

Several multivalent ligands were prepared by various research groups to study the chelation and aggregation effects of lectins with multimeric ligands. For the binding analysis several techniques were used, like HIA, ELLA, SPR, ITC and precipitation assays. Toone *et al.* synthesized mannose-decorated glycodendrimers **62a** - **e** and analyzed their binding capacities towards ConA by HIA, ELLA and ITC (Figure 28).[11] It was found that the

observed multivalency effects depend very much on the assay used. Multivalency effects were much smaller than observed for the AB_5 toxins. This can be explained by the divergent orientation of the mannose binding sites and the relatively small size of the prepared dendrimers. This spatial orientation of the receptor sites does not allow chelation to occur with such small ligands. Lectins have in general the capacity to aggregate and the observed differences between the assay techniques are probably caused by this aggregation between dendrimers and ConA. These aggregates may be stabilized not only by dendrimers but probably also by the competitive ligand and protein-protein interactions.



	IC_{50} (μM)	Rel. Pot. (per ligand)	IC_{50} (μM)	Rel. Pot. (per ligand)
MeαMan **25**	530	1 (1)	530	1 (1)
Monovalent **62a**	156	3.4 (3.4)	466	1.4 (1.4)
Divalent **62b**	60	4.7 (2.3)	152	4.3 (2.1)
Trivalent **62c**	98	5.4 (1.8)	188	3.4 (1.1)
Tetravalent **62d**	45	12 (3)	188	2.8 (0.7)
Hexavalent **62e**	35	15 (2.5)	92	8.5 (1.4)

Figure 28 *Mannose dendrimers prepared by Toone et al., only a small multivalency effect towards ConA was observed with ELLA*[11]

Wolfenden and Cloninger [4] have prepared large PAMAM dendrimers conjugated with up to 172 mannose residues and these were tested with a standard HIA for binding to monomeric and dimeric ConA which are stable complexes at lower pH. An increase in affinity of up to 195-fold per mannose residue was observed for monomeric ConA, which must be due to a purely statistical increase in binding probability. For dimeric ConA a 770-fold increase per mannose residue was observed which can be explained by the multivalent ligand presentation. Very recently Wittmann *et al.* published the synthesis of mono- to tetravalent

GlcNAc derivatives which showed up to 1440-fold affinity enhancement towards Wheat Germ Agglutinin (WGA).[61] The WGA lectin contains eight sugar binding sites and is therefore a better target to evaluate the chelation effect with multivalent ligands.

In general, observed affinity enhancements for dendrimer-lectin binding appear to be a result of protein-dendrimer aggregation, which is dependent on the dendrimeric architecture, valency and protein concentration. The performance of the glycodendrimers is also highly dependent on the assay, as different binding partners are immobilized and their aggregation interactions vary with each type of assay. It can be concluded that large dendrimers are necessary for multivalent ConA binding by chelation because of the distance between the mannose binding sites. In addition, lectins with smaller distances between the binding sites benefit from binding to correspondingly smaller dendrimers.

Microarray Analysis

To study the exact role of carbohydrates in biological systems, extensive research has been performed ranging from the synthesis of complex glycan structures to the development of binding assays like HIA, ITC, ELLA and SPR. Although these techniques have proven to be valuable, they can be labor-intensive, they require large amounts of both carbohydrate and proteins and they are not suitable for high-throughput assays. Microarray techniques played an important role in the development of both genomics and proteomics and it is therefore reasonable to aim at microarray technology for new developments in glycomics.

Micro array surface and ligation

Microarray chips consist of a surface coated with a well defined pattern of small or large biomolecules. These chips allow in principle the automated screening of hundreds of compounds for relevant biological interactions. Microarray technology is a well-established method in both oligonucleotide[62] and peptide research.[63] Methods like the polymerase chain reaction (for nucleotides), cloning (for peptides and proteins) and solid phase synthesis are available for a fast and reliable preparation of peptides and oligonucleotides.[64] These methods do not exist for oligosaccharides and thus the development of glycan microarrays was so far limited to structurally undefined glycans from natural sources and labor-intensive chemically synthesized glycans. Recently, improved synthetic methods for

carbohydrate synthesis were developed which speed up the preparation of carbohydrate derivatives. The automated solid phase synthesizer developed by Seeberger *et al.* has dramatically decreased the time scale and in the group of Kahne *et al.* a 1300-member glyco-library has been synthesized.[65] Also, recently developed one-pot glycosylation sequences[66] will greatly stimulate the development of glycan microarrays. During the last decade the first publications on glycan microarrays have appeared and several new methods have been developed.[67] Much attention has been paid to the binding of carbohydrates to legume lectins. Lectins exhibit a distinct selectivity towards certain carbohydrate ligands but the affinity is generally low. As a proof of principle often the selective binding of lectins to their ligands is investigated. Several groups described arrays of mono- and disaccharides to discriminate between binding of different legume lectins.[68, 69, 65h]

The type of surface material and the method of glycan attachment are the major differences between microarray techniques. Most important is the surface attachment, which can be either covalently or noncovalently. Whether a ligation method is suitable for a particular glycan is dependent on the structure, the availability and the size of the glycan. A selected method always has to be compatible with the functional groups present within the glycan and the glycan source, which is either isolated from nature or chemically constructed. Several microarray surfaces have been introduced for both non-covalent and covalent immobilization, including microtiter plates, nitrocellulose membranes, functionalized glass slides, self assembled monolayers on gold and microspheres. These surfaces can directly be used for noncovalent attachment, for example plastic microtiter plates can be used as hydrophobic surface for the attachment of neoglycolipids.[65f] Indirect use involves covalent or noncovalent coating of a surface with a polymer, like dextran, BSA or streptavidin to mimic a biological environment. This bio-surface mimicry allows the introduction of a variety of functionalities which can be used for ligand immobilization. Thiol-containing molecules are well known for their ability to stick to gold surfaces. This principle is used on gold-coated surfaces, by the introduction of a spacer-molecule with a thiol at one side and a second functional group at the other side of the molecule. Most microarray procedures are based on the immobilization on glass- and gold-coated slides.

Detection of binding to a microarray surface is crucial and needs to be very sensitive because of the small amounts of material present on a surface. Several detection methods

have been developed, based on fluorescence detection. Proteins are often available as their Fluoresceine, Alexa and Cy3 conjugate. Protein labeling with fluorescent dyes can be easily performed.

Noncovalent Attachment

Noncovalent attachment is based on the association of glycans or glycosphingolipids to an appropriately prepared surface (Figure 29). Bacterial polysaccharides and proteoglycans bind for example well to nitrocellulose[70] and oxidized polystyrene.[71] Selective adsorption of glycans with hydrophobic tails (lipids, alkyl chains) to polystyrene, nitrocellulose or polyvinylidene is also possible (**A**).[65b,f] This absorption phenomenon is even better with glycans attached to perfluoro alkyl tails (**B**) for example (Figure 29).[68] Glycan derivatives containing these chains were adsorbed on a fluoroalkylsilane functionalized glass surface, and now even detergents can be used during washing steps which has improved the signal to noise ratio. Noncovalent binding based on the extremely strong streptavidin-biotin affinity has also been used and has proven to be an efficient and powerful adsorption method (**C**).[72]

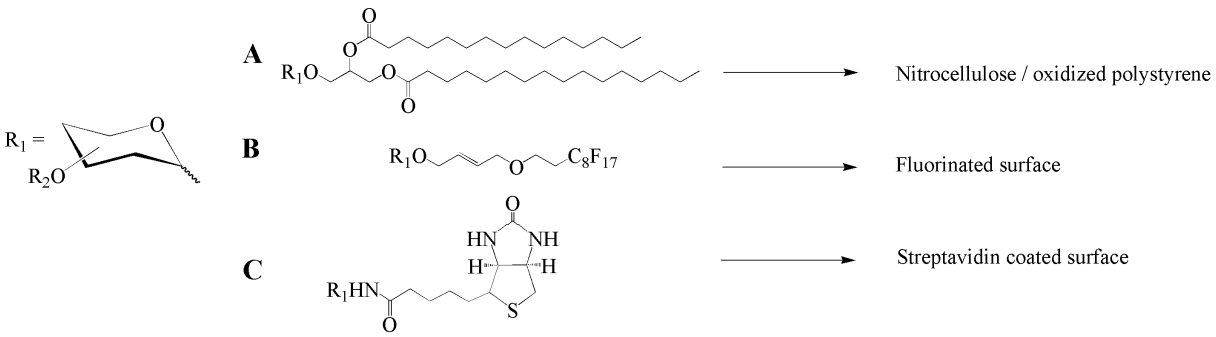


Figure 29 *Noncovalent immobilization methods used in microarray technology*

Covalent Attachment

Covalent attachment of glycans is a more robust method and is increasingly gaining interest. Covalent attachment is fueled by recent developments of selective ligation methods like the Staudinger ligation and the copper-catalyzed alkyne-azide cycloaddition, also called the "click" reaction. Glycans need to be decorated with a chemical functional group which selectively reacts with an appropriate activated surface (Figure 30). Established methods are the thiol/maleimide addition (**D**)[73] and the *N*-hydroxysuccinimide

mediated amide formation (**E**). Housemann and Mrksich applied a Diels-Alder cyclo addition as the immobilization strategy (**F**).[74] A carbohydrate conjugated with a cyclopentadiene smoothly reacted with a quinone-modified self-assembled monolayer. The "click" reaction was also used for an immobilization strategy (**G**).[75] Dotan *et al.* have explored the use of *p*-aminophenyl glycosides (H). [76] Glycosides reacted selectively with a cyanuric chloride covered glass surface and the microarrays were used for the detection of glycan antibodies. Finally Waldmann *et al.* have developed an immobilization method based on the successful Staudinger Ligation (**I**).[77] Phosphine derivatized glass slides were selectively reacted with azide-containing carbohydrates. The formed amide bond is stable and the phosphine byproduct was easily removed by washing steps.

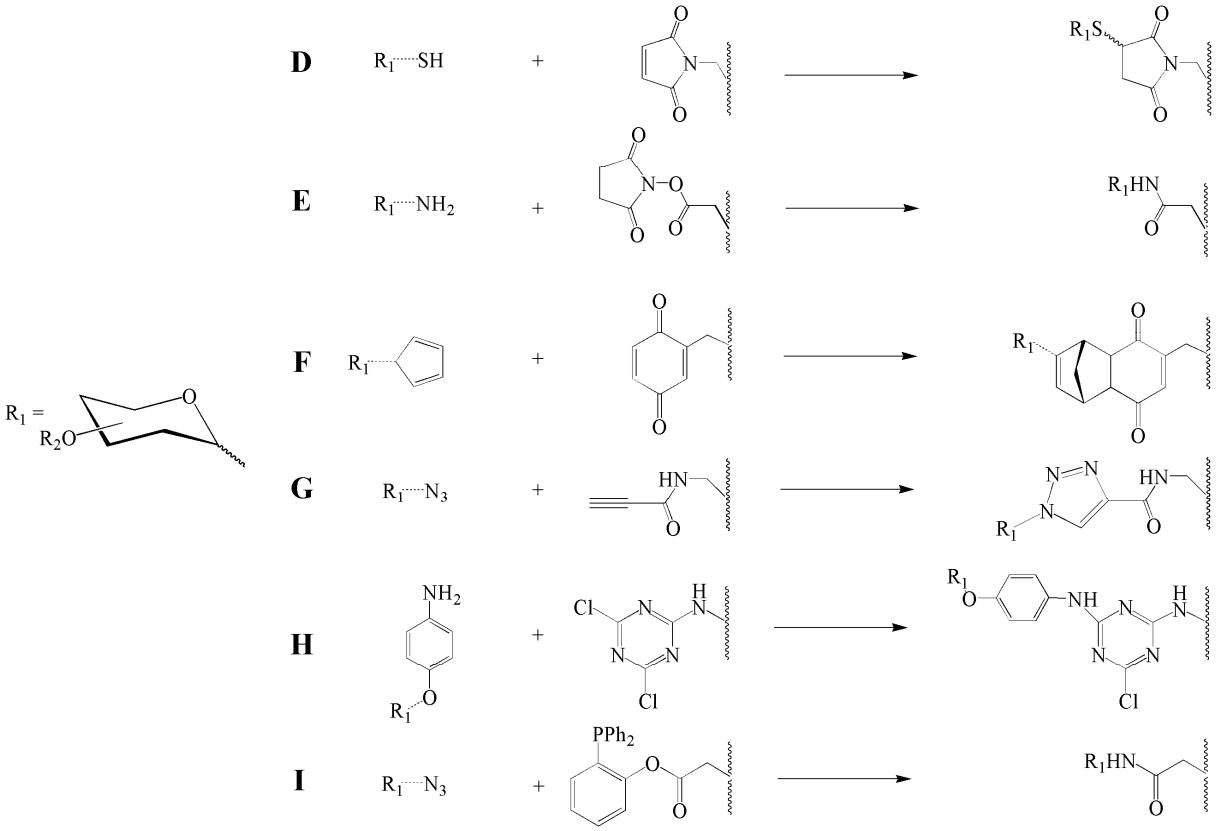


Figure 30 *Common used chemo selective covalent ligation methods*

Other research groups explored the use of unselective immobilization methods. Reactive species randomly reacted to give an appropriately coated surface. This type of ligation can be performed with a reactive group connected to the surface or to the carbohydrate ligand. Often carbene and nitrene precursors (Figure 31) were used because they can be activated upon irradiation with UV light. Carbene immobilization reactions were performed with a

chemically activated surface (**J**); an aryl trifluoro aziridine surface reacts randomly with a polysaccharide.[66] This method is valuable for covalent immobilization of large oligosaccharides. For small synthetic oligosaccharide sequences, a different method is required. Mono- and disaccharides were linked to an aryl azide unit which was allowed to react via highly reactive nitrene species with a dextran functionalized array surface (**K**) (Figure 31).[78]

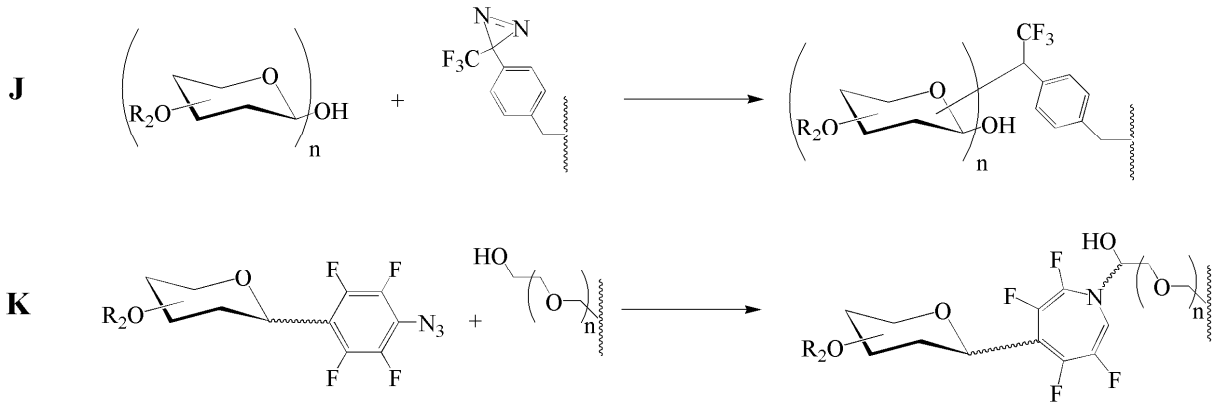


Figure 31 *Covalent ligation methods; random attachment*

Glycans present in nature are structurally complex and can be released from proteins and lipids by treatment with a specific glycosidase yielding an oligosaccharide with a free reducing end. Ligation of this oligosaccharide to a surface can be achieved by reductive amination followed by direct or indirect attachment. In general, isolated glycans from natural sources have a disadvantage; often heterogeneous glycan mixtures are obtained. This can give rise to problems with the reproducibility and therefore conclusions about glycan binding can not always be drawn. Also, it is often not known which part of the glycan is responsible for biological affinity. Organic synthesis by chemical and enzymatic steps is a reliable tool for the preparation of clearly defined both natural and unnatural glycan sequences. Relatively large quantities of structurally well-defined oligosaccharides can be obtained and a conjugation handle can conveniently be introduced during the synthesis. Driven by the need for new synthetic diverse carbohydrates, Seeberger *et al.* used the recently developed solid phase approach for the synthesis of heparin oligosaccharides with different chain length.[79] Binding studies with fibroblast growth factors FGF-1 and FGF-2 showed that FGF-2 prefers binding to higher generation heparin sequences while FGF-1 also showed binding to a 2,4-*O*-disulfated monosaccharide. Enzymatic steps are,

although restricted by substrate specificities, often combined with synthetic routes, especially for the introduction of sialic acid residues. Sialic acid is frequently present at the non-reducing end of glycoproteins and glycolipids, thus playing an important role in biological recognition processes. The chemical introduction of sialic acid residues is still a challenge while the enzymatic method, using a sialyltransferase, efficiently introduces a sialic acid to a carbohydrate sequence.

Microarrays can also be used for the detection of pathogen binding. Bacteria were labeled by cell permeable fluorescent dye's. Disney and Seeberger used mannose recognizing *E. coli* (ORN178) and a mutated *E. coli* strain (ORN209) which exhibited a lower affinity for mannose.[80] This difference in affinity to mannose was clearly observed from a mannose microarray.

As the field of glycan arrays is still expanding, there is a need for new diverse glycan libraries that can be screened in a high-throughput fashion. A combination of the recently developed synthetic and enzymatic methods will definitively speed up the generation of these libraries. Major progress has been made by the Consortium of Functional Glycomics.[81] Still there is room for improvement and major challenge will be the development of microarrays for the detection of glyco-specific antibodies, pathogenic bacteria and other disease-linked carbohydrate binding proteins that favor multivalent ligand presentation.

Outline of this Thesis

The research described so far in this chapter, deals with the most important developments in carbohydrate chemistry. However there is still room for improvement, both from a synthetic point of view and the multivalent binding analysis. This thesis covers improved synthetic methods for the construction of oligosaccharides and glycodendrimers. Several glycodendrimers have been prepared and they were analyzed for a possible multivalency effect with established methods but also novel micro array technology was introduced as a tool for high throughput screening.

Chapter 2 deals with solid phase synthesis of oligosaccharides. A new synthetic strategy for the *on-bead* synthesis of oligosaccharides was developed. Key element was the differentiation between hydroxyl functions using selective C-6 chemistry; the bulky trityl group was used for differentiation between the hydroxyl groups. After protecting group manipulations, easily accessible glycosyl building blocks were used for glycosylation. β(1,6) linked di- and trisaccharides were obtained. Despite this successful approach other protecting group manipulations have not been performed successfully.

Chapter 3 involves a new synthetic route towards galabiose dendrimers. A biocatalytic conversion was used for the cleavage of a galacturonic acid polymer. Both dimer and trimer were converted to a galabiose building block and a galactose trisaccharide respectively. The building blocks were used for the construction of novel *S. suis* adhesion inhibitors and they were evaluated for their anti-adhesion properties. The developed synthetic route showed several practical and economical advantages over the previously reported synthesis.

Both chapters 4 and 5 deal with the development of multivalent inhibitors capable of binding Cholera Toxin. Chapter 4 describes the use of the natural GM1os ligand. Dendrimers with optimized spacer arms were synthesized and azide functionalized GM1os was coupled to the dendritic scaffolds via “click” chemistry. The compounds were tested for their inhibition properties with an ELLA. The octavalent GM1 derivative showed a 47,500 fold improved affinity for CT compared to GM1os. A comparable trend was observed for GM2 dendrimers. Chapter 5 describes the synthesis of easy accessible galactose building blocks which were ligated to dendrimers via “click” chemistry. The dendrimers were tested for their inhibition capacity against Cholera Toxin. Both tetra and octavalent galactose dendrimers showed an affinity for CT comparable with GM1os, thereby showing that relative simple synthetic multivalent compounds can compete with natural CT ligands. Besides this, a series of divalent compounds was prepared with different spacer lengths and these compounds were used for identification of the most optimal spacer length. None of the synthesized compounds showed good inhibition properties, indicating that the used spacers were too short for bridging the GM1 binding sites.

Finally chapter 6 describes the application of novel flow-through microarray technology for binding studies with multivalent mannose compounds. Amine-terminated mannose dendrimers were synthesized by "click" ligation and conjugated to a maleimide-coated aluminium oxide surface. Both ConA and the GNA were tested for multivalent binding. As expected no multivalency effect was observed for binding to ConA. Binding towards GNA was shown to benefit from a multivalent display as an improved binding towards the tetravalent and octavalent dendrimers was observed compared to the monovalent and divalent dendrimers.

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81 http://www.functionalglycomics.org

Chapter 2

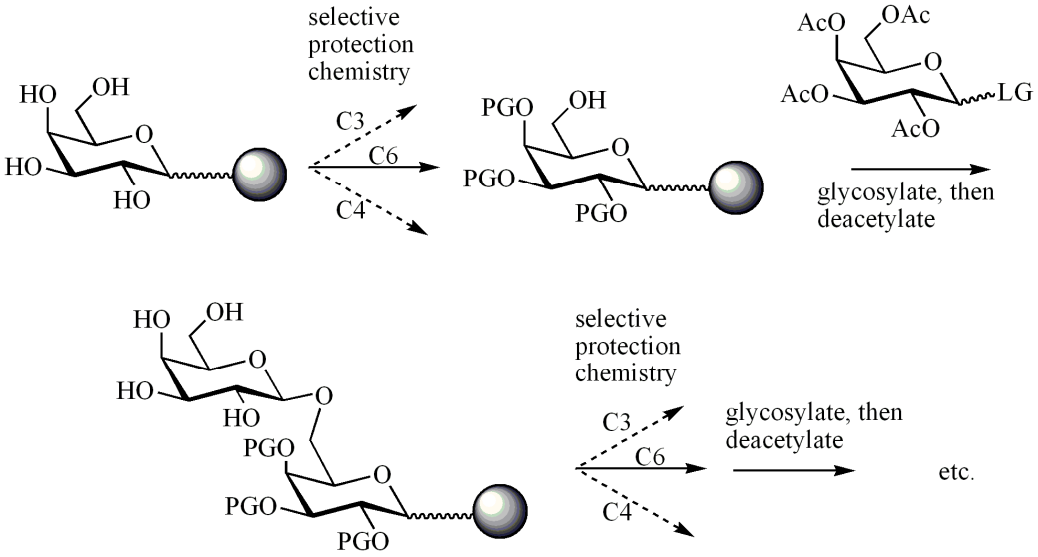
Solid-phase carbohydrate synthesis via *on-bead* protecting group chemistry

The results in this chapter have been published:

Hilbert M. Branderhorst, Rob M. J. Liskamp, Roland J. Pieters; Solid-phase carbohydrate synthesis via *on-bead* protecting group chemistry; *Tetrahedron* **2007**, 4290-4296

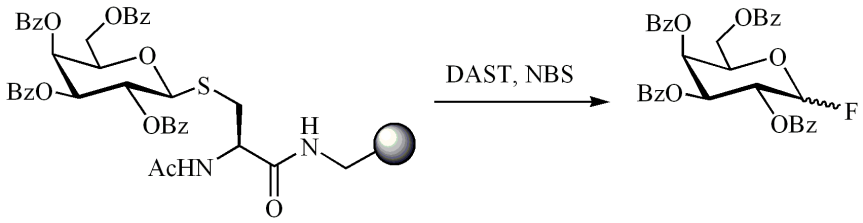
Introduction

The difficulties in synthesizing complex synthetic carbohydrates limits our understanding of their roles in biological systems and also limits carbohydrate-based drug development. Carbohydrates are nevertheless emerging as a class of compounds which play crucial roles in major diseases such as (avian) flu,[1] AIDS,[2] and cancer.[3] The shift of the synthesis of oligomeric nucleotides and peptides to the solid-phase, and subsequent automation, has led to major steps forward in their ease of preparation and application in biology. For carbohydrates, a universal solid phase synthetic method is more difficult but would have a great scientific impact. Solid phase synthesis of carbohydrates was first reported in 1971[4] and since then much progress was made.[5] The recent automation of the process represents a milestone.[6] All published strategies have in common that they are based on a 2-step protocol which includes an *on-bead* glycosylation step and an *on-bead* deprotection step. This means that strategically protected carbohydrate donors are used for glycosylation which, after coupling, can selectively be deprotected at the desired position to afford a new glycosyl acceptor for further elaboration. Although connecting the building blocks one by one is a rapid procedure, the synthesis of all the desired building blocks comprises a major effort to be customized for each desired target carbohydrate.



Scheme 1. *General solid phase synthesis of carbohydrates including a selective on-bead protection step followed by glycosylation, deprotection and reiteration. A selective C6 protection step is depicted as this selective step is used in this chapter.*

In response to this, we opted to explore the use of resin-bound unprotected sugars to be selectively converted to acceptors by *on-bead* selective chemistry commonly used in solution (Scheme 1). In principle, a single carbohydrate can thus be converted into several disaccharides depending on the protecting chemistry used. Such selective chemistry would have to be sequence independent, and based on an excess of reagents to push reactions to completion. If these procedures prove to be simple and compatible with automation, many complex sugars, including branched ones, can be made from a few building blocks by reiteration of the steps. To take the first steps towards this rather distant goal, we started with the well-studied selective C-6 chemistry, based on the bulky 4,4-dimethoxytrityl (DMT) protecting group that can selectively be introduced on primary hydroxyl groups, even when excess of DMT-Cl was used. The DMT-group was chosen for the mild cleavage conditions (1% dichloroacetic acid in CH_2Cl_2) and the visible color release by the DMT-cation that can be used for verification. As a linker, the trifunctional amino acid cysteine **1** was chosen. Its sulphur atom can be linked to the sugar anomeric center.[7] After all synthetic procedures, this thioglycosidic linkage can selectively be cleaved by *N*-bromosuccinimide (NBS) and a variety of nucleophiles.[8] The cleavage conditions we used involved DAST (diethylaminosulfur trifluoride), which yielded the anomeric fluorides that can be further functionalized (Scheme 2). Another attractive feature of the cysteine linker is its amino group that was Fmoc protected, and thus a useful tool for determination of the loading upon deprotection. Furthermore, the carboxylic acid function of cysteine was used for coupling the construct to an amine functionalized polystyrene resin.

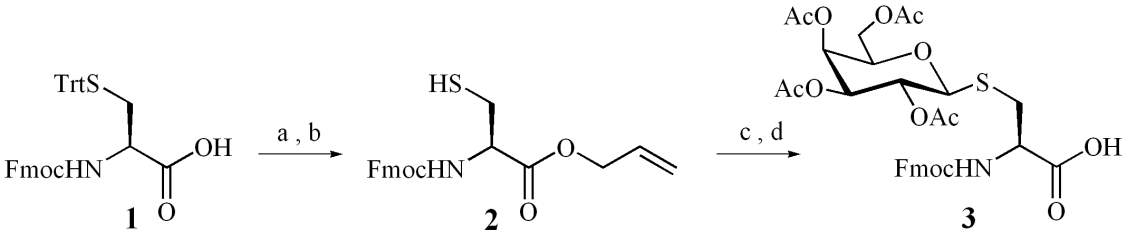


Scheme 2. *Cleavage conditions used for the cysteine bound carbohydrates from the resin by DAST and NBS.*

Results and Discussion

S-Linked Galactoside Acceptor Route

The *S*-linked amino acid **3** was synthesized starting from commercially available Fmoc-Cys[S-trityl]-OH **1** (Scheme 3). Its carboxylic acid group was first protected as the allyl ester by allyl bromide and K_2CO_3 as the base. The trityl group was removed under acidic conditions and the acetylated carbohydrate was introduced by the action of $SnCl_4$ as previously reported.[9] The allyl ester was removed by $Pd(PPh_3)_4$ using anilinium toluenesulphinate as a scavenger, yielding **3**.

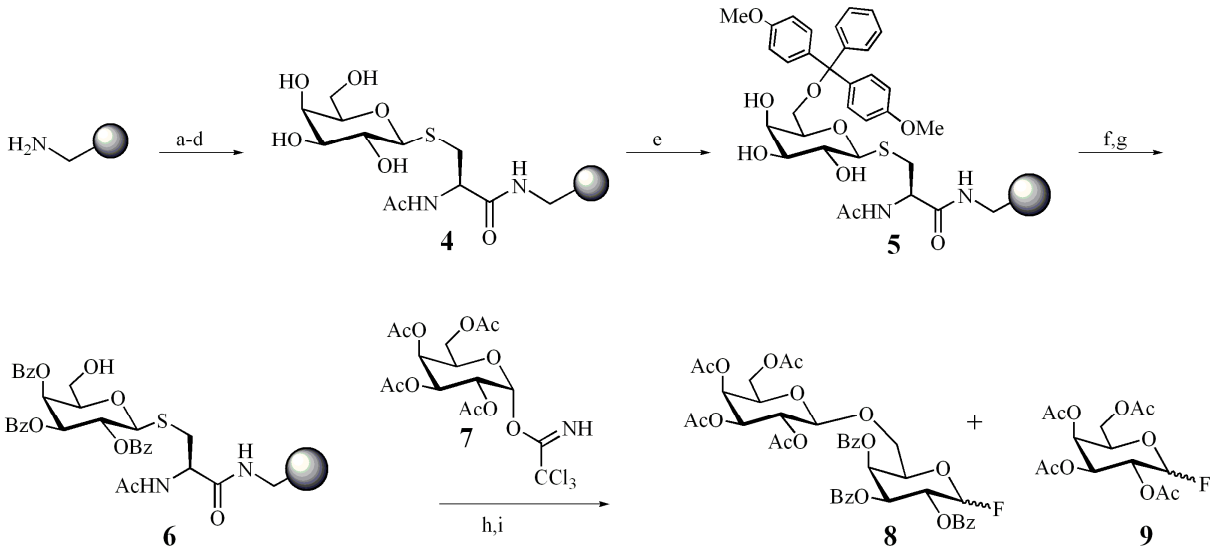


Scheme 3. *Synthesis of amino acid **3**; reagents and conditions: a) allyl bromide, K_2CO_3, DMF; b) TFA, tri-ethyl silane, CH_2Cl_2, 64% over 2 steps; c) Galactose penta-acetate, $SnCl_4$, CH_2Cl_2; d) $Pd(PPh_3)_4$, anilinium toluenesulphinate, quant. over 2 steps.*

Amino acid **3** was coupled to an aminomethylated polystyrene resin using the HATU coupling reagent and DiPEA (Scheme 4). By Fmoc deprotection analysis, a resin loading of 0.70 mmol/g was determined. The Fmoc group was cleaved by 20% piperidine in NMP and the free amine was subsequently acetylated with acetic anhydride. The carbohydrate was deacetylated using 5% N_2H_4 in DMF to yield resin **4**. To verify its identity and evaluate the DAST/NBS cleavage method, the carbohydrate was fully benzoylated with benzoyl chloride in pyridine. Subsequent treatment of the resin with NBS (5 equiv.) and DAST (5 equiv.) for 10 min afforded the desired anomeric fluoride (Scheme 2).

Introduction of the DMT-group was achieved by treating resin **4** with 10 equiv. of DMT-Cl in pyridine. The remaining hydroxyl functions of resin **5** were capped with benzoyl chloride in pyridine followed by cleavage of the DMT group with 1 % dichloro acetic acid in CH_2Cl_2 to yield resin **6**. To verify the identity of this resin, part of it was acetylated (Ac_2O) and cleaved (DAST/NBS) to afford the expected intermediate. This confirmation set the stage for the glycosylation reaction of **6** for which galactose donor **7** (5 equiv.) was used in

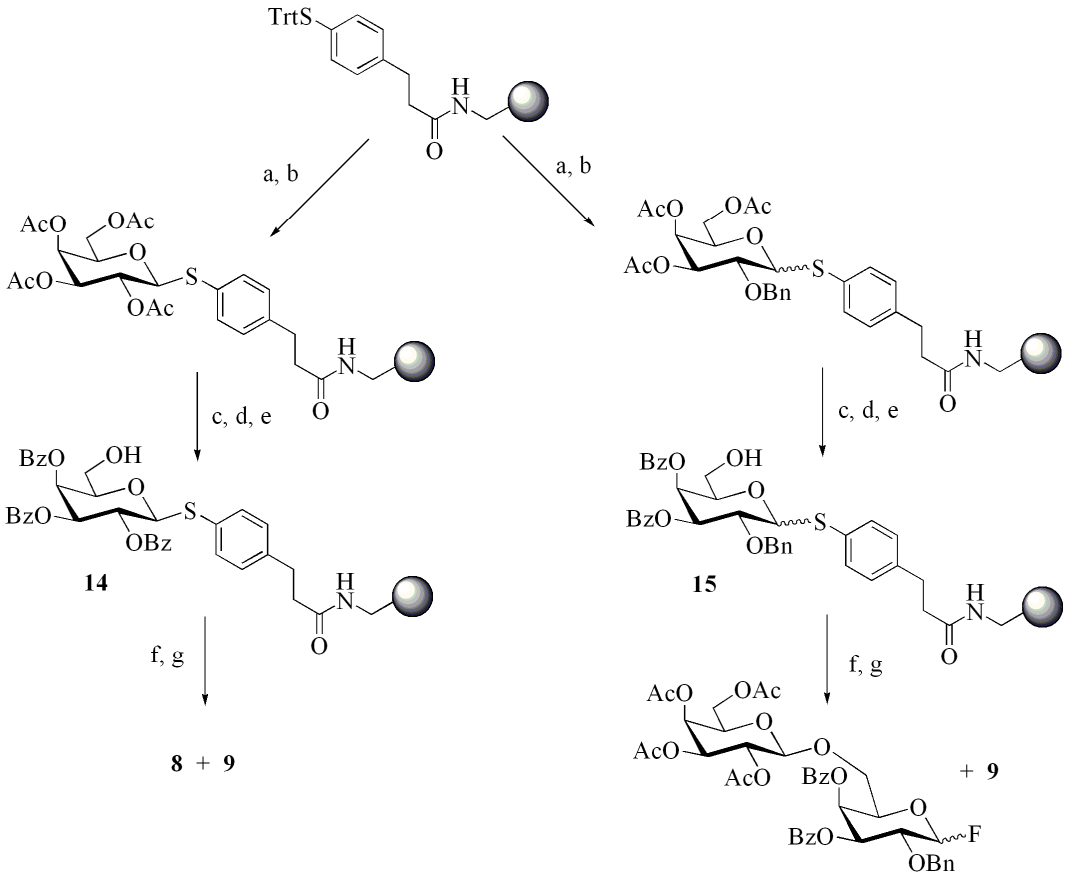
the presence of TMSOTf (0.3 equiv.) as a promoter. The procedure was run twice and was followed by NBS/DAST cleavage of the products from the resin. Product analysis revealed that indeed the desired disaccharide **8** was obtained but also the peracetylated glycosyl fluoride **9**. The reaction was run several times and the ratio of **8**:**9** varied widely between 1:1 to 9:1. The formation of **9** can be explained by an unwanted aglycon transfer mechanism, as has been noticed before for reactions in solution.[10] It is believed to result from the relatively high nucleophilicity of the sulphur atom that can to some extent outcompete the free OH of the acceptor. Modifications of the glycosylation conditions, could not prevent the formation of **9**. Furthermore the use of the analogous perbenzylated donor did not yield any product.



Scheme 4. *Reaction conditions: a)* ***3****, HATU, DiPEA; b) piperidine, NMP; c)* Ac_2O*, pyridine; d)* N_2H_4*, DMF; e) DMT-Cl, pyridine; f) benzoyl chloride, pyridine; g) 1% dichloro acetic acid,* CH_2Cl_2*; h)* ***7****, TMSOTf,* CH_2Cl_2*; i) DAST, NBS.*

In solution, the unwanted aglycon transfer was suppressed by changing from an anomeric thioalkyl group to a thioaryl group.[10a] Furthermore, changing the protecting group pattern was also shown to suppress the transfer.[10b] To this end, two new resin bound acceptors **14** and **15** were prepared as shown in Scheme 5. The first modification was the introduction of an arylthio linkage as in **14**. In **15**, an additional modification was made. Replacing the participating benzoyl group for a non-participating benzyl group was expected to disfavor the aglycon transfer reaction. Both resins were prepared from the commercially available 3-

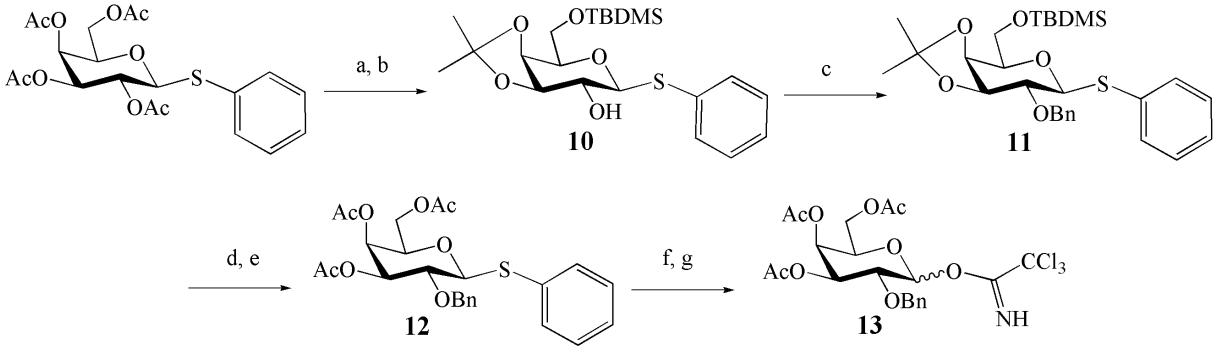
[4-(tritylmercapto)phenyl]-propionyl aminomethyl functionalized polystyrene resin, starting with removal of its trityl group. Subsequent glycosylation with glycosyl donors **7** and **13** (Scheme 6) and deacetylation followed by the C-6 chemistry protocol of Scheme 4, yielded the two acceptor resins **14** and **15**. The glycosylation of these resins with **7** as before, followed by NBS/DAST cleavage yielded the disaccharide **8** but unfortunately also the aglycon transfer product **9**. In both cases **9** was obtained in the range of 40-80%.



Scheme 5. *Reaction conditions: a)TFA/CH_2Cl_2; b) per acetylated galactose, $BF_3.Et_2O$ / **13**, TMSOTf; c) N_2H_4, DMF; d) i. DMT-Cl, pyridine; ii. benzoyl chloride, pyridine; e) 1% dichloro acetic acid, CH_2Cl_2; f) **7**, TMSOTf, CH_2Cl_2; g) DAST, NBS.*

Glycosyl donor **13** (scheme 6) was synthesized from available phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside. After deacetylaion with NaOMe in MeOH the isopropylidene bridge was introduced with 2,2-dimethoxypropane in DMF by *p*-TsOH catalysis. Selective silylation gave **10** which was benzylated with benzyl bromide afforded **11**. Both temporary protecting groups were removed under acidic conditions and the hydroxyl groups were acetylated in pyridine and acetic anhydride. Thioglycoside **12** was

hydrolyzed by NBS in acetone/H_2O and the anomeric position was converted to trichloroacetimidate **13** by DBU and trichloro acetonitrile.

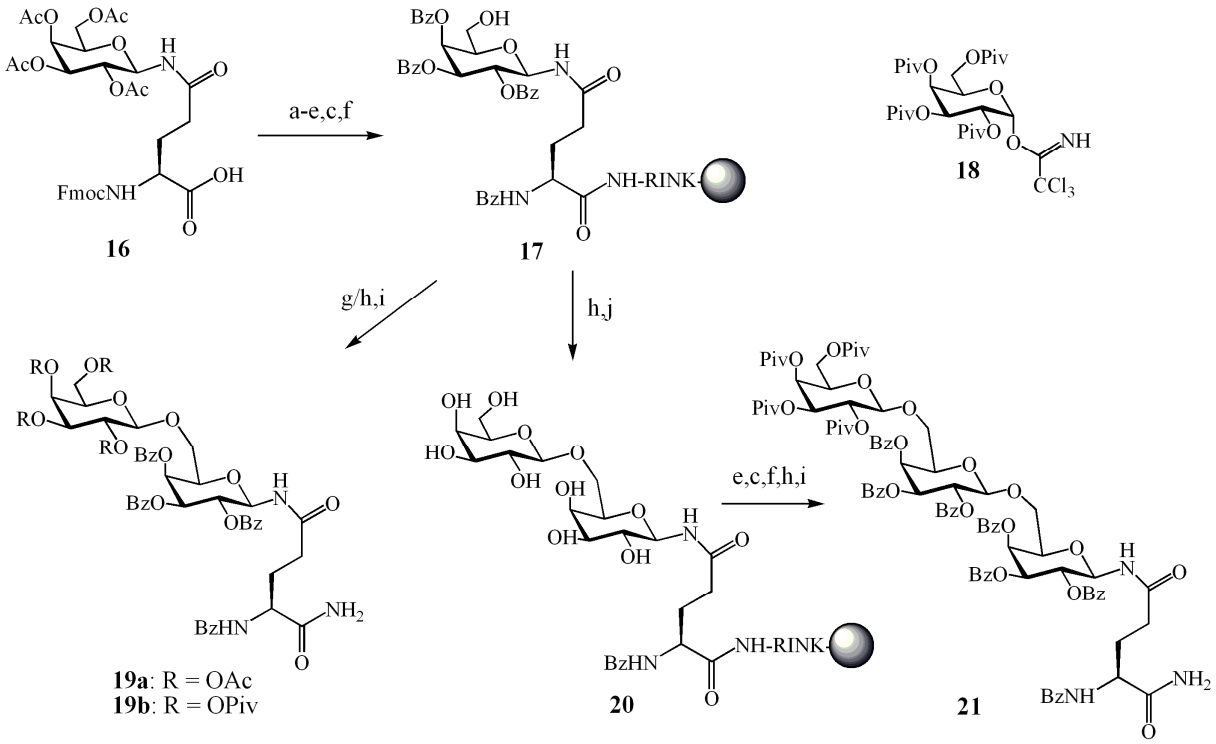


Scheme 6. *Reaction conditions: a) i. NaOMe, MeOH; ii. 2,2-dimethoxypropane, p-TsOH, DMF, 79%; b) tert-butyl dimethyl silyl chloride, Et_3N, DMAP, CH_2Cl_2, 92%; c) benzyl bromide, NaH, DMF, 95%; d) p-TsOH, MeOH/CH_2Cl_2; e) acetic anhydride, pyridine, 87% over 2 steps; f) NBS, acetone/H_2O, 77%; g) trichloro acetonitrile, DBU, CH_2Cl_2, 94%.*

N-Linked Galactoside Acceptor Route

In order to avoid the aglycon transfer, a more stable glycosidic linkage was used based on an anomeric glutamine linkage to the resin. While this modification no longer allows anomeric manipulation/conjugation of the synthesized carbohydrate, the glycopeptides that can result from this pathway are highly versatile and useful building blocks. Firstly, building block **16** [11] was coupled to a Rink amide linker of a polystyrene resin (Scheme 7). The attached glyco-amino acid is cleavable at the α-carboxy function by TFA. The coupling of **16** to the resin was performed by HATU and DiPEA. The subsequent reaction was a piperidine-mediated Fmoc cleavage. A loading of 0.48 mmol/g was determined at this stage. The liberated amine was capped with benzoyl chloride and the acetates were removed by 5% N_2H_4 in DMF. Selective tritylation of the primary hydroxyls was found to work best using 5 equiv. DMT-Cl in pyridine as a solvent. As before, all remaining hydroxyl functions were capped by benzoylation and the primary hydroxyl was liberated by dichloro acetic acid to yield resin **17**. Glycosylation of the resulting acceptor **17** with donor **7** (5 equiv.) and TMSOTf (0.3 equiv.) as a promoter yielded the expected disaccharide **19a** which was isolated by silica chromatography. The product was obtained in ca. 25% yield (i.e. 86% per reaction step). Since ortho ester formation is a possible side reaction, the glycosylation was repeated with pivaloyl protected donor **18**, which is less prone to ortho

ester formation. Again the desired disaccharide (**19b**) was obtained after HPLC purification in a slightly higher overall yield of 30% (i.e. 88% per reaction step). Using this strategy the procedure was reiterated for the synthesis of a trisaccharide. The resin bound disaccharide was subjected to NaOMe deacylation to give **20**, followed by the selective C-6 chemistry reaction sequence and, after glycosylation with **18** the desired trisaccharide **21**, was isolated after cleavage from the resin and straightforward HPLC purification.



Scheme 7. *Reaction conditions: a) Rink-polystyrene resin, HATU, DiPEA, DMF; b) piperidine, NMP; c) benzoyl chloride, pyridine; d) N_2H_2, DMF; e) DMT-Cl, pyridine; f) 1% dichloro acetic acid, CH_2Cl_2; g)* ***7****, TMSOTf, CH_2Cl_2, 0°C; h)* ***18****, TMSOTf, CH_2Cl_2; i) TFA, H_2O (95/5); j) NaOMe, MeOH*

Conclusions

The solid phase synthesis of 1,6-linked galactosides proved possible with resin-bound unprotected sugar **4** as the starting point. Selective resin compatible protection chemistry involving the C6 primary hydroxyl yielded acceptors that could be glycosylated. The synthesis greatly benefited in the practical sense, from being performed *on-bead* and therefore the steps require only washing procedures. Glycosylation thus led to glutamine-linked disaccharides, and finally a glutamine-linked trisaccharide preparation demonstrated

that reiteration of the procedure was possible. The synthesis with cysteine as the linking moiety connecting the sugar to the resin suffered from unwanted aglycon transfer, even remedies that had been effective in solution were not able to fully prevent this transfer. Recently reported insights suggest that the use of two methyl groups flanking the sulfur on the aromatic ring would be sufficient to prevent the transfer.[10c] When glutamic acid was used to link the sugar, stable glycopeptidic products were obtained from the applied reaction sequence that could be cleaved from the resin via the amino acid's carboxyl group. The work indicates that protecting group chemistry can be performed on-resin and has the potential for the synthesis of a wide variety of oligosaccharides from a limited set of glycosyl donor molecules. To make this chemistry applicable it needs to be extended to selectively produce other acceptors *on-bead*. As such the versatile 4,6-*O*-benzylidene group was successfully generated *on-bead* using an excess of reagents (not shown), although reproducibility needs to be improved. In conclusion, the presented work is an initial step in the exploration of *on-bead* carbohydrate synthesis that goes beyond glycosylation and deprotection steps, and may ultimately reduce the labor involved in the building block synthesis.

Experimental Section

General Remarks

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and were stored on molecular sieves (4Å). Pyridine was purchased from Acros (Geel, Belgium) and stored over molecular sieves (4Å). Both aminomethyl polystyrene resin and Rink-amide functionalized aminomethyl polystyrene resin were obtained from Rapp Polymere (Tübingen, Germany). 3-[4-(Tritylmercapto)phenyl]-propionyl aminomethyl functionalized polystyrene resin was obtained from Novabiochem (Läufelfingen, Switzerland). A solution of Ac_2O (4.72 mL), DiPEA (2.18 mL) and HOBt (230 mg) in NMP (100 mL) was used as the capping mixture. TLC was performed on Merck precoated Silica 60 plates. Spots were visualized by UV light and by 10% H_2SO_4 in MeOH. Analytical HPLC runs were performed on a Shimadzu automated HPLC system equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories) and a UV/VIS detector operating at 220 and 254 nm. Preparative HPLC runs

were performed on a Gilson HPLC workstation. ^{1}H NMR (300MHz) and ^{13}C NMR (75MHz) were performed on a Varian G-300 spectrometer and ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) were recorded on a Varian INOVA-500 spectrometer. Chemical shifts are given in ppm relative to TMS (^{1}H, 0.00 ppm) and $CDCl_3$ (^{13}C, 77.0 ppm). Assignments of the NMR spectra of **19** and **20** were made on the basis of their HSQC, HMBC and TOCSY spectra.

Fmoc-Cys-OAllyl (2): To a solution of Fmoc-Cys[Trt]-OH (5.86 g, 10 mmol) and K_2CO_3 (1.38 g, 10 mmol) in dry DMF (50 mL) was added allyl bromide (1.74 mL, 20 mmol). TLC showed complete conversion after 2 h. The mixture was concentrated *in vacuo* and then taken up in EtOAc (100 mL). The organic solution was washed with 1M $KHSO_4$ (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. Column chromatography on silica gel (hex/EtOAc, 7/1 → 2/1) yielded Fmoc-Cys[Trt]-OAllyl as a white foam. ^{1}H NMR was consistent with ref. 12. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.76 – 7.19 (23 H, m, CH_{arom}), 5.92 – 5.81 (1H, m, OCH_2CHCH_2), 5.33 (1H, d, Fmoc NH, *J* = 0.9 Hz), 5.27 – 5.23 (2H, m, OCH_2CHCH_2), 4.62 (d, 2H, OCH_2CHCH_2), 4.42 – 4.35 (3H, m, Fmoc CH_2, CHα), 4.23 (1H, t, Fmoc CH, *J* = 6.9 Hz)) and 2.66 (2H, m, CH_2β). To a solution of Fmoc-Cys[Trt]-OAllyl (≈ 9 mmol) and tri-ethyl silane (7.26 mL, 45 mmol) in CH_2Cl_2 (100 mL) was added TFA (3.35 mL, 45 mmol) drop wise over 5 min and the mixture was stirred for additional 2 h. TLC (hex/EtOAc 3/1) showed completion of the reaction. The mixture was concentrated *in vacuo* and co-evaporation with toluene, ethanol and CH_2Cl_2 yielded crude **2**. Silica gel chromatography (CH_2Cl_2/hex, 4/1 → 9/1) yielded the product as a white amorphous solid (2.45 g, 64% over 2 steps). ^{1}H and ^{13}C NMR was consistent with ref. 13. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.77 –7.32 (8H, m, CH_{arom}), 5.97 – 5.88 (1H, m, OCH_2CHCH_2), 5.71 (1H, br d, Fmoc NH, *J* = 7.4 Hz), 5.39 – 5.27 (2H, m, OCH_2CHCH_2), 4.71 – 4.63 (3H, m, OCH_2CHCH_2 and CHα), 4.44 (2H, d, Fmoc CH_2, *J* = 1.1 Hz), 4.23 (1H, t, Fmoc CH, *J* = 6.9 Hz), 3.05 – 2.99 (2H, m, CH_2β) and 1.37 (1H, t, SH, *J* = 8.8 Hz)). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 141.3, 127.8, 127.1, 125.1 and 120.0 (C_{arom}), 131.2 (OCH_2CHCH_2), 119.4 (OCH_2CHCH_2), 67.1 (OCH_2CHCH_2), 66.5 (Fmoc CH_2), 55.2 (Cα), 47.1 (Fmoc CH) and 27.2 (CH_2β).

Fmoc-Cys[Gal(OAc)$_4$]-OH (3): To a solution of **2** (950 mg, 2.48 mmol) and β-D-Galactose penta-acetate (1.93 g, 4.95 mmol) in dry CH_2Cl_2 (40 mL) was added drop wise $SnCl_4$ (0.42 mL, 3.72 mmol). Immediately a yellow color appeared. The mixture was stirred for 4 h. TLC (hex/EtOAc, 1/1) indicated the complete conversion of **2** to a single product. The mixture was diluted with CH_2Cl_2 (100 mL) and washed with 5% $NaHCO_3$ solution (75 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Column chromatography on silica gel was used to purify Fmoc-Cys[Gal(OAc)$_4$]-OAllyl but the excess of galactose penta-acetate could not completely be removed. ^{1}H and ^{13}C NMR was consistent with ref. 14. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.76 –7.27 (8H, m, CH_{arom}), 5.99 (1H, br d, Fmoc NH, *J* = 7.8 Hz), 5.95 – 5.84 (1H, m, OCH_2CHCH_2), 5.37 (1H, d, H-4, $J_{3,4}$ = 3.9 Hz), 5.34 – 5.18 (3H, m, OCH_2CHCH_2 and H-2), 5.01 (1H, dd, H-3, $J_{2,3}$ = 10.2 Hz, $J_{3,4}$ = 3.3 Hz), 4.66 (2H, d, OCH_2CHCH_2), 4.60 – 4.54 (1H, m, CHα), 4.45 (1H, d, H-1, $J_{1,2}$ = 9.9 Hz), 4.39 – 4.34 (2H, m, Fmoc CH_2), 4.25 (1H, t, Fmoc CH, *J* = 6.6 Hz), 4.05 (2H, d, 2 H-6, $J_{5,6}$ = 6.3 Hz), 3.76 (1H, t, H-5, $J_{5,6}$ = 6.3 Hz), 3.29 (1H, dd, CHβ, *J* = 3.9 Hz, *J* = 14.7 Hz), 3.06 (1H, dd, CHβ, *J* = 6.9 Hz, *J* = 14.1 Hz), 2.12, 2.06, 2.00 and 1.93 (4 x 3H, 4 x s, $C(O)CH_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.3, 169.9 and 169.7 ($C(O)CH_3$), 155.9 (OC(O)NH), 141.3, 127.7, 127.1, 125.0 and 120.0 (C_{arom}), 131.3 (OCH_2CHCH_2), 118.9 (OCH_2CHCH_2), 83.5 (*C*-1), 71.5, 66.8, 66.7 and 66.3 (C-2, C-3, C-4 and C-5), 67.0 (OCH_2CHCH_2), 66.3 (Fmoc CH_2), 61.5 (*C*-6), 55.2 (CHα), 47.0 (Fmoc CH), 31.8 (CH_2β) and 20.5 ($C(O)CH_3$). A solution of crude Fmoc-Cys[Gal(OAc)$_4$]-OAllyl (≈ 2.48 mmol) and anilinium toluenesulfinate (0.68 g, 2.73 mmol) was stirred in dry THF (40 mL) under an argon atmosphere. $Pd(PPh_3)_4$ (80 mg, 0.07 mmol) was added under argon. After stirring for 2 h, TLC indicated 100 % conversion. The bright yellow mixture was concentrated *in vacuo*, dissolved in CH_2Cl_2 (100 mL), and washed twice with 1M HCl (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. The product was isolated by column chromatography on silica gel (hex/EtOAc, 1/1 → EtOAc/MeOH, 1/1) as a yellow foam (1.656 g, 99% over 2 steps). ^{1}H and ^{13}C NMR was consistent with ref. 9. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.75 –7.35 (8H, m, CH_{arom}), 6.11 (1H, br d, Fmoc NH, *J* = 7.5 Hz), 5.38 (1H, d, H-4, $J_{3,4}$ = 2.7 Hz), 5.20 (1H, t, H-2, *J* = 9.9 Hz), 5.02 (1H, dd, H-3, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 3.3 Hz), 4.56 – 4.47 (3H, m, Fmoc CH_2 and H-1), 4.36 (1H, m, CHα), 4.23 (1H, t, Fmoc CH, *J* = 6.3 Hz), 4.12 (1H, dd, H-6a, *J* = 7.2 Hz, *J* = 11.4 Hz), 4.02 (1H, dd, H-6b, *J* = 6.3 Hz, *J* = 11.4 Hz), 3.29 (1H, dd, CHβ, *J* = 4.2 Hz, *J* = 14.7

Hz), 3.10 (1H, dd, CH*H*β, *J* = 6.0 Hz, *J* = 14.1 Hz), 2.12, 2.04, 1.99 and 1.93 (4 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.8, 170.2 170.0 and 169.8 (*C*(O)CH_3), 141.2, 127.7, 127.1, 124.8 and 119.9 (C_{arom}), 84.3 (C-1), 74.3, 71.5, 67.1 and 66.9 (C-2, C-3, C-4, C-5), 66.8 (Fmoc CH_2), 61.5 (C-6), 53.9 (CHα), 46.9 (Fmoc CH), 31.8 (CH_2β) and 20.7 and 20.5 (C(O)*C*H_3).

Phenyl 6-*O*-*tert*-butyldimethylsilyl-3,4-*O*-isopropylidene-1-thio-β-D-galactopyranoside (10): A solution of phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside (6.56 g, 14.9 mmol) in MeOH (50 mL) was treated with a 30 % NaOMe solution in MeOH. After completion the reaction mixture was neutralized with Dowex H^+, filtered and concentrated to a white solid. The product was dissolved in dry DMF (100 mL), 2,2-dimethoxypropane (37 mL, 298 mmol) was added. The pH was adjusted at 2 with *p*-TsOH. After 16 h TLC showed the reaction to be complete. Et_3N was added and the solution was concentrated. The product was isolated by column chromatography (CH_2Cl_2/MeOH, 1/0 → 9/1) and obtained as a clear glass (3.76 g, 79 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.54 – 7.28 (5 H, m, CH_{arom}), 4.48 (1H, d, H-1, $J_{1,2}$ = 10.3 Hz), 4.17 (1H, d, H-4, $J_{3,4}$ = 3.5 Hz), 4.11 (1H, t, H-3, *J* = 6.5 Hz), 3.97 (1H, dd, H-5, *J* = 7.0 Hz, *J* = 12.0 Hz), 3.87 and 3.80 (2 x 1H, 2 x d, 2 x H-6, *J* = 7 Hz, *J* = 11.5 Hz), 3.57 (1H, t, H-2, $J_{1,2}$ = 9.5 Hz), 2.87 and 2.50 (2 x 1H, 2 x s, 2 x OH), 1.41 and 1.33 (2 x 3H, 2 x s, $(CH_3O)_2C$). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 132.2, 132.0, 129.0 and 127.9 (C_{arom}), 110.3 ($(CH_3O)_2C$), 87.5 (C-1), 79.2, 76.8, 73.7 and 71.3 (C-2, C-3, C-4, C-5), 62.3 C-6), 27.9 and 26.2 ($(CH_3O)_2C$). To the pure product (3.67 g, 11.75 mmol) in CH_2Cl_2 (150 mL) was added DMAP (431 mg, 3.53 mmol), Et_3N (8.26 mL, 59 mmol) and *tert*-butyldimethyl silyl chloride (2.66 g, 17.6 mmol). The solution was stirred at r.t. for 20 h. The mixture was concentrated, taken up in EtOAc (200 mL), washed with 5 % $NaHCO_3$ (100 mL) and H_2O (100 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. Column chromatography (hex/EtOAc, 4/1 → 2/1) afforded **11** as a white solid (4.59 g, 92%). ^{1}H NMR (500 MHz, $CDCl_3$):): δ = 7.56 – 7.26 (5 H, m, CH_{arom}), 4.46 (1H, d, H-1, $J_{1,2}$ = 10.3 Hz), 4.23 (1H, dd, H-4, *J* = 2.0 Hz, *J* = 5.5 Hz), 4.07 (1H, t, H-3, *J* = 6.5 Hz), 3.88 (2H, d, 2 x H-6, *J* = 6.0 Hz), 3.87 – 3.81 (1H, m, H-5), 3.60 – 3.54 (1H, m, H-2), 2.53 (1H, s, OH), 1.44 and 1.33 (2 x 3H, 2 x s, $(CH_3O)_2C$), 0.90 (9H, s, $Si(CH_3)_2C(CH_3)_3$) and 0.09 (6H, s, $Si(CH_3)_2C(CH_3)_3$. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 132.6, 132.2, 128.9 and 127.9 (C_{arom}), 110.0 ($(CH_3O)_2C$), 88.3 (C-1), 79.0, 76.8, 73.1 and

71.7 (C-2, C-3, C-4, C-5), 62.2 (C-6), 28.1 and 26.3 (($CH_3O)_2C$), 25.8 ($Si(CH_3)_2C(CH_3)_3$) and 18.2 ($Si(CH_3)_2C(CH_3)_3$).

Phenyl 2-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-3,4-*O*-isopropylidene-1-thio-β-D-galactopyranoside (11): To a solution of **10** (4.59 g, 10.81 mmol) in dry DMF (50 mL) at 0°C was added NaH (60 % in oil, 476 mg, 11.9 mmol). After 15 min benzylbromide (1.94 mL, 16.22 mmol) was added dropwise. After 3 h TLC revealed the reaction to be complete. The mixture was concentrated *in vacuo* at 60 °C, the residue was taken up in CH_2Cl_2 (200 mL) and washed with H_2O (100 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. Product **11** was isolated by column chromatography (hex/EtOAc, 9/1 → 8/1) as a white foam (5.28 gr, 95 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.60 – 7.29 (10 H, m, CH_{arom}), 4.89 and 4.75 (2 x 1H, 2 x d, OCH_2Ph, J = 11.0 Hz), 4.68 (1H, d, H-1, $J_{1,2}$ = 9.8 Hz), 4.30 (2 H, s, H-4, H-3), 3.92 (2H, d, 2 x H-6, J = 7 Hz), 3.85 (1H, t, H-5, J = 6 Hz), 3.59 – 3.56 (1H, m, H-2), 1.47 and 1.40 (2 x 3H, 2 x s, $(CH_3O)_2C$), 0.95 (9H, s, $Si(CH_3)_2C(CH_3)_3$) and 0.13 (6H, s, $Si(CH_3)_2C(CH_3)_3$. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 137.9, 134.1, 131.6, 128.7, 128.2, 128.2, 127.6 and 127.2 (C_{arom}), 109.8 (($CH_3O)_2C$), 86.5 (C-1), 79.6, 78.3, 76.8 and 73.2 (C-2, C-3, C-4, C-5) 62.2 (C-6), 73.4 (OCH_2Ar), 27.8 and 26.3 (($CH_3O)_2C$), 25.8 ($Si(CH_3)_2C(CH_3)_3$) and 18.2 ($Si(CH_3)_2C(CH_3)_3$).

Phenyl 2-*O*-benzyl-3,4,6-tri-O-acetyl-1-thio-β-D-galactopyranoside (12): Compound **11** (5.28 g, 10.21 mmol) was stirred in a mixture of CH_2Cl_2 and MeOH (100 mL, 1/1, v/v). The pH was adjusted at 2 by *p*-TsOH. After 24 h of stirring the starting material has disappeared and a new spot with low R_f value was formed. After neutralization with Et_3N the solution was concentrated and subjected to column chromatography (CH_2Cl_2/MeOH, 1/0 → 19/1). The white product obtained was dissolved in pyridine (100 mL) and Ac_2O (50 mL) was added. The mixture was stirred for 3 days. The solution was concentrated at 60 °C after which the crude product was taken up in CH_2Cl_2 (200 mL). After successively washing with 5% $NaHCO_3$ (100 mL) and brine (100 mL) the organic layer was dried with Na_2SO_4, filtered and concentrated. Compound **12** was obtained as white solid (4.32 g, 87%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.60 – 7.26 (10 H, m, CH_{arom}), 5.40 (1H, d, H-4, $J_{3,4}$ = 2.5 Hz), 5.02 (1H, dd, H-3, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 3.5 Hz), 4.86 and 4.59 (2 x 1H, 2 x d, OCH_2Ar, J = 11.0 Hz), 4.73 (1H, d, H-1, $J_{1,2}$ = 9.8 Hz), 4.18 and 4.11 (2 x 1H, 2 x dd, 2 x

H-6, *J* = 7.0 Hz, *J* = 11.0 Hz), 3.90 (1H, m, H-5), 3.74 (1H, m, H-2), 2.12, 2.03 and 1.92 (3 x 3H, 3 x s, C(O)CH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.4, 170.1 and 169.9 (*C*(O)CH_3), 137.7, 133.1, 132.2, 128.9, 128.3 and 127.9 (C_{arom}), 87.8 (C-1), 75.5 (O*C*H_2Ar), 75.2, 74.1, 76.7 and 61.7 (C-2, C-3, C-4, C-5, C-6) and 20.6 (C(O)*C*H_3).

3,4,6-tri-*O*-acetyl-2-*O*-benzyl-α/β-D-galactopyranosyl trichloroacetimidate (13): To a mixture of **12** (4.32 g, 8.85 mmol) in acetone (100 mL), CH_2Cl_2 (25 mL) and H_2O (10 mL) was added NBS (7.87 g, 44 mmol). A orange colour was formed immediately which disappeared after 5 min. The reaction was stirred for 18 h, TLC showed full conversion. The mixture was concentrated, taken up in CH_2Cl_2 (200 mL) and washed with H_2O (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. Column chromatography (hex/EtOAc, 2/1) yielded the pure product as a clear glass (2.69 g, 77%, α/β 1/1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.35 – 7.32 (5 H, m, CH_{arom}) *α-anomer*: 5.42 (1H, s, H-4), 5.32 (2H, m, H-1α, H-3), 4.69 and 4.65 (2 x 1H, 2 x d, OC*H*$_2$Ar, *J* = 11.7 Hz), 4.42 (1H, t, H-5, *J* = 6.8 Hz), 4.12 and 4.06 (2 x 1H, 2 x d, 2 x H-6, J = 6.3 Hz), 3.84 (1H, dd, H-2, $J_{1,2}$ = 2.9 Hz, $J_{2,3}$ = 10.3 Hz); *β-anomer*: 5.35 (1H, s, H-4), 4.98 (1H, dd, H-3, $J_{2,3}$ = 10.25 Hz, $J_{3,4}$ = 2.4 Hz), 4.78 (1H, d, H-1β, $J_{1,2}$ 7.3 Hz), 4.12 and 4.06 (2 x 1H, 2 x dd, 2 x H-6, *J* = 6.3 Hz), 3.90 (1H, m, H-5), 3.61 (1H, t, H-2, *J* = 7.8 Hz), 2.12, 2.03 and 1.92 (3 x 3H, 3 x s, C(O)CH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.6, 170.2, 170.2 and 170.1 (*C*(O)CH_3), 138.1, 137.5, 128.5, 128.3, 128.2, 128.0 and 127.7 (C_{arom}), 97.5 and 91.6 (C-1 α /β), 73.3 (O*C*H_2Ar), 74.7, 73.8, 73.3, 72.2, 72.0, 70.6, 70.5, 69.4, 68.4, 67.5 and 66.3 (C-2, C-3, C-4, C-5), 61.8 and 61.7 (C-6) and 20.6 (C(O)*C*H_3). To a stirred solution of the intermediate (2.69 g, 6.69 mmol) and trichloro acetonitrile (2.04 mL, 20.4 mmol) in dry CH_2Cl_2 (150 mL) at 0°C under N_2 flow was added drop wise DBU (0.20 mL, 1.36 mmol). After 5 h TLC showed the formation of the α- and β product. The mixture was concentrated *in vacuo* and subjected to column chromatography (hex/EtOAc, 3/1 → 2/1). Glycosyl donor **13** was isolated as a clear glass (3.46 g, 94%, α/β 9/1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.67 (1H, s, C(NH)CCl_3), 7.34 – 7.27 (5 H, m, CH_{arom}), 6.56 (1H, d, H-1, $J_{1,2}$ 2.9 Hz), 5.53 (1H, d, H-4, $J_{3,4}$ = 2.0 Hz), 5.37 (1H, dd, H-3, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 2.9 Hz), 4.70 and 4.65 (2 x 1H, 2 x d, OCH_2Ar, *J* = 11.7 Hz), 4.42 (1H, t, H-5, *J* = 6.3 Hz), 4.17 – 4.02 (3H, m, 2 x H-6, H-2), 2.03, 2.00 and 1.99 (3 x 3H, 3 x s, C(O)CH_3). ^{13}C NMR (125

MHz, $CDCl_3$): δ = 170.1, 169.9 and 169.8 (*C*(O)CH_3), 160.9 (*C*(NH)CCl_3), 137.5, 128.2, 128.2, 127.7, 127.6 and 127.3 (C_{arom}), 94.4 (C-1), 90.8 (OC(NH)*C*Cl_3), 72.8 (O*C*H_2Ar), 72.5, 69.3, 68.7 and 67.6 (C-2, C-3, C-4, C-5), 61.2 (C-6), 20.6, 20.5 and 20.4 (C(O)*C*H_3).

General Procedure for Solid Phase Sugar Deacetylation (Procedure A): The resin was treated with 5% $N_2H_4 \cdot H_2O$ in DMF (4 x 30 min) and subsequently washed with DMF and CH_2Cl_2 (each 4 x, 2 min).

General protocol for Solid Phase C6-OH Acceptor Synthesis (Procedure B): The resin with an unprotected galactose moiety was washed with an appropriate volume of pyridine (3 x 2 min). A solution of 4,4-dimethoxytrityl chloride (5-10 equiv.) in dry pyridine was added to the resin and N_2 was bubbled through the mixture for 4 h. Then the resin was washed with CH_2Cl_2 (6 x 2 min). The resin was swollen again in pyridine and benzoyl chloride (10 equiv. per OH) was added slowly. N_2 was bubbled through the mixture for 1 h followed by a washing step with CH_2Cl_2 (4 x 2 min). The benzoylation step was repeated. The resin was treated several times with a 1% dichloro acetic acid solution in CH_2Cl_2 (2 min each) until no color release was observed. This was followed by washing with CH_2Cl_2 (6 x 2 min) and Et_2O (2 x 2 min). The resin was dried under high vacuum.

General Procedure for Solid Phase Glycosylations (Procedure C): In a flask the resin (100-300 mg) was combined with the glycosyl donor (5 equiv.). An appropriate volume of dry CH_2Cl_2 was added and the mixture was kept under an N_2 atmosphere and cooled to 0 °C. After 30 min TMSOTf (0.3 equiv.) was added. The mixture was slowly stirred at 0 °C for 1 h followed by 1 h at r.t. A drop of Et_3N was added to neutralize the mixture after which the resin was transferred to a solid phase syringe and washed 5 times with CH_2Cl_2. The glycosylation procedure was subsequently repeated.

General Procedure for Cleavage of the Thioglycosides from the Resin by DAST/NBS (Procedure D): The resin was swollen in an appropriate amount of CH_2Cl_2, and DAST (5 equiv.) was added followed by NBS (5 equiv.). N_2 was bubbled through the mixture and after 30 min the mixture was filtered and the resin was washed with CH_2Cl_2 (4 x 2 min).

The filtrate was concentrated to afford the crude anomeric fluorides, which were purified by silica gel column chromatography.

General Procedure for Cleavage of the Rink linker (Procedure E): The resin was shaken in a mixture of 95 % TFA and 5% H_2O for 2 h. The resin was filtered off and washed with CH_2Cl_2 (5 x). The filtrate was concentrated to obtain the crude products which were purified by silica gel column chromatography followed by HPLC.

Resin (4): A mixture of **3** (775 mg, 1.15 mmol), HATU (437 mg, 1.15 mmol) and DiPEA (0.38 mL, 2.30 mmol) in dry NMP (10 mL) was added to aminomethylated polystyrene resin (500 mg, 0.58 mmol NH_2). The mixture was shaken for 20 h and the resin was filtered, washed with NMP and CH_2Cl_2 (3 x 10 mL, 2 min each). The Kaiser test indicated the reaction to be complete. The remaining amines were capped with capping reagent (10 mL) for 1 h. After a washing procedure with NMP and CH_2Cl_2 (both 3 x 10 mL, 2 min each, i.e. general washing procedure) the Fmoc group was cleaved by treatment with 20 % piperidine in NMP (3 x 10 mL, each 20 min). After a general NMP/CH_2Cl_2 washing a Kaiser test showed the presence of free amines which were acetylated twice (1.5 h each) by a mixture of pyridine (7.5 mL) and Ac_2O (5 mL). After washing no free amines were present as shown by the Kaisertest. Hydroxyl deprotection was performed according to procedure A.

Resin (6): Resin **4** was treated by procedure B to yield resin **6**.

Resin (14): In a solid phase reaction tube commercially available 3-[4-(tritylmercapto)phenyl]propionyl aminomethyl functionalized polystyrene resin (1.00 g, 0.80 mmol), was treated several times (5 min each) with a 1:1 mixture of CH_2Cl_2 and TFA until the release of the yellow trityl cation had ceased. The resin was washed with CH_2Cl_2 (6 x) and Et_2O (2x) and dried for 2 h. In a flask the resin was combined with peracetylated galactopyranose (1.72 g, 4.40 mmol), CH_2Cl_2 (5 mL) was added followed by $BF_3{\cdot}Et_2O$ (0.56 mL, 4.40 mmol). The reaction mixture was stirred for 20 h. Then the resin was filtered off and washed with CH_2Cl_2 (4 x 2 min) and Et_2O (2 x 2 min). The glycosylation procedure was repeated to guarantee a full loading of the resin. The resin was subjected to

the deacetylation procedure A. A 50 mg sample was benzoylated and cleaved from the resin to indicate a loading of 0.8 mmol/g. Finally, procedure B was followed.

Resin (15): Detritylation was performed as described for the synthesis of resin **14**. The 3-[4-(tritylmercapto)phenyl]propionyl aminomethyl functionalized polystyrene resin (0.44 mmol) was combined with donor **13** (476 mg, 0.88 mmol). To this CH_2Cl_2 (15 mL) was added and the reaction mixture was cooled to 0°C under an N_2 atmosphere. After 30 min TMSOTf (17 µL, 0.09 mmol) was added and the mixture was stirred slowly for 3 h. Then the mixture was warmed to r.t. and stirred additionally for 20 h. The resin was transferred to a solid phase reaction vessel and washed with CH_2Cl_2 (4x) and Et_2O (2x). The glycosylation procedure was repeated to guarantee a full loading of the resin. The resin was subjected to the deacetylation procedure A. A 50 mg sample was benzoylated and cleaved from the resin to indicate a loading of 0.5 mmol/g. Finally, procedure B was followed.

Fluoro (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-α/β-D-galactopyranoside (8): Either resin **6**, or **14** or **15** (100-300 mg) was glycosylated according to procedure C and the product was cleaved from the resin according to procedure D, to yield monosaccharide **9** as a clear glass and the disaccharide **8** as a white foam. Compound **8** (α:β = 1:1): ^{1}H NMR (500 MHz, $CDCl_3$): 8.08 – 7.27 (15H, m, CH_{arom}), 6.07 (0.5H, d, H-1α, $J_{H,F}$ = 53 Hz), 5.98 – 5.84 (2 H, m, H-2 and H-4), 5.70 – 5.58 (1H, 2 x m, H-3), 5.63 and 5.53 (0.5H, 2 x d, H-1β, $J_{H,F}$ = 51.8 Hz, $J_{1,2}$ = 6.4 Hz), 5.36 (1H, s, H-4'), 5.20 (1H, t, H-2', $J_{2',3'}$ = 9.8 Hz), 5.02 (1H, d, H-3', $J_{2',3'}$= 10.3 Hz), 4.58 – 4.52 (1H, m, H-1'), 4.65 and 4.30 (1H, 2 x bs, H-5), 4.09 – 3.83 (5H, m, H-5', 2 x H-6 and 2 x H-6'), 2.15, 2.07, 2.01 and 1.99 (4 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): 170.2, 170.1 and 169.6 (*C*(O)CH_3), 165.4, 165.2 and 165.1 (*C*(O)Ar), 133.8 – 128.3 (C_{arom}), 101.5 (C-1'), 73.4, 73.3, 70.7, 69.9, 69.6, 68.5, 67.6, 66.9, 66.8 and 61.1 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5', C-6, C-6') and 20.6 (*C*(O)CH_3).

Fluoro 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (9): Compound **9** (only β isomer): NMR is consistent with ref. 15. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.42 (1H, s, H-4), 5.36 – 5.29 (1H, m, H-2), 5.25 (1H, dd, $J_{1,2}$ = 7.2 Hz, $J_{H,F}$ = 49.2 Hz), 5.05 (1H, d, H-3, *J* = 4.5

Hz), 4.21 (2H, d, 2 x H-6, J = 3.9 Hz), 4.06 (1H, t, H-5, J = 3.6 Hz), 2.18, 2.11, 2.07 and 2.01 (4 x 3H, 4 x s, C(O)CH_3).

Resin (17): Fmoc loaded Rink amino methyl polystyrene resin (1.00 g, 0.78 mmol NHFmoc) was treated with 20% piperidine in NMP (3 x 10 mL, 15 min each). The resin was washed with NMP (4 x 10 mL, 2 min each), and CH_2Cl_2 (4 x 10 mL, 2 min each). Kaiser test indicated that the reaction was complete. To the resin was added a mixture of **16** (1.09 g, 1.56 mmol), HATU (593 mg, 1.556 mmol) and DiPEA (515 µL, 3.12 mmol) in dry NMP (10 mL). The resin was shaken for 20 h and the resin was filtered and washed with NMP (4 x 10 mL, 2 min each), CH_2Cl_2 (4 x 10 mL, 2 min each) and Et_2O (2 x 10 mL, 2 min each). Standard Fmoc analysis revealed a loading of 0.48 mmol/g. Although the Kaiser test revealed a complete coupling, a capping reaction (10 mL capping solution) was performed for 1h. The Fmoc group was cleaved by piperidine as described above. The resin was swollen in dry pyridine (10 mL) and benzoyl chloride (0.91 mL, 7.8 mmol) was added. N_2 was bubbled through the mixture for 1 h followed by washing with CH_2Cl_2 (6 x 10 mL, 2 min each) and Et_2O (2 x 10 mL, 2 min each). Hydroxyl deprotection was performed according to procedure A, selective protecting group chemistry was performed according to procedure B.

2,3,4,6-tetra-*O*-pivaloyl-α/β-D-galactopyranosyl trichloroacetimidate (18): A solution of phenyl 1-thio-β-D-galactopyranoside (3.92 g, 14.4 mmol), DMAP (catalytic) was stirred in dry pyridine (100 mL) and pivaloyl chloride (13.8 mL, 115 mmol) was added drop wise. The reaction was heated to 100 ˚C and stirred for 18h. After concentrating at 60 ˚C, crude product was taken up in EtOAc (200 mL), washed with 1M NaOH (100 mL) and with 1M HCl (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. Silica gel chromatography (hex/EtOAc, 9/1 → 4/1) yielded phenyl 2,3,4,6-tetra-*O*-pivaloyl-1-thio-β-D-galactopyranoside as a white solid (8.23 g, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.53 – 7.30 (5H, m, CH_{arom}), 5.41 (1H, s, H-4, $J_{3,4}$ = 3.0 Hz), 5.22 (1H, t, H-2, J = 9.6 Hz), 5.13 (1H, dd, H-3, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 2.7 Hz), 4.72 (1H, d, H-1, $J_{1,2}$ = 9.9 Hz), 4.18 (1H, dd, H-5, J = 9.3 Hz, J = 13.5 Hz), 4.05 – 3.98 (2H, m, 2 x H-6), 1.22, 1.19, 1.17 and 1.09 (4 x 9H, 4 x s, C(O)C(CH_3)$_3$). Intermediate (8.23 g, 13.5 mmol) was dissolved in EtOAc (100 mL), acetone (25 mL) and H_2O (25 mL) were added followed by *N*-

bromosuccinimide (9.63 g, 54 mmol). After 1 h. the reaction mixture was diluted with EtOAc (100 mL), washed with 5% $NaHCO_3$ (100 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. Silicagel chromatography (hex/EtOAc, 5/1) yielded hemi acetal as clear glass (4.61g, 66%). The compound was dissolved in dry CH_2Cl_2 (150 mL) and trichloro acetonitrile (2.68 mL, 27 mmol) was added. After cooling to 0 ˚C under a N_2 flow DBU (0.40 mL, 2.7 mmol) was added drop wise and the reaction was kept at 0 ˚C for 2 hours and 2 hours at r.t. The reaction mixture was concentrated and **18** was isolated by silica gel chromatography (hex/EtOAc, 9/1) as white foam (4.79 g, 81%). NMR was consistent with ref. 16. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.67 (1H, s, C(NH)CCl_3), 6.62 (1H, d, H-1, $J_{1,2}$ = 3.3 Hz), 5.58 – 5.53 (2H, m, H-3, H-4), 4.43 (1H, t, H-2, $J_{1,2}$ = 3.6 Hz, $J_{2,3}$ = 10.5 Hz), 4.49 (1H, t, H-5, J = 6.6 Hz), 4.15 – 4.02 (2H, m, 2 x H-6), 1.28, 1.16, 1.15 and 1.14 (4 x 9H, 4 x s, C(O)C$(CH_3)_3$). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.0, 177.6, 177.4 and 177.1 (*C*(O)C$(CH_3)_3$), 161.0 (*C*(NH)CCl_3), 114.9 (C(NH)*C*Cl_3), 93.7 (C-1), 70.0, 67.9, 67.6and 67.1 (C-2, C-3, C-4, C-5), 61.8 (C-6), 39.3, 39.1 and 39.0 (C(O)*C*$(CH_3)_3$), 27.4, 27.3, 27.3 and 27.2 (C(O)C(*C*$H_3)_3$).

(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzoyl-*N*-(carbox-amido-*N*-benzoyl-L-glutam-5-oyl))-β-D-galactopyranosylamine (19a): Glycosylation of resin **17** with **7** according to procedure C, followed by cleavage from the resin by procedure E yielded **19a** (white solid) (16.4 mg, 25%): ν_{max}(KBr) = 3500 – 3100 (br), 2924, 1734, 1262, 1070 and 711 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.10 – 7.23 (22H, m, CH_{arom}, NH_2), 6.74 (1H, bt, C(O)NHC-1), 5.92 (1H, dd, H-4, $J_{3,4}$ = 3.0 Hz), 5.73 (1H, dd, H-3, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 3.5 Hz), 5.67 (1H, t, H-2, $J_{1,2}$ = 9.0 Hz), 5.61 (1H, t, H-1, $J_{1,2}$ = 8.5 Hz), 5.36 (1H, s, NHBz), 5.32 (1H, d, H-4', $J_{3,4}$ = 3.0 Hz), 5.17 (1H, dd, H-2', $J_{2',3'}$ = 10.5 Hz, $J_{1',2'}$ = 8.0 Hz), 4.99 (1H, dd, H-3', $J_{2',3'}$= 10.0 Hz, $J_{3',4'}$= 3.0 Hz), 4.58 (1H, d, H-1', $J_{1',2'}$ = 8.0 Hz), 4.65 – 4.61 (1H, m, CHα), 4.26 (1H, t, H-5, $J_{5,6}$ = 6.0 Hz), 4.08 – 3.83 (4H, m, H-5', 2 x H-6' and 2 x H-6), 2.59 - 2.51 and 2.42 - 2.27 (2 x 1H, 2 x m, CH_2γ), 2.10 - 1.94 (2H, m, CH_2β), 2.13, 2.06, 1.98 and 1.97 (4 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR (125.5 MHz, $CDCl_3$): δ = 170.7, 170.5, 170.3 and 169.3 (*C*(O)CH_3, *C*(O)NHC-1), 133.3 – 128.3 (C_{arom}), 100.9 (C-1'), 78.9 (*C*-1), 75.5 (C-5), 71.5 (C-3), 70.8 (C-2), 70.8 (C-3'), 70.6 (C-5'), 68.0 (C-4), 68.2 (C-2'), 66.3 (H-6'), 66.7 (C-4'), 60.7 (H-6), 52.7 (Cα), 32.7 (CH_2γ), 21.4, 20.5

and 20.3 (C(O)*C*H_3); HRMS for $C_{53}H_{55}N_3O_{20}$ (M, 1053,338): found $[M + H]^+$ 1054.295, calcd. 1054.347.

(2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzoyl-*N*-(carboxamido-*N*-benzoyl-L-glutam-5-oyl)-β-D-galactopyranosylamine (19b): Glycosylation of resin **17** with **18** according to procedure C, followed by cleavage from the resin by procedure E yielded **19b** (white solid) (21.8 mg, 30%): ν_{max}(KBr) = 3500 – 2800 (br), 1730, 1684, 1533, 1451, 1267, 1107 and 709 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 8.10 – 7.22 (22H, m, C_{arom}, NH_2), 6.85 (1H, bt, (CO)NHC-1), 5.90 (1H, dd, H-4, $J_{3,4}$ = 3.5 Hz, $J_{4,5}$ = 1.0 Hz), 5.72 (1H, dd, H-3, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 3.5 Hz), 5.67 (1H, dd, H-2, $J_{1,2}$ = 8.5 Hz, $J_{2,3}$ = 10.0 Hz), 5.64 (1H, s, NHBz), 5.61 (1H, t, H-1, $J_{1,2}$ = 8.5 Hz), 5.35 (1H, d, H-4', $J_{3',4'}$ = 3.5 Hz), 5.19 (1H, dd, H-2', $J_{1',2'}$= 8.0 Hz, $J_{2',3'}$ = 10.5 Hz), 5.09 (1H, dd, H-3', $J_{2',3'}$ = 10.5, Hz, $J_{3',4'}$ = 3.5 Hz), 4.62 (1H, d, H-1', $J_{1',2'}$ = 8.0 Hz), 4.61 (1H, m, CHα), 4.26 – 4.23 (1H, m, H-5), 4.01 – 3.89 (4H, m, H-5', H-6a and 2 x H-6'), 3.76 (1H, dd, H-6b, *J* = 7.0 Hz, 11.0 Hz), 2.56 – 2.50 and 2.42 – 2.37 (2 x 1H, 2 x m, CH_2γ), 2.12 – 2.05 and 2.00 - 1.94 (2 x 1H, 2 x m, CH_2β), 1.24, 1.16, 1.10 and 1.10 (4 x 9H, 4 x s, C(O)C$(CH_3)_3$). ^{13}C NMR (125.5 MHz, $CDCl_3$): δ = 177.9, 177.5, 177.0 and 176.8 (*C*(O)C$(CH_3)_3$), 167.9, 165.4, 165.3, 165.2 and 165.1 (*C*(O)Ar), 165.2 (*C*(O)NHC-1),130.0 – 128.3 (C_{arom}), 100.9 (C-1'), 79.0 (*C*-1), 75.2 (C-5), 71.0 (C-3'), 71.0 (C-5'), 70.8 (C-3), 70.8 (C-2), 68.7 (C-4), 68.6 (C-2'), 67.6 (C-6'), 66.8 (C-4'), 61.0 (C-6), 52.5 (Cα), 38.7, 38.8 and 39.1 (C(O)*C*$(CH_3)_3$), 32.8 (CH_2γ), 27.1 (CH_2β), 27.2, 27.1 and 27.1 (C(O)C(*C*$H_3)_3$). HRMS for $C_{65}H_{79}N_3O_{20}$ (M, 1221.526): found $[M + H]^+$ 1222.538, calcd. 1222.534.

(2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-benz-oyl-β-D-galactopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzoyl-*N*-(carbamoyl-*N*-benz-oyl-L-glutam-5-oyl)-β-D-galactopyranosylamine (21): Glycosylation of resin **17** with **18** according to procedure C**.** The resin (208 mg) was subsequently treated with a 1% NaOMe solution in 20% MeOH/dioxane (6 x 5 mL, 20 min each). The resin was then washed with CH_2Cl_2 (6 x 5 mL, 2 min each) and Et_2O (2 x 5 mL, 2 min each). Introduction of the DMT-group was performed according to procedure B followed by glycosylation with **18** according to procedure C. Finally, cleavage from the resin was performed by procedure E and

purification by HPLC to yield **21** (white solid) (1.9 mg, 2%, non-optimized): ν_{max}(KBr) = 3500 – 3100 (br), 2965, 2929, 1734, 1281, 1107 and 710 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.08 – 7.22 (37H, m, CH_{arom}, NH_2), 6.81 (1H, s, C(O)NH), 5.94 (1H, dd, H-4, $J_{3,4}$ = 3.0 Hz, $J_{4,5}$ = 1.0 Hz), 5.82 (1H, dd, H-4', $J_{3',4'}$ = 3.0 Hz, $J_{4',5'}$ = 0.5 Hz), 5.67 (1H, dd, H-2', $J_{1',2'}$ = 8.0 Hz, $J_{2',3'}$ = 10.5 Hz), 5.66 (1H, dd, H-3', $J_{2',3'}$ = 11.0 Hz, $J_{3',4'}$ = 3.5 Hz), 5.61 (1H, t, H-2, $J_{1,2}$ = 8.5 Hz,), 5.56 – 5.53 (3H, m, H-1, NH, H-3), 5.30 (1H, d, H-4'', $J_{3'',4''}$ = 3.5 Hz), 5.11 (1H, dd, H-2'', $J_{1'',2''}$ = 8.0 Hz, $J_{2'',3''}$ = 10.5 Hz), 5.02 (1H, dd, H-3'', $J_{2'',3''}$ = 10.5 Hz, $J_{3'',4''}$ = 3.5 Hz), 4.90 (1H, d, H-1', $J_{1',2'}$ = 7.5 Hz), 4.61 – 4.57 (1H, m, CHα), 4.28 (1H, d, H-1'', $J_{1'',2''}$ = 7.5 Hz), 4.19 (1H, t, H-5, $J_{5,6}$ = 6.0 Hz), 4.04 – 4.00 (3H, m, H-5'', H-6a, H-6a'), 3.89 – 3.77 (5H, m, H-5', H-6b, H-6b', 2 x H-6''), 2.56 – 2.50 and 2.39 – 2.34 (2 x 1H, 2 x m, $CH_2\gamma$), 2.17 – 2.10 and 2.05 - 1.97 (2 x 1H, 2 x m, $CH_2\beta$), 1.22, 1.11, 1.09 and 1.08 (4 x 9H, 4 x s, C(O)C$(CH_3)_3$). ^{13}C NMR (125.5 MHz, $CDCl_3$): δ = 177.8, 177.3, 176.9 and 176.6 (*C*(O)C$(CH_3)_3$), 167.7, 165.3 and 165.1 (*C*(O)Ar), 133.8 – 127.3 (C_{arom}), 101.0 (C-1''), 100.5 (C-1'), 79.1 (C-1), 75.0 (C-5), 72.9 (C-5'), 71.7 (C-3), 71.6 (C-3'), 70.9 (C-3''), 70.8 (C-5''), 70.5 (C-2), 69.6 (C-2'), 68.7 (C-2''), 68.4 (C-4), 68.3 (C-4'), 67.4 (C-6), 66.6 (C-4''), 66.4 (C-6'), 60.7 (C-6''), 52.6 (Cα), 38.7, 38.8 and 39.1 (C(O)*C*$(CH_3)_3$), 32.7 ($CH_2\gamma$), 27.1 ($CH_2\beta$), 27.1 and 27.0 (C(O)C(*C*$H_3)_3$). HRMS for $C_{92}H_{101}N_3O_{28}$ (M, 1695.657): found $[M + Na]^+$ 1718.638, calcd. 1718.647.

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Chapter 3

Synthesis of multivalent *S. suis* adhesion inhibitors by enzymatic cleavage of polygalacturonic acid

The results in this chapter have been published:

Hilbert M. Branderhorst, Raymond Kooij, Annika Salminen, Lieneke H. Jongeneel, Christopher J. Arnusch, Rob M. J. Liskamp, Jukka Finne, Roland J. Pieters; Synthesis of multivalent *Streptococcus suis* adhesion inhibitors by enzymatic cleavage of polygalacturonic acid; *Organic and Biomolecular Chemistry* **2008**, *6*, 1425-1434

Introduction

The preparation of carbohydrates and their conjugates has become an important field for the study of biological processes and the development of therapeutic lead structures[1]. Many methods for selective protection schemes and for the stereoselective introduction of glycosidic bonds have been developed over the years.[2] While a synthetic strategy is often the most desired preparation method, a biocatalytic degradation of readily available natural carbohydrates or carbohydrate polymers can be advantageous. In this chapter we report on a synthetic route to galabiose derivative **1** and its subsequent conjugation to dendrimers by 'click chemistry'. A route was used involving enzymatic degradation of polygalacturonic acid and subsequent chemical modification steps. In our research program on bacterial anti-adhesion compounds,[3,4] the galabiose derivative **1** (Figure 1a) became of interest as its azido function allows convenient conjugation to dendrimers using our microwave heated "click" protocol.[5] The galabiose sequence shows strong binding to the bacterial Gram-positive pathogen *Streptococcus suis*[6] that can cause meningitis, septicemia, and pneumonia in pigs and also meningitis in humans.[7,8] Multivalent presentation of the disaccharide sequence on dendritic scaffolds greatly enhances their binding potency.[4b,9] Other important biological targets that bind to the galabiose epitope are the uropathogenic *E. coli* via its PapG adhesin,[4d,10] the bacterium *Pseudomonas aeruginosa* via its PA-IL lectin[11] and the Shiga like Toxin, a toxin of the AB_5 family produced by *E. coli*.[12] The triazole linkage between the dendritic scaffold and the galabiose that is described here is very similar to the previously used[4b,d] amide connection. (Figure 1b).

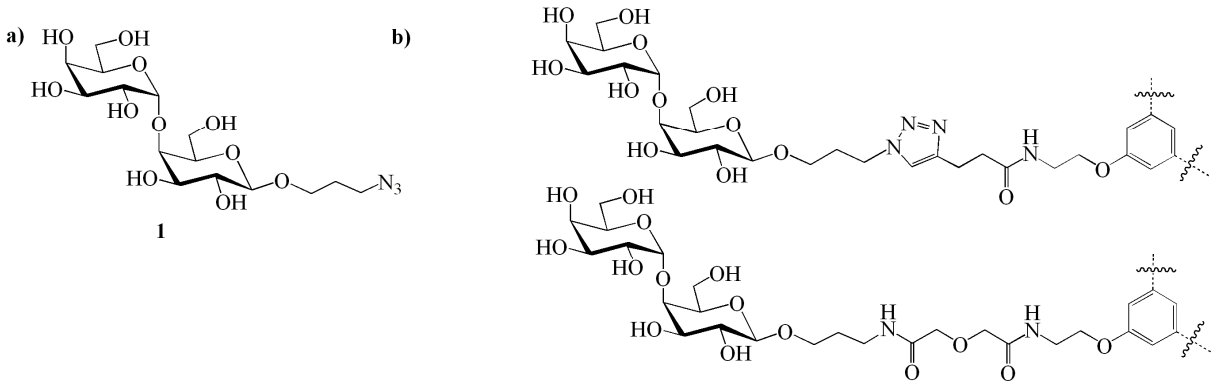


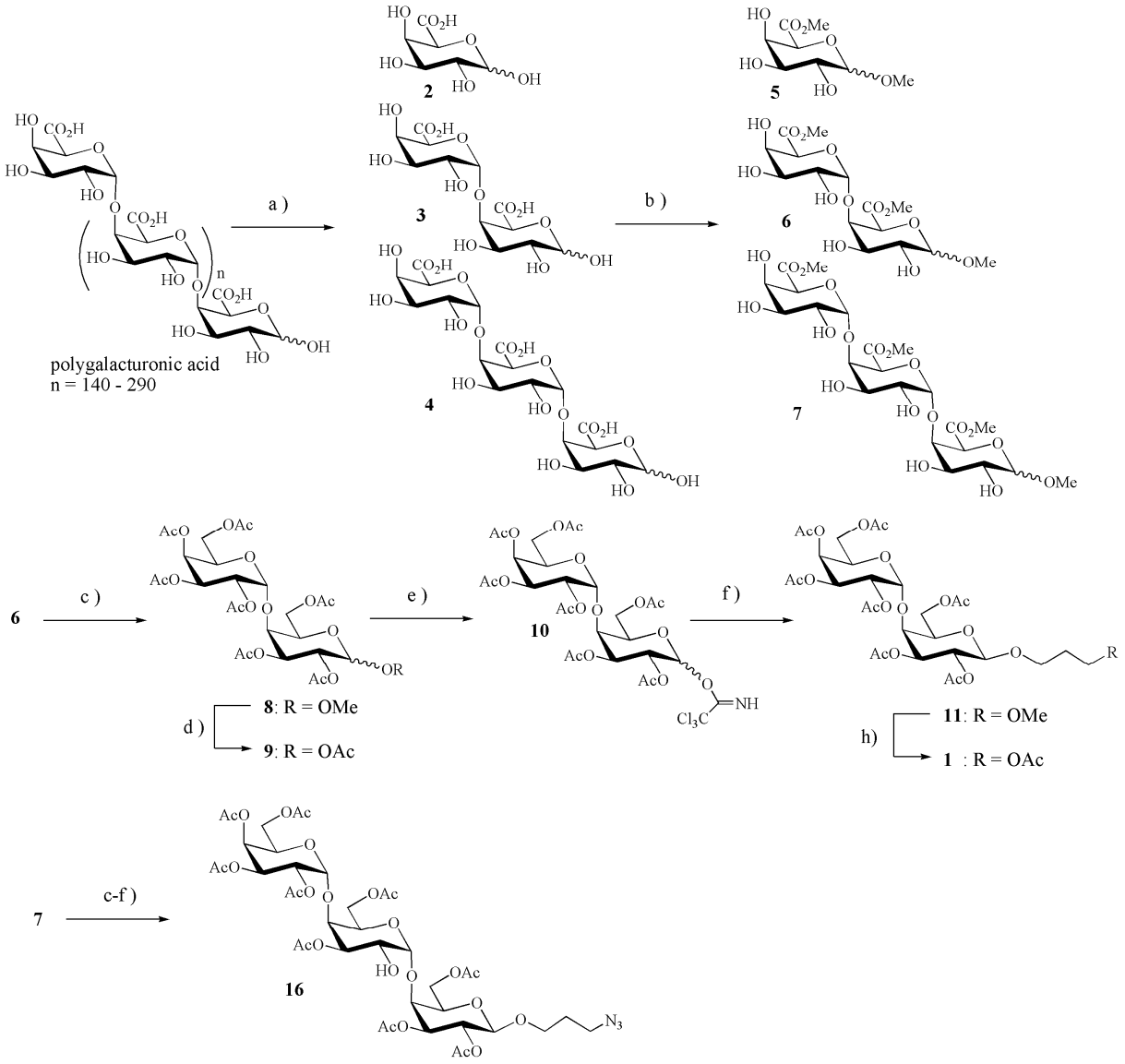
Figure 1. *a) Desired Galabiose building block for conjugation via "click" chemistry; b) triazole linkage to dendritic structures compare to previously used linkage (ref. [4b,d]).*

For the synthesis of the galabiose unit our previous fully synthetic strategy was applied.[4b] While this route did produce the target product, its many steps make it less ideal for large scale synthesis. Alternative routes towards galabiose based on an enzymatic cleavage of polygalacturonic acid were earlier described in the literature.[13] The work described in this chapter represents an optimization and an extension of earlier research, describing the discovery of a widely available highly effective enzyme and easy conjugation procedures. Furthermore, besides the desired disaccharide also the α(1,4) linked galactopyranosyl trisaccharide was obtained which would require an even lengthier multistep synthetic route for its preparation than the galabiose disaccharide.

Results and Discussion

The procedure started with the enzymatic cleavage of commercially available pectin-derived polygalacturonic acid, a polymer consisting of α(1,4)linked galacturonic acid moieties. The polymer was treated with the pectinase from *Rhizopus oryzae* and separately also with the pectinase from *Aspergillus niger* in sodium acetate buffer at pH 4.5 (Scheme 1). Both enzymes showed the expected cleavage activity but in the case of the *Rhizopus oryzae* pectinase the relative amount of the desired disaccharide **3** versus monosaccharide **2** and trisaccharide **4** was much higher and was estimated by TLC to be ca. 4:4:2 (for **3**:**2**:**4**) while for the *Aspergillus niger* pectinase this ratio was ca. 2:4:4. For this reason the pectinase from *Rhizopus oryzae* was chosen for further use. Initial attempts were made to separate the obtained mixture of mono-, di and trisaccharides by ion exchange chromatography using a formic acid gradient,[13a] however on a 10 gram scale this became impractical. Better and faster separation was achieved by standard silica gel chromatography after converting the free sugars **2-4** to their methyl acetals (α/β ratio ≈ 3/1) and the methyl esters **5-7**, using the crude mixture and Dowex-H^+ catalysis in MeOH. After separation, the disaccharide **6** was reduced by $NaBH_4$ and its hydroxyls were subsequently acetylated (Ac_2O, pyridine). The anomeric methoxy group was replaced with an acetate by treatment with H_2SO_4 in acetic anhydride to give **9**. The disaccharide **9** was converted to glycosyl donor **10** by selective anomeric deacetylation using hydrazine acetate followed by standard introduction of the trichloroacetimidate moiety. Coupling of the 3-bromo propanol spacer was achieved by TMSOTf catalysis (Scheme 3) to give **11** which was subjected to a nucleophilic substitution by NaN_3 in DMF to give the desired target compound **1**. The

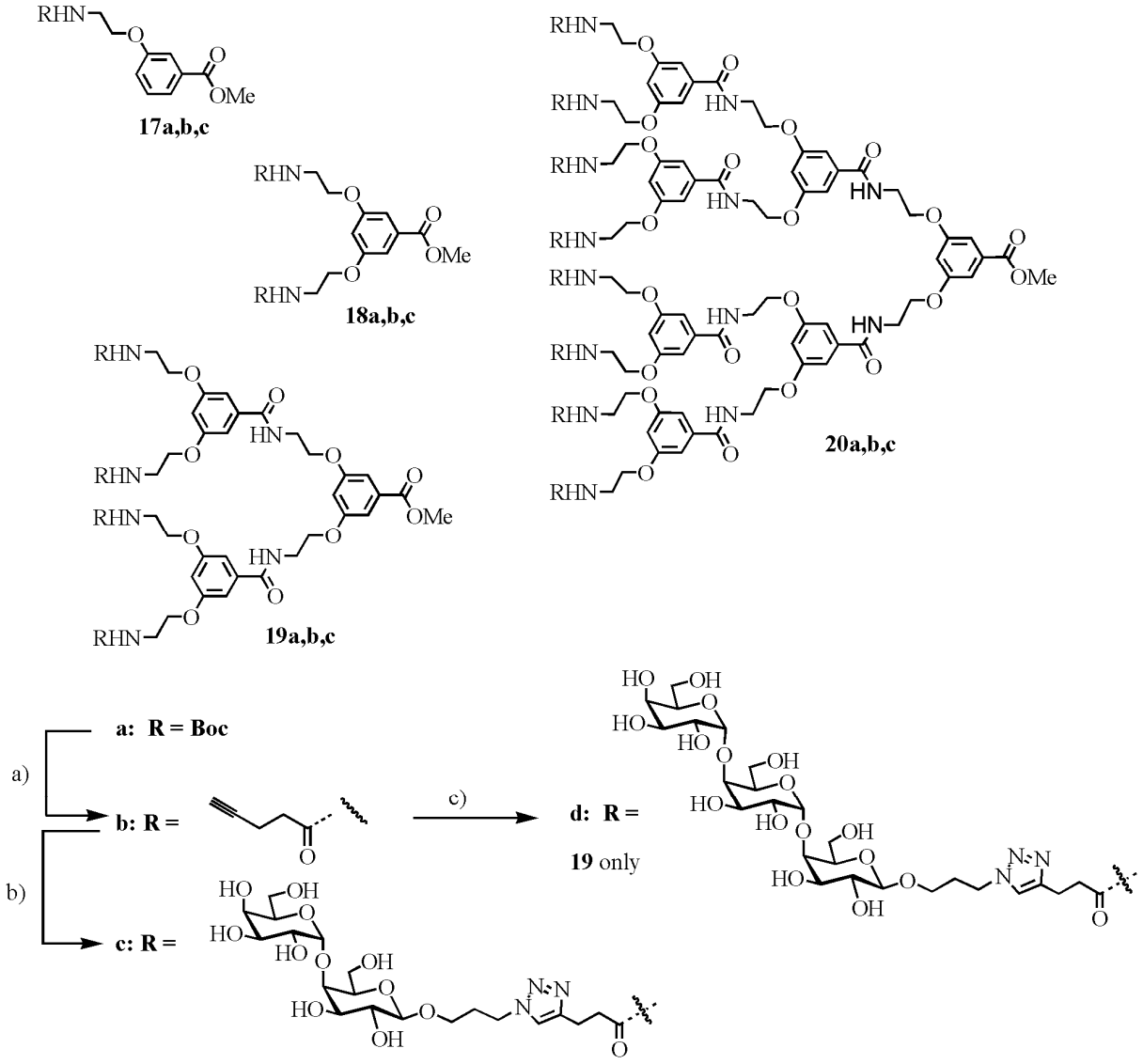
described procedure was also applied to the galacturonic acid trisaccharide **7** to give **16** in comparable yields.



Scheme 1. *Building Block synthesis, reagents and conditions a) Pectinase, pH=4.5, 40˚C; b) MeOH, Dowex-H$^+$, then separate on silica, 28% for* ***6****, 12% for* ***7****; c) i. $NaBH_4$, MeOH, ii. Ac_2O, pyridine, 75 % d) H_2SO_4, Ac_2O, quant.; e) i. N_2H_4:AcOH, DMF, ii. Cl_3CCN, DBU, CH_2Cl_2, 64 % for* ***10****, 42% for* ***14****; f) $HO(CH_2)_3Br$, TMSOTf, 58 % for* ***11****, 55% for* ***15*** *d) NaN_3, DMF, quant. for* ***1****, 94% for* ***16****.*

After optimizing the synthesis of the galabiose building blocks, they were conjugated to the dendritic scaffolds. For this purpose the so-called "click" reaction, the copper(I) catalyzed [3+2] cyclo addition between azides and alkynes,[14] was used with our previously reported conditions.[5] The dendrimers **17a**-**20a**[15] were subjected to Boc cleavage, followed by BOP

mediated coupling of 4-pentynoic acid (Scheme 2). Subsequent conjugation of **17-20b** was performed with the galabiose building block **1** using Cu(I) catalysis and microwave irradiation at 80 ˚C. Tetravalent dendrimer **19b** was also conjugated with the trisaccharide **16** (Scheme 2) to give **19d**. The obtained dendrimers were deacetylated with NaOMe conditions to give **17c**-**20c** and **19d**.



Scheme 2. *Glycodendrimer synthesis, reagents and conditions: a) i. TFA/CH_2Cl_2; ii. 4-pentynoic acid, BOP, DiPEA, 70- 95%; b) i.* ***1****, $CuSO_4$, sodium ascorbate, DMF, 66-84%, ii. NaOMe, MeOH, 35-77%; c) i.* ***16****, $CuSO_4$, sodium ascorbate, DMF, 79%, ii. NaOMe, MeOH, 57%.*

The resulting galabiose dendrimers were tested for their interactions with the *S. suis* adhesin by evaluating their inhibitory capacity of hemagglutination inhibition as previously

described.[4b,9] The minimal inhibitory concentration (MIC) values were determined and depicted in Table 1. The results showed a clear multivalency effect. These results were comparable with our previously reported data, describing low nanomolar inhibition for the multivalent galabiose conjugates.[4b] The tetravalent galabiose dendrimer **19c** showed essentially the same MIC value as the tetravalent reference compound with the amide linkage (Figure 1b) as it was retested in the present study, indicating no influence of the structure of the linking moiety. The tested tetravalent galactose trisaccharide **19d**, showed significantly reduced inhibitory potency in comparison to the tetravalent galabiose compound **19c**, i.e. 48 nM vs. 2.4 nM.

Table 1. *Inhibition of the S. suis D282 mediated Hemagglutination of Human Erythrocytes by Galabiose derivatives*

compound	valency	MIC (nM)	Rel. pot.[a] (per sugar)[b]
MONO 17c	1	400	1 (1)
Di 18c	2	8	50 (25)
TETRA 19c	4	2.4	167 (42)
OCTA 20c	8	3.9	100 (13)
TETRA 19d	4	48	

[a] Relative potency = IC_{50}(monovalent)/IC_{50}(multivalent ligand). [b] Relative potency per sugar = relative potency/valency

Conclusions

The preparation of derivatives of galabiose and the corresponding trisaccharide were described involving a biocatalytic polysaccharide degradation step followed by chemical modification steps. The key step in this procedure is the efficient enzymatic cleavage of the cheap and commercial available polygalacturonic acid by a readily available pectinase enzyme. Methylation of the crude cleavage mixture allowed efficient separation by standard silica gel chromatography. Both disaccharide and trisaccharide were thus obtained and subsequently subjected to a limited number of chemical modifications leading to the desired galabiose building block and a galactose trisaccharide. Compared to previous reports, both the purification and ligation were optimized and this route is a good alternative for the fully chemical synthesis approach and it does not require expensive chemical reagents. Besides the desired disaccharide also the corresponding trisaccharide was obtained, which would require many steps by complete chemical synthesis. Ligation of the carbohydrate building blocks to a series of alkyne functionalized dendrimers was

achieved by "click" chemistry and was found to give excellent coupling yields. The newly prepared galabiose dendrimers showed comparable *S. suis* inhibition capacities with our previously reported compounds as was reflected in the MIC values. The tetravalent trisaccharide inhibitor was found not to be as effective as the galabiose disaccharide. This revised partial enzymatic preparation method is attractive for the preparation of larger quantities of anti-adhesion compounds with relatively low costs and efforts. It is thus well-suited to further pursue the anti-adhesion strategy with multivalent carbohydrates[16], as an alternative intervention method against microbial pathogens in animal models.

Experimental Section

General Remarks

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. All solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and were stored on molecular sieves (4Å). Acetic anhydride and pyridine were purchased from Acros (Geel, Belgium). Polygalacturonic acid and both pectinases from *Aspergillus niger* and *Rhizopus oryzae* were obtained from Fluka. TLC was performed on Merck precoated Silica 60 plates. Spots were visualized by dipping in 10% H_2SO_4 in MeOH followed by heating. Microwave reactions were carried out in a dedicated microwave oven, i.e. the Biotage Initiator. The microwave power was limited by temperature control once the desired temperature was reached. A sealed vessel of 2–5 mL was used. Preparative HPLC runs were performed on an Applied Biosystems workstation. Elution was effected using a gradient of 5% MeCN and 0.1% TFA in H_2O to 5% H_2O and 0.1% TFA in MeCN. Analytical HPLC runs were performed on a Shimadzu automated HPLC system equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories) and a UV/VIS detector operating at 220 and 254 nm. ^1H NMR (300MHz) and ^{13}C NMR (75.5 MHz) were performed on a Varian G-300 spectrometer. Chemical shifts are given in ppm relative to TMS (^1H, 0.00 ppm), CD_3OD (^1H, 3.31 ppm), $CDCl_3$ (^{13}C, 77.0 ppm), D_2O (^1H, 4.80 ppm), CD_3OD (^{13}C, 49.2 ppm) or acetone (215.94 ppm in D_2O). Exact masses were measured by nano electro spray time-of-flight mass spectrometry on a Micromass LC TOF mass spectrometer. Gold-coated capillaries were loaded with 1 μL of sample (concentration 20 μM) dissolved in a 1:1 (v/v) mixture of CH_3CN / H_2O with 0.1% formic acid. NaI or poly(ethylene glycol) (PEG) was added as

internal standard. The capillary voltage was set between 1100 and 1350 V, and the cone voltage was set at 30 V. Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI TOF) MS was recorded on a Shimadzu Axima-CFR with α-cyano-4-hydroxycinnamic acid or sinapic acid as a matrix. Insulin and adrenocorticotropin fragment 18-39 (Acth) were used for calibration.

Enzymatic cleavage of polygalacturonic acid: A solution of polygalacturonic acid (10.0 g, ≈ 57 mmol monosaccharide) in aqueous NaOAc (0.1 M, 500 mL) was adjusted to pH = 4.5 with NaOH (4M). Pectinase (*Rhizopus oryzae*, 250 mg or *Aspergillus niger*, 250 mg) was added to the polymer suspension and the resulting mixture was allowed to stir for 24 h at 40˚C. TLC (formic acid/n-BuOH/H_2O, 6/4/1) showed the formation of the mono-, di- and tri-galacturonic acid (R_f values resp. 0.35, 0.28 and 0.23). The reaction was terminated by stirring for 5 min at 100˚C. The reaction mixture was concentrated and co-evaporated with MeOH to dryness. The crude mixture was suspended in MeOH (250 mL) and Dowex-H^+ (30 g) was added. The mixture was refluxed for 18 h, the Dowex resin was filtered off and the crude mixture was concentrated. Silica gel chromatography (EtOAc/MeOH/H_2O, 7/2/1 → 3/2/1) was used to separate the mono- di- and trigalacturonic acid methyl esters **5**, **6** and **7**.

Methyl [methyl (methyl α-D-galactopyranosyluronate)-(1→4)-α/β-D-galacto-pyranoside] uronate (6): Disaccharide **6** was obtained as a white foam (3.26 g, 28 %). ^{1}H NMR (300 MHz, CD_3OD): δ = 5.11 (1H, d, *J* = 1.8 Hz), 4.51 (0.66H, d, H-1 α, $J_{1,2}$ = 0.9 Hz), 4.37 (1H, d, *J* = 3.0 Hz), 4.20 (1H, m), 3.90 (1H, dd), 3.81 and 3.75 (2 x s, 2 x 3H, C(O)OCH_3), 3.59 (0.6H, s, OCH_3 β) and 3.42 (2.4H, s, OCH_3 α). ^{13}C NMR (CD_3OD, 75.5 MHz): δ = 171.9 and 170.9 (C-6, C-6'), 105.9 (C-1 β), 102.4 and 101.9 (C-1', C-1 α), 80.5, 79.6, 74.7, 73.5, 73.0, 72.0, 71.3, 70.9, 70.0 and 69.9 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 58.0 and 56.6 (OCH_3 α/β), 53.2 and 52.7 (C(O)O*C*H_3). HRMS for $C_{15}H_{24}O_{13}$ (M, 412.1217): found $[M + Na]^+$ 435.0811, calcd. 435.1115.

Methyl [methyl (methyl α-D-galactopyranosyluronate)-(1→4)-(methyl α-D-galacto-pyranosyluronate)-(1→4)-α/β-D-galacto-pyranoside] uronate (7): Trisaccharide **7** was obtained as an amorphous solid (1.35 g, 12%). ^{1}H NMR (300 MHz, CD_3OD): δ = 5.15

(1H, d, *J* = 1.8 Hz), 5.10 (1H, dd, *J* = 1.2 Hz), 4.51 (0.70H, s, H-1 α), 4.39 – 4.36 (2H, m), 4.20 – 4.18 (1H, m), 3.90 (1H, t), 3.87 (1H, t), 3.80, 3.78 and 3.75 (3 x 3H, 3 x s, C(O)OCH_3). 3.51 (1.0H, s, OCH_3 β) and 3.42 (2.5H, s, OCH_3 α). ^{13}C NMR (CD_3OD, 75.5 MHz): δ = 171.9, 171.3 and 170.9 (C-6, C-6', C-6''), 105.9 (C-1 β), 102.2 and 102.0 (C-1 α, C-1', C-1''), 80.6, 80.3, 79.7, 74.7, 72.9, 72.2, 72.0, 71.3, 70.9 and 70.1 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 58.0 and 56.6 (OCH_3 α/β), 53.1, 53.0 and 52.7 (C(O)O*C*H_3). HRMS for $C_{22}H_{34}O_{19}$ (M, 602.1694): found $[M + Na]^+$ 625.1316, calcd. 625.1592.

Methyl (2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α/β-D-galactopyranoside (8): To a solution of digalacturonic acid methyl ester **6** (3.26 g) in MeOH (150 mL) was added $NaBH_4$ (4.0 g). The solution was stirred for 78 h. After neutralization with Dowex-H^+ the mixture was filtered and concentrated to dryness. Crude disaccharide was suspended in pyridine (100 mL) and Ac_2O (50 mL) was added. The reaction mixture was stirred for 18 h and concentrated *in vacuo* at 60°C. Crude product was taken up in EtOAc (250 mL) and washed with aqueous NaOH (1M, 100 mL) aqueous HCl (1M, 100 mL), and brine (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. Silica gel chromatography (hexane/EtOAc, 1/1 → 1/2) was used to obtain pure disaccharide **6** as a white foam (3.85 g). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.56 (1H, d, H-4', $J_{3',4'}$ = 3.0 Hz), 5.39 (1H, dd, H-3', $J_{2',3'}$ = 11.7 Hz, $J_{3',4'}$= 3.3 Hz), 5.25 – 5.18 (3H, m, H-3, H-2, H-2'), 4.98 (2H, m, H-1, H-1'), 4.54 (1H, t, H-5, $J_{5,6}$ = 6.6 Hz), 4.17 – 4.06 (6H, m, H-4, H-5', 2 x H-6, 2 x H-6'), 3.52 (0.6H, s, OCH_3 β), 3.42 (2.4H, s, OCH_3 α), 2.14, 2.13, 2.09, 2.08, 2.07, 2.03 and 1.99 (7 x 3H, 7 x s, C(O)CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 170.7, 170.4, 170.2, 170.1 169.9 and 169.9 (*C*(O)CH_3), 101.7 (C-1 β), 99.0 (C-1'), 97.1 (C-1 α), 72.7, 71.8, 69.4, 68.5, 68.2, 67.7, 67.3 and 66.9 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 62.3, 61.9, 60.6 and 60.4 (C-6, C-6'), 55.4 and 55.2 (OCH_3 α/β), and 20.6 (C(O)*C*H_3). HRMS for $C_{27}H_{38}O_{18}$ (M, 650.2058): found $[M + Na]^+$ 673.1956, calcd. 672.9821.

Acetyl (2,3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α/β-D-galactopyranoside (**9**): To a cooled solution of **8** (3.80 g, 5.84 mmol) in Ac_2O (45 mL) was added concentrated H_2SO_4 (150 μL). The solution was stirred at 0°C for 2 h, diluted with

CH_2Cl_2 (150 mL) and quenched with aqueous $NaHCO_3$ (5%, 10 mL). The reaction mixture was concentrated to dryness at 60 ˚C. The residue was taken up in EtOAc (200 mL) and washed with aqueous NaOH (1M, 75 mL) and brine (75 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to yield **9** as a white foam (3.83 g, 97 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.37 (1H, d, H-1α, $J_{1,2}$ = 3.6 Hz), 5.57 (1H, dd, H-4', $J_{3',4'}$ = 3.0 Hz, $J_{4',5'}$= 3.3 Hz), 5.43 – 5.35 (2H, m, H-3' and H-3), 5.26 – 5.19 (2H, m, H-2', H-2), 5.02 (H, d, H-1', $J_{1',2'}$ = 3.6 Hz), 4.52 (1H, t, H-5, $J_{5,6}$ = 7.8 Hz), 4.38 – 4.05 (6H, m, H-4, H-5', 2 x H-6', 2 x H-6), 2.16, 2.14, 2.11, 2.11, 2.07, 2.03, 2.03 and 1.99 (8 x 3H, 8 x s, C(O)CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 170.5, 170.5, 170.3, 170.1, 169.8, 169.4, 169.0 and 168.8 (*C*(O)CH_3), 99.1 and 89.8 (C-1 and C-1'), 70.3, 69.2, 68.4, 68.2, 67.7, 67.2, 67.1 and 66.1 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 61.5 and 60.5 (C-6, C-6'), 20.9, 20.7 and 20.6 (C(O)*C*H_3). HRMS for $C_{28}H_{38}O_{19}$ (M, 678.2007): found $[M + Na]^+$ 701.1905, calcd. 701.1530.

(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α-D-galacto-pyranosyl trichloroacetimidate (10): A solution of **9** (4.50 g, 6.64 mmol) and N_2H_4.HOAc (672 mg, 7.29 mmol) in dry DMF (50 mL) was stirred at r.t. for 70 h. The mixture was concentrated in vacuo at 60˚C, taken up in EtOAc (100 mL) and washed twice with brine (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. The obtained white foam (4.24 g, quantitative) and trichloroacetonitrile (2.0 mL, 20 mmol) were dissolved in dry CH_2Cl_2 (50 mL) and the solution was cooled to 0˚C. DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene, 301 µL, 2.0 mmol) was added and the solution was stirred at 0˚C for 1 h followed by 20 h at r.t. The reaction mixture was concentrated and product **10** was isolated by silica gel chromatography (hex/EtOAc, 1/1) as a slightly yellow foam (3.30 g, 64%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.69 (1H, s, OCNHCCl_3), 6.61 (1H, d, H-1α, $J_{1,2}$ = 3.6 Hz), 5.57 (1H, d, H-4', $J_{3',4'}$ = 3.0 Hz), 5.42 (1H, dd, H-2, $J_{1,2}$ = 3.6 Hz, $J_{2,3}$ = 11.3 Hz), 5.37 – 5.28 (2H, m, H-3, H-3'), 5.24 (1H, dd, H-2', $J_{1',2'}$ = 3.6 Hz, $J_{2',3'}$ = 11.0 Hz), 5.03 (1H, m, H-1', $J_{1',2'}$ = 3.6 Hz), 4.53 (1H, t, H-5, $J_{5,6}$ = 6.0 Hz), 4.38 – 4.28 (3H, m, H-4, 2 x H-6'), 4.16 – 4.11 (3H, m, H-5', 2 x H-6), 2.12, 2.11, 2.04 and 2.00 (7 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 170.4, 170.2, 170.0, 169.8 and 168.6 (*C*(O)CH_3), 160.7 (O*C*(NH)CCl_3), 98.9 and 93.5 (C-1, C-1'), 90.8 (OCNH*C*Cl_3), 70.5, 69.3, 68.1, 67.7, 67.2, 67.1 and 66.7 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 61.8 and 60.6 (C-6, C-6'), 20.9, 20.6 and 20.4 (C(O)*C*H_3).

3-bromopropyl (2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranoside (11): A solution of **10** (1.22 g, 1.56 mmol) and 3-bromopropanol (0.68 mL, 7.8 mmol) in CH_2Cl_2 (30 mL) was stirred under N_2 at 0°C. TMSOTf (91 μL, 0.47 mmol) was added and the mixture was stirred at 0°C for 1 h. The mixture was warmed to r.t. and after 1 h the reaction was quenched with Et_3N and concentrated in vacuo. Silica gel chromatography (hex/EtOAc, 3/2 → 1/1) afforded pure **11** (0.77 g, 65 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.57 (1H, dd, H-4', $J_{3',4'}$ = 3.0 Hz, $J_{4',5'}$= 1.1 Hz), 5.39 (1H, dd, H-3', $J_{2',3'}$ = 11.0 Hz, $J_{3',4'}$= 3.3 Hz), 5.22 – 5.14 (2H, m, H-2, H-2'), 4.93 (1H, d, H-1', $J_{1',2'}$ = 3.6 Hz), 4.82 (1H, dd, H-3, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 10.7 Hz), 4.48 (1H, d, H-1, $J_{1,2}$ = 8.3 Hz), 4.54 – 4.43 (2H, m, H-5', H-6a), 4.20 – 4.10 (3H, H-6b and 2 x H-6'), 4.07 (1H, d, H-4, $J_{3,4}$ = 2.8 Hz), 4.03 – 4.96 and 3.73 – 3.65 (each 1H, 2 x m, CH_2O_{Gal}), 3.79 (1H, t, H-5, $J_{5,6}$ = 5.0 Hz), 3.50 (2H, dd, CH_2Br, J = 5.5 Hz, J = 7.4 Hz), 2.20 – 2.02 (2H, m, $OCH_2C\mathit{H}_2CH_2Br$), 2.13, 2.11, 2.08, 2.07, 2.04 and 1.99 (7 x 3H, 7 x s, $C(O)CH_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.5, 170.1, 169.8 and 169.2 ($\mathit{C}(O)CH_3$), 101.4 (C-1), 99.4 (C-1'), 72.6, 71.9, 68.7, 68.5, 67.8, 67.3 and 67.0 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 67.1 ($O\mathit{C}H_2CH_2CH_2Br$), 61.9 and 60.5 (C-6, C-6'), 32.3 (CH_2Br), 30.3 ($OCH_2\mathit{C}H_2CH_2Br$) and 20.6 ($C(O)\mathit{C}H_3$). HRMS for $C_{29}H_{41}BrO_{18}$ (M, 756.1476): found $[M + Na]^+$ 779.1374, calcd. 779.0682.

3-azidopropyl (2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranoside (1): A solution of **11** (1.21 g, 1.60 mmol) and NaN_3 (0.52 g, 8 mmol) in dry DMF (20 mL) was stirred at 100°C for 20 h. The solution was concentrated in vacuo at 60°C, taken up in EtOAc (150 mL) and washed with brine (75 mL). The organic phase was dried (Na_2SO_4), filtered and concentrated. Product **1** was lyophilized from MeCN/H_2O and was obtained as white powder (1.09 g, 95%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.50 (1H, dd, H-4', $J_{3',4'}$ =3.0 Hz, $J_{4',5'}$= 1.1 Hz), 5.32 (1H, dd, H-3', $J_{2',3'}$ = 11.0 Hz, $J_{3',4'}$= 3.3 Hz), 5.15 – 5.08 (2H, m, H-2, H-2'), 4.93 (1H, d, H-1', $J_{1',2'}$ = 3.6 Hz), 4.75 (1H, dd, H-3, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 2.8 Hz), 4.42 (1H, d, H-1, $J_{1,2}$ = 7.8 Hz), 3.91 – 3.88 and 3.57 – 3.54 (each 1H, 2 x m, CH_2O_{Gal}), 3.79 (1H, t, H-5, $J_{5,6}$ = 6.6 Hz), 3.34 (2H, t, CH_2N_3, J = 6.6 Hz), 2.06, 2.04, 2.01, 2.00, 1.99, 1.97 and 1.92 (7 x 3H, 7 x s, $C(O)CH_3$) and 1.90 – 1.77 (2H, m, $OCH_2C\mathit{H}_2CH_2N_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.4, 170.1, 169.8 and 169.1 ($\mathit{C}(O)CH_3$), 101.1 (C-1), 99.4 (C-1'), 72.6, 71.9, 68.6, 67.8, 67.3 and 67.0 (C-2, C-2',

C-3, C-3', C-4, C-4', C-5, C-5'), 66.2 ($OCH_2CH_2CH_2N_3$), 61.9 and 60.4 (C-6, C-6'), 48.0 (OCH_2), 28.9 (CH_2N_3) and 20.8 ($C(O)CH_3$). HRMS for $C_{29}H_{41}N_3O_{18}$ (M, 719.2385): found $[M + Na]^+$ 742.2283, calcd. 742.1694.

Methyl (2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-(2,3,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α/β-D-galactopyranoside (12): To a solution of trigalacturonic acid methyl ester **7** (1.35 g) in MeOH (100 mL) was added $NaBH_4$ (2.0 g). The solution was stirred for 78 h. After neutralization with Dowex-H^+ the mixture was filtered and concentrated to dryness. Crude trisaccharide was suspended in pyridine (50 mL) and Ac_2O (25 mL) was added. The reaction mixture was stirred for 18 h and concentrated *in vacuo* at 60˚C. Crude product was taken up in EtOAc (250 mL) and washed with aqueous NaOH (1M, 100 mL) aqueous HCl (1M, 100 mL), and brine (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. Silica gel chromatography (hexane/EtOAc, 1/2 → 1/4) was used to obtain pure trisaccharide **12** as a white foam (2.30 g). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.56 (1H, dd, H-4'', $J_{3'',4''}$ = 3.0 Hz, $J_{4'',5''}$ = 1.4 Hz), 5.40 (1H, dd, H-3'', $J_{2'',3''}$ = 11.3 Hz, $J_{3'',4''}$ = 3.3 Hz), 5.33 (1H, dd, H-3', $J_{2',3'}$ = 11.3 Hz, $J_{3',4'}$ = 3.3 Hz), 5.25 (1H, t, H-2'', J = 2.8 Hz), 5.21 – 5.16 (3H, m, H-2, H-2', H-3), 5.00 – 4.98 (2H, m, H-1', H-1''), 4.95 (1H, d, H-1, $J_{1,2}$ = 3.6 Hz), 4.54 (1H, t, H-5, $J_{5,6}$ = 6.6 Hz), 4.46 – 3.99 (10 H, m, H-4, H-4', H-5', H-5'', 2 x H-6, 2 x H-6', 2 x H-6''), 3.51 (0.6H, s, OCH_3 β), 3.41 (2.2H, s, OCH_3 α), 2.14, 2.13, 2.10, 2.10, 2.09, 2.08, 2.07, 2.05, 2.04 and 1.99 (10 x 3H, 10 x s, $C(O)CH_3$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 170.5, 170.2, 170.1 and 170.0 ($C(O)CH_3$), 101.8 (C-1 β), 99.2 and 99.1 (C-1', C-1''), 97.1 (C-1 α), 72.5, 72.0, 69.3, 69.2, 68.8, 68.5, 68.3, 67.8, 67.7, 67.3, and 66.9 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 62.5, 62.1, 61.1 and 60.3 (C-6, C-6', C-6''), 56.6 and 55.3 (OCH_3 α/β), 20.9, 20.7 and 20.6 ($C(O)CH_3$). HRMS for $C_{39}H_{54}O_{26}$ (M, 938.2903): found $[M + Na]^+$ 961.2786, calcd. 961.2801.

Acetyl (2,3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α/β-D-galactopyranoside (13): To a cooled solution of **12** (2.25 g, 2.40 mmol) in Ac_2O (20 mL) was added concentrated H_2SO_4 (60 µL). The solution was stirred at 0˚C for 2 hours, diluted with CH_2Cl_2 (100 mL) and quenched with aqueous $NaHCO_3$ (5%, 5 mL). The reaction mixture was concentrated to

dryness at 60 ˚C. Crude product was taken up in EtOAc (200 mL) and washed with NaOH (1M, 75 mL) and brine (75 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. After lyophilization **13** was obtained as white powder (2.08 g, 90%) ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.37 (1H, d, H-1α, $J_{1,2}$ = 3.6 Hz), 5.56 (1H, d, H-4'', $J_{3'',4''}$ = 2.2 Hz), 5.42 – 5.20 (6H, m, H-3, H-3', H-3'', H-2, H-2', H-2''), 5.00 – 4.98 (2H, 2 x d, H-1', H-1'', $J_{1',2'}$= 3.6 Hz, $J_{1'',2''}$ = 3.6 Hz), 4.53 (1H, t, H-5, $J_{5,6}$ = 6.6 Hz), 4.46 – 4.00 (10 H, m, H-4, H-4', H-5', H-5'', 2 x H-6, 2 x H-6', 2 x H-6''), 2.15, 2.14, 2.13, 2.11, 2.10, 2.08, 2.07, 2.05, 2.04, 2.01 and 1.99 (11 x 3H, 11 x s, C(O)CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 170.7, 170.5, 170.3 , 170.2, 170.1, 169.6 and 168.9 (*C*(O)CH_3), 99.3 and 89.8 (C-1, C-1', C-1''), 70.5, 69.3, 69.1, 68.9, 68.3, 67.8, 67.4, 66.9 and 66.3 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 61.9, 61.0 and 60.4 (C-6, C-6', C-6'') 20.9and 20.6 (C(O)*C*H_3). HRMS for $C_{40}H_{54}O_{27}$ (M, 966.2852): found $[M + Na]^+$ 989.2119, calcd. 989.2750.

(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-α-D-galacto-pyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α/β-D-galactopyranosyl trichloroacetimidate (14): A solution of **13** (2.50 g, 2.60 mmol) and N_2H_4.HOAc (262 mg, 2.85 mmol) in dry DMF (20 mL) was stirred at r.t. for 70 h. The mixture was concentrated in vacuo at 60˚C, taken up in EtOAc (150 mL) and washed twice with brine (75 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. The obtained white foam (2.22 g, 93%) and trichloroacetonitrile (0.72 mL, 7.2 mmol) were dissolved in dry CH_2Cl_2 (50 mL) and the solution was cooled to 0˚C under a N_2. 1,8-Diazabicyclo[5.4.0]undec-7-ene (108 µL, 0.72 mmol) was added and the solution was stirred at 0˚C for 1 h followed by 2 h at r.t.. The reaction mixture was concentrated and product **14** was isolated by silica gel chromatography (hex/EtOAc, 2/3 → 1/3) as a white amorphous solid (1.17 g, 42%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.69 (1H, s, OCNHCCl_3), 6.60 (1H, d, H-1α, $J_{1,2}$ = 3.3 Hz), 5.56 (1H, d, H-4'', $J_{3'',4''}$ = 2.8 Hz), 5.42 – 5.20 (6H, m, H-2, H-2', H-2'', H-3, H-3', H-3''), 5.00 and 4.99 (2 x 1H, 2 x d, H-1', H-1'', $J_{1',2'}$ = 3.0 Hz, $J_{1'',2''}$ = 2.8 Hz), 4.53 (1H, t, H-5, $J_{5,6}$ = 7.2 Hz), 4.46 – 4.02 (10H, m, H-4, H-4', H-5', H-5'', 2 x H-6, 2 x H-6', 2 x H-6''), 2.14, 2.12, 2.10, 2.09, 2.06, 2.05, 2.04, 2.04, 2.02 and 1.99 (10 x 3H, 10 x s, C(O)CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 170.4, 170.2, 170.1, 169.9 and 169.6 (*C*(O)CH_3), 160.7 (OC(NH)CCl_3), 99.3 and 99.1 (C-1', C-1''), 93.5 (C-1) 90.7

(OCNH*C*Cl_3), 70.6, 69.2, 68.9, 68.2, 67.7, 67.3, 66.9 and 66.7 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 62.0, 61.2 and 60.3 (C-6, C-6', C-6''), 20.9 and 20.5 (C(O)*C*H_3).

3-bromopropyl (2,3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranoside (15): A solution of **14** (1.17 g, 1.09 mmol) and 3-bromopropanol (0.48 mL, 5.5 mmol) in CH_2Cl_2 (25 mL) was stirred under N_2 at 0˚C. TMSOTf (63 µL, 0.33 mmol) was added and the mixture was stirred at 0˚C for 1 h. The mixture was warmed to r.t. and after 1 h the reaction was quenched with Et_3N and concentrated in vacuo. Silica gel chromatography (hex/EtOAc, 2/3 → 1/3) afforded pure **15** as white foam (607 mg, 55 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.56 (1H, dd, H-4'', $J_{3'',4''}$ = 3.3 Hz, $J_{4'',5''}$ = 1.1 Hz), 5.39 (1H, dd, H-3'', $J_{2'',3''}$ = 11.0 Hz, $J_{3'',4''}$ = 3.3 Hz), 5.33 – 5.21 (3H, m, H-2', H-2'', H-3'), 5.00 and 4.98 (each 1H, 2 x d, H-1' and H-1'', $J_{1',2'}$ = 3.6 Hz, $J_{1'',2''}$ = 3.3 Hz), 4.86 (1H, dd, H-3, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 2.8 Hz), 4.49 (1H, d, H-1, $J_{1,2}$ = 7.7 Hz), 4.54 – 4.05 (9H, m, H-4, H-5', H-5'', 2 x H-6, 2 x H-6', 2 x H-6''), 4.31 (1H,d, H-4', $J_{3',4'}$= 1.9 Hz), 4.02 – 3.96 and 3.75 – 3.66 (2 x 1H, 2 x m, CH_2O_{Gal}), 3.82 (1H, t, H-5, $J_{5,6}$ = 6.0 Hz), 3.50 (2H, dd, CH_2Br, *J* = 5.5 Hz, *J* = 6.6 Hz), 2.14, 2.11, 2.11, 2.10, 2.09, 2.07, 2.07, 2.06, 2.03 and 1.99 (10 x 3H, 10 x s, C(O)CH_3) and 2.20 – 2.05 (2H, m, $OCH_2CH_2CH_2N_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.4, 170.1, 170.0, 169.9, 169.8 and 168.9 (*C*(O)CH_3), 101.1 (C-1), 99.1 and 99.0 (C-1', C-1''), 72.2, 71.8, 69.1, 68.7, 68.4, 68.0, 67.8, 67.5, 67.1 and 66.6 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5' , C-5''), 66.8 ($OCH_2CH_2CH_2$Br), 62.0, 60.9 and 60.2 (C-6, C-6', C-6''), 32.1 (CH_2Br), 30.1 ($OCH_2CH_2CH_2$Br), 20.7, 20.6 and 20.4 (C(O)*C*H_3). HRMS for $C_{41}H_{57}BrO_{26}$ (M, 1044.2321): found $[M + Na]^+$ 1067.1964, calcd 1067.2159.

3-azidopropyl (2,3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranoside (16): A solution of **15** (607 mg, 0.60 mmol) and NaN_3 (195 mg, 3 mmol) in dry DMF (20 mL) was stirred at 100˚C for 20 h. The solution was concentrated in vacuo at 60˚C, taken up in EtOAc (50 mL) and washed twice with brine (25 mL). The organic phase was dried (Na_2SO_4), filtered and concentrated. After lyophilization **16** was obtained as white powder (548 mg, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.56 (1H, dd, H-4'', $J_{3'',4''}$ = 3.3 Hz), 5.39 (1H, dd, H-3'', $J_{2'',3''}$ = 11.0 Hz, $J_{3'',4''}$ = 3.3 Hz), 5.32– 5.22 (3H, m, H-2', H-2'', H-3'), 5.17 (1H, dd, H-2,

$J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 10.7 Hz), 4.99 and 4.98 (each 1H, 2 x d, H-1', H-1'', $J_{1',2'}$ = 3.6 Hz, $J_{1'',2''}$ = 3.3 Hz), 4.83 (1H, dd, H-3, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 2.8 Hz), 4.46 (1H, d, H-1, $J_{1,2}$ = 7.7 Hz), 4.56 – 4.02 (9H, m, H-4, H-5', H-5'', 2 x H-6, 2 x H-6', 2 x H-6''), 4.30 (1H, d, H-4', $J_{3,4}$ = 1.7 Hz), 3.98 – 3.92 and 3.64 – 3.66 (2 x 1H, 2 x m, CH_2O_{Gal}), 3.79 (1H, t, H-5, $J_{5,6}$ = 6.6 Hz), 3.38 (2H, t, CH_2N_3, J = 6.6 Hz), 2.14, 2.11, 2.10, 2.09, 2.08, 2.07, 2.06, 2.05, 2.03 and 1.99 (10 x 3H, 10 x s, C(O)CH_3) and 1.90 – 1.82 (2H, m, $OCH_2CH_2CH_2N_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.6, 170.2, 170.0 and 169.0 (C(O)CH_3), 101.0 (C-1), 99.3 and 99.2 (C-1', C-1''), 72.4, 72.0, 69.2, 68.9, 68.5, 68.2, 68.0, 67.7, 67.3 and 66.8 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 66.0 ($OCH_2CH_2CH_2N_3$), 62.1, 61.0 and 60.3 (C-6, C-6', C-6''), 47.9 (CH_2N_3), 28.9 ($OCH_2CH_2CH_2N_3$) and 20.5 (C(O)CH_3). HRMS for $C_{41}H_{57}N_3O_{26}$ (M, 1007.3230): found $[M + Na]^+$ 1030.2924, calcd. 1030.3128.

Monovalent pentynoic acid dendrimer (17b): Compound **17a** (295 mg, 1.0 mmol) was stirred in a mixture of CH_2Cl_2/TFA (1/1, v/v, 20 mL) with a trace of H_2O for 3 h. The reaction mixture was then concentrated to dryness and taken up in CH_2Cl_2 (10 mL). BOP (487 mg, 1.1 mmol) and 4-pentynoic acid (108 mg, 1.1 mmol) were added followed by DiPEA (0.55 mL, 3.3 mmol). The mixture was stirred for 72 h and concentrated in vacuo. The crude mixture was taken up in EtOAc (50 mL) and washed twice with $KHSO_4$ (1M, 15 mL), twice with NaOH (1M, 15 mL) and with brine (15 mL). Pure product was obtained as a white solid (192 mg, 70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.64 (1H, m, CH_{arom}-6), 7.55 (1H, s, CH_{arom}-2), 7.35 (1H, t, CH_{arom}-5), 7.11 (1H, dd, CH_{arom}-4), 6.12 (1H, bs, C(O)NH), 4.10 (2H, t, OCH_2CH_2NH, J = 5.4 Hz), 3.92 (3H, s, OCH_3), 3.74 – 3.68 (2H, m, OCH_2CH_2NH), 2.59 – 2.53 and 2.48 – 2.42 (2 x 2H, 2 x m, C(O)CH_2CH_2CCH) and 1.94 (1H, t, CH_2CH_2CCH). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 171.3 (C(O)NH), 166.8 (C(O)OCH_3), 158.6 (C_{arom}-3), 131.8 (C_{arom}-1), 129.8 (C_{arom}-5), 122.7 (C_{arom}-6), 119.8 (C_{arom}-4), 115.1 (C_{arom}-2), 69.6 (CH_2CH_2CCH), 67.3 (OCH_2CH_2NH), 52.4 (C(O)OCH_3), 39.2 (OCH_2CH_2NH), 35.5 (C(O)CH_2CH_2CCH).) and 15.1 (C(O)CH_2CH_2CCH).

Divalent pentynoic acid dendrimer (18b): Divalent dendrimer **18a** (454 mg, 1.0 mmol) was stirred in a mixture of CH_2Cl_2/TFA (1/1, v/v, 16 mL) with a trace of H_2O for 3 h. The reaction mixture was then concentrated to dryness and taken up in CH_2Cl_2 (5 mL). BOP (0.97 g, 2.2 mmol) and 4-pentynoic acid (215 mg, 2.2 mmol) were added followed by

DiPEA (1.10 mL, 6.6 mmol). The reaction mixture was stirred for 18 h and concentrated. Crude product was taken up in EtOAc (100 mL) and washed with $KHSO_4$ (1M, 50 mL), NaOH (1M, 50 mL) and brine (50 mL). Dendrimer **18b** was obtained after silica gel chromatography (EtOAc) as a white solid (380 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.19 (2H, d, CH_{arom}-2,6), 6.65 (1H, s, CH_{arom}-4), 6.05 (2H, bs, C(O)NH), 4.09 – 4.06 (4H, m, OCH_2CH_2NH), 3.91 (3H, s, OCH_3), 3.73 – 3.67 (4H, m, OCH_2CH_2NH), 2.57 – 2.51 and 2.46 – 2.40 (2 x 4H, 2 x m, $C(O)CH_2CH_2CCH$) and 1.95 (2H, t, CH_2CH_2CCH, *J* = 2.1 Hz). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 171.4 (C(O)NH), 166.7 ($C(O)OCH_3$), 159.7 (C_{arom}-3,5), 132.5 (C_{arom}-1), 108.4 (C_{arom}-2,6), 106.6 (C_{arom}-4), 69.7 (CH_2CH_2CCH), 67.4 (OCH_2CH_2NH), 52.6 ($C(O)OCH_3$), 39.1 (OCH_2CH_2NH), 35.5 ($C(O)CH_2CH_2CCH$) and 15.1 ($C(O)CH_2CH_2CCH$).

Tetravalent pentynoic acid dendrimer (19b): Tetravalent dendrimer **19a** (1099 mg, 1.0 mmol) was stirred in a mixture of CH_2Cl_2/TFA (1/1, v/v, 20 mL) with a trace of H_2O for 1 h. The reaction mixture was then concentrated to dryness and taken up in CH_2Cl_2 (10 mL), BOP (2.65 g, 6.0 mmol) and 4-pentynoic acid (589 mg, 6.0 mmol) were added followed by DiPEA (1.98 mL, 12.0 mmol). The reaction mixture was stirred for 18 h and concentrated at 60°C. Dendrimer **19b** was obtained after silica gel chromatography (CH_2Cl_2/MeOH/DMF, 94/5/1 → 85/10/5) as a white solid (826 mg, 81%, small contamination). ^{1}H NMR (300 MHz, DMSO): δ = 8.66 (2H, s, C(O)NH), 8.15 (4H, t, C(O)NH), 7.09 (2H, m, CH_{arom}-2,6), 7.04 (4H, s, CH_{arom}-2',6'), 6.84 (1H, t, CH_{arom}-4), 6.65 (2H, t, CH_{arom}-4'), 4.16 (4H, t, OCH_2CH_2NH, *J* = 5.4 Hz), 4.01 (8H, t, OCH_2CH_2NH, *J* = 5.4 Hz), 3.83 (3H, s, OCH_3), 3.64 – 3.58 (4H, m, OCH_2CH_2NH), 3.45 – 3.39 (8H, m, OCH_2CH_2NH), 2.73 (4H, t, CH_2CH_2CCH, *J* = 2.1 Hz) and 2.39 – 2.27 (16H, m, $C(O)CH_2CH_2CCH$). ^{13}C NMR (75.5 MHz, DMSO): δ = 170.6 (C(O)NH), 165.9 ($C(O)OCH_3$), 159.7 (C_{arom}-3,5), 159.4 (C_{arom}-3',5'), 136.2 (C_{arom}-1'), 131.6 (C_{arom}-1), 107.7 (C_{arom}-2,6), 106.0 (C_{arom}-2',6'), 104.1 (C_{arom}-4'), 83.7 (CH_2CH_2CCH), 71.2 (CH_2CH_2CCH), 66.6 (OCH_2CH_2NH), 52.3 ($C(O)OCH_3$), 38.2 (OCH_2CH_2NH), 34.0 ($C(O)CH_2CH_2CCH$) and 14.2 ($C(O)CH_2CH_2CCH$).

Octavalent pentynoic acid dendrimer (20b): Octavalent dendrimer **20a** (597 mg, 0.25 mmol) was stirred in a mixture of CH_2Cl_2/TFA (1/1, v/v, 10 mL) with a trace of H_2O for 1 h. The reaction mixture was then concentrated to dryness and taken up in dry DMF (5 mL),

BOP (1.33 g, 3 mmol) and 4-pentynoic acid (295 mg, 3 mmol) were added followed by DiPEA (0.99 mL, 6 mmol). The reaction mixture was stirred for 18 h and concentrated at 60°C. Silica gel chromatography (CH_2Cl_2/MeOH/DMF, 94/5/1 → 85/10/5) was used for purification although a small impurity remains present (621 mg, ≈ quantitative). ^{1}H NMR (300 MHz, DMSO): δ = 8.69 (6H, s, C(O)NH), 8.16 (8H, t, C(O)NH), 7.07 – 7.04 (14H, m, CH_{arom}-2,6, 2',6', 2'',6''), 6.82 (1H, s, CH_{arom}-4), 6.70 (2H, bs, CH_{arom}-4'), 6.64 (4H, d, CH_{arom}-4''), 4.14 (12H, bs, OC*H*$_2$CH$_2$NH), 4.01 (16H, s, OC*H*$_2$CH$_2$NH), 3.80 (3H, s, OCH_3), 3.61 (12H, d, OCH_2CH_2NH), 3.42 (16H, d, OCH_2CH_2NH), 2.72 (8H, bs, CH_2CH_2CCH) and 2.35 – 2.29 (32H, m, C(O)C*H*$_2$C*H*$_2$CCH). ^{13}C NMR (75.5 MHz, DMSO): δ = 170.6 (C(O)NH), 165.9 (*C*(O)OCH_3), 159.4 (C_{arom}-3'',5''), 136.2 (C_{arom}-1''), 106.0 (C_{arom}-2'',6''), 104.1 (C_{arom}-4''), 83.7 (CH_2CH_2*C*CH), 71.2 (CH_2CH_2C*C*H), 66.6 (O*C*H$_2$CH$_2$NH), 53.4 (C(O)O*C*H$_3$), 40.3 (OCH_2*C*H$_2$NH), 34.0 (C(O)*C*H$_2$CH$_2$CCH) and 14.2 (C(O)CH$_2$*C*H$_2$CCH).

General "Click" procedure: Dendrimer was mixed with the galabiose azide (1.5 eq per alkyne), $CuSO_4.5H_2O$ (0.15 eq per alkyne) and sodium ascorbate (0.3 eq per alkyne) in 1% H_2O/DMF. The mixture was heated under microwave irradiation to 80°C for 20 min. The reaction mixture was concentrated in vacuo at 60°C and after silica gel chromatography the dendrimer was obtained.

General deacetylation procedure: A solution of the dendrimer in MeOH (5 mL) was treated with NaOMe in MeOH (30% wt solution, 50 µL) for 5 h. The solution was neutralized by Dowex-H$^+$, filtered and concentrated in vacuo. Deprotected dendrimers were purified using preparative HPLC. Pure products were obtained after lyophilisation.

Monovalent Galabiose dendrimer (17c): The general "click" procedure was applied. Monovalent galabiose derivative was obtained as a white foam (179 mg, 72%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.65 (1H, d, CH_{arom}-6), 7.53 (1H, t, CH_{arom}-2), 7,35 (1H, t, CH_{arom}-5), 7.09 (1H, dd, CH_{arom}-4), 6.34 (1H, bs, NHCO), 5.58 (1H, dd, H-4', $J_{3',4'}$ =3.0 Hz, $J_{4',5'}$ = 1.1 Hz), 5.41 (1H, dd, H-3', $J_{2',3'}$ = 11.0 Hz, $J_{3',4'}$= 3.3 Hz), 5.23– 5.16 (2H, m, H-2, H-2'), 5.00 (1H, d, H-1', $J_{1',2'}$ = 3.9Hz), 4.80 (1H, dd, H-3, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 2.7 Hz), 4.45 (1H, d, H-1, $J_{1,2}$ = 7.70 Hz), 4.55 – 4.35, 4.21 – 4.04 and 3.90 – 3.76 (5H, 6H, 2H, 3 x m, H-1, H-4, H-5', H-6, H-6', OC*H*$_2$CH$_2$NH, $CH_2N_{triazole}$, CH_2O_{Gal}), 3.91 (3H, s, OCH_3), 3.69 – 3.63

(2H, bs, OCH_2CH_2NH), 3.49 – 3.40 (1H, m, H-5), 3.06 and 2.67 (2 x bs, each 2H, $C(O)CH_2CH_2C_{triazole}$), 2.20 – 2.02 (2H, m, $OCH_2CH_2CH_2N_{triazole}$), 2.14, 2.10, 2.09, 2.08, 2.06, 2.04 and 1.99 (7 x 3H, 7 x s, $C(O)CH_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.5, 170.4, 170.1, 169.9 and 169.2 ($C(O)CH_3$), 166.6 ($C(O)OCH_3$), 158.3 (C_{arom}-3), 131.4 (C_{arom}-1), 129.4 (C_{arom}-5), 122.3 (C_{arom}-6), 119.6 (C_{arom}-4), 114.7 (C_{arom}-2), 101.0 (C-1), 99.4 (C-1'), 72.6, 71.9, 68.5, 67.7, 67.2 and 67.0 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 66.8 ($OCH_2CH_2CH_2N_{triazole}$), 65.6 ($OCH_2CH_2NH$), 61.9 and 60.4 (C-6, C-6'), 52.1 ($C(O)OCH_3$), 46.6 ($CH_2N_{triazole}$), 38.8 and 30.0 ($C(O)CH_2CH_2C_{triazole}$), 20.8 and 20.6 ($C(O)CH_3$). HRMS for $C_{44}H_{58}N_4O_{22}$ (M, 994.3543): found $[M + H]^+$ 995.3600, calcd. 995.3621. The general deacetylation procedure yielded compound **17c** as a clear oil (30.7 mg, 35%). ^1H NMR (300 MHz, D_2O): δ = 7.72(1H, bs, $CH_{triazole}$), 7.61 (2H, s, CH_{arom}-6), 7.46 – 7.40 (2H, m, CH_{arom}-2,5), 7.14 (1H, d, CH_{arom}-4), 4.97 (1H, d, H-1', $J_{1',2'}$ = 3.6 Hz), 4.39 – 4.27 (6H, m), 4.39 – 4.31 (2H, m), 4.22 (2H, t), 4.02 - 3.97 (4H, m), 3.94 – 3.67 (14H, m), 3.56 – 3.42 (5H, m), 2.98 and 2.60 (2 x 2H, 2 x bt, $C(O)CH_2CH_2C_{triazole}$) and 2.00 (2H, bt, $OCH_2CH_2CH_2N_{triazole}$). ^{13}C NMR (75.5 MHz, D_2O): δ = 175.6 (C(O)NH), 169.4 ($C(O)OCH_3$), 158.9 (C_{arom}-3), 131.5 (C_{arom}-1), 130.8 (C_{arom}-5), 123.1 (C_{arom}-6), 121.0 (C_{arom}-4), 115.6 (C_{arom}-2), 103.7 (C-1), 101.0 (C-1'), 77.9, 77.7, 75.7, 73.1, 71.6, 71.4, 69.8 and 69.6 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 67.5 ($OCH_2CH_2CH_2N_{triazole}$), 67.0 ($OCH_2CH_2NH$), 61.2 and 60.7 (C-6, C-6'), 53.4 ($C(O)OCH_3$), 47.7 ($CH_2N_{triazole}$), 39.4 and 35.7 ($C(O)CH_2CH_2C_{triazole}$) and 21.7 ($OCH_2CH_2CH_2N_{triazole}$). HRMS for $C_{30}H_{44}N_4O_{15}$ (M, 700.2803): found $[M + H]^+$ 701.0662, calcd. 701.2881.

Divalent Galabiose dendrimer (18c): The general "click" procedure was applied. Protected divalent galabiose dendrimer was obtained as a white foam (155 mg, 84%). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.44 (2H, bs, $CH_{triazole}$), 7.15 (2H, s, CH_{arom}-2,6), 6.67 (1H, s, CH_{arom}-4), 6.62 (2H, bs, NHCO), 5.58 (2H, dd, H-4', $J_{3',4'}$ = 2.5 Hz), 5.41(2H, dd, H-3', $J_{2',3'}$ = 11.3 Hz, $J_{3',4'}$ = 3.3 Hz), 5.23– 5.16 (4H, m, H-2, H-2'), 5.00 (2H, d, H-1', $J_{1',2'}$ = 3.6Hz), 4.81 (2H, dd, H-3, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 2.5 Hz), 4.47 (2H, d, H-1, $J_{1,2}$ = 7.70 Hz), 4.55 – 4.35, 4.17 – 4.00 and 3.87 – 3.75 (10H, 12H, 4H, 3 x m, H-1, H-4, H-5', H-6, H-6', OCH_2CH_2NH, $CH_2N_{triazole}$, CH_2O_{Gal}), 3.90 (3H, s, $C(O)OCH_3$),3.65 (4H, bs, OCH_2CH_2NH), 3.50 – 3.42 (1H, m, H-5), 3.06 and 2.67 (2 x bs, each 4H, $C(O)CH_2CH_2C_{triazole}$), 2.20 – 2.02 (4H, m, $OCH_2CH_2CH_2N_{triazole}$), 2.14, 2.10, 2.09, 2.08, 2.06, 2.04 and 1.99 (7 x 6H, 7 x s,

$C(O)CH_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.2, 170.1, 170.0, 169.8, 169.6 and 168.9 ($C(O)CH_3$), 166.1 ($C(O)OCH_3$), 159.2 (C_{arom}-3,5), 131.7 (C_{arom}-1), 107.7 (C_{arom}-2,6), 106.4 (C_{arom}-4), 100.7 (C-1), 99.1 (C-1'), 72.3, 71.6, 68.3, 67.5, 67.0 and 66.8 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 66.6 ($OCH_2CH_2CH_2N_{triazole}$), 65.4 ($OCH_2CH_2NH$), 61.7 and 60.2 (C-6, C-6'), 51.9 ($C(O)OCH_3$), 38.5 and 29.7 ($C(O)CH_2CH_2C_{triazole}$), 20.5 and 20.3 ($C(O)CH_3$). HRMS for $C_{80}H_{108}N_8O_{42}$ (M, 1852.6561): found $[M + Na]^+$ 1875.6348, calcd. 1875.6459. The general deacetylation procedure yielded compound **18c** as a white foam (66.3 mg, 49%). ^1H NMR (300 MHz, D_2O): δ = 7.75 (2H, bs, $CH_{triazole}$), 7.05 (2H, s, CH_{arom}-2,6), 6.62 (1H, s, CH_{arom}-4), 4.97 (2H, s, H-1'), 4.39 – 4.27 (6H, m), 4.02 - 3.70 (25H, m), 3.56 – 3.53 (8H, m), 2.97 and 2.60 (2 x 4H, 2 x bs, $C(O)CH_2CH_2C_{triazole}$) and 2.02 (4H, bs, $OCH_2CH_2CH_2N_{triazole}$). ^{13}C NMR (75.5 MHz, D_2O): δ = 175.7 (C(O)NH), 168.9 ($C(O)OCH_3$), 160.1 (C_{arom}-3,5), 132.3 (C_{arom}-1), 108.9 (C_{arom}-2,6), 107.5 (C_{arom}-4), 103.7 (C-1), 101.0 (C-1'), 77.9, 77.7, 75.9, 75.6, 73.2, 73.0, 71.5, 69.9 and 69.5 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 67.7 ($OCH_2CH_2CH_2N_{triazole}$), 67.1 ($OCH_2CH_2NH$), 61.3 and 60.8 (C-6, C-6'), 47.6 ($CH_2N_{triazole}$), 39.4 and 35.8 ($C(O)CH_2CH_2C_{triazole}$) and 21.9 ($OCH_2CH_2CH_2N_{triazole}$). HRMS for $C_{52}H_{80}N_8O_{28}$ (M, 1264.5082): found $[M + Na]^+$ 1287.1271, calcd. 1287.4980.

Tetravalent Galabiose dendrimer (19c): The general "click" procedure was applied. Protected tetravalent galabiose dendrimer **19c** was obtained as a white foam (151 mg, 78%). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.44 (4H, bs, $CH_{triazole}$), 7.36 (2H, bs, C(O)NH), 7.15 (2H, s, CH_{arom}-2,6), 6.94 (8H, bs, CH_{arom}-2',6' and C(O)NH), 6.73 (1H, bs, CH_{arom}-4), 6.52 (2H, bs, CH_{arom}-4'), 5.57 (4H, dd, H-4', $J_{3',4'}$ = 2.2 Hz), 5.41 (4H, dd, H-3', $J_{2',3'}$ = 11.0 Hz, $J_{3',4'}$= 3.3 Hz), 5.23– 5.15 (8H, m, H-2, H-2'), 5.00 (4H, d, H-1', $J_{1',2'}$ = 3.7Hz), 4.81 (4H, dd, H-3, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 2.4 Hz), 4.54 – 4.35, 4.21 – 4.08, 3.98 and 3.87 – 3.80 (22H, 22H, 8H, 12H, 3 x m, 1 x bs, H-1, H-4, H-5', H-6, H-6', OCH_2CH_2NH, OCH_2CH_2NH, $CH_2N_{triazole}$, CH_2O_{Gal}), 3.87 (3H, s, OCH_3), 3.57 (6H, bs, OCH_2CH_2NH), 3.45 (4H, bs, H-5) 3.03 and 2.66 (2 x bs, 2 x 8H, $C(O)CH_2CH_2C_{triazole}$), 2.20 – 2.00 (8H, m, $OCH_2CH_2CH_2N_{triazole}$), 2.13, 2.10, 2.08, 2.07, 2.05, 2.04 and 1.99 (7 x 12H, 7 x s, $C(O)CH_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.5, 170.4, 170.0, 169.9 and 169.2 ($C(O)CH_3$), 167.9 (C(O)NH), 166.4 ($C(O)OCH_3$), 159.5 (C_{arom}-3,5 and C_{arom}-3',5'), 136.3 (C_{arom}-1'), 131.9 (C_{arom}-1), 108.1 (C_{arom}-2,6), 106.5 (C_{arom}-4), 106.0 (C_{arom}-2',6'), 104.8

(C_{arom}-4'), 100.9 (C-1), 99.4 (C-1'), 72.5, 71.8, 68.6, 68.4, 67.7, 67.2 and 67.0 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 66.7 (O*C*$H_2$$CH_2$$CH_2$$N_{triazole}$), 65.6 (O*C*$H_2$$CH_2$NH), 61.8 and 60.4 (C-6, C-6'), 52.2 (C(O)O*C*H_3), 46.7 ($CH_2N_{triazole}$), 39.5 and 38.6 (OCH$_2$*C*H_2NH), 38.6 and 30.0 (C(O)*C*H_2*C*$H_2$$C_{triazole}$), 20.8 and 20.6 (C(O)*C*H_3). HRMS for $C_{170}H_{226}N_{18}O_{86}$ (M, 3895.3864): found $[M + 3Na]^{3+}$ 1321.7095, calcd. 1321.4519. The general deacetylation procedure yielded compound **19c** as a white foam (64.3 mg, 74%). ^{1}H NMR (300 MHz, D_2O): δ = 7.65 (4H, s, $CH_{triazole}$), 6.79 (6H, s, CH_{arom}-2,6, 2',6'), 6.48 (3H, t, CH_{arom}-4, 4'), 4.95 (4H, s, H-1'), 4.37 – 4.27 (16H, m), 4.01 – 3.45 (84H, m), 2.89 and 2.52 (2 x 8H, 2 x bs, C(O)C*H*$_2$C*H*$_2$$C_{triazole}$) and 2.00 (8H, m, OCH$_2$C*H*$_2$$CH_2$$N_{triazole}$). ^{13}C NMR (75.5 MHz, D_2O): δ = 175.4 and 169.6 (C(O)NH), 168.2 (*C*(O)OCH$_3$), 160.0 (C_{arom}-3',5'), 159.8 (C_{arom}-3,5), 133.0 (C_{arom}-1), 131.7 (C_{arom}-1'), 108.6 (C_{arom}-2,6), 106.9 (C_{arom}-2',6'), 105.3 (C_{arom}-4'), 103.6 (C-1), 100.9 (C-1'), 77.8, 77.7, 73.0, 71.5, 71.3, 69.7 and 69.5 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 67.5 (O*C*$H_2$$CH_2$$CH_2$$N_{triazole}$), 67.0 (O*C*$H_2$$CH_2$NH), 61.2 and 60.7 (C-6, C-6'), 47.5 ($CH_2N_{triazole}$), 39.2 and 35.7 (C(O)*C*H_2*C*$H_2$$C_{triazole}$) and 21.7 (OCH$_2$*C*$H_2$$CH_2$$N_{triazole}$). MALDI TOF MS for $C_{114}H_{170}N_{18}O_{58}$ (M, 2719.0906): found $[M + Na]^+$ 2743.20, calcd. 2743.65.

Octavalent Galabiose dendrimer (20c): The general "click" procedure was applied. Protected octavalent galabiose dendrimer was obtained as a white foam (131 mg, 66%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.68 (8H, bs, $CH_{triazole}$), 7.46 and 7.30 (8H and 6H, 2 x bs, C(O)NH), 7.07 (CH_{arom}-2,6), 6.89 (12H, s, CH_{arom}-2',6', 2'',6''), 6.69 (1H, s, CH_{arom}-4), 6.40 (6H, bs, CH_{arom}-4', CH_{arom}-4''), 5.57 (4H, d, H-4', $J_{3',4'}$= 2.7 Hz), 5.41 (4H, dd, H-3', $J_{2',3'}$ = 11.0 Hz, $J_{3',4'}$ = 3.3 Hz), 5.23– 5.15 (8H, m, H-2, H-2'), 5.00 (4H, d, H-1', $J_{1',2'}$ = 3.7 Hz), 4.83 (4H, d, H-3, $J_{2,3}$ = 10.8 Hz), 4.55 – 4.30, 4.20 – 4.00 and 3.90 – 3.70 (40H, 45H, 46H, 3 x m, H-1, H-4, H-5', H-6, H-6', $CH_2N_{triazole}$,OC*H*$_2$CH$_2$NH, OCH$_2$C*H*$_2$NH, CH_2O_{Gal}, C(O)OCH$_3$), 3.53 (16H, bs, OCH$_2$C*H*$_2$NH), 3.45 (8H, m, H-5), 2.19 – 2.01 (16H, m, OCH$_2$C*H*$_2$$CH_2$$N_{triazole}$), 2.14, 2.13, 2.10, 2.08, 2.05, 2.04 and 1.99 (7 x 12H, 7 x s, C(O)CH$_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.5, 170.3, 170.0, 169.9 and 169.2 (*C*(O)CH$_3$), 167.6 (C(O)NH), 166.4 (*C*(O)OCH$_3$), 159.4 (C_{arom}-3,5, 3',5', 3'',5''), 136.3 (C_{arom}-1', 1''), 131.9 (C_{arom}-1), 108.2, 106.0 and 104.4 (C_{arom}-4, 4', 4'', C_{arom}-2,6, 2',6', 2'',6''), 100.9 (C-1), 99.4 (C-1'), 72.5, 71.8, 68.6, 68.4, 67.7, 67.2 and 67.0 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 66.7 (O*C*$H_2$$CH_2$$CH_2$$N_{triazole}$), 65.7 (O*C*$H_2$$CH_2$NH), 61.8 and

60.4 (C-6, C-6'), 30.0 and 29.5 (C(O)$CH_2CH_2C_{triazole}$) and 20.5 (C(O)CH_3). The general deacetylation procedure yielded compound **20c** as a white foam (54.5 mg, 77%). ^{1}H NMR (300 MHz, D_2O): δ = 7.67 (8H, s, $CH_{triazole}$), 6.73 (10H, s, CH_{arom}-2,6, 2',6', 2'',6''), 6.42 (6H, t, CH_{arom}-4, 4', 4''), 4.95 (8H, d, H-1', $J_{1,2}$ = 3.6 Hz), 4.37 – 4.30 (25H, m), 4.01 – 3.43 (140H, m), 2.87 and 2.51 (2 x 16H, 2 x bs, C(O)CH_2C$H_2$$C_{triazole}$) and 2.01 (16H, m, $OCH_2CH_2CH_2N_{triazole}$). ^{13}C NMR (75.5 MHz, D_2O): δ = 175.2, 169.3 and 169.1 (C(O)NH), 167.9 (*C*(O)OCH_3), 159.9 (C_{arom}-3'',5''), 103.6 (C-1), 100.9 (C-1'), 77.8, 77.7, 75.7, 73.0, 71.5, 71.3, 69.7 and 69.5 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 67.5 ($OCH_2CH_2CH_2N_{triazole}$), 67.0 ($OCH_2CH_2NH$), 61.2 and 60.7 (C-6, C-6'), 47.5 ($CH_2N_{triazole}$), 39.2 and 35.5 (C(O)$CH_2CH_2C_{triazole}$) and 21.7 ($OCH_2CH_2CH_2N_{triazole}$). MALDI TOF MS for $C_{238}H_{350}N_{38}O_{118}$ (M, 5628.2555): found $[M + Na]^+$ 5654.46, calcd. 5654.50.

Tetravalent trisaccharide dendrimer (19d): The general "click" procedure was applied. Protected tetravalent dendrimer was obtained as a white foam (201 mg, 79%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.45 (4H, bs, $CH_{triazole}$), 7.36 (2H, bs, C(O)NH), 7.14 (2H, s, CH_{arom}-2,6), 6.94 (8H, bs, CH_{arom}-2',6' and C(O)NH), 6.70 (1H, bs, CH_{arom}-4), 6.48 (2H, bs, CH_{arom}-4'), 5.56 (4H, d, H-4'', $J_{3'',4''}$ = 2.7 Hz), 5.40 (4H, dd, H-3'', $J_{2'',3''}$ = 11.1 Hz, $J_{3',4'}$= 3.3 Hz), 5.31– 5.15 (12H, m, H-2, H-2', H-2''), 5.00 (4H, d, H-1'', $J_{1'',2''}$ = 3.6 Hz), 4.97 (4H, d, H-1', $J_{1',2'}$ = 1.8 Hz), 4.81 (4H, d, H-3, $J_{2,3}$ = 9.9 Hz), 4.56 – 4.30, 4.22 – 3.98 and 3.82 – 3.75 (25H, 24H, 8H, 3 x m, H-1, H-4, H-4', H-5', H-5'', H-6, H-6', H-6'', O$CH_2$$CH_2$NH, OCH_2CH_2NH, $CH_2N_{triazole}$, CH_2O_{Gal}), 3.87 (3H, s, OCH_3), 3.57 (6H, bs, OCH_2CH_2NH), 3.44 (4H, bs, H-5), 3.01 and 2.65 (2 x 8H, 2 x bs, C(O)CH_2C$H_2$$C_{triazole}$), 2.20 – 2.00 (8H, m, $OCH_2CH_2CH_2N_{triazole}$), 2.14, 2.12, 2.09, 2.07, 2.06, 2.03 and 1.99 (10 x 12H, 10 x s, C(O)CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.6, 170.4, 170.2, 170.1, 169.9 and 169.2 (*C*(O)CH_3), 167.2 (C(O)NH), 166.4 (*C*(O)OCH_3), 159.5 (C_{arom}-3'',5''), 136.4 (C_{arom}-1''), 131.7 (C_{arom}-1), 108.3, 108.0, 106.3, 106.1 and 104.7 (C_{arom}), 101.0 (C-1), 99.6 and 99.3 (C-1', C-1''), 72.5, 72.3, 72.1, 71.9, 69.0, 68.7, 68.4, 67.8, 67.5 and 66.8 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 65.6 (OCH_2CH_2NH), 62.1, 61.0 and 60.3 (C-6, C-6', C-6''), 52.1 (C(O)OCH_3), 46.5 ($CH_2N_{triazole}$), 39.5 and 38.6 (OCH_2CH_2NH), 35.3 and 30.0 (C(O)$CH_2CH_2C_{triazole}$), 20.6 and 20.5 (C(O)CH_3). HRMS for $C_{218}H_{290}N_{18}O_{118}$ (M, 5047.7245): found $[M + Na]^+$ 5072.99, calcd. 5073.6748. General deacetylation procedure yielded compound **19d** as a white foam (28.4 mg, 57%). ^{1}H NMR

(300 MHz, D_2O): δ = 7.70 (4H, s, $CH_{triazole}$), 6.84 (2H, s, CH_{arom}-2,6), 6.79 (4H,s, CH_{arom}-2',6'), 6.51 (3H, t, CH_{arom}-4, 4'), 5.01 – 4.97 (8H, m, H-1', H-1''), 4.41 (4H, t), 4.29 (16H, m), 4.11 – 3.60 (86H, m), 3.55 – 3.40 (18H, m), 2.91 and 2.54 (2 x 8H, 2 x bs, $C(O)CH_2CH_2C_{triazole}$) and 2.00 (8H, m, $OCH_2CH_2CH_2N_{triazole}$). ^{13}C NMR (75.5 MHz, D_2O): δ = 175.3 and 169.7 (C(O)NH), 168.3 ($C(O)OCH_3$), 160.0 (C_{arom}-3',5'), 159.8 (C_{arom}-3,5), 136.2 (C_{arom}-1'), 106.8 (C_{arom}), 103.5 (C-1), 101.1 and 100.8 (C-1', C-1''), 79.6, 77.5, 75.6, 72.8, 71.6, 71.4, 69.8, 69.5 and 69.2 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 67.4 ($OCH_2CH_2CH_2N_{triazole}$), 66.9 ($OCH_2CH_2NH$), 61.2 and 60.5 (C-6, C-6', C-6''), 47.7 ($CH_2N_{triazole}$), 39.2 and 35.4 ($C(O)CH_2CH_2C_{triazole}$), 30.1 (OCH_2CH_2NH) and 21.5 ($OCH_2CH_2CH_2N_{triazole}$). MALDI TOF MS for $C_{138}H_{210}N_{18}O_{78}$ (M, 3367.3019): found $[M + Na]^+$ 3391.87, calcd. 3392.21.

Hemagglutination assay: Hemagglutination assays were performed as described previously.[4b] Briefly, equal volumes of bacteria and 5% sialidase-treated human erythrocytes were mixed and hemagglutination was visually recorded after 1 h incubation on ice. For inhibition assays, 2-fold dilutions of galabiose compounds (25 µL) were mixed with bacteria (25 µL). After 5 min of incubation at room temperature, 50 µL of the erythrocytes was added. The hemagglutination was recorded as described and the MIC values (the lowest concentration completely inhibiting the hemagglutination) were recorded.

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Chapter 4

Strong inhibition of Cholera Toxin binding by multivalent GM1 derivatives

The results in this chapter have been published:

Aliaksei V. Pukin, Hilbert M. Branderhorst, Cristina Sisu, Carel A. G. M. Weijers, Michel Gilbert, Rob M. J. Liskamp, Gerben M. Visser, Han Zuilhof, Roland J. Pieters; Strong Inhibition of Cholera Toxin by Multivalent GM1 Derivatives; *ChemBioChem*, **2007**, *8*, 1500-1503

Introduction

Proteins often bind to their carbohydrate ligands in a multivalent manner.[1] Cholera Toxin (CT) is a prime example of a multimeric protein and is capable of binding simultaneously to the carbohydrate moieties of five GM1 gangliosides. The binding of the five B subunits is of critical importance for the internalization[2] and subsequent disease process of this AB_5 toxin.[3] The development of strong binding agents to the toxin is of interest for the development of disease prevention/treatment, but also for the detection of toxin in patient samples[4] or in materials suspect of terrorist origin.[5] Furthermore, CT serves as a benchmark case to test multivalent strategies. As such, several systems have been reported that showed varying degrees of multivalency effects.[6] For example, a multivalent conjugated version of the GM1 oligosaccharide (GM1os) was prepared in very small quantities from isolated GM1 via reductive amination, and found to enhance binding up to 250-fold.[6a] Studies with the smaller Shiga-like toxin,[7] the *E. coli* heat-labile enterotoxin and the CTB pentamer (CTB_5), indicated that long spacers are beneficial for strong binding.[6e,8] A pentavalent version of conjugated *m*-nitrophenyl α-D-galactopyranoside that contained long spacer arms, surpassed GM1os in affinity (relative potency 2.8), although the compound was unstable in water.[6e] While a pentavalent presentation of ligands seemed essential for effective inhibition of AB_5 toxins, octavalent glycodendrimers were shown to be even more effective.[9] In this chapter we report on highly effective inhibitors of CTB_5 due to a combination of three factors in the inhibitor design: 1) the use of the authentic GM1 oligosaccharide sequence as the optimal monovalent ligand, 2) the use of a multivalent dendritic scaffolds, and 3) the use of elongated spacer arms of optimal length.[6e] The combined effects led to inhibitors that were up to an unprecedentedly 47,500-fold more potent per GM1os ligand than a monovalent GM1os conjugate.

Results and Discussion

GM1os conjugates **1** and **2** (Figure 1) and related GM2os conjugate **3** were prepared on a 100 mg scale via a route containing chemical synthesis and enzymatic steps.[10] The C11 tail of **1** and **3** contains an azido group to enable its conjugation via "click" chemistry,[11] a method which extends the range of organic and inorganic substrates to which oligosaccharides can be attached significantly.[12]

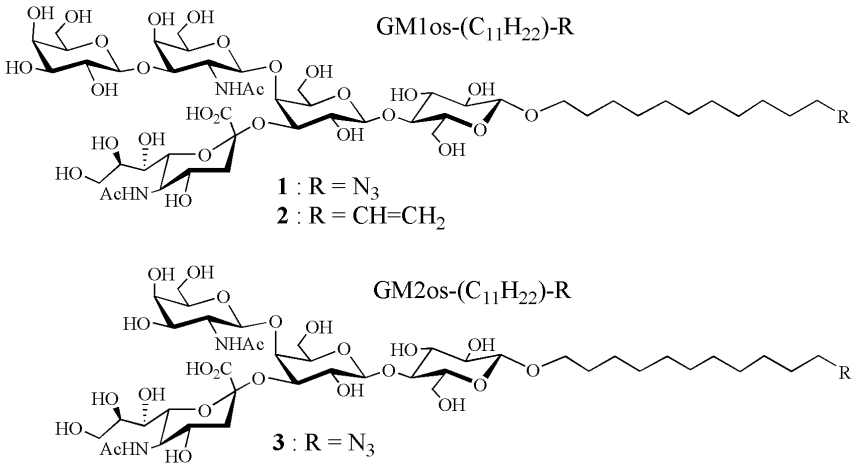
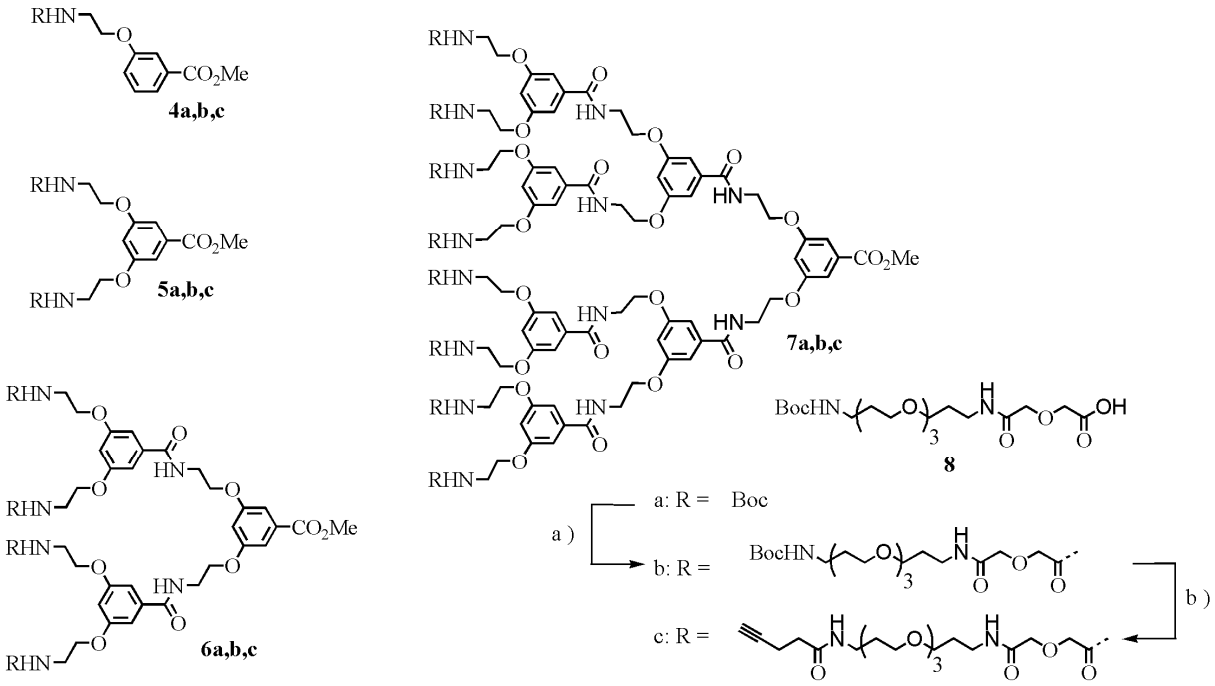


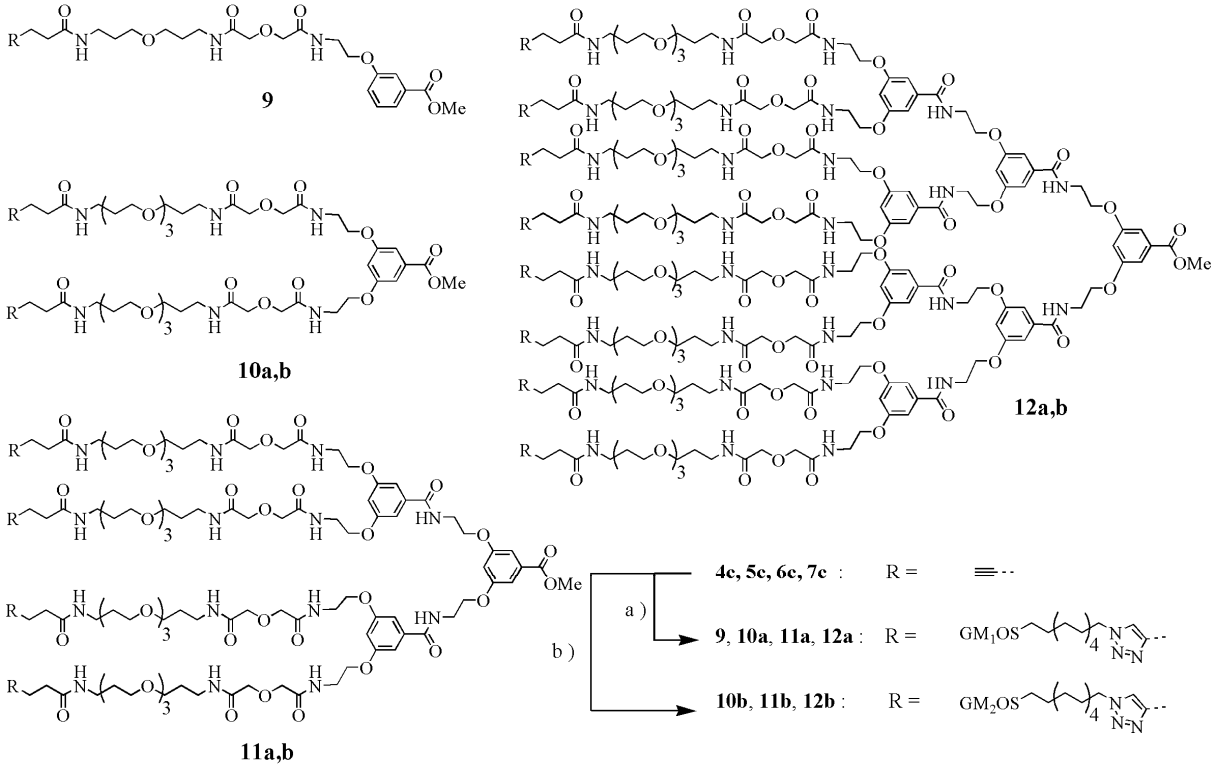
Figure 1. *Structures of the functionalized GM1os and GM2os*

The dendritic scaffolds **5c**, **6c, 7c** and the monovalent reference compound **4c** were derived from **4a**, **5a**, **6a** and **7a**.[6b,d,13,14] These dendrimers were subjected to Boc deprotection conditions and spacer unit **8** was introduced by standard coupling conditions. Intermediates **4b**, **5b**, **6b** and **7b** were obtained, subjected to Boc deprotection conditions and functionalized with alkynes by coupling to 4-pentynoic acid (Scheme 1).



Scheme 1. *Synthesis of the Dendrimers. Reagents and conditions: a) i: TFA, CH_2Cl_2, quant.; ii:* ***8****, HATU, DiPEA, quant. for* ***4b****, 97% for* ***5b****, 38% for* ***6b****, 96% for* ***7b****; b) TFA, CH_2Cl_2, quant.; ii: 4-pentynoic acid, HATU, DiPEA, 46% for* ***4c,*** *60% for* ***5c****, 87% for* ***6c****, 56% for* ***7c****.*

In the subsequent step **1** and the alkynes **4c**, **5c**, **6c** and **7c** were exposed to $CuSO_4$ and sodium ascorbate in DMF/H_2O (1:1) at 80°C with microwave heating for 20 min according to reported conditions (Scheme 2).[15] These conditions facilitated efficient coupling and provided products **9**, **10a**, **11a** and **12a** in good isolated yield after HPLC purification. It is worth noticing that analytical HPLC analysis showed the reaction between **1** and **6c** to form **11a** to be, in fact, complete after 1 min! GM2os conjugates **10b, 11b** and **12b** were prepared in a similar fashion.



Scheme 2. *Synthesis of the ligands. Reagents and conditions: GM1 serie)* ***1, 4c/5c/6c/7c****, $CuSO_4$, sodium ascorbate, DMF/H_2O, 57% for* ***9****, 78% for* ***10a,*** *76% for* ***11a,*** *44% for* ***12a.*** *GM2 serie)* ***3, 5c/6c/7c*** *$CuSO_4$, sodium ascorbate, DMF/H_2O, 64% for* ***10b****, 37% for* ***11b,*** *34% for* ***12b****.*

In order to evaluate the inhibitory potency of the inhibitors an ELISA type assay was used.[6d,16] In this assay, wells of a 96-well plate were coated with GM1 ganglioside, and after blocking with BSA, horseradish peroxidase (HRP)-conjugated CTB_5 was allowed to bind to the surface in the presence or absence of inhibitors. In this assay the monovalent GM1os derivative **2** exhibited an IC_{50} in the micromolar range (19 μM, Table 1). Since the GM1os oligosaccharide is reported to have a K_d of 43 nM,[17] the high inhibitory

concentration of the conjugated GM1os **2** indicates that the toxin binds strongly to the ELISA plate due to multivalent binding. The role of the aglycon part beyond the C_{11} tail was found to be negligible, since monovalent **9** showed a similar IC_{50} as **2** i.e. in the micromolar range. The divalent **10a** was subsequently measured, and found to have an IC_{50} that was almost 4 orders of magnitude lower, i.e. 2.0 nM. The tetravalent **11a** gave an IC_{50} which was an additional order of magnitude lower (0.23 nM). Finally for the octavalent **12a** the IC_{50} was found to be 50 pM. Control experiments with non-functional dendrimers (**4c**, **5c** and **6c**) showed no measurable inhibition for all concentrations tested (up to 100 μM).

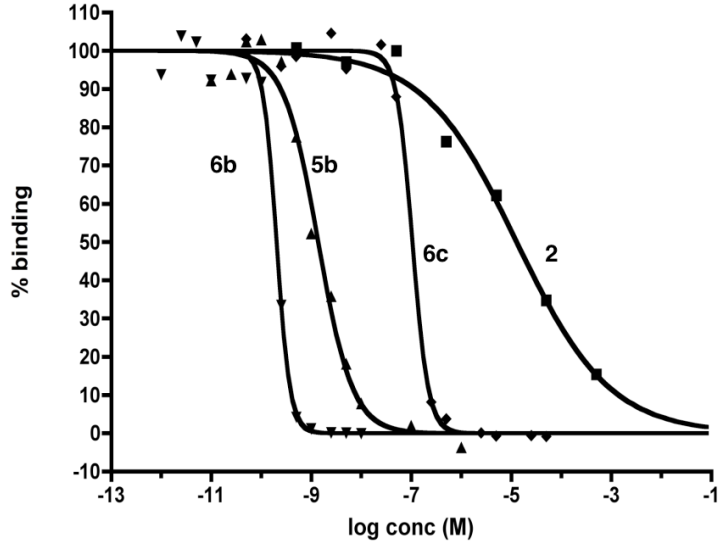


Figure 2. *Observed data points and fitted curves of the inhibition of the shown compounds on the CT binding to GM1-coated plates.*

The prepared compounds exhibited unprecedented affinities, not only because the strongest known CT ligands (GM1os) were used, but also because these ligands were combined with the strongest multivalency effects observed for the CTB pentamer. For tetravalent **11a**, each of the GM1os moieties bound 47,500-fold stronger than monovalent **2**. Experiments with GM2os were performed to confirm the multivalency effects with a similar but weaker ligand. Monovalent GM2os was not measured because an expected low affinity indicated that it would take too much material to perform the assay. However, divalent **10b** was prepared and its inhibitory potency could just be observed with an IC_{50} of 2 mM. A large jump in potency was observed when moving to tetravalent **11b** (19,000-fold) having an

IC_{50} of 0.1 μM. This jump was almost three orders of magnitude larger than in the GM1os series. The octavalent **12b** was shown to be a weaker inhibitor than its tetravalent counterpart, while in the GM1 series the octavalent inhibitor was still moderately better (2.3 fold per sugar).

Table 1. *Inhibitory potency of the CTB_5 inhibitors*

Compound	valency	IC_{50} (M)[a]	rel. pot.[b] (per sugar)	Hill coefficient
GM1os derivatives				
2	1	1.9 (±0.6) x 10^{-5}	1 (1)	0.5
9	1	7 (±3) x 10^{-6}	2,7 (2.7)	0.5
10a	2	2 (±1) x 10^{-9}	9,500 (4,750)	1.0
11a	4	2.3 (±0.7) x 10^{-10}	83,000 (20,750)	3.0
12a	8	5 (±1) x 10^{-11}	380,000 (47,500)	1.7
GM2os derivatives				
10b	2	2 (±1) x 10^{-3}	1	n.d.
11b	4	1.05 (±0.02) x 10^{-7}	19,000	2.8
12b	8	4 (±1) x 10^{-7}	5,000	1.4

[a] Determined in an ELISA experiment with 0.43 nM CTB_5-HRP and wells coated with 0.2 μg GM1. [b] relative potency of compounds **2** and **10b** were taken as 1.

In the assay, more information about the cooperativity of binding can be obtained from the Hill coefficients. Interestingly, the monovalent compounds showed negative cooperativity with a Hill coefficient of around 0.5; after one molecule of **2** has bound to CTB_5, subsequent molecules of **2** bind less strongly. For the divalent **10a** this value was close to 1, but the tetravalent **11a** and **11b** both showed steeper inhibition curves and thus positive cooperativity, with Hill coefficients of around 3 (Figure 2). For the octavalent compounds **12a** and **12b** the Hill coefficients were lowered to ca. 1.5. Cooperativity of CT binding has previously been observed.[18] The observations made here possibly indicate cooperative binding of the tetravalent inhibitors to CTB_5, but also the aggregation phenomena may

explain the difference in Hill coefficients. Further studies are needed to uncover the origin of the difference in the Hill slope.

Conclusions

In short, multivalent GM1os and GM2os compounds were prepared via efficient "click" chemistry to dendritic scaffolds with extended arms. The unambiguously characterized compounds clearly reveal strong multivalent binding to CTB_5, with an unparalleled value of at least 380,000-fold stronger for octavalent GM1os conjugate **12a** than monovalent GM1os derivatives, which are known to have K_d's that are already in the nM range (K_d GM1os = 43 nM[17]). This opens up possibilities for e.g. development of very sensitive sensor applications,[19] such as also relevant for other oligosaccharide-related medical problems.[20]

Experimental Section

General Remarks

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Solvents were purchased from Biosolve (Valkenswaard, The Netherlands). Microwave reactions were carried out in a dedicated microwave oven, i.e. the Biotage Initiator. The microwave power was limited by temperature control once the desired temperature was reached. A sealed vessel of 2 – 5 mL was used. Analytical HPLC runs were performed on a Shimadzu automated HPLC system with a reversed phase column (Alltech, Adsorbosphere C8, 90 Å, 5μm, 250x4.6 mm) equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories) and a UV/VIS detector operating at 220 and 254 nm. Preparative HPLC runs were performed on a Applied Biosystems workstation. Elution was effected using a gradient of 5% MeCN and 0.1% TFA in H_2O to 5% H_2O and 0.1% TFA in MeCN. ^1H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) were performed on a Varian G-300 spectrometer. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) were performed on a Bruker DPX 400 spectrometer. Exact masses were measured by nanoelectrospray time-of-flight mass spectrometry on a Micromass LC TOF mass spectrometer. Gold-coated capillaries were loaded with 1 μL of sample (concentration 2 μM) dissolved in a 1:1 (v/v) mixture of CH_3CN-H_2O with 0.1% formic acid. NaI or poly(ethylene glycol) (PEG) was added as internal standard. The capillary voltage was set

between 1100 and 1350 V, and the cone voltage was set at 30 V. Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI TOF) MS were recorded on a Shimadzu Axima-CFR with α-cyano-4-hydroxycinnamic acid or sinapic acid as a matrix. Insulin and adrenocorticotropin fragment 18-39 (Acth) were used for calibration.

Monovalent compound (4b)[6d]: To a solution of **4a**[21] dendrimer (591 mg, 2 mmol) in CH_2Cl_2 (5 mL) with a trace of H_2O was added TFA (5 mL). The solution was stirred at rt for 1 h followed by concentration to dryness. The product was dissolved in CH_2Cl_2 (10 mL) and acid **8** (960 mg, 2.2 mmol) and HATU (836 mg, 2.2 mmol) were subsequently added followed by DiPEA (0.99 mL, 6.0 mmol). The reaction mixture was stirred for 2 h. The reaction mixture was concentrated in vacuo and silica gel chromatography (CH_2Cl_2/MeOH, 19/1 → 9/1) was used for purification (1.24 g, quant., small impurity). ^{1}H NMR was consistent with ref 6d.

Monovalent compound (4c): To a solution of **4b** (1.24 g, 2 mmol) in CH_2Cl_2 (5 mL) with a trace of H_2O was added TFA (5 mL). After 1 h the mixture was concentrated to dryness and dissolved in CH_2Cl_2 (10 mL). HATU (836 mg, 2.2 mmol), 4-pentynoic acid (294 mg, 3.0 mmol) and DiPEA (0.99 mL, 6.0 mmol) were subsequently added and the reaction mixture was stirred for 18 h. Silica gel chromatography (CH_2Cl_2/MeOH, 19/1 → 9/1) was used for purification followed by size exclusion chromatography (MeOH) to afford pure product **4c** (552 mg, 46% over 2 steps). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.65 (1H, d, CH_{arom}-6), 7.55 (1H, s, CH_{arom}-2), 7.35 (1H, t, CH_{arom}-5), 7.10 (1H, dd, CH_{arom}-4), 7.44, 7.20 and 6.60 (3 x 1H, 3 x bt, 3 x C(O)NH), 4.15 – 4.11 (2H, m, OCH_2CH_2NH), 4.08 and 4.04 (2 x 2H, s, $OCH_2C(O)$), 3.91 (3H, s, $C(O)OCH_3$), 3.74 – 3.72 (2H, m, $OCH_2CH_2NHC(O)$), 3.61 – 3.53 (12H, m, OCH_2), 3.41 – 3.35 (4H, m, $CH_2NHC(O)$), 2.54 – 2.48 and 2.42 – 2.37 (2 x 2H, 2 x m, CH_2CH_2CCH), 2.00 (1H, t, CH_2CCH) and 1.83 – 1.72 (8H, m, $OCH_2CH_2CH_2NH$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 171.0, 169.0 and 168.4 (C(O)NH), 166.7 ($C(O)OCH_3$), 158.4 (C_{arom}-3), 131.5 (C_{arom}-1), 129.5 (CH_{arom}-5), 122.4 (CH_{arom}-6), 119.6 (CH_{arom}-4), 114.8 (CH_{arom}-2), 83.2 (CH_2CCH), 71.0 (CH_2CCH), 70.3 – 69.1 (OCH_2), 66.6 (OCH_2CH_2NH), 52.2($C(O)OCH_3$), 38.5 (OCH_2CH_2NH), 37.8 and 37.3 ($CH_2NHC(O)$), 35.3 (CH_2CH_2CCH), 29.0 and 28.8 ($OCH_2CH_2CH_2NH$) and 14.8 (CH_2CCH). HRMS for $C_{29}H_{43}N_3O_{10}$ (M, 593.2948): found $[M + Na]^+$ 616.2775, calcd. 616.2846.

Divalent dendrimer (5b)[6d]: To a solution of **5a**[13] dendrimer (250 mg, 0.55 mmol) in CH_2Cl_2 (10 mL) with a trace of H_2O was added TFA (5 mL). The solution was stirred at rt for 1 h followed by concentration to dryness. A solution of acid **8** (602 mg, 1.38 mmol) in CH_2Cl_2 (10 mL) was added to the dendrimer, subsequently followed by HATU (525 mg, 1.38 mmol) and DiPEA (0.68 mL, 4.13 mmol). The reaction mixture was stirred for 70 h. The reaction mixture was concentrated in vacuo and Silica gel chromatography (CH_2Cl_2/MeOH, 19/1 → 9/1) was used for purification (580 mg, 97 %, small impurity). ^{1}H NMR was consistent with ref 6d: ($CDCl_3$, 300 MHz): δ = 7.40 and 7.24 (2 x 2H, 2 x bs, C(O)NH), 7.16 (2H, d, CH_{arom}-2,6), 6.66 (1H, t, CH_{arom}-4), 5.06 (NHBoc), 4.12 – 4.10 (4H, m, OC*H*$_2$CH$_2$NH), 4.08 and 4.04 (2 x 4H, 2 x s, $OCH_2C(O)$), 3.89 (3H, s, $C(O)OCH_3$), 3.73 – 3.68 (4H, m, OCH_2CH_2NHC(O)), 3.64 – 3.49 (24H, m, OCH_2), 3.43 – 3.37 (4H, m, C*H*$_2$NHC(O)), 3.23 – 3.17 (4H, m, C*H*$_2$NHBoc), 1.83 – 1.72 (8H, m, $OCH_2CH_2CH_2NH$) and 1.43 (18H, s, $C(O)OC(CH_3)_3$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 169.3 and 168.6 (C(O)NH), 166.4 (*C*(O)OCH_3), 159.5 (C_{arom}-3,5), 156.1 (*C*(O)$OC(CH_3)_3$), 132.1 (C_{arom}-1), 108.1 (CH_{arom}-2,6), 106.5 (CH_{arom}-4), 78.9 (C(O)O*C*$(CH_3)_3$), 71.0 – 69.3 (OCH_2), 66.7 (O*C*H$_2$CH$_2$NH), 52.2 (C(O)O*C*H$_3$), 38.4 (CH_2NHBoc), 37.5 (OCH_2CH_2NH), 29.6 and 28.9 ($OCH_2CH_2CH_2NH$) and 28.4 ($C(O)OC(CH_3)_3$).

Divalent dendrimer (5c): To a solution of **5b** (300 mg, 0.27 mmol) in CH_2Cl_2 (10 mL) with a trace of H_2O was added TFA (5 mL). After 4 h of stirring the mixture was concentrated to dryness and dissolved in CH_2Cl_2 (5 mL) and DMF (5 mL). HATU (260 mg, 0.68 mmol), 4-pentynoic acid (67 mg, 0.68 mmol) and DiPEA (225 µL, 1.36 mmol) were subsequently added and the reaction mixture was stirred for 18 h. Silica gel chromatography (CH_2Cl_2/MeOH, 19/1 → 4/1) was used for purification followed by size exclusion chromatography (MeOH) to afford pure product (170 mg, 60%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.58 and 7.33 (2 x H, 2 x t, C(O)NH), 7.17 (2H, d, CH_{arom}-2,6), 6.67 (3H, bt, CH_{arom}-4 and C(O)NH), 4.13 – 4.09 (4H, m, OC*H*$_2$CH$_2$NH), 4.08 and 4.03 (2 x 4H, 2 x s, $OCH_2C(O)$), 3.90 (3H, s, $C(O)OCH_3$), 3.71 – 3.63 (4H, m, OCH_2CH_2NHC(O)), 3.62 – 3.53 (24H, m, OCH_2), 3.42 – 3.32 (8H, m, C*H*$_2$NHC(O)), 2.54 – 2.48 and 2.41 – 2.36 (2 x 2H, 2 x m, C*H*$_2$C*H*$_2$CCH), 2.01 (2H, t, CH_2CCH) and 1.83 – 1.72 (8H, m, $OCH_2CH_2CH_2NH$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 171.1, 169.2 and 168.6 (C(O)NH), 166.4 (*C*(O)OCH_3), 159.5 (C_{arom}-3,5), 132.1 (C_{arom}-1), 108.1 (CH_{arom}-2,6), 106.6 (CH_{arom}-4), 83.2 (CH_2CCH), 70.9 (CH_2CCH), 70.3 – 69.2 (OCH_2), 66.7 (O*C*H$_2$CH$_2$NH), 52.3

(C(O)O*C*H_3), 38.4 (OCH$_2$*C*H$_2$NH), 37.7 and 37.2 (*C*H$_2$NHC(O)), 35.2 (*C*H$_2$CH$_2$CCH), 29.0 and 28.8 (OCH$_2$*C*H$_2$CH$_2$NH) and 14.8 (*C*H$_2$CCH). HRMS for $C_{50}H_{78}N_6O_{18}$ (M, 1050.537): found $[M + Na]^+$ 1073.559, calcd. 1073.5271.

Tetravalent dendrimer (6b)[6d]: To a solution of **6a**[13] dendrimer (540 mg, 0.49 mmol) in CH_2Cl_2 (20 mL) with a trace of H_2O was added TFA (10 mL). The solution was stirred at rt for 3 h followed by concentration to dryness. A solution of acid **8** (1.07 gr, 2.46 mmol) in CH_2Cl_2 (10 mL) was added to the dendrimer, subsequently followed by HATU (936 mg, 2.46 mmol) and DiPEA (1.06 mL, 6.4 mmol). The reaction mixture was stirred for 20 h. The reaction mixture was concentrated in vacuo and silica gel chromatography (CH_2Cl_2/MeOH, 9/1 → 4/1) was used for purification (442 mg, 38 %). ^{1}H NMR was consistent with ref 6d: ($CDCl_3$, 300 MHz): δ = 7.83, 7.63 and 7.44 (2, 4 and 4H, 3 x bt, C(O)NH), 7.15 (2H, d, CH$_{arom}$-2,6), 6.98 (2H, d, CH$_{arom}$-2',6'), 6.68 (1H, t, CH$_{arom}$-4), 6.51 (1H, t, CH$_{arom}$-4'), 5.19 (NHBoc), 4.17 (4H, bs, OC*H*$_2$CH$_2$NH), 4.04 and 4.00 (2 x 24H, 2 x bs, OCH$_2$C(O) and OC*H*$_2$CH$_2$NH), 3.87 (3H, s, C(O)OCH$_3$), 3.80 – 3.79 (4H, m, OCH$_2$C*H*$_2$NH), 3.62 – 3.48 (48H, m, OCH$_2$), 3.39 – 3.33 (8H, m, C*H*$_2$NHC(O)), 3.21 – 3.17 (8H, m, C*H*$_2$NHBoc), 1.81 – 1.68 (16H, m, OCH$_2$C*H*$_2$CH$_2$NH) and 1.43 (36H, s, C(O)OC(CH$_3$)$_3$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 169.2, 168.5 and 167.3 (C(O)NH), 166.4 (*C*(O)OCH$_3$), 159.5 (C$_{arom}$-3,5), 159.3 (C$_{arom}$-3',5'), 156.0 (*C*(O)OC(CH$_3$)$_3$), 136.3 (C$_{arom}$-1), 131.8 (C$_{arom}$-1'), 107.9 (CH$_{arom}$-2,6), 106.4 (CH$_{arom}$-4), 106.1(CH$_{arom}$-2',6'), 104.3 (CH$_{arom}$-4'), 78.7 (C(O)O*C*(CH$_3$)$_3$), 70.7 – 69.1 (OCH$_2$), 66.5 and 66.3 (O*C*H$_2$CH$_2$NH), 52.1 (C(O)O*C*H$_3$), 39.4 and 38.2 (OCH$_2$*C*H$_2$NH), 37.0 (CH$_2$NHBoc), 29.5 and 28.8 (OCH$_2$*C*H$_2$CH$_2$NH) and 28.2 (C(O)OC(*C*H$_3$)$_3$).

Tetravalent dendrimer (6c): To a solution of **6b** (442 mg, 0.186 mmol) in CH_2Cl_2 (10 mL) with a trace of H_2O was added TFA (5 mL). After 4 h stirring the mixture was concentrated to dryness and dissolved in CH_2Cl_2 (10 mL) and DMF (5 mL). HATU (354 mg, 0.93 mmol), 4-pentynoic acid (91 mg, 0.93 mmol) and DiPEA (463 μL, 2.80 mmol) were subsequently added and the reaction mixture was stirred for 20 h. Silica gel chromatography (CH_2Cl_2/MeOH, 9/1 → 1/1) was used for purification, followed by Sephadex size exclusion chromatography (MeOH) to afford pure product (369 mg, 87%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.70, 7.61 and 7.39 (2, 4 and 4H, 3 x t, C(O)NH), 7.17 (2H, d, CH$_{arom}$-2,6), 6.98 (4H, d, CH$_{arom}$-2',6'), 6.75 – 6.71 (5H, m, CH$_{arom}$-4 and C(O)NH),

6.54 (2H, t, CH_{arom}-4'), 4.21 – 4.17 and 4.08 – 4.04 (4 and 8H, 2 x m, OCH_2CH_2NH), 4.04 and 4.00 (2 x 8H, 2 x s, $OCH_2C(O)$), 3.88 (3H, s, $C(O)OCH_3$), 3.83 – 3.78 and 3.68 – 3.63 (4 and 8H, 2 x m, $OCH_2CH_2NHC(O)$), 3.62 – 3.51 (48H, m, OCH_2), 3.39 – 3.29 (16H, m, $CH_2NHC(O)$), 2.51 – 2.46 and 2.40 – 2.35 (2 x 8H, 2 x m, CH_2CH_2CCH), 2.02 (4H, t, CH_2CCH) and 1.81 – 1.70 (16H, m, $OCH_2CH_2CH_2NH$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 171.2, 169.3, 168.5 and 167.4 (C(O)NH), 166.6 ($C(O)OCH_3$), 159.7 (C_{arom}-3,5), 159.6 (C_{arom}-3',5'), 136.6 (C_{arom}-1'), 132.0 (C_{arom}-1), 108.2 (CH_{arom}-2',6'), 106.7 (CH_{arom}-2,6), 106.3 (CH_{arom}-4'),104.5 (CH_{arom}-4), 83.2 (CH_2CCH), 70.9 (CH_2CCH), 70.3 – 69.2 (OCH_2), 66.7 and 66.5 (OCH_2CH_2NH), 52.3 ($C(O)OCH_3$), 39.6 and 38.4 (OCH_2CH_2NH), 37.7 and 37.2 ($CH_2NHC(O)$), 35.2 (CH_2CH_2CCH), 29.1 and 28.9 ($OCH_2CH_2CH_2NH$) and 14.8 (CH_2CCH). HRMS for $C_{110}H_{166}N_{14}O_{38}$ (M, 2291.149): found $[M + Na]^+$ 2314.415, calcd. 2314.1385.

Octavalent dendrimer (7b): A solution of **7a**[13] (533 mg, 0.223 mmol) was stirred in CH_2Cl_2 (5 mL), TFA (5 mL) and a trace of H_2O for 1 h. This solution was concentrated to dryness and taken up in DMF (6 mL). Spacer **8** (1.16 g, 2.67 mmol) and HATU (1.02 g, 2.67 mmol) were added subsequently followed by DiPEA (882 µL, 5.34 mmol). This solution was stirred for 18 h and concentrated in vacuo at 60°C. Silica gel chromatography (CH_2Cl_2/MeOH, 19/1 → 4/1) afforded elongated dendrimer **7b** (slightly contaminated) as yellow foam (1.06 g, 96 %). ^{1}H NMR was consistent with ref 1: ($CDCl_3$, 300 MHz): δ = 7.96, 7.73 and 7.50 (6H, 8H, 8H, 3 x bs, 22 x C(O)NH), 7.14 (2H, s, CH_{arom}-2,6), 6.98 (4H, s, CH_{arom}-2',6'), 6.91 (8H, s, CH_{arom}-2'',6''), 6.64 (1H, bs, CH_{arom}-4), 6.49 (2H, bs, CH_{arom}-4'), 6.41 (4H, bs, CH_{arom}-4''), 5.13 (8H, s, NHBoc), 3.70 (60H, bs, OCH_2CH_2NH and $OCH_2C(O)$), 3.85 (3H, s, $C(O)OCH_3$), 3.77 - 3.68 (28H, m, OCH_2CH_2NH), 3.59 – 3.47 (96H, m, OCH_2), 3.33 - 3.31 (16H, m, CH_2NHBoc), 3.20 – 3.11 (16H, m, $CH_2NHC(O)$), 1.76 – 1.68 (32H, m, $OCH_2CH_2CH_2NH$) and 1.41 (72H, s, $C(O)OC(CH_3)_3$).

Octavalent dendrimer (7c): To a solution of **7b** (1.06 g, 0.21 mmol) in CH_2Cl_2 (5 mL) with a trace of H_2O was added TFA (5 mL). After 1 h stirring the mixture was concentrated to dryness and dissolved in DMF (5 mL). HATU (958 mg, 2.52 mmol), 4-pentynoic acid (247 mg, 2.52 mmol) and DiPEA (830 µL, 5.04 mmol) were subsequently added and the reaction mixture was stirred for 18 h. After concentration at 60°C silica gel chromatography (CH_2Cl_2/MeOH, 19/1 → 1/1) was used for purification. Pure product **7c** was obtained as

white foam (600 mg, 56 % over 2 steps). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.21, 8.02, 7.75 and 7.04 (6, 8, 8 and 8 H, 4 x bs, 30 x C(O)NH), 7.02 and 6.93 (14H, 2 x bs, CH_{arom}-2,6, CH_{arom}-2',6', CH_{arom}-2'',6''), 6.61 (1H, bs, CH_{arom}-4), 6.48 (2H, bs, CH_{arom}-4'), 6.41 (4H, bs, CH_{arom}-4''), 3.97 (60H, bs, OCH_2CH_2NH and $OCH_2C(O)$), 3.82 (3H, s, $C(O)OCH_3$), 3.59 -3.48 (124H, m, $OCH_2CH_2NHC(O)$ and OCH_2), 3.29 – 3.28 (32H, m, $CH_2NHC(O)$), 2.48 – 2.43 and 2.38 – 2.35 (2 x 16H, 2 x m, CH_2CH_2CCH), 2.02 (8H, t, CH_2CCH, J = 2.4 Hz) and 1.73 (32H, bs, $OCH_2CH_2CH_2NH$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 171.4, 169.7, 169.1 and 167.8 (C(O)NH), 166.6 ($C(O)OCH_3$), 159.6 (C_{arom}-3',5'), 159.5 (C_{arom}-3'', 5''), 136.2 (C_{arom}-1''), 131.8 (C_{arom}-1), 106.1 (CH_{arom}-2'',6''), 104.6 (CH_{arom}-4''), 83.2 (CH_2CCH), 70.7 (CH_2CCH), 70.2, 69.8, 69.4 and 69.2 (OCH_2), 66.3 (OCH_2CH_2NH), 53.4 ($C(O)OCH_3$), 39.6 and 38.4 (OCH_2CH_2NH), 37.3 and 36.7 ($CH_2NHC(O)$), 35.1 (CH_2CH_2CCH), 29.1 and 28.9 ($OCH_2CH_2CH_2NH$) and 14.8 (CH_2CCH). MALDI TOF for $C_{230}H_{342}N_{30}O_{78}$ (M, 4772.3717): found $[M + Na]^+$ 4797.93, calcd. 4798.32.

Click chemistry general procedure: Alkyne dendrimer (1-5 mM), sugar azide (1.5 equiv / alkyne), $CuSO_4{\cdot}5H_2O$ (1 equiv / alkyne) and sodium ascorbate (1 equiv / alkyne) were dissolved in DMF/H_2O (1/1, v/v). The mixture was heated under microwave irradiation at 80°C for 20 min. The reaction mixture was concentrated *in vacuo* at 60°C; the product was purified by preparative HPLC and lyophilized from H_2O/MeCN**.**

Monovalent GM1 compound (9): A solution of **4c** (10 mg, 16.8 μmol), pentasaccharide **1** (30 mg, 25 μmol), $CuSO_4.5H_2O$ (4.2 mg, 16.8 μmol) and sodium ascorbate (3.3 mg, 16.8 μmol) in H_2O/DMF (1/1, v/v, 4 mL) was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed conversion of the monovalent compound to a new peak. The reaction mixture was concentrated and the residue was subjected to preparative HPLC purification. The product was obtained after lyophilization as a glass (17 mg, 57 %). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 400 MHz): δ = 8.41 (1H, t, C(O)NH), 8.17 (1H, t, C(O)NH), 8.06 (1H, d, NHAc), 7.84 (1H, t, C(O)NH), 7.66 (1H, s, $CH_{triazole}$), 7.59 (1H, d, NHAc), 7.50 (1H, m, CH_{arom}-2), 7.46 (1H, d, CH_{arom}-6), 7.40 (1H, t, CH_{arom}-5), 7.19 (1H, dd, CH_{arom}-4), 4.49 (1H, d, $H_{Gal'}$-1), 4.47 (1H, d, H_{Gal}-1), 4.39 (1H, d, H_{Glc}-1), 4.27 (2H, t), 4.17 (2H, t), 4.11 (2H, m), 4.05 and 3.99 (2 x 2H, 2 x s, $OCH_2C(O)$), 3.87 (3H, s, $C(O)OCH_3$), 3.33 - 3.29 (3H, m), 3.24 - 3.21 (3H, m), 3.11 - 3.07 and 2.94 - 2.90 (2 x 2H, 2 x m, $CH_2CH_2C_{triazole}$), 2.61 (1H, dd, H_{NeuAc}-3), 2.51 (2H, t), 1.99 and 1.96 (2 x 3H, 2 x s,

NHC(O)CH_3), 1.89 (1H, m, H_{NeuAc}-3), 1.78 - 1.67 (4H, m, $OCH_2CH_2CH_2NH$), 1.60 - 1.49 and 1.23 - 1.08 (4H and 14H, 2 x m, $CH_2CH_2CH_2$). ^{13}C NMR (H_2O/D_2O, 9/1, v/v, 100 MHz): δ = 175.5, 175.2, 174.9, 174.3, 172.1 and 171.6 (COOH and C(O)NH), 168.5 (C(O)OCH_3), 158.5 (C_{arom}-3), 146.3 ($C_{triazole}$-4), 131.2 (C_{arom}-1), 130.5 (CH_{arom}-5), 123.6 ($C_{triazole}$-5), 122.9 (CH_{arom}-6), 120.7 (CH_{arom}-4), 115.4 (CH_{arom}-2), 105.1 ($C_{Gal'}$-1), 102.9 (C_{Gal}-1), 102.8 (C_{GalNAc}-1), 102.4 (C_{Gluc}-1), 101.8 (C_{NeuAc}-2), 80.6 (C_{GalNAc}-3), 79.0 (C_{Gluc}-4), 77.4 (C_{Gal}-4), 75.2 ($C_{Gal'}$-5), 75.1 (C_{Gluc}-5), 74.8 (C_{Gal}-3), 74.72 (C_{Gluc}-3), 74.70 (C_{GalNAc}-5), 74.4 (C_{Gal}-5), 73.4 (C_{NeuAc}-6), 73.1 (C_{Gluc}-2), 72.8 ($C_{Gal'}$-3), 72.5 (C_{NeuAc}-7), 71.0 ($C_{Gal'}$-2), 70.9 (CH_2OC_{Gluc}-1), 70.3 (C_{NeuAc}-8), 70.2 (OCH_2), 70.0 (OCH_2), 69.8 (OCH_2), 69.1, 69.0 (C_{Gal}-2 and C_{NeuAc}-7), 68.9 (OCH_2), 68.7 (OCH_2), 68.4 (C_{GalNAc}-4), 68.2 (C_{NeuAc}-4), 67.1 (OCH_2CH_2NH), 63.1 (C_{NeuAc}-9), 61.4 (C_{GalNAc}-6), 61.3 ($C_{Gal'}$-6), 60.9 (C_{Gluc}-6), 60.5 (C_{Gal}-6), 53.2 (C(O)OCH_3), 51.9 (C_{GalNAc}-2), 51.5 (C_{NeuAc}-5), 50.7 ($CH_2N_{triazole}$), 38.9 (C_{NeuAc}-3), 37.3 and 36.7 (OCH_2CH_2NH and CH_2NHC(O)) 35.5 ($CH_2CH_2C_{triazole}$), 29.7, 29.2, 29.12, 29.06, 29.0, 28.97, 28.6, 28.5, 25.9 and 25.5 ($CH_2CH_2CH_2$), 22.9 (C_{GalNAc}-NHC(O)CH_3), 22.4 (C_{NeuAc}-NHC(O)CH_3) and 21.3 ($CH_2CH_2C_{triazole}$). HRMS for $C_{77}H_{126}N_8O_{39}$ (M, 1786.8122): found $[M - H]^-$ 1785.722, calcd. 1785.804.

Divalent GM1 dendrimer (10a): A solution of **5c** (3.8 mg, 3.6 μmol), pentasaccharide **1** (13 mg, 10.8 μmol), $CuSO_4.5H_2O$ (1.8 mg, 7.2 μmol) and sodium ascorbate (1.4 mg, 7.2 μmol) in H_2O/DMF (1/1, v/v, 2 mL) was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed conversion of the dendrimer to a new peak. The reaction mixture was concentrated and the residue was subjected to preparative HPLC purification. The product was obtained after lyophilization as a white fluffy compound (9.8 mg, 78 %). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 400 MHz): δ = 8.45 (2H, t, C(O)NH), 8.22 (2H, t, C(O)NH), 8.08 (2H, d, NHAc), 7.87 (2H, t, C(O)NH), 7.73 (2H, s, $CH_{triazole}$), 7.47 (2H, d, CH_3C(O)NH), 7.17 (2H, d, CH_{arom}-2,6), 6.78 (1H, m, CH_{arom}-4), 4.54 (2H, d, $H_{Gal'}$-1), 4.52 (2H, d, H_{Gal}-1), 4.43 (2H, d, H_{Glc}-1), 4.32 (4H, t), 4.10 and 4.06 (2 x 4H, 2 x s, OCH_2C(O)), 3.41 - 3.34 (6H, m), 3.30 - 3.25 (6H, m), 3.14 and 2.97 (2 x 4H, 2 x t, $CH_2CH_2C_{triazole}$), 2.66 (2H, dd, H_{NeuAc}-3), 2.56 (4H, t), 2.03 and 2.00 (2 x 6H, 2 x s, NHC(O)CH_3), 1.94 (2H, m, H_{NeuAc}-3), 1.80 - 1.73 (8H, m, $OCH_2CH_2CH_2NH$), 1.66 - 1.52 and 1.30 - 1.08 (8H and 28H, 2 x m, $CH_2CH_2CH_2$). ^{13}C NMR (H_2O/D_2O, 9/1, v/v, 100 MHz): δ = 175.5, 175.2, 174.9, 174.3, 172.1 and 171.6 (COOH and C(O)NH), 168.5 (C(O)OCH_3), 159.9 (C_{arom}-3,5), 146.4 ($C_{triazole}$-4), 132.0 (C_{arom}-1), 123.6 ($C_{triazole}$-5), 108.8 (CH_{arom}-2,6), 107.1 (CH_{arom}-4), 105.1

($C_{Gal'}$-1), 102.9 (C_{Gal}-1), 102.8 (C_{GalNAc}-1), 102.4 (C_{Gluc}-1), 101.8 (C_{NeuAc}-2), 80.6 (C_{GalNAc}-3), 79.0 (C_{Gluc}-4), 77.4 (C_{Gal}-4), 75.2 ($C_{Gal'}$-5), 75.1 (C_{Gluc}-5), 74.8 (C_{Gal}-3), 74.72 (C_{Gluc}-3), 74.70 (C_{GalNAc}-5), 74.4 (C_{Gal}-5), 73.4 (C_{NeuAc}-6), 73.1 (C_{Gluc}-2), 72.8 ($C_{Gal'}$-3), 72.5 (C_{NeuAc}-7), 71.0 ($C_{Gal'}$-2), 70.9 (CH_2OC_{Gluc}-1), 70.3 (C_{NeuAc}-8), 70.2 (OCH_2), 70.0 (OCH_2), 69.8 (OCH_2), 69.0 (C_{Gal}-2), 68.9 (OCH_2), 68.7 (OCH_2), 68.4 (C_{GalNAc}-4), 68.2 (C_{NeuAc}-4), 67.1 (OCH_2CH_2NH), 63.1 (C_{NeuAc}-9), 61.4 (C_{GalNAc}-6), 61.3 ($C_{Gal'}$-6), 60.9 (C_{Gluc}-6), 60.5 (C_{Gal}-6), 53.2 ($C(O)OCH_3$), 51.9 (C_{GalNAc}-2), 51.5 (C_{NeuAc}-5), 50.7 ($CH_2N_{triazole}$), 38.9 (C_{NeuAc}-3), 37.3 and 36.7 (OCH_2CH_2NH and $CH_2NHC(O)$) 35.5 ($CH_2CH_2C_{triazole}$), 29.7, 29.2, 29.12, 29.06, 29.0, 28.97, 28.6, 28.5, 25.9 and 25.5 ($CH_2CH_2CH_2$), 22.9 (C_{GalNAc}-$NHC(O)CH_3$), 22.4 (C_{NeuAc}-$NHC(O)CH_3$) and 21.3 ($CH_2CH_2C_{triazole}$). HRMS for $C_{146}H_{244}N_{16}O_{76}$ (M, 3437.572): found $[M + 3H]^{3+}$ 1147.27, calcd. 1146.86.

Divalent GM2 dendrimer (10b): A solution of **5c** (9.5 mg, 9.0 μmol), tetrasaccharide **3** (28.8 mg, 28.0 μmol), $CuSO_4.5H_2O$ (2.3 mg, 9.0 μmol) and sodium ascorbate (1.8 mg, 9.0 μmol) in H_2O/DMF (1/1, v/v, 2 mL) was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed conversion of the dendrimer to a new peak. The reaction mixture was concentrated and the residue was subjected to preparative HPLC purification. The product was obtained after lyophilization as a white fluffy compound (19.0 mg, 68 %). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 400 MHz): δ = 8.44 (2H, t, C(O)NH), 8.23 (2H, t, C(O)NH), 8.08 (2H, d, NHAc), 7.87 (2H, t, C(O)NH), 7.71 (2H, s, $CH_{triazole}$), 7.36 (2H, d, NHAc), 7.16 (2H, d, CH_{arom}-2,6), 6.78 (1H, m, CH_{arom}-4), 4.52 (2H, d, H_{Gal}-1), 4.43 (2H, d, H_{Glc}-1), 4.31 (4H, t), 4.11 and 4.07 (2 x 4H, 2 x s, $OCH_2C(O)$), 3.39 - 3.34 (6H, m), 3.31 - 3.26 (6H, m), 3.17 - 3.12 and 2.98 - 2.95 (2 x 4H, 2 x m, $CH_2CH_2C_{triazole}$), 2.66 (2H, dd, H_{NeuAc}-3), 2.55 (4H, t), 2.03 and 2.01 (2 x 6H, 2 x s, $NHC(O)CH_3$), 1.92 (2H, m, H_{NeuAc}-3), 1.79 - 1.74 (8H, m, $OCH_2CH_2CH_2NH$), 1.66 - 1.51 and 1.35 - 1.04 (8H and 28H, 2 x m, $CH_2CH_2CH_2$). ^{13}C NMR (H_2O/D_2O, 9/1, v/v, 100 MHz): δ = 175.5, 175.2, 174.9, 174.3, 172.1 and 171.6 (COOH and C(O)NH), 168.5 ($C(O)OCH_3$), 159.9 (C_{arom}-3,5), 146.4 ($C_{triazole}$-4), 132.0 (C_{arom}-1), 123.5 ($C_{triazole}$-5), 108.9 (CH_{arom}-2,6), 107.2 (CH_{arom}-4), 103.1 (C_{Gal}-1), 103.0 (C_{GalNAc}-1), 102.5 (C_{Gluc}-1), 101.9 (C_{NeuAc}-2), 79.0 (C_{Gluc}-4), 77.5 (C_{Gal}-4), 75.1 (C_{Gal}-3), 75.0 (C_{Gluc}-5), 74.8 (C_{Gluc}-3), 74.4 (C_{GalNAc}-5), 73.5 (C_{Gal}-5), 73.3 (C_{NeuAc}-6), 72.7 (C_{Gluc}-2), 71.8 (C_{GalNAc}-3), 71.0 (CH_2OC_{Gluc}-1), 70.5 (C_{NeuAc}-8), 70.3 (OCH_2), 70.0 (OCH_2), 69.8 (OCH_2), 69.1 (2C, C_{NeuAc}-7 and C_{Gal}-2), 68.9 (OCH_2), 68.7 (OCH_2), 68.5 (C_{GalNAc}-4), 68.3 (C_{NeuAc}-4), 67.2 (OCH_2CH_2NH), 63.4 (C_{NeuAc}-9), 61.6 (C_{GalNAc}-6), 61.0 (C_{Gluc}-6), 60.6 (C_{Gal}-

6), 53.3 (C(O)O*C*H$_3$), 52.8 (C$_{GalNAc}$-2), 52.1 (C$_{NeuAc}$-5), 50.7 (*C*H$_2$N$_{triazole}$), 39.0 (C$_{NeuAc}$-3), 37.4 and 36.8 (OCH$_2$*C*H$_2$NH and *C*H$_2$NHC(O)), 35.6 (*C*H$_2$CH$_2$C$_{triazole}$), 29.8, 29.3, 29.2, 29.1, 29.0, 28.9, 28.7, 28.5, 26.0 and 25.5 (CH$_2$*C*H$_2$CH$_2$), 23.1 (C$_{GalNAc}$-NHC(O)*C*H$_3$), 22.5 (C$_{NeuAc}$-NHC(O)*C*H$_3$) and 21.4 (CH$_2$*C*H$_2$C$_{triazole}$). HRMS for C$_{134}$H$_{224}$N$_{16}$O$_{66}$ (M, 3113.4664): found [M – 2H]$^{2-}$ 2139.08, calcd. 2138.00.

Tetravalent GM1 dendrimer (11a): A solution of **6c** (4.1 mg, 1.8 μmol), pentasaccharide **1** (13.0 mg, 10.8 μmol), CuSO$_4$.5H$_2$O (1.8 mg, 7.2 μmol) and sodium ascorbate (1.4 mg, 7.2 μmol) in H$_2$O/DMF (1/1, v/v, 2 mL) was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed conversion of the dendrimer to a new peak. The reaction mixture was concentrated and the residue was subjected to preparative HPLC purification. The product was obtained after lyophilization as a white fluffy compound (9.7 mg, 76 %). ^{1}H NMR (H$_2$O/D$_2$O, 9/1, v/v, 400 MHz): δ = 8.56 (2H, t, C(O)NH), 8.41 (4H, t, C(O)NH), 8.21 (4H, t, C(O)NH), 8.07 (4H, d, NHAc), 7.87 (4H, t, C(O)NH), 7.70 (4H, s, CH$_{triazole}$), 7.48 (4H, d, NHAc), 7.05 (2H, m, CH$_{arom}$-2,6), 6.93 (4H, m, CH$_{arom}$-2',6'), 6.66 (3H, m, CH$_{arom}$-4, 4'), 4.52 (4H, d, H$_{Gal'}$-1), 4.49 (4H, d, H$_{Gal}$-1), 4.39 (4H, d, H$_{Glc}$-1), 4.24 (8H, t), 4.07 and 4.03 (2 x 8H, 2 x s, OCH$_2$C(O)), 3.39 - 3.33 (12H, m), 3.29 - 3.22 (12H, m), 3.17 - 3.10 and 2.95 - 2.90 (2 x 8H, 2 x m, C*H*$_2$C*H*$_2$C$_{triazole}$), 2.65 (4H, dd, H$_{NeuAc}$-3), 2.53 (8H, t), 2.01 and 1.99 (2 x 12H, 2 x s, NHC(O)C*H*$_3$), 1.93 (4H, m, H$_{NeuAc}$-3), 1.76 - 1.68 (16H, m, OCH$_2$C*H*$_2$CH$_2$NH), 1.64 - 1.59, 1.53 - 1.45 and 1.22 - 0.95 (8H, 8H and 56H, 3 x m, CH$_2$C*H*$_2$CH$_2$). ^{13}C NMR (H$_2$O/D$_2$O, 9/1, v/v, 100 MHz): δ = 175.5, 175.2, 174.9, 174.3, 172.1 and 171.6 (COOH and C(O)NH), 168.5 (*C*(O)OCH$_3$), 159.9 (C$_{arom}$-3,5), 146.4 (C$_{triazole}$-4), 132.0 (C$_{arom}$-1), 123.6 (C$_{triazole}$-5), 108.8 (CH$_{arom}$-2,6), 107.1 (CH$_{arom}$-4), 105.1 (C$_{Gal'}$-1), 102.9 (C$_{Gal}$-1), 102.8 (C$_{GalNAc}$-1), 102.4 (C$_{Gluc}$-1), 101.8 (C$_{NeuAc}$-2), 80.6 (C$_{GalNAc}$-3), 79.0 (C$_{Gluc}$-4), 77.4 (C$_{Gal}$-4), 75.2 (C$_{Gal'}$-5), 75.1 (C$_{Gluc}$-5), 74.8 (C$_{Gal}$-3), 74.72 (C$_{Gluc}$-3), 74.70 (C$_{GalNAc}$-5), 74.4 (C$_{Gal}$-5), 73.4 (C$_{NeuAc}$-6), 73.1 (C$_{Gluc}$-2), 72.8 (C$_{Gal'}$-3), 72.5 (C$_{NeuAc}$-7), 71.0 (C$_{Gal'}$-2), 70.9 (*C*H$_2$OC$_{Gluc}$-1), 70.3 (C$_{NeuAc}$-8), 70.2 (OCH$_2$), 70.0 (OCH$_2$), 69.8 (OCH$_2$), 69.0 (C$_{Gal}$-2 and C$_{NeuAc}$-7), 68.9 (OCH$_2$), 68.7 (OCH$_2$), 68.4 (C$_{GalNAc}$-4), 68.2 (C$_{NeuAc}$-4), 67.1 (O*C*H$_2$CH$_2$NH), 63.1 (C$_{NeuAc}$-9), 61.4 (C$_{GalNAc}$-6), 61.3 (C$_{Gal'}$-6), 60.9 (C$_{Gluc}$-6), 60.5 (C$_{Gal}$-6), 53.2 (C(O)O*C*H$_3$), 51.9 (C$_{GalNAc}$-2), 51.5 (C$_{NeuAc}$-5), 50.7 (*C*H$_2$N$_{triazole}$), 38.9 (C$_{NeuAc}$-3), 37.3 and 36.7 (OCH$_2$*C*H$_2$NH and *C*H$_2$NHC(O)) 35.5 (*C*H$_2$CH$_2$C$_{triazole}$), 29.7, 29.2, 29.12, 29.06, 29.0 and 28.9 (CH$_2$*C*H$_2$CH$_2$), 22.9 (C$_{GalNAc}$-

NHC(O)CH_3), 22.4 (C_{NeuAc}-NHC(O)CH_3) and 21.3 ($CH_2CH_2C_{triazole}$). HRMS for $C_{302}H_{498}N_{34}O_{154}$ (M, 7065.2182): found $[M + 4H + 2Na]^{6+}$ 1185.64, calcd. 1185.87.

Tetravalent GM2 dendrimer (11b): A solution of **6c** (7.4 mg, 3.2 μmol), tetrasaccharide **3** (20 mg, 19.4 μmol), $CuSO_4.5H_2O$ (3.2 mg, 12.8 μmol) and sodium ascorbate (2.5 mg, 12.8 μmol) in H_2O/DMF (1/1, v/v, 2 mL) was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed conversion of the dendrimer to a new peak. The reaction mixture was concentrated and the residue was subjected to preparative HPLC purification. The product was obtained after lyophilization as a white fluffy compound (7.6 mg, 37 %). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 400 MHz): δ = 8.57 (2H, t, C(O)NH), 8.41 (4H, t, C(O)NH), 8.22 (4H, t, C(O)NH), 8.07 (4H, d, NHAc), 7.88 (4H, t, C(O)NH), 7.71 (4H, s, $CH_{triazole}$), 7.37 (4H, d, NHAc), 7.04 (2H, m, CH_{arom}-2,6), 6.95 (4H, m, CH_{arom}-2',6'), 6.66 (3H, m, CH_{arom}-4, 4'), 4.51 (4H, d, H_{Gal}-1), 4.40 (4H, d, H_{Glc}-1), 4.25 (8H, t), 4.08 and 4.04 (2 x 8H, 2 x s, $OCH_2C(O)$), 3.39 - 3.35 (12H, m), 3.30 - 3.24 (12H, m), 3.17 - 3.12 and 2.97 - 2.94 (2 x 8H, 2 x m, $CH_2CH_2C_{triazole}$), 2.67 (4H, dd, H_{NeuAc}-3), 2.55 (8H, t), 2.03 and 2.01 (2 x 12H, 2 x s, NHC(O)CH_3), 1.94 (4H, m, H_{NeuAc}-3), 1.76 - 1.67 (16H, m, $OCH_2CH_2CH_2NH$), 1.64 - 1.60, 1.52 - 1.48 and 1.23 - 0.96 (8H, 8H and 56H, 3 x m, $CH_2CH_2CH_2$). ^{13}C NMR (H_2O/D_2O, 9/1, v/v, 100 MHz): δ = 175.4, 175.2, 174.6, 174.0, 172.0 and 171.4 (COOH and C(O)NH), 168.9 ($C(O)OCH_3$), 160.0 (C_{arom}-3,5), 146.4 ($C_{triazole}$-4), 131.8 (C_{arom}-1), 123.5 ($C_{triazole}$-5), 115.2 (CH_{arom}-2,6), 106.8 (CH_{arom}-4), 103.1 (C_{Gal}-1), 103.0 (C_{GalNAc}-1), 102.6 (C_{Gluc}-1), 101.6 (C_{NeuAc}-2), 79.0 (C_{Gluc}-4), 77.3 (C_{Gal}-4), 75.1 (C_{Gal}-3), 75.0 (C_{Gluc}-5), 74.9 (C_{Gluc}-3), 74.4 (C_{GalNAc}-5), 73.5 (C_{Gal}-5), 73.3 (C_{NeuAc}-6), 72.6 (C_{Gluc}-2), 71.8 (C_{GalNAc}-3), 70.9 (CH_2-OC_{Gluc}1), 70.4 (C_{NeuAc}-8), 70.3 (OCH_2), 70.0 (OCH_2), 69.8 (OCH_2), 69.1 (C_{Gal}-2), 69.0 (C_{NeuAc}-7), 68.9 (OCH_2), 68.7 (OCH_2), 68.5 (C_{GalNAc}-4), 68.3 (C_{NeuAc}-4), 67.0 (OCH_2CH_2NH), 63.4 (C_{NeuAc}-9), 61.6 (C_{GalNAc}-6), 61.0 (C_{Gluc}-6), 60.6 (C_{Gal}-6), 53.2 ($C(O)OCH_3$), 52.9 (C_{GalNAc}-2), 52.1 (C_{NeuAc}-5), 50.8 ($CH_2N_{triazole}$), 38.9 (C_{NeuAc}-3), 37.4, 36.9 and 36.8 (OCH_2CH_2NH and $CH_2NHC(O)$), 35.5 ($CH_2CH_2C_{triazole}$), 29.9, 29.4, 29.3, 29.2, 29.1, 28.8, 26.1 and 25.7 ($CH_2CH_2CH_2$), 23.1 (C_{GalNAc}-NHC(O)CH_3), 22.5 (C_{NeuAc}-NHC(O)CH_3) and 21.4 ($CH_2CH_2C_{triazole}$). HRMS for $C_{278}H_{458}N_{34}O_{134}$ (M, 6417.0069): found $[M + 5Na]^{5+}$ 1184.90, calcd. 1184.41.

Octavalent GM1 dendrimer (12a): A solution of **7c** (6.6 mg, 1.4 μmol), pentasaccharide **1** (20.0 mg, 16.6 μmol), $CuSO_4.5H_2O$ (5.5 mg, 22.0 μmol) and sodium ascorbate (4.6 mg, 23.0 μmol) in H_2O/DMF (1/1, v/v, 3 mL) was heated under microwave irradiation at 80 ˚C for 40 min. Analytical HPLC showed conversion to a new peak. The reaction mixture was concentrated and the residue was subjected to preparative HPLC purification. The product was obtained after lyophilization as a white fluffy compound (8.5 mg, 44%). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 400 MHz): δ = 8.44 (6H, bs, C(O)NH), 8.30 (8H, bs, C(O)NH), 8.09 (8H, bs, NHAc), 7.96 (8H, d, C(O)NH), 7.75 (8H, s, $CH_{triazole}$), 7.53 (bs, C(O)NH), 7.34 (8H, d, NHAc), 6.92, 6.81 and 6.52 (m, CH_{arom}) 4.41, 4.29, 3.23 (16H, m), 3.13 (16H, m), 3.02 (16H, m), 2.81 (16H, m), 2.54 (8H, m, H_{NeuAc}-3), 2.40 (16H, m), 1.91 and 1.88 (each 24H, s, NHC(O)CH_3), 1.60, 1.50, 1.37 and 0.90 (16H,16H, 16H and 112H, 4 x m, $CH_2CH_2CH_2$). ^{13}C NMR (H_2O/D_2O, 9/1, v/v, 100 MHz): δ = 175.4, 175.1, 174.6, 174.4, 171.9, 171.4 (COOH and C(O)NH),168.5 (*C*(O)OCH_3),160.0 (C_{arom}-3,5),146.5 ($C_{triazole}$-4), 136.1 and 126.3 (C_{arom}), 123.2 ($C_{triazole}$-5), 106.7 (C_{arom}), 105.1 ($C_{Gal'}$-1), 103.0 (C_{Gal}-1), 102.9 (C_{GalNAc}-1), 102.6 (C_{Gluc}-1), 102.0 (C_{NeuAc}-2), 80.8 (C_{GalNAc}-3), 79.1 (C_{Gluc}-4), 77.6 (C_{Gal}-4), 75.3 ($C_{Gal'}$-5), 75.1 (C_{Gluc}-5), 75.0 (C_{Gluc}-5, 74.8 (C_{Gal}-3), 74.5 (C_{Gal}-5), 73.5 (C_{NeuAc}-6), 73.3 (C_{Gluc}-2), 73.0, 72.8 ($C_{Gal'}$-3), 71.2 ,70.9 (*C*H_2-OC_{Gluc}1), 70.5 (C_{NeuAc}-8), 70.3 (OCH_2), 70.0 (OCH_2), 69.8 (OCH_2), 69.2 (C_{Gal}-2 and C_{NeuAc}-7), 69.1, 68.9 and 68.7 (OCH_2), 68.5, 68.4 and 66.9 (O*C*H_2CH_2NH), 63.4 (C_{NeuAc}-9), 61.6 (C_{GalNAc}-6), 61.4 ($C_{Gal'}$-6), 61.1 (C_{Gluc}-6), 60.7 (C_{Gal}-6), 52.1 (C_{GalNAc}-2), 51.6 (C_{NeuAc}-5), 50.5 (*C*$H_2N_{triazole}$), 39.0 (C_{NeuAc}-3), 37.3, 36.9 and 36.8 (OCH_2*C*H_2NH and *C*H_2NHC(O)), 35.7 (*C*$H_2CH_2C_{triazole}$), 29.9, 29.4, 29.3, 29.2, 28.8, 26.2 and 25.7 (CH_2*C*H_2CH_2), 23.1 (C_{GalNAc}-NHC(O)*C*H_3), 22.5 (C_{NeuAc}-NH(O)*C*H_3) and 21.6 (CH_2*C*$H_2C_{triazole}$).

Octavalent GM2 dendrimer (12b): A solution of **7c** (6.6 mg, 1.4 μmol), tetrasaccharide **3** (17.1 mg, 16.6 μmol), $CuSO_4.5H_2O$ (5.5 mg, 22.0 μmol) and sodium ascorbate (4.6 mg, 23.0 μmol) in H_2O/DMF (1/1, v/v, 3 mL) was heated under microwave irradiation at 80 ˚C for 40 min. Analytical HPLC showed conversion to a new peak. The reaction mixture was concentrated and the residue was subjected to preparative HPLC purification. The product was obtained after lyophilization as a white fluffy compound (7.6 mg, 42%). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 400 MHz): δ = 8.44 (6H, bs, C(O)NH), 8.30 (8H, bs, C(O)NH), 8.09 (8H, bs, NHAc), 7.96 (8H, d, C(O)NH), 7.75 (8H, s, $CH_{triazole}$), 7.53 (bs, C(O)NH), 7.34

(8H, d, NHAc), 6.92, 6.81 and 6.52 (m, CH_{arom}) 4.41, 4.29, 3.23 (16H, m), 3.13 (16H, m), 3.02 (16H, m), 2.81 (16H, m), 2.54 (8H, m, H_{NeuAc}-3), 2.40 (16H, m), 1.91 and 1.88 (each 24H, s, NHC(O)CH_3), 1.60, 1.50, 1.37 and 0.90 (16H,16H, 16H and 112H, 4 x m, $CH_2CH_2CH_2$). ^{13}C NMR (H_2O/D_2O, 9/1, v/v, 100 MHz): δ = 175.4, 175.1, 174.6, 174.4, 171.9, 171.4 (COOH and C(O)NH),168.5 (*C*(O)OCH_3),160.0 (C_{arom}-3,5),146.5 ($C_{triazole}$-4), 136.1 and 126.3 (C_{arom}), 123.2 ($C_{triazole}$-5), 106.7 (C_{arom}), 103.0 (C_{Gal}-1), 102.9 (C_{GalNAc}-1), 102.6 (C_{Gluc}-1), 102.0 (C_{NeuAc}-2), 80.8 (C_{GalNAc}-3), 79.1 (C_{Gluc}-4), 77.6 (C_{Gal}-4), 75.1 (C_{Gluc}-5), 75.0 (C_{Gluc}-5, 74.8 (C_{Gal}-3), 74.5 (C_{Gal}-5), 73.5 (C_{NeuAc}-6), 73.3 (C_{Gluc}-2), 71.2 ,70.9 (*C*H_2-OC_{Gluc}1), 70.5 (C_{NeuAc}-8), 70.3 (OCH_2), 70.0 (OCH_2), 69.8 (OCH_2), 69.2 (C_{Gal}-2 and C_{NeuAc}-7), 69.1, 68.9 and 68.7 (OCH_2), 68.5, 68.4 and 66.9 (O*C*H_2CH_2NH), 63.4 (C_{NeuAc}-9), 61.6 (C_{GalNAc}-6), 61.1 (C_{Gluc}-6), 60.7 (C_{Gal}-6), 52.1 (C_{GalNAc}-2), 51.6 (C_{NeuAc}-5), 50.5 (*C*$H_2N_{triazole}$), 39.0 (C_{NeuAc}-3), 37.3, 36.9 and 36.8 (OCH_2*C*H_2NH and *C*H_2NHC(O)), 35.7 (*C*$H_2CH_2C_{triazole}$), 29.9, 29.4, 29.3, 29.2, 28.8, 26.2 and 25.7 (CH_2*C*H_2CH_2), 23.1 (C_{GalNAc}-NHC(O)*C*H_3), 22.5 (C_{NeuAc}-NH(O)*C*H_3) and 21.6 (CH_2*C*$H_2C_{triazole}$).

GM1 / GM2 ELISA experiments: A 96 wells plate (F96 Maxisorp™, Nunc) was coated with a 100 µL solution of GM1 (2 µg/mL) (Sigma-Aldrich) in PBS at 37 °C for 16 h. Unattached ganglioside was removed by washing with PBS (2 x 450 µL) after which remaining binding sites were blocked by incubation with 100 µL 1% (w/v) BSA in PBS for 30 min at 37 °C. After blocking the plate was washed with PBS (3 x 450 µL). Samples of Cholera Toxin B Subunit horseradish peroxidase conjugate (Sigma-Aldrich) and inhibitor in 0.1% BSA, 0.05 % Tween 20 in PBS were incubated at rt for 2 h and were then transferred to the coated plate. After 30 min incubation the solution was removed followed by washing steps with 0.1 % BSA, 0.05 % Tween-20 in PBS (3 x 450 µL). To identify toxin binding to surface bound GM1 the wells were treated with a freshly prepared *o*-phenylenediamine (OPD) solution (100 µL) (25 mg OPD.2HCl, 7.5 mL 0.1M citric acid, 7.5 mL 0.1M sodium citrate and 6 µL of a 30% H_2O_2 solution) and the colour forming reaction was quenched after 15 min with H_2SO_4 (50 µL) (2.5 M). The absorbance at 490 nm was measured by a µQuant plate reader.

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Chapter 5

Strong inhibition of Cholera Toxin binding by galactose dendrimers

Part of the results in this chapter have been published:

Hilbert M. Branderhorst, Rob M. J. Liskamp, Gerben M. Visser, Roland J. Pieters; Strong inhibition of Cholera Toxin binding by galactose dendrimers; *Chem. Commun.* **2007**, 5043-5045

Introduction

Cholera Toxin (CT) is the causative agent of cholera and this disease still causes major problems in the developing world, with over 100,000 reported cases per year.[1] CT is a member of the AB_5 toxin family, and its B-subunits bind to the oligosaccharide of the GM1 ganglioside (GM1os) molecules present on the cell surfaces of the intestines. This binding process leads to toxin internalization[2] followed by disease initiation caused by the A-subunit.[3] Inhibitors of B-subunit binding have therapeutic potential and they may also be useful for toxin detection either in patient samples[4] or in materials suspect of terrorist origin.[5] Considering the pentameric architecture of the B subunit (CTB_5) to which up to five GM1 oligosaccharide ligands can simultaneously bind, a multivalency approach is particularly attractive for inhibitor design. Several multivalent inhibitors have been designed for CT and related proteins of the AB_5 family.[6] Recently we reported on a series of GM1os conjugated dendrimers which showed unprecedented affinity enhancements of up to 380,000 fold beyond monovalent GM1os derivatives.[7]

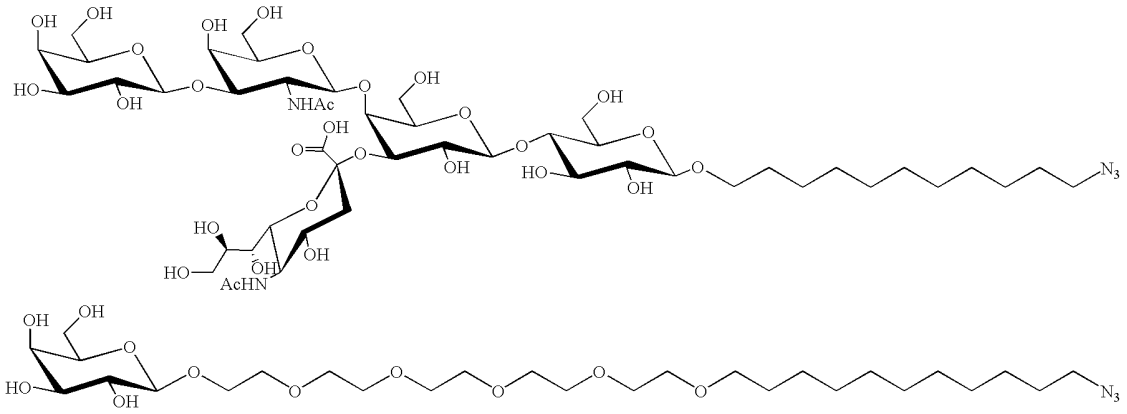
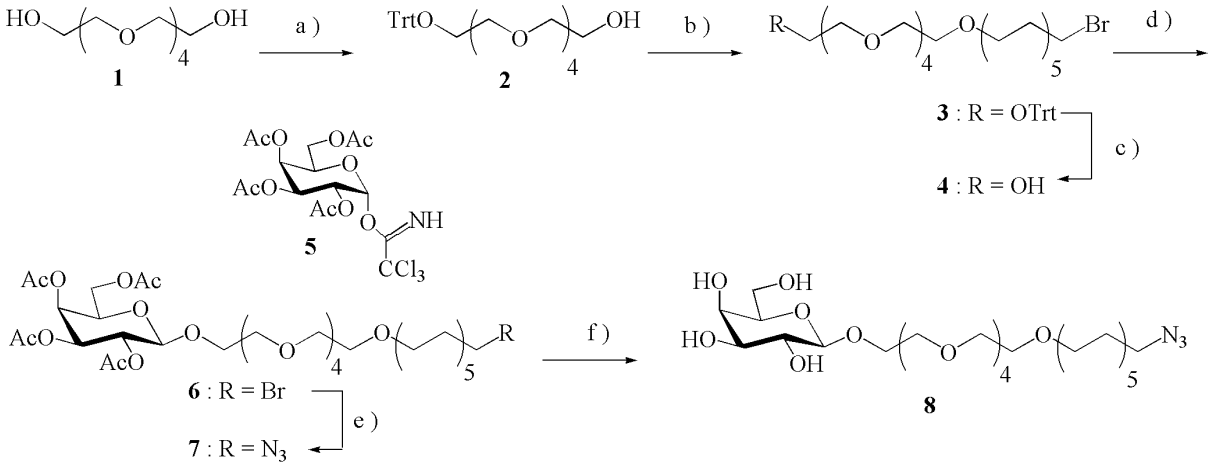


Figure 1. *Structure of the galactose building block used here (bottom) to mimic some of the features of the GM1os building block (top, ref. 7), such as the terminal galactose moiety, the spacer length and its hydro- and lipophilicity.*

The strong binding was attributed to the combined use of the strong GM1os ligand and its multivalent presentation on elongated dendrimers.[6e] While these GM1os-dendrimer conjugates were very potent, the goal still remains to prepare inhibitors based on cheap bulk sugars like galactose with potencies equal or better than the endogenous GM1os ligand. The synthesis of GM1os is complex and not suitable to be performed on an industrial scale.

The design lessons learned in the GM1os study[7] were applied towards the development of galactose-based multivalent CTB_5 inhibitors. In this chapter we report inhibitors that exhibited CTB_5 inhibitory potencies comparable to GM1os derivatives. Galactose was used as the CTB_5 ligand, since this is by far the most important residue for binding within GM1os.[8] Galactose was outfitted with a poly-ethylene glycol unit, to crudely mimic the other sugar rings of the GM1os (Figure 1). Furthermore, a lipophilic part was attached in order to keep this factor the same as in our GM1os based system.[7] Next to the use of the optimized galactose building block combined with the long armed dendrimer we also explored the use of a series of divalent galactose dendrimers with increasing length of the dendritic arms. The optimal spacer length between two CTB_5 ligands was already determined by Hol[6e] for a pentavalent scaffold and squaric acid-based spacers. The galactose building block and a series of divalent dendrimers were constructed from easily available starting materials. The synthesized divalent galactose dendrimers were tested for their inhibition capacity.

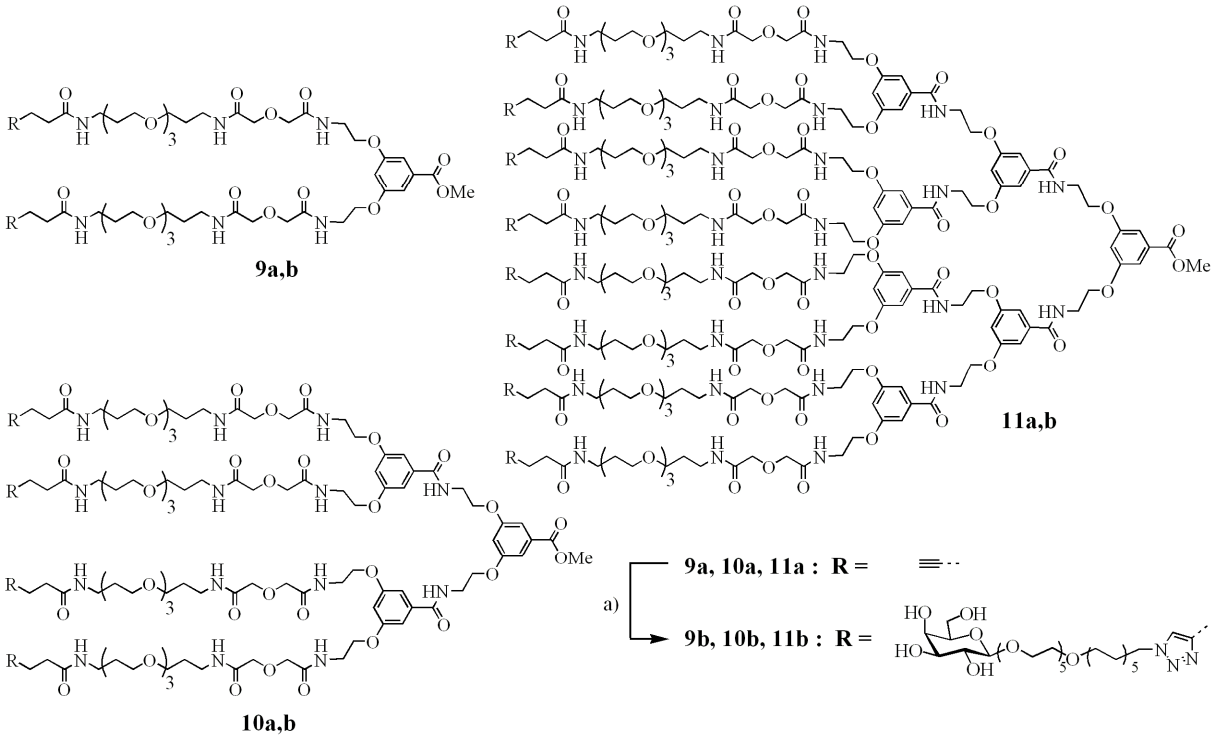


Scheme 1. *Synthesis of galactose building block* ***8****; reagents and conditions: a) trityl chloride, pyridine, 72%; b) NaH, Br(CH₂)₁₁Br, DMF, 66%; c) TsOH, MeOH, 95%; d)* ***5****, BF₃·Et₂O, toluene, 44%; e) NaN₃, DMF, 84%; f) NaOMe, MeOH, 72%.*

Results and Discussion

Building block **8** was prepared as is shown in Scheme 1. The synthesis started with penta-ethylene glycol **1**, which was mono tritylated to give **2**. The chain was elongated via an S_N2 reaction with 1,11-dibromo undecane and the trityl group was cleaved by *p*-TsOH in MeOH to give **4**. Introduction of the galactose moiety was achieved with galactosyl donor **5**

and $BF_3.OEt_2$ as the promotor. An azide function for 'click' conjugation[9] was introduced by reaction of **6** with NaN_3 in DMF at elevated temperature and as the final step the acetyl protecting groups were removed by NaOMe in MeOH to afford the desired compound **8**. Ligation of **8** to dendrimers **9a**, **10a** and **11a**[7] (Scheme 2) was performed using "click" chemistry.[9] Our recently developed protocol involving microwave heating at 80 ˚C was used[10] and the products **9b**, **10b** and **11b** were obtained after preparative HPLC purification in good yields and purity.



Scheme 2. *Synthetic galactose dendrimers; reagents and conditions: a)* ***8****,* $CuSO_4$*, sodium ascorbate, DMF/*H_2O*, 70% for* ***9b****, 78% for* ***10b****, 77% for* ***11b****.*

The glycodendrimers were tested for Cholera Toxin inhibition capacities in a well established ELISA like assay.[11,7] A GM1 ganglioside coated 96 well plate was allowed to bind horseradisch peroxidise (HRP)-conjugated CTB_5 (CTB_5-HRP) and this binding was inhibited with a concentration range of mono- to octavalent inhibitors (Table 1). In this assay galactose and the monovalent galactose derivative **12** exhibited the expected weak inhibitory power with IC_{50} values of 240 and 80 mM respectively. The divalent dendrimer **9b** showed a large increase in potency with an IC_{50} value of 130 μM. Tetravalent compound **10b** showed a further increase in potency (IC_{50} 25 μM) and the affinity of

octavalent compound **11b** increased only by another factor of ca. 2 (IC_{50} 12 μM). The potency increase of divalent **9b** is remarkable.

Table 1. *Inhibitory potencies of the CTB_5 inhibitors*

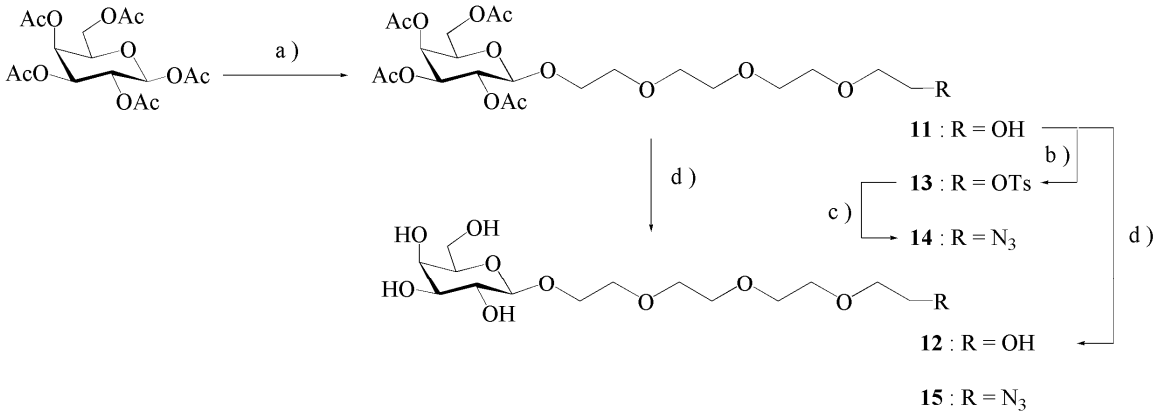
Compound	valency	IC_{50} (M)[a]	rel. pot.[b] (per sugar)
Galactose	1	2.4 (±0.5) x 10^{-1}	1 (1)
12 (scheme 3)	1	8.0 (±0.2) x 10^{-2}	3 (3)
9b	2	1.3 (±0.2) x 10^{-4}	1,846 (923)
10b	4	2.5 (±0.4) x 10^{-5}	9,600 (2,400)
11b	8	1.2 (±0.3) x 10^{-5}	20,000 (2,500)
GM1os-$C_{11}H_{21}$	1	1.9 (±0.6) x 10^{-5}	12,632 (12,632)

[a] Determined in an ELISA experiment with 0.43 nM CTB_5-HRP and wells coated with 0.2 μg GM1. [b] relative potency of galactose was taken as 1.

However, compound **9b** still binds weaker than the monovalent GM1os derivative. The compounds of higher valency **10b** and **11b** showed IC_{50} values in the same range as the GM1os derivative. The multivalency effect as expressed by the relative potency per sugar, still increased from di- to tetravalent (923 vs. 2400), while it remained basically the same at the octavalent stage (2500). Additional experiments including the use of complementary experimental methods will likely uncover the nature of the multivalency effects, i.e. chelation, aggregation or a combination thereof. In comparison to reported multivalent galactose-based CTB_5 inhibitors the present system compares favourably. A reported pentavalent galactose based system still fell short by a factor of ca. 80 relative to GM1.[6e] Another decavalent system seemed to come close, but no direct comparison with GM1os or its derivatives was made.[6c]

Synthesis of a range of divalent galactose dendrimers started with the preparation of galactose building block **15**. Per acetylated galactose reacted with tetra ethylene glycol under $BF_3.Et_2O$ activation to give **11**, part of which was deacetylated to give monovalent reference compound **12**. Tosylation of **11** with *p*-tosyl chloride and Et_3N in CH_2Cl_2 was followed by substitution of the tosylate by NaN_3 in DMF at elevated temperature. Final

deacetylation with NaOMe yielded galactoside **15**. Ligation of **15** to the divalent dendrimers **16a** - **19a** was achieved using the previously described "click" procedure (Scheme 4). The desired dendrimers were obtained in reasonable yields and good purity. The dendrimers were tested for their inhibition capacities using the standard ELISA like assay.



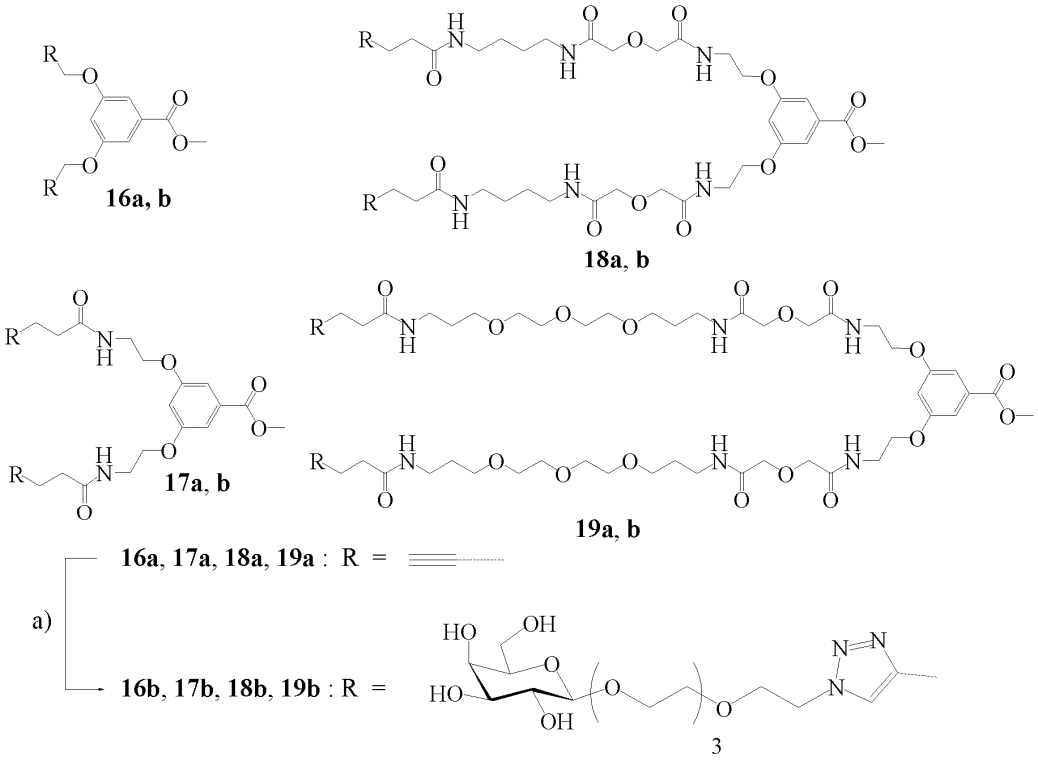
Scheme 3. *Synthesis of the Galactose building block **15**. Reagents and conditions: a) tetra ethylene glycol, $BF_3.Et_2O$, CH_2Cl_2, 62%; b) p-tosyl chloride, Et_3N, CH_2Cl_2, 74%; c) NaN_3, DMF, 94%; d) NaOMe, MeOH, quant.*

Although the divalent dendrimers performed much better over monovalent compound **12**, no increase in affinity has been observed with increasing spacer length. In fact **16b** and **18b** showed the lowest IC_{50} value (5.7 mM and 4.1 mM respectively) which is considerably higher compared to **9b**. The IC_{50} value of **17b** and **19b** could not be measured but they were found to be at least higher then 10 mM.

Conclusions

To conclude, galactose-containing building blocks were synthesized in a straightforward synthesis from galactose. This building block was efficiently coupled to di-, tetra- and octavalent dendrimers via 'click' chemistry. The potencies are very high, considering that **10b** and **11b** showed roughly equal IC_{50} values to the GM1-os derivative in this assay. This result is an important step towards low cost potent ligands for cholera toxin with applications in therapy and detection. Short divalent galactose dendrimers **16b** - **19b** were prepared in a similar fashion but it was shown that they can not compete with the long armed divalent **9b**. We can there for conclude that, for strong multivalent chelation, the

effective length of the dendritic arms must at least be able to bridge the distance between two galactose binding sites. Also the composition of the spacer could explain the difference in affinity, the hydrophobic C11 spacer could possibly increase the affinity.



Scheme 4. *Synthesis divalent galactose dendrimers. Reagents and conditions: a)* ***15****, $CuSO_4$, sodium ascorbate, DMF/H_2O, 46% for* ***16b****, 58% for* ***17b****, 53% for* ***18b****, 42% for* ***19b****.*

Experimental Section

General Remarks

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Solvents were purchased from Biosolve (Valkenswaard, The Netherlands). TLC was performed on Merck precoated Silica 60 plates. Spots were visualized by UV light and by 10% H_2SO_4 in MeOH. Microwave reactions were carried out in a dedicated microwave oven, i.e. the Biotage Initiator. The microwave power was limited by temperature control once the desired temperature was reached. A sealed vessel of 2 – 5 mL was used. Analytical HPLC runs were performed on a Shimadzu automated HPLC system with a reversed phase column (Alltech, Adsorbosphere C8, 90 Å, 5µm, 250x4.6

mm) equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories) and a UV/VIS detector operating at 220 and 254 nm. Preparative HPLC runs were performed on a Applied Biosystems workstation. Elution was effected using a gradient of 5% MeCN and 0.1% TFA in H_2O to 5% H_2O and 0.1% TFA in MeCN. ^1H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) were performed on a Varian G-300 spectrometer. Acetone (215.94 ppm) was used as reference for measurements in D_2O and H_2O. Exact masses were measured by nanoelectrospray time-of-flight mass spectrometry on a Micromass LC TOF mass spectrometer. Gold-coated capillaries were loaded with 1 μL of sample (concentration 2 μM) dissolved in a 1:1 (v/v) mixture of CH_3CN-H_2O with 0.1% formic acid. NaI or poly(ethylene glycol) (PEG) was added as internal standard. The capillary voltage was set between 1100 and 1350 V, and the cone voltage was set at 30 V. Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI TOF) MS were recorded on a Shimadzu Axima-CFR with α-cyano-4-hydroxycinnamic acid or sinapic acid as a matrix. Insulin and adrenocorticotropin fragment 18-39 (Acth) were used for calibration.

H-(OCH_2CH_2)$_5$-OTrityl (2): To a solution of penta ethylene glycol (6.35 mL, 30 mmol) in dry pyridine (100 mL) was added trityl chloride (2.79 g, 10 mmol) and the mixture was stirred for 70 h. The reaction mixture was concentrated to dryness at 60 °C, taken up in EtOAc (250 mL) and washed with H_2O (100 mL), NaOH (1M, 100 mL) and brine (100 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. Silica gel chromatography (EtOAc / MeOH, 1/0 → 19/1) yielded **2** as clear oil (3.45 g, 72 % based on trityl chloride). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.48 – 7.44 and 7.31 – 7.18 (5 and 10H, 2 x m, CH_{arom}), 3.70 – 3.63 (16H, m, OCH_2), 3.58 – 3.54 (2H, m, CH_2OH) and 3.23 (2H, t, CH_2OTrt, J = 5.4 Hz). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.0, 128.6, 127.6 and 126.8 (C_{arom}), 86.4 (O*C*Ph_3), 72.4, 70.5 and 70.2 (OCH_2), 63.2 (CH_2OTrt) and 61.6 (CH_2OH). HRMS for $C_{29}H_{36}O_6$ (M, 480.2512): found $[M + Na]^+$ 503.3338, calcd. 503.2410.

Br-($C_{11}H_{22}$)-(OCH_2CH_2)$_5$-OTrityl (3): A solution of **2** (3.45 g, 7.2 mmol) in dry DMF (25 mL) at 0 ˚C was treated with NaH (60% in oil, 400 mg, 21.6 mmol) and stirred for 30 min. This solution added in 15 min to a solution of 1,11-dibromoundecane (5.10 mL, 21.6 mmol) in DMF (25 mL) at 0 ˚C. The final solution was slowly warmed to rt and stirred for

18 h. The reaction mixture was concentrated at 60 ˚C, taken up in EtOAc (200 mL) and washed with H_2O (100 mL) and brine (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. Silica gel chromatography (hex/EtOAc, 3/1 → 1/1) yielded **3** as a clear oil (3.12 g, 61% based on **2**). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.48 – 7.44 and 7.30 – 7.17 (5 and 10H, 2 x m, CH_{arom}), 3.68 - 3.60 (16H, m, OCH_2), 3.59 – 3.53 (2H, m, $CH_2OC_{11}H_{22}Br$), 3.43 (2H, t, $OCH_2CH_2CH_2$, *J* = 6.9 Hz), 3.36 (2H,t, CH_2Br, *J* = 6.9 Hz), 3.25 (2H, t, CH_2OTrt, *J* = 5.1 Hz), 1.87 – 1.78 (2H, m, CH_2CH_2Br), 1.59 – 1.54 (2H, m, $OCH_2CH_2CH_2$) and 1.27 (14H, s, C_7H_{14}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.0, 128.5, 127.6 and 126.7 (C_{arom}), 86.3 ($OCPh_3$), 71.3, 70.4 and 69.9 (OCH_2), 63.2 (CH_2OTrt), 33.8, 32.6, 29.5, 29.3, 28.5, 28.0 and 25.9 ($C_{11}H_{22}Br$). HRMS for $C_{40}H_{57}O_6Br$ (M, 712.3339): found $[M + Na]^+$ 735.4650, calcd. 735.3237.

Br-($C_{11}H_{22}$)-(OCH_2CH_2)$_5$-OH (4): A solution of **3** (2.79 g, 3.9 mmol) and *p*-toluene sulphonic acid in MeOH (40 mL) was stirred for 18 h. The reaction mixture was neutralised with aqueous $NaHCO_3$ and concentrated. **4** was obtained after silica gel chromatography (CH_2Cl_2/MeOH, 1/0 → 19/1) as clear oil (1.75 g, 95 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.71 - 3.55 (16H, m, OCH_2), 3.46 – 3.38 (4H, m, $OCH_2CH_2CH_2$, CH_2Br,), 3.21 (1H, bs, CH_2OH), 1.87 – 1.80 (2H, m, CH_2CH_2Br), 1.59 – 1.54 (2H, m, $OCH_2CH_2CH_2$) and 1.27 (14H, s, C_7H_{14}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 72.3, 71.1, 70.2, 69.9 and 69.7 (OCH_2), 61.2 (CH_2OH), 33.6, 32.4, 29.2, 28.4, 27.8 and 25.7 ($C_{11}H_{22}$). HRMS for $C_{21}H_{43}O_6Br$ (M, 470.2243): found $[M + Na]^+$ 493.3121, calcd. 493.2141.

Br-($C_{11}H_{22}$)-(OCH_2CH_2)$_5$ 2,3,4,6-tri-*O*-acetyl-β-D-galactopyranoside (6): A solution of **4** (136 mg, 0.67 mmol) and 2,3,4,6-tri-*O*-acetyl-α-D-galactosyl trichloro acetimidate **5** (492 mg, 1 mmol) in dry toluene (10 mL) was stirred at 0 ˚C under a N_2 flow for 15 min. $BF_3.Et_2O$ (170 µL, 1.34 mmol) was added and the reaction was stirred at 0 ˚C for 1 h. After neutralisation with Et_3N the mixture was concentrated and subjected to silica gel chromatography (hex/EtOAc, 1/3 → 0/1). Product **6** was obtained as clear oil (236 mg, 44%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.39 (1H, d, H-4, $J_{3,4}$ = 3.6 Hz), 5.20 (1H, dd, H-2, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.2 Hz), 5.02 (1H, dd, H-3, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.6 Hz), 4.58 (1H, d, H-1, $J_{1,2}$ = 8.1 Hz), 4.15 (2H, dd, H-6ab, *J* = 2.4 Hz, *J* = 6.6 Hz), 3.98 – 3.90 (2H, m, $CHHO_{Gal}$ and H-5), 3.78 – 3.72 (1H, m, $CHHO_{Gal}$), 3.65 -

3.56 (18H, m, OCH_2), 3.48 – 3.39 (4H, m, $OCH_2CH_2CH_2$, CH_2Br,), 2.15, 2.06, 2.05 and 1.98 (4 x 3H, 4 x s, $C(O)CH_3$), 1.88 – 1.80 (2H, m, CH_2CH_2Br), 1.59 – 1.54 (2H, m, $OCH_2CH_2CH_2$) and 1.28 (14H, s, C_7H_{14}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.3, 170.0 and 169.4 ($C(O)CH_3$), 101.2 (C-1), 71.0, 68.6, 67.1 and 66.8 (C-2, C-3, C-4, C-5), 71.4, 70.5, 69.9 and 69.0 (OCH_2), 61.2 (C-6), 34.0, 32.7, 29.4, 28.6, 28.1 and 26.0 ($C_{11}H_{22}$) and 20.7 ($C(O)CH_3$). HRMS for $C_{35}H_{61}O_{15}Br$ (M, 800.3194): found $[M + H]^+$ 801.4191, calcd. 801.3272.

N_3-($C_{11}H_{22}$)-(OCH_2CH_2)$_5$ 2,3,4,6-tri-*O*-acetyl-β-D-galactopyranoside (7): A solution of **6** (264 mg, 0.33 mmol) and NaN_3 (107 mg, 1.65 mmol) was stirred in dry DMF at 100 ˚C for 20 h. The solution was concentrated at 60 ˚C, taken up in CH_2Cl_2 and filtered. Silica gel chromatography (hex/EtOAc, 1/7 → 0/1) yielded azide **7** (211 mg, 84%). ^1H NMR (300 MHz, $CDCl_3$): δ = ^1H NMR (300 MHz, $CDCl_3$): δ = 5.39 (1H, d, H-4, $J_{3,4}$ = 3.3 Hz), 5.20 (1H, dd, H-2, $J_{1,2}$ = 8.1 Hz, $J_{2,3}$ = 10.5 Hz), .02 (1H, dd, H-3, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.6 Hz), 4.59 (1H, d, H-1, $J_{1,2}$ = 8.1 Hz), 4.16 (2H, dd, H-6ab, *J* = 2.4 Hz, *J* = 6.9 Hz), 3.98 – 3.90 (2H, m, $CHHO_{Gal}$ and H-5), 3.79 – 3.73 (1H, m, $CHHO_{Gal}$), 3.66 - 3.57 (18H, m, OCH_2), 3.46 (2H, t, $OCH_2CH_2CH_2$, *J* = 6.9 Hz), 3.27 (2H, t, CH_2N_3, *J* = 6.9 Hz), 2.15, 2.06, 2.05 and 1.98 (4 x 3H, 4 x s, $C(O)CH_3$), 1.62 – 1.55 (2H, m, $OCH_2CH_2CH_2$) and 1.28 (14H, s, C_8H_{16}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.1, 170.0, 169.9 and 169.3 ($C(O)CH_3$), 101.1 (C-1), 70.9, 68.5, 67.0 and 66.7 (C-2, C-3, C-4, C-5), 71.3, 70.4, 69.9 and 68.9 (OCH_2), 61.1 (C-6), 51.3 (CH_2N_3), 29.2, 28.9, 28.7, 26.5 and 25.9 ($C_{11}H_{22}$) and 20.5 ($C(O)CH_3$). HRMS for $C_{35}H_{61}O_{15}N_3$ (M, 763.4103): found $[M + H]^+$ 786.5811, calcd. 764.8779.

N_3-($C_{11}H_{22}$)-(OCH_2CH_2)$_5$ β-D-galactopyranoside (8): A solution of **7** (200 mg, 0.26 mmol) in MeOH was treated with NaOMe (30% wt in MeOH, 50 µL). After 2 h the solution was neutralised with Dowex-H^+, filtered, concentrated and silica gel chromatography (CH_2Cl_2/MeOH, 9/1 → 4/1) was used as final purification to yield **8** was clear oil (119 mg, 72%). ^1H NMR (300 MHz, $CDCl_3$): δ = ^1H NMR (300 MHz, $CDCl_3$): δ = 4.52 (bs, 1H), 4.22 (1H, bd, H-1, $J_{1,2}$ = 7.2 Hz), 3.95 (1H, bs), 3.73 - 3.63 (2H, m), 3.58 - 3.46 (18H, m, OCH_2), 3.37 (2H, t, $OCH_2CH_2CH_2$, *J* = 6.9 Hz), 3.19 (2H, t, CH_2N_3, *J* = 7.2 Hz), 1.55 – 1.29 (2H, m, $OCH_2CH_2CH_2$) and 1.21 (14H, s, C_8H_{16}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 103.4 (C-1), 74.5, 73.4, 71.0 and 68.6 (C-2, C-3, C-4, C-5), 71.4, 70.3 and 68.4

(OCH_2), 61.1 (C-6), 51.3 (CH_2N_3), 29.3, 29.0, 28.7, 26.6 and 25.9 ($C_{11}H_{22}$). HRMS for $C_{27}H_{53}N_3O_{11}$ (M, 595.3680): $[M + Na]^+$ found 618.3063, calcd. 618.3578.

Divalent galactose dendrimer (9b): A solution of **9a** (30 mg, 29 μmol), **1** (51 mg, 86 μmol), $CuSO_4.5H_2O$ (7.1 mg, 29 μmol) and sodium ascorbate (5.6 mg, 29 μmol) was heated in DMF/H_2O (1/1, v/v, 5 mL) under microwave irradiation at 80 ˚C for 20 min. The reaction mixture was concentrated at 60 ˚C and subjected to HPLC purification. Divalent dendrimer **9b** was obtained as oil (44.5 mg, 70%). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 500 MHz): δ = 8.51 (2H, bt, C(O)NH), 8.30 (2H, bt, C(O)NH), 7.97 (2H, bt, C(O)NH), 7.79 (2H, s, $CH_{triazole}$), 7.11 (2H, s, CH_{arom}-2,6), 6.77 (1H, s, CH_{arom}-4), 4.42 (2H, d, H-1, $J_{1,2}$ = 6.0 Hz), 4.33 (4H, t, $OCH_2C_{10}H_{20}$), 4.12 and 4.07 (2 x 4H, 2 x s, $OCH_2C(O)$), 3.94 - 3.50 (50H, m), 3.45 (4H, t, $CH_2NHC(O)$), 3.40 (4H, t, $CH_2NHC(O)$), 3.30 (4H, bd, $CH_2N_{triazole}$), 3.18 (4H, t, $CH_2NHC(O)$), 2.99 and 2.58 (2 x 4H, 2 x t, $CH_2CH_2C_{triazole}$), 1.77 (8H, m, $OCH_2CH_2CH_2NH$), 1.66 (4H, t, $OCH_2CH_2C_9H_{18}$), 1.51 (4H, t, $C_9H_{18}CH_2CH_2N_{triazole}$) and 1.25 - 1.10 (28H, m, C_7H_{14}). ^{13}C NMR (D_2O, 75.5 MHz): δ = 174.9, 172.3 and 171.8 (C(O)NH), 168.4 ($C(O)OCH_3$), 160.4 (C_{arom}-3,5), 146.9 ($C_{triazole}$-4), 132.2 (C_{arom}-1), 123.7 ($C_{triazole}$-5), 109.2 (CH_{arom}-2,6), 107.3 (CH_{arom}-4), 103.8 (C-1), 76.0 73.6 71.6 (C-2, C-3, C-4, C-5), 71.9, 70.5, 69.4 and 69.2 (OCH_2), 67.5 (OCH_2CH_2NH), 61.8 (C-6), 51.0 ($C(O)OCH_3$), 39.2 and 37.1 (OCH_2CH_2NH and $CH_2NHC(O)$) 36.0 ($CH_2CH_2C_{triazole}$), 29.7, 29.3, 28.5, 26.7 and 26.3 ($CH_2CH_2CH_2$) and 22.0 ($CH_2CH_2C_{triazole}$). MALDI TOF for $C_{104}H_{184}N_{12}O_{40}$ (M, 2241.2733): found $[M + Na]^+$ 2265.16, calcd 2265.6199.

Tetravalent galactose dendrimer (10b): A solution of **10a** (20 mg, 9 μmol), **1** (31 mg, 52 μmol), $CuSO_4.5H_2O$ (4.4 mg, 17 μmol) and sodium ascorbate (3.5 mg, 17 μmol) was heated in DMF/H_2O (1/1, v/v, 3 mL) under microwave irradiation at 80 ˚C for 20 min. The reaction mixture was concentrated at 60 ˚C and subjected to HPLC purification. Tetravalent dendrimer **10b** was obtained as oil (31.7 mg, 78%). ^{1}H NMR (H_2O/D_2O, 9/1, v/v, 500 MHz): δ = 8.68 (2H, t, C(O)NH), 8.50 (4H, t, C(O)NH), 8.30 (4H, t, C(O)NH), 7.97 (4H, t, C(O)NH), 7.76 (4H, s, $CH_{triazole}$), 7.03 (2H, s, CH_{arom}-2,6), 7.00 (4H, s, CH_{arom}-2',6'), 6.67 (3H, bs, CH_{arom}-4, 4'), 4.42 (4H, d, H-1, $J_{1,2}$ = 6.0 Hz), 4.28 (8H,bs t, $OCH_2C_{10}H_{20}$), 4.10 and 4.06 (2 x 4H, 2 x s, $OCH_2C(O)$), 3.94 - 3.73 (16H, m), 3.72 - 3.61 (44H, m), 3.60 - 3.52 (32H, m), 3.50 (8H, bs, $CH_2N_{triazole}$), 3.46 - 3.36 (12H, m $CH_2NHC(O)$), 3.28 (8H, bs, $CH_2NHC(O)$), 3.18 (8H, bd, $CH_2NHC(O)$), 2.97 and 2.57 (2 x 8H, bs and bt,

$CH_2CH_2C_{triazole}$), 1.78 - 1.70 (16H, m, $OCH_2CH_2CH_2NH$), 1.66 (8H, t, $OCH_2CH_2C_9H_{18}$), 1.48 (8H, t, $C_9H_{18}CH_2CH_2N_{triazole}$) and 1.20 - 1.05 (56H, m, C_7H_{14}). ^{13}C NMR (D_2O, 75.5 MHz): δ = 174.7, 172.1 and 171.6 (C(O)NH), 169.0 ($C(O)OCH_3$), 160.5 (C_{arom}-3',5''), 146.9 ($C_{triazole}$-4), 118.2 (CH_{arom}) 103.8 (C-1), 76.0, 73.6, 71.6 and 69.5 (C-2, C-3, C-4 and C-5), 71.9, 70.5 and 69.4 (OCH_2), 67.4 (OCH_2CH_2NH), 61.8 (C-6), 51.1 ($C(O)OCH_3$), 39.2 and 37.2 (OCH_2CH_2NH and $CH_2NHC(O)$) 36.0 ($CH_2CH_2C_{triazole}$), 29.9, 29.4, 28.5, 26.8 and 26.4 ($CH_2CH_2CH_2$) and 22.0 ($CH_2CH_2C_{triazole}$). MALDI TOF for $C_{218}H_{378}N_{26}O_{82}$ (M, 4672.6208): found $[M + Na]^+$ 4698.12, calcd 4698.45.

Octavalent galactose dendrimer (11b): A solution of **11a** (20 mg, 4 μmol), **1** (30 mg, 50 μmol), $CuSO_4.5H_2O$ (4.2 mg, 17 μmol) and sodium ascorbate (3.3 mg, 17 μmol) was heated in DMF/H_2O (1/1, v/v, 3 mL) under microwave irradiation at 80 ˚C for 20 min. The reaction mixture was concentrated at 60 ˚C and subjected to HPLC purification. Octavalent dendrimer **11b** was obtained as oil (31 mg, 77%). ^1H NMR (H_2O/D_2O, 9/1, v/v, 500 MHz): δ = 8.66 (4H, bs, C(O)NH), 8.49 (8H, bs, C(O)NH), 8.30 (8H, bs, C(O)NH), 8.06 (2H, bs, C(O)NH), 7.97 (8H, bs, C(O)NH), 7.75 (8H, s, $CH_{triazole}$), 6.96 (14H, bs, CH_{arom}-2,6, 2',6', 2'',6''), 6.64 (7H, bs, CH_{arom}-4, 4', 4''), 4.42 (8H, d, H-1, $J_{1,2}$ = 6.0 Hz), 4.27 (16H,bs, $OCH_2C_{10}H_{20}$), 4.12 and 4.02 (16H, m, $OCH_2C(O)$), 3.93 - 3.72 (16H, m), 3.72 - 3.61 (82H, m), 3.61 - 3.50 (60H, m), 3.48 (16H, bs, $CH_2N_{triazole}$), 3.46 - 3.36 (28H, m $CH_2NHC(O)$), 3.26 (16H, bs, $CH_2NHC(O)$), 3.16 (16H, bs, $CH_2NHC(O)$), 2.96 and 2.56 (2 x 16H, 2 x bs, $CH_2CH_2C_{triazole}$), 1.74 (32H, bs, $OCH_2CH_2CH_2NH$), 1.64 (16H, bt, $OCH_2CH_2C_9H_{18}$), 1.46 (16H, bs, $C_9H_{18}CH_2CH_2N_{triazole}$) and 1.20 - 1.05 (112H, m, C_7H_{14}). ^{13}C NMR (D_2O, 75.5 MHz): δ = 174.4, 171.9 and 171.4 (C(O)NH), 168.4 ($C(O)OCH_3$), 160.3 (C_{arom}-3'',5''), 103.6 (C-1), 75.8 73.4 and 71.4 (C-2, C-3, C-4, C-5), 71.7, 70.3, 69.2 and 69.1 (OCH_2), 61.6 (C-6), 50.8 ($CH_2N_{triazole}$), 37.0 ($CH_2NHC(O)$) 29.7, 29.2, 26.8 and 26.3 ($CH_2CH_2CH_2$) and 21.9 ($CH_2CH_2C_{triazole}$). MALDI TOF for $C_{446}H_{766}N_{54}O_{166}$ (M, 9535.3158): found $[M + Na]^+$ 9561.58, calcd. 9562.11.

HO-$(CH_2CH_2O)_4$-β-D-galactopyranoside (11): A solution of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranoside (3.90 g, 10.0 mmol) and tetraethylene glycol (6.91 mL, 40 mmol) in dry CH_2Cl_2 (50 mL) was cooled to 0 °C and $BF_3.Et_2O$ (6.34 mL, 50 mmol) was added in 2 min. The mixture was stirred overnight, neutralized with Et_3N, diluted with CH_2Cl_2 (200 mL), washed with aqueous $NaHCO_3$ (5%, 125 mL), H_2O (125 mL) and brine (125 mL).

The organic layer was dried over Na_2SO_4, filtered, and concentrated. Silica gel chromatography (CH_2Cl_2/MeOH, 19/1) of the residue gave the desired compound as a slightly yellow syrup (3.24 g, 62%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.39 (1H, dd, H-4, $J_{3,4}$ = 3.6 Hz, $J_{4,5}$ = 1.2 Hz), 5.20 (1H, dd, H-2, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.5 Hz), 5.03 (1H, dd, H-3, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.3 Hz), 4.59 (1H, d, H-1, $J_{1,2}$ = 7.8 Hz), 4.16 (2H, dd, H-6ab, *J* = 3.6 Hz, *J* = 6.9 Hz), 3.98 - 3.91 (2H, m, CH*H*O$_{Gal}$ and H-5), 3.79 - 3.60 (15H, m, CH*H*O$_{Gal}$, OCH_2), 2.80 (1H, bs, OH), 2.15, 2.07, 2.05 and 1.98 (4 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.2, 170.1, 170.0 and 169.3 (*C*(O)CH_3), 101.1 (C-1), 70.7, 68.7 and 66.9 (C-2, C-3, C-4, C-5), 72.3, 70.5, 70.4, 70.1, 68.8 and 63.4 (OCH_2), 61.5 (CH_2OH), 61.1 (C-6), 20.6 and 20.5 (C(O)*C*H_3). HRMS for $C_{22}H_{36}O_{14}$ (M, 524.2105): found $[M + Na]^+$ 547.1472, calcd. 547.2003.

Monovalent galactose reference (12): Galactoside **11** (1.05 g, 2.0 mmol) was stirred in MeOH (10 mL) and NaOMe (50 µL, 30% wt in MeOH) was added. After 1 h the reaction mixture was neutralized with Dowex-H^+, filtered and concentrated. Small impurities were removed by silica gel chromatography (EtOAc/MeOH/H2O, 5/2/1) to give **12** (531 mg, 75%). ^{1}H NMR (300 MHz, CD_3OD): δ = 4.26 (1H, d, H-1, $J_{1,2}$ = 6.9 Hz), 4.05 – 3.98 (1H, m), 3.83 (1H, s, H-4), 3.79 - 3.60 (15H, m, OCH_2) and 3.58 - 3.50 (5H, m). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 105.1 (C-1), 76.7, 74.9, 72.6 and 70.3 (C-2, C-3, C-4, C-5), 73.7, 71.5 and 69.7 (OCH_2), 62.6 and 62.3 (CH_2OH, C-6). HRMS for $C_{14}H_{28}O_{10}$ (M, 356.1682): found $[M + Na]^+$ 379.1194, calcd. 379.1580.

TsO-(CH_2CH_2O)$_4$ 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (13): To a solution of **11** (2.20 g, 4.2 mmol) and *p*-toluenesulfonyl chloride (1.40 g, 7.4 mmol) in dry CH_2Cl_2 (50 mL) was added Et_3N (1.18 mL, 8.4 mmol). The reaction was stirred for 30h and was concentrated in vacuo. The crude product was taken up in EtOAc (100 mL) and washed with NaOH (1M, 50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. Product **13** was isolated after silica gel chromatography (hex/EtOAc, 1/3 → 0/1) as a clear oil (2.10 g, 74%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.80 and 7.35 (2 x 2H, 2 x d, CH_{arom}, *J* = 8.3 Hz, *J* = 8.0 Hz), 5.39 (1H, dd, H-4, $J_{3,4}$ = 3.6 Hz), 5.20 (1H, dd, H-2, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.5 Hz), 5.03 (1H, dd, H-3, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.3 Hz), 4.57 (1H, d, H-1, $J_{1,2}$ = 7.8 Hz), 4.18 - 4.13 (4H, m, 2 x H-6, CH_2OTs), 3.98 - 3.91

(2H, m, CH*H*O_{Gal} and H-5), 3.79 - 3.59 (13H, m, CH*H*O_{Gal}, OCH_2), 2.15, 2.05, 2.05 and 1.99 (4 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.1 and 169.4 (*C*(O)CH_3), 144.7 (C_{arom}-4), 129.7 (C_{arom}-3,5), 127.9 (C_{arom}-2,6), 101.2 (C-1), 70.8, 68.7 and 67.0 (C-2, C-3, C-4, C-5), 70.6, 70.4, 70.2, 69.1, 69.0 and 68.6 (OCH_2), 61.2 (C-6), 21.5 and 20.6 (C(O)*C*H_3). HRMS for $C_{29}H_{42}O_{16}S$ (M, 678.2194): found $[M + Na]^+$ 701.2092, calcd. 701.0314.

N_3-(CH_2CH_2O)$_4$ 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (14): A solution of tosylate **13** (2.10 g, 3.09 mmol) and NaN_3 (975 mg, 15.5 mmol) was heated in DMF (5 mL) at 80˚C for 20 h. The DMF was evaporated at 60˚C in vacuo and the residue was taken up in EtOAc (100 mL) and washed with NaOH (1M, 50 mL) and brine 50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. Silica gel chromatography (hex/EtOAc, 2/1 → 0/1) yielded azide **14** as a clear oil (1.59 g, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.39 (1H, dd, H-4, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ = 0.9 Hz), 5.20 (1H, dd, H-2, $J_{1,2}$ = 8.1 Hz, $J_{2,3}$ = 10.5 Hz), 5.03 (1H, dd, H-3, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.6 Hz), 4.59 (1H, d, H-1, $J_{1,2}$ = 8.1 Hz), 4.19 - 4.10 (2H, m, 2 x H-6), 3.99 - 3.89 (2H, m, CH*H*O_{Gal} and H-5), 3.79 - 3.62 (13H, m, CH*H*O_{Gal}, OCH_2), 3.39 (2H, t, CH_2N_3, *J* = 5.4 Hz), 2.15, 2.06, 2.05 and 1.99 (4 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.3, 170.1, 170.0 and 169.3 (*C*(O)CH_3), 101.2 (C-1), 70.8, 68.7 and 67.0 (C-2, C-3, C-4, C-5), 70.6, 70.2, 69.9 and 68.9 (OCH_2), 61.1 (C-6), 50.6 (CH_2N_3) and 20.5 (C(O)*C*H_3). HRMS for $C_{22}H_{35}N_3O_{13}$ (M, 549.2170): found $[M + Na]^+$ 571.9808, calcd. 572.2068.

N_3-(CH_2CH_2O)$_4$ β-D-galactopyranoside (15): A solution of **14** (1.0 g, 1.82 mmol) in MeOH (10 mL) was treated with NaOMe (50 μL, 30% wt in MeOH). The solution was stirred for 1 h, neutralized by Dowex-H^+, filtered and concentrated to dryness to give **15** as clear oil (696 mg, quant.). ^{1}H NMR (300 MHz, CD_3OD): δ = 4.19 (1H, d, H-1, $J_{1,2}$ = 7.2 Hz), 3.98 - 3.90 (m, 1H), 3.75 (1H, d, H-4, $J_{3,4}$ = 3.0 Hz), 3.70 - 3.55 (16H, m, OCH_2), 3.50 - 3.38 (4H, m) and 3.31 (2H, t, CH_2N_3, *J* = 5.2 Hz). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 105.2 (C-1), 76.8, 75.0, 72.7 and 70.4 (C-2, C-3, C-4, C-5), 71.7, 71.3 and 69.8 (OCH_2), 62.7 (C-6) and 51.9 (CH_2N_3). HRMS for $C_{14}H_{27}O_9$ (M, 381.3789): found $[M + Na]^+$ 404.0090, calcd. 404.1645.

Divalent galactose dendrimer (16b): A solution of **16a** (25 mg, 100 μmol), **15** (115 mg, 300 μmol), $CuSO_4.5H_2O$ (25 mg, 100 μmol) and sodium ascorbate (20 mg, 100 μmol) was heated in DMF/H_2O (1/1, v/v, 5 mL) under microwave irradiation at 80 ˚C for 20 min. The reaction mixture was concentrated at 60 ˚C and subjected to HPLC purification. Divalent dendrimer was isolated as white hygroscopic material (46 mg, 46%). ^{1}H NMR (300 MHz, D_2O): δ = 8.15 (2H, s, $CH_{triazole}$), 7.14 (2H, s, CH_{arom}-2,6), 6.80 (1H, s, CH_{arom}-4), 4.61 (4H, bt, $OCH_2C_{triazole}$, J = 3.9 Hz), 4.35 (2H, d, H-1, $J_{1,2}$ = 7.8 Hz), 4.02 - 3.90 (6H, m), 3.86 (3H, s, $OC(O)CH_3$) and 3.78 - 3.49 (38H, m). ^{13}C NMR (D_2O, 75.5 MHz): δ = 168.7 (C(O)OCH_3), 159.5 (C_{arom}-3,5), 143.7 ($C_{triazole}$-4), 132.4 (C_{arom}-1), 126.4 ($C_{triazole}$-5), 109.8 (CH_{arom}-2,6), 108.3 (CH_{arom}-4), 103.7 (C-1), 75.9, 73.5, 71.5 and 69.4 (C-2, C-3, C-4, C-5), 70.5, 70.3, 69.5 and 69.3 (OCH_2), 62.1 ($OCH_2C_{triazole}$), 61.7 (C-6), 53.6 ($C(O)OCH_3$) and 50.9 ($CH_2N_{triazole}$). HRMS for $C_{42}H_{66}N_6O_{22}$ (M, 1006.4230): found $[M+Na]^+$ = 1029.1206, calcd. 1029.4028.

Divalent galactose dendrimer (17b): A solution of **17a** (31 mg, 75 μmol), **15** (86 mg, 75 μmol), $CuSO_4.5H_2O$ (19 mg, 75 μmol) and sodium ascorbate (15 mg, 75 μmol) was heated in DMF/H_2O (1/1, v/v, 5 mL) under microwave irradiation at 80 ˚C for 20 min. The reaction mixture was concentrated at 60 ˚C and subjected to HPLC purification. Divalent dendrimer was isolated as white hygroscopic material (51 mg, 58%). ^{1}H NMR (D_2O, 300 MHz): δ = 7.80 (2H, s, $CH_{triazole}$), 7.11 (2H, d, CH_{arom}-2,6, J = 1.6 Hz), 6.67 (1H, s, CH_{arom}-4), 4.40 - 4.34 (6H, m, H-1, OCH_2CH_2NH), 4.06 - 3.53 (51H,m), 3.01 and 2.63 (2 x 4H, 2 x t, $CH_2CH_2C_{triazole}$, J = 6.3 Hz, J = 6.6 Hz). ^{13}C NMR (D_2O, 75.5 MHz): δ = 175.4 (C(O)NH), 168.9 (C(O)OCH_3), 160.0 (C_{arom}-3,5), 132.2 (C_{arom}-1), 124.5 ($C_{triazole}$-5), 108.9 (CH_{arom}-2,6), 107.5 (CH_{arom}-4), 103.5 (C-1), 75.7, 73.3 71.4 and 69.2 (C-2, C-3, C-4, C-5), 70.2, 70.1, 70.0 and 69.1 (OCH_2), 67.5 (OCH_2CH_2NH), 61.5 (C-6), 53.4 ($C(O)OCH_3$), 50.6 ($CH_2N_{triazole}$), 39.2 (OCH_2CH_2NH) 35.4 ($CH_2CH_2C_{triazole}$) and 21.4 ($CH_2CH_2C_{triazole}$). HRMS for $C_{50}H_{80}N_8O_{24}$ (M, 1176.5285): found $[M+Na]^+$ = 1199.149, calcd. 1199.5183.

Divalent galactose dendrimer (18b): A solution of **18a** (39 mg, 50 μmol), **15** (57 mg, 150 μmol), $CuSO_4.5H_2O$ (13 mg, 50 μmol) and sodium ascorbate (10 mg, 50 μmol) was heated in DMF/H_2O (1/1, v/v, 5 mL) under microwave irradiation at 80 ˚C for 20 min. The reaction mixture was concentrated at 60 ˚C and subjected to HPLC purification. Divalent

dendrimer was isolated as white hygroscopic material (41 mg, 53%). ^{1}H NMR (D_2O, 300 MHz): δ = 7.88 (2H, s, $CH_{triazole}$), 7.06 (2H, d, CH_{arom}-2,6, *J* = 1.9 Hz), 6.70 (1H, s, CH_{arom}-4), 4.58 (4H, t, OCH_2CH_2NH, *J* = 4.7 Hz), 4.38 (2H, d, H-1, $J_{1,2}$ = 7.7 Hz), 4.10 and 4.04 (2 x 4H, 2 x s, $OCH_2C(O)$), 4.14 - 3.49 (51H, m), 3.14 and 3.07 (2 x 4H, 2 x bt, $CH_2NHC(O)$), 2.98 and 2.56 (2 x 4H, 2 x t, $CH_2CH_2C_{triazole}$, *J* = 7.5 Hz, *J* = 7.2 Hz) and 1.35 (8H, bs, $NHCH_2C_2H_4$). ^{13}C NMR (D_2O, 75.5 MHz): δ = 174.9, 172.5 and 171.8 (C(O)NH), 168.7 ($C(O)OCH_3$), 160.0 (C_{arom}-3,5), 146.3 ($C_{triazole}$-4), 132.1 (C_{arom}-1), 124.9 ($C_{triazole}$-5), 109.0 (CH_{arom}-2,6), 107.5 (CH_{arom}-4), 103.5 (C-1), 75.8, 73.3 71.4 and 69.3 (C-2, C-3, C-4, C-5), 70.5, 70.2, 70.1 and 69.2 ($C(O)CH_2O$, OCH_2), 67.3 (OCH_2CH_2NH), 61.6 (C-6), 53.4 ($C(O)OCH_3$), 51.0 ($CH_2N_{triazole}$), 39.8 and 39.1 ($CH_2NHC(O)$) 35.5 ($CH_2CH_2C_{triazole}$), 26.3 ($NHCH_2C_2H_4$) and 21.4 ($CH_2CH_2C_{triazole}$). HRMS for $C_{66}H_{108}N_{12}O_{30}$ (M, 1548.7294): found $[M+Na]^+$ = 1571.2144, calcd. 1571.7192.

Divalent galactose dendrimer (19b): A solution of **19a** (26 mg, 25 μmol), **15** (29 mg, 75 μmol), $CuSO_4.5H_2O$ (6 mg, 25 μmol) and sodium ascorbate (5 mg, 25 μmol) was heated in DMF/H_2O (1/1, v/v, 5 mL) under microwave irradiation at 80 ˚C for 20 min. The reaction mixture was concentrated at 60 ˚C and subjected to HPLC purification. Divalent dendrimer was isolated as white hygroscopic material (19 mg, 42%). ^{1}H NMR (D_2O, 300 MHz): δ = 7.92 (2H, s, $CH_{triazole}$), 7.16 (2H, d, CH_{arom}-2,6, *J* = 2.2 Hz), 6.78 (1H, s, CH_{arom}-4), 4.61 (4H, t, OCH_2CH_2NH, *J* = 4.2 Hz), 4.40 (2H, d, H-1, $J_{1,2}$ = 7.8 Hz), 4.18 (4H, t), 4.10 and 4.04 (2 x 4H, 2 x s, $OCH_2C(O)$), 3.96 - 3.90 (9H, m), 3.82 - 3.52 (54H, m), 3.43 (4H, t, $CH_2N_{triazole}$, *J* = 6.3 Hz), 3.27 and 3.18 (2 x 4H, 2 x t, $CH_2NHC(O)$, *J* = 6.6 Hz, *J* = 6.6 Hz), 3.01and 2.59 (2 x 4H, 2 x t, $CH_2CH_2C_{triazole}$, *J* = 7.2 Hz, *J* = 7.2 Hz) and 1.77 – 1.65 (8H, m, $OCH_2CH_2CH_2NH$). ^{13}C NMR (D_2O, 75.5 MHz): δ = 174.9, 172.5 and 171.9 (C(O)NH), 168.8 ($C(O)OCH_3$), 160.0 (C_{arom}-3,5), 146.2 ($C_{triazole}$-4), 132.2 (C_{arom}-1), 123.2 ($C_{triazole}$-5), 109.1 (CH_{arom}-2,6), 107.7 (CH_{arom}-4), 103.5 (C-1), 75.8 73.3 and 71.3 (C-2, C-3, C-4, C-5), 70.5, 70.2, 69.9, 69.2, 69.1 and 68.9 (OCH_2), 67.3 (OCH_2CH_2NH), 61.6 (C-6), 53.4 ($C(O)OCH_3$), 51.0 ($CH_2N_{triazole}$), 39.1 and 36.9 ($CH_2NHC(O)$) 35.4 ($CH_2CH_2C_{triazole}$), 28.8 ($OCH_2CH_2CH_2NH$) and 21.3 ($CH_2CH_2C_{triazole}$). HRMS for $C_{78}H_{132}N_{12}O_{36}$ (M, 1812.8867): found $[M+Na]^+$ = 1835.4349, calcd. 1835.8765.

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Chapter 6

Multivalent carbohydrate recognition on a glycodendrimer-functionalized flow-through chip

The results in this chapter have been published:

H. M. Branderhorst, R. Ruijtenbeek, R. M. J. Liskamp, R. J. Pieters; Multivalent Carbohydrate Recognition on a Glycodendrimer-functionalized Flow-through Chip; *ChemBioChem* **2008***, 9*, 1836-1844

Introduction

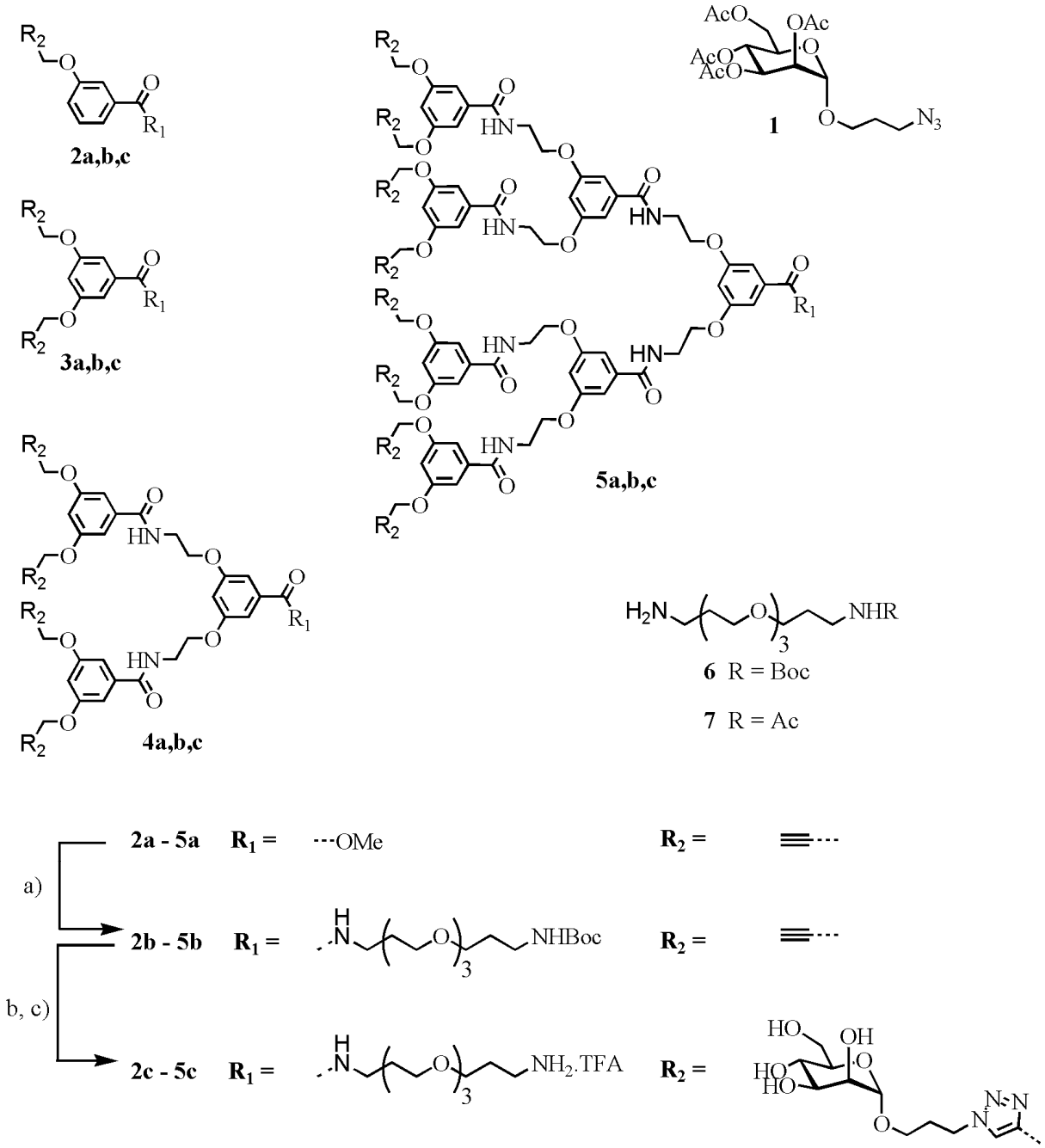
Carbohydrate recognition is increasingly believed to be a crucial event in many biological process including the development of diseases such as (avian) flu,[1] AIDS,[2] and cancer.[3] Increasing the knowledge of the carbohydrate-mediated communication between cells and preventing pathological consequences, are important goals in science.[4] For DNA[5] and peptide/protein[6] recognition studies, biochips have been developed which have greatly increased the efficiency due to miniaturization and the high through-put characteristics of such microarrays. To apply such biochips for studies in carbohydrate recognition is a logical progression and increasingly papers are appearing that develop and/or apply carbohydrate microarrays.[7] Reports have described the selective detection of carbohydrate binding proteins, mostly lectins, on chips of various designs.[8] In general carbohydrates bind only weakly to their complementary proteins. To achieve biologically relevant binding, multivalency[9] is often involved in natural carbohydrate recognition processes. In order to effectively interfere with multivalent protein-carbohydrate interactions it was found that multivalent inhibitors can be much more potent than their monovalent counterparts. A multivalent display of synthetic carbohydrate ligands has proven to be an effective inhibitor design for blocking e.g. lectins,[10] AB_5 toxins[11] and bacteria.[12] In these cases a chelation type of mechanism is the likely cause of the enhancements, which can be very large and exceed a factor 10^4-10^5. Examples in which chelation cannot play a role for geometric reasons typically show more moderate enhancements below a factor of 100, except when large extended arrays are used.[13] Despite successes many features of multivalent carbohydrate binding require further study, such as the degree to which multivalency effects vary with the architecture of the protein (complex) and that of the multivalent ligand. We here take a step in that direction by adding the efficiency of microarrays to the study of multivalent interactions. The present system enables the systematic screening of carbohydrate binding proteins and can rapidly determine if they favor a multivalent carbohydrate display and also to what extent. Furthermore, selected binding parameters including kinetic rate constants can be deduced from the obtained data due to the ability to monitor the binding in real time.

In order to do so, a flow-through microarray technology was used with multivalent ligands spotted onto aluminium oxide[14] porous chips.[15] The use of porous chips has several

advantages. It allows the analyte solution to be pumped up and down to avoid diffusion limitations. Furthermore the binding process of the fluorescent protein to the chip can be monitored in real time. This in contrast to the use of conventional chips where only an end-point determination is possible after all non-bound fluorescent material has been washed away. The real time monitoring is possible because the liquid containing the fluorescent components is pumped up and down through the micro channels, unique for this chip material, which is controlled by air pressure control below the chip. Periodic pictures are taken by a CCD camera when the fluid with the non-bound fluorescent components is temporarily positioned below the chip where it will not be pictured by the camera. In this way only fluorescence resulting from binding is detected. Another advantage of the three-dimensional porous material is that its internal surface to which ligands can be attached is ca. 500-fold larger than for two-dimensional spots on a glass surface. This high loading capacity has advantages for detecting weak binding processes. Additionally it is advantageous for our purpose since it allows us to place the ligands far apart to avoid undesirable multivalency effects due to proteins bridging between ligands, which would mask the intended multivalency effects in the binding to glycodendrimers. In our studies we coupled mono- to octavalent mannose ligands to the chip surface and we observed binding of the fluorescently labeled lectins Concanavalin A (ConA) from the Jack bean seeds and the Galanthus Nivalis Agglutinin (GNA) from the snowdrop bulb. Distinct multivalent binding of the higher generation mannose dendrimers towards the GNA lectin was observed.

Results

In order to prepare the glycodendrimer chips, first dendrimers were prepared based on the 3,5-di-(2-aminoethoxy)-benzoic acid repeating unit.[16] In order to attach the dendrimers to the chip surface, its core carboxylate was utilized for the attachment of a spacer. The spacer terminated in an amine for conjugation at pH 9 to the chip-displayed maleimide function. The carbohydrates were attached to the dendrimer arm by ‘click’ chemistry[17] prior to attachment of the whole construct to the chip.



Scheme 1. *Synthesis of dendrimers; reagents and conditions: a) i. NaOH, dioxane, MeOH, H_2O; ii.* ***6****, BOP, DiPEA, CH_2Cl_2/DMF, 40–95%; b)* ***1****, $CuSO_4$, sodium ascorbate, DMF, 65–80 %; c) i: NaOMe, MeOH, ii. TFA, H_2O, quant.*

Synthesis of mannose dendrimers and attachment to the chip: Mannose azide building block **1**[18] and alkyne-functionalized dendrimers **2a-5a**[19] were prepared as previously reported (Scheme 1). Dendrimers **2a-5a** were treated with Tesser's base,[20] followed by coupling to amine **6** with BOP as the coupling reagent. Decoration of the dendrimers with mannose moieties was achieved using "click" chemistry under microwave irradiation, with

$CuSO_4$ / sodium ascorbate as the Cu(I) source.[21] To this end dendrimers **2b-5b** were treated with **1**. The obtained glycodendrimers were deacetylated by NaOMe after which the Boc group was removed with TFA. The pure **2c**-**5c** were obtained after preparative HPLC purification and were satisfactorily characterized by NMR and MS analysis.

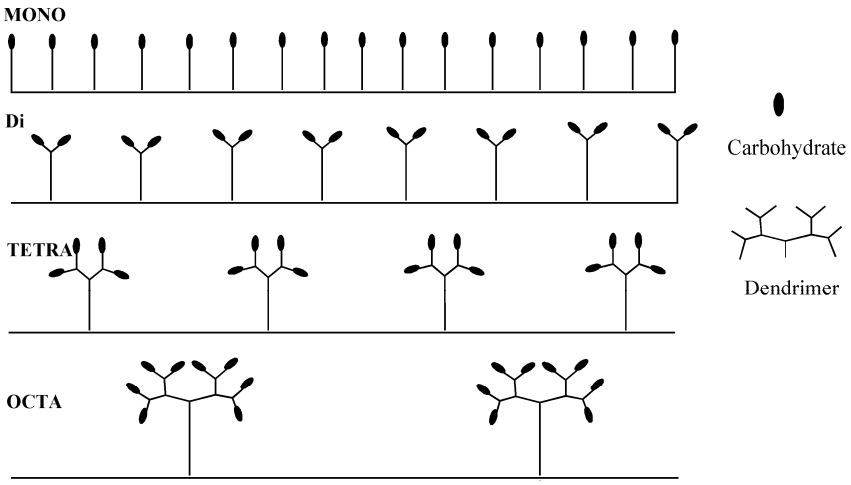


Figure 1. *Microarray surfaces with identical mannose contents, but different valencies.*

Initial experiments on the chip were performed with dendrimers **2c**-**5c** that reacted with the maleimide-functionalized chip surface at pH 9. The composition of the spotting solutions was chosen to have an equal mannose concentration in all solutions, despite the valency differences, i.e. the dendrimer concentrations were corrected for their valency. We compared e.g. spots made from a 100 μM spotting solution of monovalent **2c** to those made from a 12.5 μM spotting solution of octavalent **5c**, to yield chip surfaces as schematically shown in Figure 1. In a separate preparation we also corrected for the difference in amine concentrations of the spotting solutions by adding the non-sugar amine **7**, however this did not make a significant difference in the binding studies (Figure 2) and was therefore not applied for the studies described below.

Binding experiments: The dendrimer solutions with varying concentrations and valencies were printed on to the microarray slides, using piezoelectric spotting of 330 pL per spot. A concentration range was applied from 0.1 to 5 mM mannose, corrected for the valency of the dendrimers, as mentioned. Each array slide contained spots in quadruplicate.

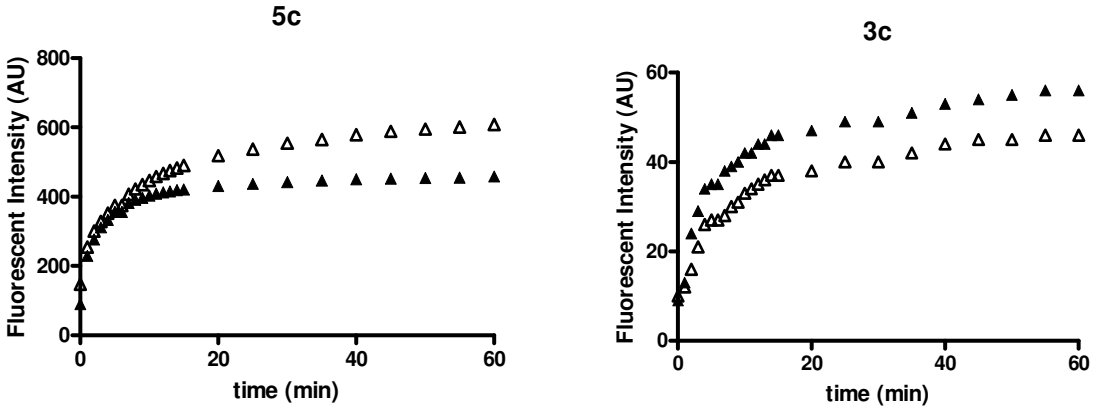


Figure 2. *Binding progress curves of ConA to chips displaying the octavalent* ***5c*** *and the divalent* ***3c*** *with (solid symbols) or without (open symbols) the cospotted amine* ***7****, to equalize the amine concentrations of the spotting solutions. No major differences are observed.*

The experimental binding protocol started with the blocking of the non-functionalized area with BSA. A concentration range of FITC-labelled ConA was applied to the chips and the fluorescent signal was periodically recorded for 2 hours (Figure 3), the fluorescent signal was quantified, averaged for the same spots and converted to progress curves (Figure 4). As a negative control fluorescently labeled BS-I (*Bandeiraea simplicifolia* agglutinin) was used which is specific for β-D-galactose residues. We did not observe any binding of this lectin to the array surface.

From the ConA binding curves (Figure 4) we can clearly see the binding event of the lectin to the mono- to octavalent dendrimers as a function of time. It is also clear that the signal increases with the sugar density on the chip, a consequence of the more concentrated spotting solutions (Figure 3). A linear correlation between the spotting concentration and the observed equilibrium signal due to ConA binding was observed up to a 1 mM spot concentration of mannose residues (Figure 5). This indicated that with the concentrations of dendrimer-linked amine used, we were not close to occupying all the maleimide sites.

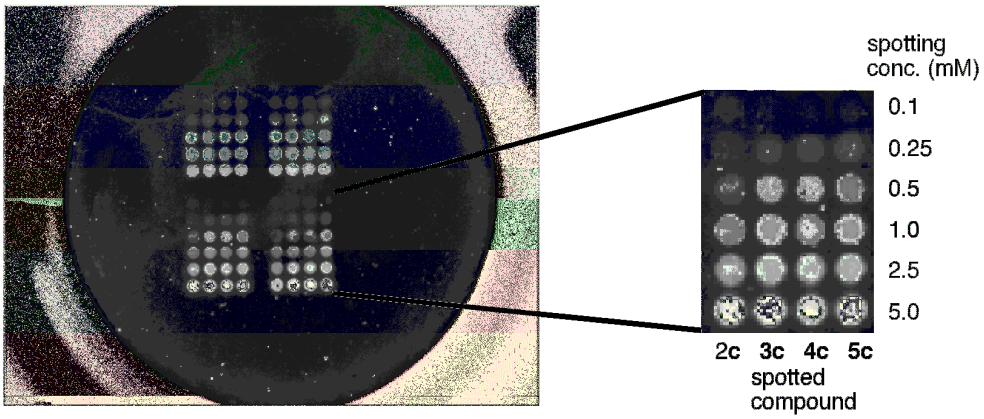


Figure 3. *FITC-labelled ConA binds to the chip surface. In each of the four identical blocks the spotting concentration increases from top to bottom while the valency increases from left (MONO* ***2c****) to right (OCTA* ***5c****).*

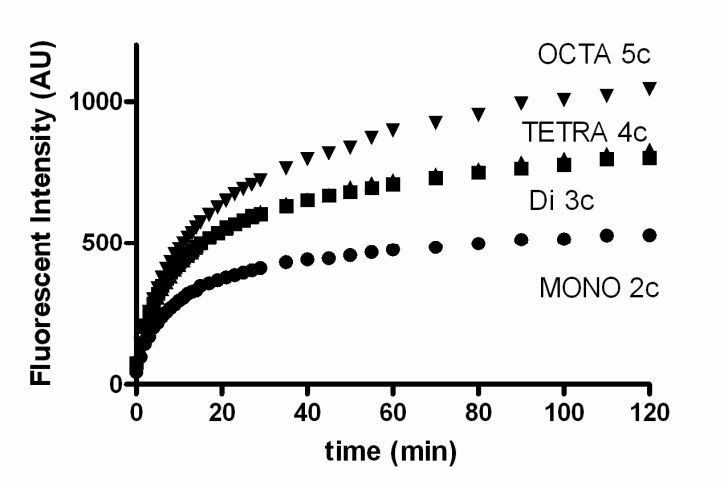


Figure 4. *Progress curves for the microarray experiment with attached mono- to octavalent compounds* ***2c-5c*** *(spotting concentrations 0.5 mM in mannose) with ConA (monomer concentration 98 nM).*

Furthermore, with the spotting concentration of 0.5 mM in mannose residues, as used in the evaluations below, the calculated minimal intermolecular distance of the surface bound ligands indicates that this is non-bridgeable by ConA.[22] The observed multivalency effect observed for ConA was relatively small. The final equilibrium signals only varied by a factor of two favoring the octavalent presentation. Furthermore, the binding kinetics were similar for all compounds. By fitting the progress curves to a simple receptor-ligand interaction model i.e. a one phase exponential association model, observed rate constants of binding k_{obs} were obtained. These were all within the 0.20-0.23 min^{-1} range for the mono-

to the octavalent glycodendrimer-functionalized surfaces. Subsequently globally fitting the progress curves obtained from several ConA concentrations in the 0.2-1.0 μM range, yielded the kinetic binding parameters k_{on} and k_{off} and the related kinetically determined dissociation constant K_d (Table 1) for each of the four valencies.

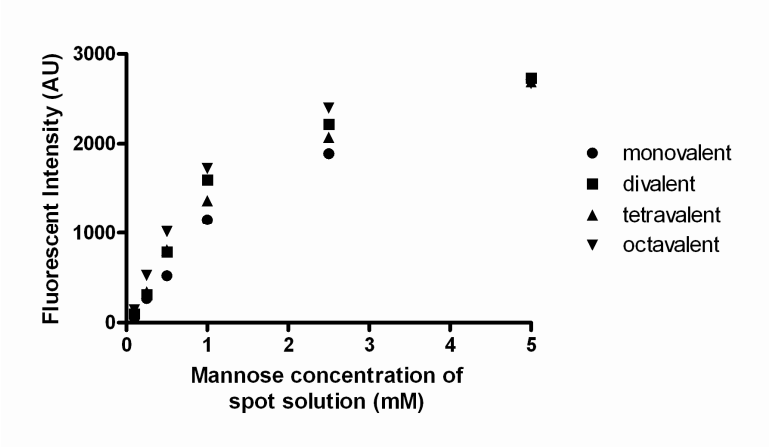


Figure 5. *Observed binding for ConA (10 μg/mL) against the different spotting concentrations; a linear trend was observed at spot concentrations between 0.1 and 1.0 mM.*

In order to see if reasonable numbers were obtained the K_d's were also determined using equilibrium end-values and a Langmuir binding isotherm. As can be seen in the table, the K_d values determined by these two methods were in the same range. The K_d for the octavalent **5c** covered surface is the lowest, followed by tetra- and divalent **3c** and **4c** which are very similar and the monovalent **2c** covered surface interacted the weakest. The same trend can be seen from the progress curves in Figure 4, illustrating the value of the technology as a rapid screening method to evaluate multivalency effects.

Subsequently, a higher valency lectin, the *Galanthus Nivalis Agglutinin* (GNA) was studied. This lectin is tetrameric with three binding sites per subunit, so twelve in total, several of them spaced closely together. The close spacing should allow chelation by the multivalent ligands and thus strong binding enhancement due to multivalency was expected. The monovalent affinity for mannose derivatives, however, is a lot lower: the K_d of α methyl mannose for ConA is ca. 100 μM[23] and for GNA this value is 24 mM.[24]

Table 1. *Kinetic and thermodynamic parameters deduced from chip experiments for the association of ConA to the four different surfaces.*

compound on surface	k_{off} $(M^{-1}min^{-1})$[a]	k_{on} (min^{-1}) [a]	K_d (nM) (from binding kinetics)[b]	K_d (nM) (from equilibrium values)[c]
MONO **2c**	0.12	1.26×10^5	950	960
Di **3c**	0.08	1.76×10^5	450	510
TETRA **4c**	0.07	1.76×10^5	400	540
OCTA **5c**	0.04	1.93×10^5	210	440

[a] Derived from global fitting of progress curves for multiple ConA concentrations. [b] $K_d = k_{off}/k_{on}$. [c] Derived from fitting end-values using different ConA concentrations to a Langmuir binding isotherm.

Despite the low affinity for mannose, binding was observable and indeed a strong preference for the higher valency mannose dendrimers was observed (Figure 6). Furthermore the binding kinetics were significantly faster than for the ConA experiment. It also appears that GNA binds slightly slower to the higher valency compounds on the chip. The progress curves could not be fitted adequately to a one phase exponential association model to obtained the k_{obs}. The use of a two phase model was necessary which yielded a low k_{obs1} in the 0.03-0.08 min^{-1} range for all valencies, and also a faster k_{obs2}, which varied steadily with valency between 1.8 min^{-1} for the monovalent surface to 1.0 min^{-1} for the tetra- and octavalent compounds on the chip.

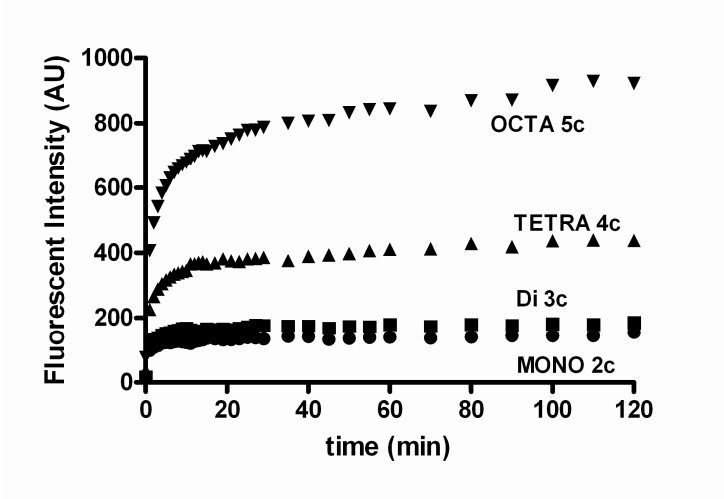


Figure 6. *Progress curves for the microarray experiment with GNA (monomer conc. 2 μM) A clear preference for the higher valency mannosides was observed.*

Inhibition experiment: The carbohydrate microarray system was also used for the evaluation of an inhibition experiment similar to those reported for other microarray systems.[8b,25] To this end the FITC-labelled ConA was incubated with the soluble inhibitor α-methyl mannose at several concentrations and added to spots of monovalent **2c**. A clear inhibition was observed that was used to determine an IC_{50} value of 400 (±100) μM (Figure 7).

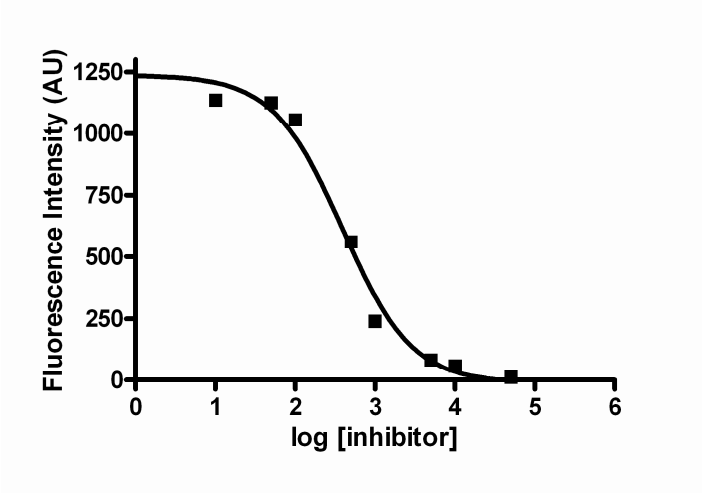


Figure 7. *Inhibition data points and fitted curve of the binding of ConA to a chip of* ***2c*** *with increasing concentrations of α-methyl mannose in solution.*

Discussion and Conclusions

Multivalent mannose dendrimers were prepared from alkyne dendrimers. The dendrimers were synthesized with an amine functional group linked to the dendrimeric core that allowed attachment to surfaces. The dendrimers based on the 3,5-di-(2-aminoethoxy)-benzoic acid repeating unit were first equipped with a mono-Boc protected diamino spacer after which the mannose residues were introduced at the periphery by the copper catalyzed "click" reaction and the compounds were fully deprotected. The mannose dendrimers were spotted onto a maleimide functionalized porous aluminium oxide surface and binding of FITC-labelled lectins to the functionalized surfaces was clearly observed in real-time using flow-through microarray technology. The flow-through microarray setup has several advantages over the ordinary microarray technologies. Monitoring of progression of protein binding is important; not only endpoints were obtained but also the binding kinetics. We demonstrated this technology for the binding of ConA and GNA towards multivalent compounds. Clear differences were seen in the responses of these proteins. ConA is a

tetramer with binding site that are separated by over 65Å and therefore too far apart to allow bridging by our dendrimers. No major multivalency effects were expected by our compounds and indeed they were not observed. In studies of related compounds multivalency effects were limited to a factor of up to ~6.[26] Kinetic data were obtained from the chip experiments due to the possibility of real-time monitoring. By fitting a series of progress curves run at different ConA concentrations k_{on} and k_{off} values were obtained as well as the related kinetically determined dissociation constant K_d. The K_d was also determined using the end points and the numbers are of the same order of magnitude. The relative magnitude of the K_d values correlate well with the progress curves of the single experiment shown in Figure 2. This underscores the value of the present methodology as a screening tool that can rapidly identify multivalency effects, even in a single experimental run (and therefore well controlled). The magnitude of the K_d values was low (200-900 nM) in comparison to reported K_d values of monovalent mannose derivatives binding to ConA, which are around 100 μM.[23,27] We speculate that the very long and very narrow pores of the aluminum oxide (0.2 μm diameter, 60 μm height) are the cause. After dissociation a rapid rebinding event is likely, resulting in a net slow k_{off}. The determined k_{off} rate was 7-fold lower than that derived from using SPR methodology[28] with immobilized yeast mannan, a system where k_{off} rates are likely already lowered due to the operative multivalency effects with the polymeric yeast mannan.

The phenomenon of enhanced binding due to the porous three-dimensional nature of the chip material is a bonus for the application of the technology as a screening tool for carbohydrate-protein interactions, which are typically weak. The combination used in this study of multivalency enhancement and the long pores make it possible to conveniently detect GNA binding, whose monovalent mannoside K_d is ca. 24 mM, by using only 2 μM of the protein (Figure 6). This results in low detection limits of weakly binding proteins. While this study was not optimized for a low detection limit, using a moderately dense substituted surface (spotting concentrations 0.5 mM in mannose) and an average shutter time of the CCD camera, ConA was detectable down to ca. 10 nM or 0.26 μg/mL. For comparison, the detection limit of an SPR based system with immobilized yeast mannan was reported with a detection limit of 0.5 μg/mL for ConA.[28]

For GNA the effect of the multivalency was markedly different than for ConA. A strong signal for the immobilized octavalent compound **5c** was observed and hardly any observable signal was seen for the mono- and divalent compounds (**2c-3c**) under the same conditions. This behavior is in line with the high-valency architecture of the tetramer comprising 12 binding sites in total with relatively closely spaced binding sites starting at around 20Å and the weak monovalent binding affinities in the millimolar range. Similar effects were also seen with Wheat Germ Agglutinin (WGA) with 8 binding sites and its binding to densely functionalized SPR chips.[29] Based on our recent experiences with multivalent binding to Cholera Toxin, the spacers used here are too short for optimal multivalent binding.[11f,30] Nevertheless strong multivalency was clearly observed.

The spotting concentrations used for the analysis of the ConA binding parameters were in the range where the fluorescent signal still increases linearly with the spotting concentrations, i.e. $\leq$ 1 mM in mannosides. For the GNA studies the higher spotting concentration of 5.0 mM in mannosides was needed to get a strong enough signal, due to the weak inherent mannose affinity of this lectin. We cannot exclude that at this concentration the packing of the monovalent **2c** is sufficiently dense to allow bridging by GNA molecules, however considering the low signal of this binding event versus the GNA binding to the octavalent **5c** spotted at only 0.625 mM, it seems highly unlikely.

In conclusion, the real-time evaluation of a multivalent carbohydrate chip as described here is a useful rapid screening method to evaluate multivalency effects in a single experiment. Extension of this study will be undertaken in the direction of other carbohydrates, other spacers and other carbohydrate binding proteins. Furthermore it is also clear that inhibition studies are also possible which provides additional potential for applications.

Experimental Section

General Remarks

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Solvents were purchased from Biosolve (Valkenswaard, The Netherlands). Microwave reactions were carried out in a dedicated microwave oven, i.e. the Biotage Initiator. The microwave power was limited by temperature control once the

desired temperature was reached. A sealed vessel of 2 – 5 mL was used. Analytical HPLC runs were performed on a Shimadzu automated HPLC system with a reversed phase column (Alltech, Adsorbosphere C8, 90 Å, 5µm, 250x4.6 mm) equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories) and a UV/VIS detector operating at 220 and 254 nm. Preparative HPLC runs were performed on a Applied Biosystems workstation. Elution was effected using a linear gradient of 5% MeCN / 0.1% TFA in H_2O to 5% H_2O / 0.1% TFA in MeCN. ^{1}H NMR (300MHz) and ^{13}C NMR (75.5MHz) were performed on a Varian G-300 spectrometer.

3-azidopropyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (1): To a stirred and cooled (0˚C) solution of per acetylated mannose (3.90 g, 10 mmol) in dry CH_2Cl_2 (150 mL) was added a solution of HBr in acetic acid (30 % solution, 10 mL). After 18 hours the mixture was poured into ice water, neutralized with $NaHCO_3$. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated. The mannosyl bromide was isolated as yellow oil (3.88 g, 94%). NMR is consistent with ref. 18. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.30 (1H, d, H-1, $J_{1,2}$ = 0.83 Hz), 5.72 (1H, dd, H-3, $J_{2,3}$ = 3.3 Hz, $J_{3,4}$ = 10.2 Hz), 5.45 (1H, dd, H-2, $J_{1,2}$ = 1.5 Hz, $J_{2,3}$ = 3.6 Hz), 5.37 (1H, t, H-4, $J_{3,4}$ = 10.2 Hz), 4.35 (1H, dd, H-6a, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 12.3 Hz), 4.15 (1H, dd, H-6b, $J_{5,6b}$ = 2.1 Hz, $J_{6a,6b}$ = 12.3 Hz), 4.25 – 4.20 (1H, m, H-5), 2.18, 2.11 2.08 and 2.01 (4 x 3H, 4 x s, C(O)CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.4, 169.6 and 169.5 (*C*(O)CH_3), 83.0 (C-1), 72.8, 72.1 67.9 and 65.2 (C-2, C-3, C-4, C-5), 61.4 (C-6), 20.7, 20.6 and 20.5 (C(O)*C*H_3). A solution of mannosyl bromide (3.88g, 9.4 mmol) and 3-bromopropanol (2.04 mL, 23.5 mmol) in dry MeCN (50 mL) was stirred and cooled to 0˚C. A solution of IBr in CH_2Cl_2 (1 M) (23.5 mL, 23.5 mmol) was added drop wise. After 1 h the solution was warmed to r.t. and stirred for additionally 18 h. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed twice with $Na_2S_2O_5$ (5%, 75 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. Column chromatography (hex/EtOAc, 2/1) gave the mannoside (2.39 g, 54%). NMR is consistent with ref. 18. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.30 – 5.24 (3H, m, H-2, H-3, H-4), 4.83 (1H, s, H-1, $J_{1,2}$ = 1.65 Hz), 4.29 (1H, dd, H-6a, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 12 Hz), 4.14 (1H, dd, H-6b, $J_{5,6b}$ = 1.8 Hz, $J_{6a,6b}$ = 12.0 Hz), 4.03 – 3.99 (1H, m, H-5), 3.91 (1H, m, OCH*H*CH_2CH_2Br), 3.62 – 3.52 (3H, m, OCH*H*CH_2C*H*$_2$Br), 2.16, 2.11, 2.05 and 1.99 (4 x 3H, 4 x s, C(O)CH_3) and 2.00 – 1.94 (2H, m, OCH_2C*H*$_2CH_2$Br). ^{13}C NMR

(75.5 MHz, $CDCl_3$): δ = 170.6, 170.0, 169.9 and 169.7 (*C*(O)CH_3), 97.6 (C-1), 69.4, 69.0, 68.6, 66.0 and 65.5 (C-2, C-3, C-4, C-5, C-6), 62.4 (CH_2O_{man}), 32.0 (CH_2Br), 30.0 ($OCH_2CH_2CH_2Br$), 20.8, 20.7 and 20.6 (C(O)*C*H_3). A solution of the bromide (2.39 g, 5.08 mmol) and NaN_3 (0.99 g, 15.24 mmol) in DMSO (30 mL) was stirred for 20 hours. Brine (100 mL) was added and the mixture was extracted with CH_2Cl_2 (3 x 150 mL). The organic phases were combined, concentrated, taken up in EtOAc (150 mL) and washed with brine (3 x 75 mL), dried over Na_2SO_4, filtered and concentrated. **1** was obtained as a yellow oil (1.83 g, 84%). NMR is consistent with ref. 18. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.32 – 5.24 (3H, m, H-2, H-3, H-4), 4.82 (1H, d, H-1, $J_{1,2}$ = 1.38 Hz), 4.28 (1H, dd, H-6a, $J_{5,6a}$ = 5.4 Hz, $J_{6a,6b}$ = 12.0 Hz), 4.12 (1H, dd, H-6b, $J_{5,6b}$ = 2.1 Hz, $J_{6a,6b}$ = 12.0 Hz), 4.00 – 3.95 (1H, m, H-5), 3.85 – 3.78 and 3.56 – 3.51 (2 x 1H, 2 x m, CH_2O_{man}), 3.44 (2H, t, CH_2N_3, *J* = 6.6 Hz), 2.16, 2.11, 2.05 and 2.00 (4 x 3H, 4 x s, C(O)CH_3) and 1.94 – 1.87 (2H, m, $OCH_2CH_2CH_2N_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.6, 170.0, 169.9 and 169.7 (*C*(O)CH_3), 97.6 (C-1), 69.4, 69.0 68.6, 66.1 and 64.8 (C-2, C-3, C-4, C-5, C-6), 62.5 (CH_2O_{man}), 48.0 (CH_2N_3), 28.6 ($OCH_2CH_2CH_2N_3$), 20.8 and 20.6 (C(O)*C*H_3).

Monovalent alkyne dendrimer (2b): A solution of **2a** (1.43 g, 7.5 mmol) was stirred in Tesser's base (50 mL) for 20 h. The reaction mixture was acidified with aqueous $KHSO_4$ (1M) to pH = 2 and concentrated in vacuo. Crude product was taken up in EtOAc (100 mL) and washed twice with H_2O (50 mL) and with brine (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give the free carboxylic acid (1.30 g, 98%). To a solution of the acid (352 mg, 2.0 mmol), spacer **6** (0.96 g, 3.0 mmol) and BOP (1.33 g, 3.0 mmol) in CH_2Cl_2 (30 mL) was added DiPEA (0.99 mL, 6.0 mmol). The reaction was stirred for 18 h. TLC (EtOAc) showed the formation of **2b**. The reaction mixture was diluted with CH_2Cl_2 (100 mL), washed with $KHSO_4$ (1M, 50 mL), $NaHCO_3$ (5%, 50 mL) and brine. The organic phase was dried over Na_2SO_4, filtered and concentrated. Silica column chromatography (EtOAc) afforded **2b** as an oil (775 mg, 81%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.45 – 7.29 (3H, m, CH_{arom}-2,5,6), 7.22 (1H, bs, C(O)NH), 7.13 (1H, bs, C(O)NH), 7.09 (1H, dd, CH_{arom}-4), 4.98 (1H, bs, NHBoc), 4.73 (2H, d, OCH_2CCH, *J* = 2.4 Hz), 3.66 – 3.45 (14H, m, CH_2O, $CH_2NHC(O)$), 3.18 (2H, q, CH_2NHBoc), 2.55 (1H, t, OCH_2CCH, *J* = 2.4 Hz), 1.93 – 1.85 and 1.76 – 1.68 (2 x 2H, 2 x m, $OCH_2CH_2CH_2NH$) and 1.42 (9H, s, $NHC(O)OC(CH_3)_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 167.0 (C(O)NH),

157.6 (NH*C*(O)C(CH_3)$_3$), 157.6 (C_{arom}-3), 136.2 (C_{arom}-1), 129.4 (C_{arom}-5), 119.8 (C_{arom}-6), 118.1 (C_{arom}-4), 113.5 (C_{arom}-2), 79.0 (OCH_2*C*CH), 78.2 (NHC(O)*C*(CH_3)$_3$), 75.8 (OCH_2C*C*H), 70.2, 70.1, 70.0, 69.8 and 69.2 (OCH_2), 55.9 (O*C*H_2CCH), 38.4 (CH_2NHBoc), 29.6 and 28.8 (OCH_2*C*H_2CH_2NH) and 28.3 (NHC(O)OC(*C*H_3)$_3$). HRMS for $C_{25}H_{38}N_2O_7$ (M, 478.2679): found $[M+Na]^+$ 501.240, calcd. 501.262.

Divalent alkyne dendrimer (3b): A solution of **3a** (1.35 g, 5.53 mmol) was stirred in Tesser's base (75 mL) for 16 h. The reaction mixture was acidified with aqueous $KHSO_4$ (1M) to pH = 2 and concentrated in vacuo. Crude product was taken up in EtOAc (100 mL) and washed with $KHSO_4$ (1M, 50 mL), brine (50 mL) and H_2O (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give the free carboxylic acid (1.27 g, quant.). To a solution of the acid (460 mg, 2.0 mmol), spacer **6** (960 mg, 3.0 mmol) and BOP (1.33 g, 3.0 mmol) in CH_2Cl_2 (25 mL) was added DiPEA (0.99 mL, 6.0 mmol). The solution was stirred for 2 h. TLC (CH_2Cl_2/MeOH, 19/1) showed full conversion. The reaction mixture was diluted with 100 ml CH_2Cl_2, washed with $KHSO_4$ (1M, 50 mL), $NaHCO_3$ (5%, 50 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. **3b** was isolated by silica column chromatography (CH_2Cl_2/MeOH, 19/1) (1.10 g, quant.). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.03 (3H, bs, CH_{arom}-2,6, NHC(O)), 6.73 (1H, t, CH_{arom}-4), 4.71 (4H, d, 2 x OC*H*$_2$CCH, *J* = 2.2 Hz), 3.67 – 3.46 (14H, m, CH_2O, CH_2NHC(O)), 3.18 (2H, q, C*H*$_2$NHBoc), 2.55 (2H, t, OCH_2CC*H*, *J* = 2.2 Hz), 1.93 – 1.85 and 1.76 – 1.68 (2 x 2H, 2 x m, OCH_2C*H*$_2$CH_2NH) and 1.41 (9H, s, NHC(O)OC(C*H*$_3$)$_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 166.8 (C(O)NH), 156.2 (NH*C*(O)OC(CH_3)$_3$), 158.6 (C_{arom}-3,5), 137.0 (C_{arom}-1), 106.7 (C_{arom}-2,6), 105.2 (C_{arom}-4), 79.2 (OCH_2*C*CH), 78.1 (NHC(O)O*C*(CH_3)$_3$), 75.9 (OCH_2C*C*H), 70.2, 70.0, 69.9 and 69.2 (OCH_2), 56.1 (O*C*H_2CCH), 38.5 (CH_2NHBoc), 29.6 and 28.8 (OCH_2*C*H_2CH_2NH) and 28.4 (NH(CO)OC(*C*H_3)$_3$). HRMS for $C_{28}H_{40}N_2O_8$ (M, 532.2785): found $[M+H]^+$ 533.108, calcd. 533.286.

Tetravalent alkyne dendrimer (4b): A solution of **4a** (680 mg, 1.0 mmol) was stirred in Tesser's base (30 mL) for 3 h. The reaction mixture was acidified with aqueous $KHSO_4$ (1M) to pH = 2 and concentrated in vacuo. Crude product was taken up in EtOAc (100 mL, with 10 mL of DMF) and washed twice with H_2O (50 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give the free carboxylic acid

(682 mg, quant.). To a solution of the acid (680 mg, 1.0 mmol), spacer **6** (480 mg, 1.5 mmol) and BOP (660 mg, 1.5 mmol) in DMF (40 mL) was added DiPEA (0.50 mL, 3.0 mmol). The solution was stirred for 3 h. The reaction mixture was concentrated in vacuo at 60˚C and pure product was obtained after silica column chromatography (EtOAc/MeOH, 1/0 → 19/1) as a white foam (385 mg, 40%). ^{1}H NMR (300 MHz, DMSO): δ = 8.66 (2H,t, C(O)NH), 8.41 (1H, t, C(O)NH), 7.12 (4H, s, CH_{arom}-2',6'), 7.04 (2H, s, CH_{arom}-2,6), 6.78 (2H, s, CH_{arom}-4'), 6.73 (1H, bs, N*H*Boc), 6.70 (1H, s, CH_{arom}-4), 4.83 (8H, d, OCH_2CCH, *J* = 2.4 Hz), 4.15 (4H, t, OCH_2CH_2N), 3.63 (4H, q, OCH_2CH_2N), 3.57 (2H, t, $CH_2NHC(O)$), 3.50 - 3.27 (16H, m, CH_2O, $CH_2NHC(O)$), 2.93 (2H, q, CH_2NHBoc), 2.50 (4H, t, OCH_2CCH, *J* = 1.8 Hz), 1.78 - 170 and 1.62 – 1.54 (2 x 2H, 2 x m, $OCH_2CH_2CH_2NH$) and 1.36 (9H, s, $NHC(O)OC(CH_3)_3$). ^{13}C NMR (75.5 MHz, DMSO): δ = 165.7 and 165.5 (C(O)NH), 159.4 (C_{arom}-3,5), 158.1 (C_{arom}-3',5'), 155.5 ($NHC(O)OC(CH_3)_3$), 136.7 (C_{arom}-1), 36.3 (C_{arom}-1'), 106.7(C_{arom}-2',6'), 105.9 (C_{arom}-2,6), 105.0 (C_{arom}-4'), 103.8 (C_{arom}-4), 78.9 (OCH_2CCH), 78.4 (OCH_2CCH), 77.4 ($NHC(O)OC(CH_3)_3$), 69.7, 69.5, 69.5, 68.2, 68.0 and 66.2 (OCH_2), 55.8 (OCH_2CCH), 37.2 ($CH_2NHC(O)$), 36.6 (CH_2NHBoc), 29.7 and 29.3 ($OCH_2CH_2CH_2NH$) and 28.2 ($NHC(O)OC(CH_3)_3$). HRMS for $C_{52}H_{62}N_4O_{14}$ (M, 966.4236): found $[M+H]^+$ 967.096, calcd. 967.434.

Octavalent alkyne dendrimer (5b): A solution of **5a** was stirred in Tesser's base. The reaction mixture was acidified with aqueous $KHSO_4$ (1M) to pH = 2 and concentrated in vacuo. Crude product was taken up in EtOAc (with 10 % of DMF) and washed twice with H_2O (50 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give the free carboxylic acid (quant.).To a solution of the acid (767 mg, 0.5 mmol), spacer **6** (240 mg, 0.75 mmol) and BOP (332 mg, 0.75 mmol) in DMF (40 mL) was added DiPEA (248 µL, 1.5 mmol). The solution was stirred for 20 hours. The reaction mixture was concentrated in vacuo at 60˚C. The reaction mixture was taken up in EtOAc (100 mL) washed with $KHSO_4$ (1M, 50 mL), $NaHCO_3$ (5%, 50 mL) and brine. The organic phase was dried over Na_2SO_4, filtered and concentrated. Silica column chromatography could not be used for purification because of solubility problems. The product was obtained after precipitation and centrifugation from DMF and EtOAc (1.55 g, 55%). ^{1}H NMR (300 MHz, DMSO): δ = 8.67 (6H, bt, C(O)NH), 8.41 (1H, t, C(O)NH), 7.13 (8H, d, CH_{arom}-2'',6''), 7.08 (4H, d, CH_{arom}-2',6'), 7.03 (2H, s, CH_{arom}-2,6), 6.79 (4H, d, CH_{arom}-4''), 6.72

(2H, s, CH_{arom}-4'), 6.68 (1H, s, CH_{arom}-4), 4.83 (16H, d, OCH_2CCH, J = 1.9 Hz), 4.14 (12H, t, OCH_2CH_2N), 3.64 - 3.27 (26H, m, CH_2O, $CH_2NHC(O)$, OCH_2CH_2N), 2.95 (2H, q, CH_2NHBoc), 2.50 (8H, t, OCH_2CCH, J = 1.7 Hz), 1.77 - 1.69 and 1.62 - 1.54 (2 x 2H, 2 x m, $OCH_2CH_2CH_2NH$) and 1.36 (9H, s, $NHC(O)OC(CH_3)_3$). ^{13}C NMR (75.5 MHz, DMSO): δ = 165.5, 165.8 and 165.9 (C(O)NH), 159.5 (C_{arom}-3,5), 159.4 (C_{arom}-3',5'), 158.2 (C_{arom}-3'',5''), 136.7 (C_{arom}-1'), 136.3 (C_{arom}-1''), 106.8(C_{arom}-2'',6''), 106.0 (C_{arom}-2',5'), 105.0 (C_{arom}-4''), 104.1 (C_{arom}-4'), 78.9 (OCH_2CCH), 78.4 (OCH_2CCH), 69.7, 69.6, 68.2, 68.1 and 66.2 (OCH_2), 55.8 (OCH_2CCH), 36.7 (CH_2NHBoc), 29.7 and 29.3 ($OCH_2CH_2CH_2NH$) and 28.2 ($NHC(O)OC(CH_3)_3$). HRMS for $C_{100}H_{106}N_8O_{26}$ (M, 1834.7218): found $[M+H]^+$ 1835.730, calcd. 1835.257.

General "click" conditions: Alkyne dendrimer, sugar azide (1.5 eq / alkyne), $CuSO_4$ (0.15 eq / alkyne) and sodium ascorbate (0.3 eq / alkyne) were dissolved in an appropriate volume of 1% H_2O in DMF. The mixture was heated under microwave irradiation to 80°C for 20 minutes. The reaction mixture was concentrated in vacuo at 60°C and the product was isolated by silica gel chromatography.

General deprotection procedure: Dendrimers were dissolved in MeOH. Catalytic NaOMe was added and the reaction was stirred until TLC showed full deacetylation. The mixture was neutralized with Dowex H^+, filtered and concentrated in vacuo. The residue was stirred in 5% H_2O in TFA for 1 h. Solvents were evaporated and the product was purified by preparative HPLC and lyophilized from H_2O/MeCN.

Monovalent mannose dendrimer (2c): "Click" reaction was performed by the general procedure. Protected monovalent mannose dendrimer was isolated by silica gel chromatography (EtOAc/MeOH, 1/0 → 9/1) (147 mg, 81%). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.64 (1H, s, $CH_{triazole}$), 7.21 – 7.42 (3H, m, CH_{arom}-2,5,6), 7.15 (bs, 1H, C(O)NH), 7.02 (1H, d, CH_{arom}-4), 5.17 – 5.23 (m, 5H, H-2, H-3, H-4, $OCH_2C_{triazole}$), 4.95 (1H, bs, NHBoc), 4.73 (1H, s, H-1), 4.47 – 4.41 (2H, m, CH_2O_{man}), 4.22 (1H, dd, H-6a, $J_{5,6a}$ = 5.2 Hz, $J_{6a,6b}$ = 12.1 Hz), 4.02 (1H, dd, H-6b, $J_{5,6b}$ = 2.5 Hz, $J_{6a,6b}$ = 12.4 Hz), 3.93 – 3.89 (1H, m, H-5), 3.71 – 3.65 (2H, m, $CH_2NH(CO)$), 3.59 – 3.35 (12H, m, CH_2O), 3.14 – 3.08 (2H, m, CH_2NHBoc), 2.21 – 2.12 (2H, m, $OCH_2CH_2CH_2N_{triazole}$), 2.09, 2.01, 1.98 and 1.92 (4 x 3H, 4 x s, $C(O)CH_3$), 1.86 – 1.79 and 1.68 – 1.60 (2 x 2H, 2 x m, $OCH_2CH_2CH_2NH$) and 1.35

(s, 9H, NHC(O)OC(CH_3)$_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.5, 170.0, 169.9 and 169.6 (*C*(O)CH_3), 166.9 (C(O)NH), 156.0 (NH*C*(O)C$(CH_3)_3$), 158.2 (C_{arom}-3), 143.7 ($C_{triazole}$-4), 136.2 (C_{arom}-1), 129.4 (C_{arom}-5), 123.1 ($C_{triazole}$-5), 119.3 (C_{arom}-6), 117.8 (C_{arom}-4), 113.4 (C_{arom}-2), 97.6 (C-1), 70.3, 70.1, 70.0 and 69.3 (OCH_2), 69.3, 68.9, 68.6, 65.9 and 64.5 (C-2, C-3, C-4, C-5, C-6), 62.3 (CH_2O_{man}), 61.8 (O*C*$H_2C_{triazole}$), 47.0 ($CH_2N_{triazole}$), 38.6 (CH_2NHBoc), 29.7 29.5 and 28.8, (OCH_2*C*H_2CH_2NH, OCH_2*C*$H_2CH_2N_{triazole}$), 28.3 (NHC(O)OC(*C*$H_3)_3$), 20.7, 20.6 and 20.5 (C(O)*C*H_3). HRMS for $C_{42}H_{63}N_5O_{17}$ (M, 909.4219): found $[M+Na]^+$ 932.3491, calcd. 932.4117. The dendrimer was deprotected according to the general deprotection procedure. **2c** was isolated as clear oil (54 mg, 88%). ^{1}H NMR (300 MHz, D_2O): δ = 8.10 (1H, s, $CH_{triazole}$), 7.47 – 7.34 (3H, s, CH_{arom}-2,5,6), 7.23 (1H, s, CH_{arom}-4), 5.26 (2H, s, $OCH_2C_{triazole}$), 4.71 (1H, s, H-1), 4.53 (2H, t, OC*H*$_2$$CH_2$NH), 3.81 – 3.40 (24H, m, H-2, H-3, H-4, H-5, H-6, OCH_2, CH_2NH, $CH_2N_{triazole}$), 3.08 (2H, t, C*H*$_2$$NH_3$), 2.23 – 2.15 (2H, m, C*H*$_2$$CH_2O_{Man}$) and 1.96 – 1.84 (4H, m, OCH_2C*H*$_2$$CH_2NH, OCH_2C*H*_2$$CH_2$NH). ^{13}C NMR (75.5 MHz, D_2O): δ = 170.5 (C(O)NH), 158.2 (C_{arom}-3), 143.7 ($C_{triazole}$-4), 136.0 (C_{arom}-1), 130.9 (C_{arom}-5), 125.9 ($C_{triazole}$-5), 121.0 (C_{arom}-6), 119.4 (C_{arom}-4), 114.6 (C_{arom}-2), 100.5 (C-1), 73.3, 71.2, 70.7 and 67.3 (C-2, C-3, C-4, C-5), 70.3, 70.2, 70.0, 69.4 and 68.9 (OCH_2), 65.0 (O*C*$H_2C_{triazole}$), 61.9 (O*C*H_2CH_2NH), 61.5 (C-6), 48.5 ($CH_2N_{triazole}$), 38.3 and 38.0 (OCH_2*C*H_2NH, OCH_2CH_2*C*H_2NH), 29.7, 28.9 and 27.1 (*C*$H_2CH_2O_{Man}$, OCH_2*C*H_2CH_2NH). HRMS for $C_{29}H_{47}N_5O_{11}$ (M, 641.3272): found $[M+H]^+$ 642.070, calcd. 642.7178.

Divalent mannose dendrimer (3c): "Click" reaction was performed by the general procedure. Protected divalent mannose dendrimer was isolated by silica gel chromatography (EtOAc/MeOH, 1/0 → 9/1) (210 mg, 75%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.65 (s, 2H, $CH_{triazole}$), 7.10 (bs, 1H, C(O)NH), 6.98 (2H, d, CH_{arom}-2,6), 6.69 (1H, t, CH_{arom}-4), 5.24 – 5.13 (6H, m, H-2, H-3, H-4), 4.99 (1H, bs, NHBoc), 4.73 (2H, s, H-1), 4.48 – 4.39 (4H, m, $OCH_2C_{triazole}$), 4.22 (1H, dd, H-6a, $J_{5,6a}$ = 5.2 Hz, $J_{6a,6b}$ = 12.4 Hz), 4.02 (1H, dd, H-6b, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 12.4 Hz), 3.91 – 3.87 (2H, m, H-5), 3.71 – 3.66 (2H, m, C*H*$_2$NHC(O)), 3.57 – 3.36 (m, 12H, CH_2O), 3.13 – 3.07 (2H, m, C*H*$_2$NHBoc), 2.20 – 2.16 (4H, m, OCH_2C*H*$_2$$CH_2N_{triazole}$), 2.09, 2.01, 1.97 and 1.93 (4 x 6H, 4 x s, C(O)C*H*$_3$), 1.86 – 1.78 and 1.65 – 1.61 (2 x 2H, 2 x m, OCH_2C*H*$_2$$CH_2$NH) and 1.35 (9H, s, NHC(O)OC(C*H*$_3)_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.5, 169.9, 169.8 and 169.6

($C(O)CH_3$), 166.7 (C(O)NH), 155.9 ($NHC(O)C(CH_3)_3$), 159.3 (C_{arom}-3,5), 143.5 ($C_{triazole}$-4), 137.1 (C_{arom}-1), 123.1 ($C_{triazole}$-5), 106.3 (C_{arom}-2,6), 104.6 (C_{arom}-4), 97.6 (C-1), 70.3, 70.1, 70.0 and 69.3 (OCH_2), 69.2, 68.9, 68.6, 65.9 and 64.5 (C-2, C-3, C-4, C-5, C-6), 62.3 (CH_2O_{man}), 61.9 ($OCH_2C_{triazole}$), 47.0 ($CH_2N_{triazole}$), 38.4 (CH_2NHBoc), 29.7, 29.5 and 28.8 ($OCH_2CH_2CH_2NH$, $OCH_2CH_2CH_2N_{triazole}$), 28.3 ($NHC(O)OC(CH_3)_3$), 20.7, 20.6 and 20.5 ($C(O)CH_3$). HRMS for $C_{62}H_{90}N_8O_{28}$ (M, 1394.5865): found $[M+Na]^+$ 1417.7437, calcd. 1417.5763. The dendrimer was deprotected according to the general deprotection procedure. **3c** was isolated as clear oil (70 mg, 91%). ^{1}H NMR (300 MHz, D_2O): δ = 8.06 (2H, s, $CH_{triazole}$), 6.96 (2H, s, CH_{arom}-2,6), 6.76 (1H, s, CH_{arom}-4), 5.16 (4H, s, $OCH_2C_{triazole}$), 4.70 (2H, s, H-1), 4.50 (4H, t, OCH_2CH_2NH), 3.80 – 3.55 (28H, m, H-2, H-3, H-4, H-5, H-6, OCH_2), 3.50 (2H, bs, CH_2NH), 3.40 (4H, t, $CH_2N_{triazole}$), 3.07 (2H, t, CH_2NH_3), 2.16 (4H, t, $CH_2CH_2O_{Man}$) and 1.94 – 1.83 (4H, m, $OCH_2CH_2CH_2NH$, $OCH_2CH_2CH_2NH$). ^{13}C NMR (75.5 MHz, D_2O): δ = 169.7 (C(O)NH), 159.5 (C_{arom}-3,5), 143.6 ($C_{triazole}$), 136.9 (C_{arom}-1), 125.9 ($CH_{triazole}$), 107.8 (C_{arom}-2,6), 106.4 (C_{arom}-4), 100.5 (C-1), 73.4, 71.3, 70.7 and 67.3 (C-2, C-3, C-4, C-5), 70.2, 70.1, 69.6 and 69.0 (OCH_2), 65.0 ($OCH_2C_{triazole}$), 62.0 (CH_2O_{Man}), 61.5 (C-6), 48.5 ($CH_2N_{triazole}$), 38.3 and 38.0 (OCH_2CH_2NH, $OCH_2CH_2CH_2NH$), 29.8, 29.0 and 27.2 ($CH_2CH_2O_{Man}$, $OCH_2CH_2CH_2NH$). HRMS for $C_{41}H_{66}N_8O_{18}$ (M, 958.4495): found $[M+H]^+$ = 959.188, calcd. 960.0129.

Tetravalent mannose dendrimer (4c): "Click" reaction was performed by the general procedure. Protected tetravalent mannose dendrimer was isolated by silica gel chromatography (CH_2Cl_2/MeOH, 9/1 →4/1) as a white foam (163 mg, 59%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.76 (4H, s, $CH_{triazole}$), 7.45, (2H, s, C(O)NH), 7.36 (1H, s, C(O)NH), 7.07 (4H, s, CH_{arom}-2',6'), 6.94 (2H, s, CH_{arom}-2,6), 6.72 (2H, s, CH_{arom}-4'), 6.59 (1H, s, CH_{arom}-4), 5.32 – 5.15 (12H, m, H-2, H-3, H-4), 4.80 (4H, s, 4 x H-1), 4.54 – 4.48 (8H, m, CH_2O_{man}), 4.29 (1H, dd, H-6a, $J_{5,6a}$ = 5.4 Hz, $J_{6a,6b}$ = 12.3 Hz), 4.08 (1H, dd, H-6b, $J_{5,6b}$ = 2.4 Hz, $J_{6a,6b}$ = 12.3 Hz), 3.96 – 4.00 (4H, m, H-5), 3.80 – 3.73 (8H, m, $CH_2N_{triazole}$), 3.60 – 3.41 (14H, m, CH_2O, $CH_2NHC(O)$), 3.18 – 3.12 (2H, m, CH_2NHBoc), 2.29 – 2.19 (8H, m, $OCH_2CH_2CH_2N_{triazole}$), 2.15, 2.08, 2.04 and 1.99 (4 x 12H, 4 x s, $C(O)CH_3$), 1.90 – 1.82 and 1.72 – 1.64 (2 x 2H, 2 x m, $OCH_2CH_2CH_2NH$) and 1.40 (9H, s, $NHC(O)OC(CH_3)_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.5, 169.9, 169.8 and 169.5 ($C(O)CH_3$), 167.1 and 166.7 (C(O)NH), 159.5 (C_{arom}-3,5), 159.2 (C_{arom}-3',5'), 155.9 ($NHC(O)C(CH_3)_3$), 143.4 ($C_{triazole}$-

4), 136.9 (C_{arom}-1), 136.4 (C_{arom}-1'), 123.3 ($C_{triazole}$-5), 106.3 (C_{arom}-2'6'), 106.0 (C_{arom}-2,6), 104.9 (C_{arom}-4'), 104.1 (C_{arom}-4), 97.5 (C-1), 78.7 (NHC(O)*C*$(CH_3)_3$), 70.1, 70.0, 69.8 and 69.8 (OCH_2), 69.2, 68.9, 68.5, 65.8 and 64.5 (C-2, C-3, C-4, C-5, C-6), 62.2 (CH_2O_{man}), 61.7 (O*C*$H_2C_{triazole}$), 47.1 ($CH_2N_{triazole}$), 29.6, 29.4 and 28.8 ($OCH_2CH_2CH_2NH$, $OCH_2CH_2CH_2N_{triazole}$), 28.2 (NHC(O)OC(*C*$H_3)_3$), 20.7, 20.6 and 20.5 (C(O)*C*H_3). HRMS for $C_{120}H_{162}N_{16}O_{54}$ (M, 2691.0422): found $[M+Na]^+$ 2714.3508, calcd. 2714.0320. The dendrimer was deprotected according to the general deprotection procedure. **4c** was isolated as a white powder (59 mg, quant.). ^{1}H NMR (300 MHz, D_2O): δ = 7.91 (4H, s, $CH_{triazole}$), 6.78 (4H, s, CH_{arom}-2',6'), 6.76 (2H, s, CH_{arom}-2,6), 6.51 (2H, s, CH_{arom}-4'), 6.39 (1H, s, CH_{arom}-4), 4.90 (8H, s, $OCH_2C_{triazole}$), 4.69 (4H, s, H-1), 4.37 (8H, bs, O*C*H_2CH_2NH), 3.96 (4H, bs, O*C*H_2CH_2NH), 3.81 – 3.46 (48H, m), 3.38 – 3.25 (8H, m, $CH_2N_{triazole}$), 3.08 (2H, t, *C*H_2NH_3), 2.06 (8H, m, *C*$H_2CH_2O_{Man}$), 1.91 (2H, q, $OCH_2CH_2CH_2NH$) and 1.74 (2H, bt, $OCH_2CH_2CH_2NH$). ^{13}C NMR (75.5 MHz, D_2O): δ = 169.1 (C(O)NH), 160.0 (C_{arom}-3,5), 159.4 (C_{arom}-3',5'), 143.5 ($C_{triazole}$-4), 136.1 (C_{arom}-1'), 125.5 ($C_{triazole}$-5), 107.2 and 105.5 (C_{arom}), 100.4 (C-1), 73.4, 71.3, 70.7 and 67.3 (C-2, C-3, C-4, C-5), 70.1, 69.2 and 68.9 (OCH_2), 64.8 (O*C*$H_2C_{triazole}$), 61.5 (C-6), 48.4 ($CH_2N_{triazole}$), 38.3 (OCH_2CH_2NH), 29.9, 29.1 and 27.1 (*C*$H_2CH_2O_{Man}$, $OCH_2CH_2CH_2NH$). HRMS for $C_{83}H_{122}N_{16}O_{36}$ (M, 1918.8208): found $[M+2H]^{2+}$ = 960.129, calcd. 959.914.

Octavalent mannose dendrimer (5c): "Click" reaction was performed by the general procedure. Protected octavalent mannose dendrimer was isolated by silica gel chromatography (CH_2Cl_2/MeOH, 1/0 → 9/1) (185 mg, 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.79 (8H, bs, $CH_{triazole}$), 7.06 (8H, bs, CH_{arom}-2'',6''), 6.88 (6H, bs, CH_{arom}-2,6, 2',6'), 6.67 (4H, bs, CH_{arom}-4''), 6.44 (3H, bs, CH_{arom}-4, 4'), 5.29 – 5.20 (24H, m, H-2, H-3, H-4), 4.80 (8H, bs, H-1), 4.50 (16H, bs, CH_2O_{man}), 4.29 (8H, bd, H-6a), 4.07 (8H, bd, H-6b), 3.99 (8H, bs, H-5), 3.74 (16H, bs, *C*$H_2N_{triazole}$), 3.59 – 3.47 (14H, m, CH_2O, *C*H_2NHC(O)), 3.13 (2H, bs, *C*H_2NHBoc), 2.23 (16H, bs, $OCH_2CH_2CH_2N_{triazole}$), 2.15, 2.07, 2.04 and 1.99 (4 x 24H, 4 x s, C(O)C*H*$_3$), 1.90 – 1.82 and 1.72 – 1.64 (2 x 2H, 2 x m, $OCH_2CH_2CH_2NH$) and 1.39 (9H, s, NHC(O)OC(C*H*$_3)_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.3, 169.7, 169.6 and 169.4 (*C*(O)CH_3), 167.1 (C(O)NH), 159.4 (C_{arom}-3,5), 159.2 (C_{arom}-3',5'), 158.9 (C_{arom}-3'',5''), 155.8 (NH*C*(O)C$(CH_3)_3$), 136.2 (C_{arom}-1''), 136.7 (C_{arom}-1'), 106.2 (C_{arom}-2'',6''), 97.4 (C-1), 78.5 (NHC(O)*C*$(CH_3)_3$), 70.2(OCH_2), 69.0, 68.8, 68.3, 65.8 and 64.5 (C-2, C-3,

C-4, C-5, C-6), 62.2 (CH_2O_{man}), 62.1 ($OCH_2C_{triazole}$), 47.8 ($CH_2N_{triazole}$), 29.5, 29.3 and 28.3 ($OCH_2CH_2CH_2NH$, $OCH_2CH_2CH_2N_{triazole}$), 28.1 ($NHC(O)OC(CH_3)_3$), 20.5 and 20.4 ($C(O)CH_3$), HRMS for $C_{236}H_{306}N_{32}O_{106}$ (M, 5283.9538): found $[M+2Na]^{2+}$ 2666.540, calcd. 2666.543. To a solution of the dendrimer (60 mg, 12 µmol) in MeOH (5 mL) and H_2O (5 ml) was added NaOMe (50 µL, 30% in MeOH) and stirred for 18h. The reaction mixture was concentrated and taken up in H_2O (5 mL) and TFA was added (5 mL). The reaction was stirred for 18 h, concentrated and subjected to preparative HPLC purification. After lyophilization **5c** was obtained as a white foam (40.2 mg, 92%). ^{1}H NMR (300 MHz, D_2O): δ = 7.88 (8H, bs, $CH_{triazole}$), 6.79 (8H, s, CH_{arom}-2'',6''), 6.69 (6H, s, CH_{arom}-2,6, 2',6'), 6.51 (2H, s, CH_{arom}-4''), 6.272H, s, CH_{arom}-4'), 6.39 (1H, s, CH_{arom}-4), 4.87 (16H, s, $OCH_2C_{triazole}$), 4.69 (8H, s, H-1), 4.34 (16H, bs, OCH_2CH_2NH), 3.81 – 3.49 (100H, m), 3.31 (16H, bs, $CH_2N_{triazole}$), 3.08 (2H, t, CH_2NH_3), 2.02 (16H, bs, $CH_2CH_2O_{Man}$) and 1.95 - 1.91 (2H, q, $OCH_2CH_2CH_2NH$). ^{13}C NMR (75.5 MHz, D_2O): δ = 168.9 (C(O)NH), 159.9 (Carom-3',5'), 159.4 (C_{arom}-3'',5''), 143.4 ($C_{triazole}$-4), 136.1 (C_{arom}-1''), 125.4 ($C_{triazole}$-5), 107.1 (C_{arom}), 100.4 (C-1), 73.4, 71.3, 70.7 and 67.3 (C-2, C-3, C-4, C-5), 70.1 (OCH_2), 64.8 ($OCH_2C_{triazole}$), 61.5 (C-6), 48.3 ($CH_2N_{triazole}$), 38.3 (OCH_2CH_2NH) and 30.8 ($CH_2CH_2O_{Man}$, $OCH_2CH_2CH_2NH$). MALDI TOF MS for $C_{167}H_{234}N_{32}O_{72}$ (M, 3839.5633): found $[M+H]^+$ 3842.4735, calcd. 3842.8235.

Microarray analysis: Microarray experiments were performed using PamChip® arrays run on a PamStation®12 instrument (PamGene B. V., 's Hertogenbosch, The Netherlands). Temperature controlled mannose chips were run in parallel by pumping the sample up and down through the 3-dimensional porous chip. Data were captured by real-time imaging of the fluorescence signal by CCD imaging. Images were analyzed by BioNavigator software (PamGene B. V., 's Hertogenbosch, The Netherlands). The fluorescent intensities were expressed as arbitrary units and the relative intensities of individual dendrimers was the average of four spots.

Detection of Dendrimer-ConA binding: A concentration range of Fitc labeled ConA (25-0.5 µg/mL) in HEPES/BSA buffer containing Ca^{2+} and Mn^{2+} (10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 100 mM NaCl, 0.1% BSA, pH = 7.5) was used for binding experiments. Determination of the observed rate constant k_{obs} was performed by fitting the

data to an exponentional association equation, within GraphPad Prism v. 4/5 the 'One phase exponential association' model was used. The kinetic parameters k_{on}, k_{off} and the related K_d were determined by a global fit of multiple binding progress curves determined by different ConA concentrations, within GraphPad Prism v. 4/5 the 'Association kinetics - Two or more conc. of hot.' model was used, globally sharing the k_{on}, k_{off} and the B_{max}. The K_d based on equilibrium end value was determined by fitting these values as a function of ConA concentration using the GraphPad Prism v. 4/5 the 'One site binding (hyperbola)' model.

Detection of Dendrimer-GNA binding: A concentration range of Fitc labeled GNA (100-5 µg/mL) in HEPES/BSA buffer (10 mM HEPES, 100 mM NaCl, 0.1% BSA, pH = 7.5) was used for binding experiments. Determination of the observed rate constant k_{obs} was performed by fitting the data to an exponential association equation, within GraphPad Prims v. 4/5 the 'Two phase exponential association' model was used.

Negative control: A concentration range of Fitc labeled BS-I (250 – 10 µg/mL) in HEPES/BSA buffer (10 mM HEPES, 100 mM NaCl, 0.1% BSA, pH = 7.5) was used for binding experiments.

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molecules. 95% attach themselves to the internal surface, i.e: $9.44 * 10^{10}$ molecules. Each molecule has this amount of surface: $5.65 * 10^{-6}$ m^2 / $9.44 * 10^{10}$ molecules = $5.99 * 10^{-17}$ m^2/molecule. The average distance between molecules on a circular area (πr^2) of $5.99 * 10^{-16}$ m^2 is twice the ratio, i.e. $8.73 * 10^{-9}$ m or 87 Å..

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Appendices

List of Abbreviations

Summary

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List of Abbreviations

δ	chemical shift
Ac	acetyl
Ac_2O	acetic anhydride
AIDS	Acquired Immuno-Deficiency Syndrome
APT	attached proton test
arom	aromatic
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BOP	(benzotriazol-1-yloxy) tris (dimethyl-amino)phosphonium hexafluoro phosphate
br	broad
BSA	Bovine Serum Albumin
BSI	Bandeirae Simplicifolia Agglutinin
Bz	benzoyl
calcd	calculated
ConA	Concanavalin A
COSY	Correlation Spectroscopy
CT	Cholera Toxin
CTB	Cholera Toxin B subunit
Cys	cysteine
d	doublet
DAST	diethylaminosulfur trifluoride
DBU	1,8-diazabicyclo-[5.4.0]-undec-7-ene
DiPEA	*N*,*N*-diisopropyl-ethylamine
DMF	*N*,*N*-dimethyl formamide
DMSO	dimethyl sulfoxide
DMT	4,4-dimethoxy trityl
E. coli	*Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
ELLA	Enzyme-Linked Lectin Assay
equiv.	equivalent(s)
ESI-MS	Electrospray Ionisation Mass Spectrometry
EtOAc	ethyl acetate
Fmoc	9-fluorenylmethyl oxycarbonyl
Gal	galactose
GalNAc	*N*-acetyl galactosamine
Glc	glucose
GM1os	monosialoganglioside oligosaccharide
GNA	Galanthus Nivalis Agglutinin
h	hours
HIA	Heamagglutination Inhibition Assay
hex	hexane
HATU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate
HIA	Heamagglutination Inhibition Assay
HMBC	Heteronuclear Multiple Bond Correlation
HRP	Horseradish Peroxidase
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	Inhibiting Concentration (50%)
ITC	Isothermal Titration Calorimetry
J	coupling constant
K_d	dissociation constant
LT	heat-labile Enterotoxin
m	multiplet
M	molar
MALDI	Matrix Assisted Laser Desorption Ionization
Man	mannose
MeOH	methanol
MIC	Minimal Inhibitory Concentration
MS	mass spectrometry

NeuAc	*N*-acetylneuraminic acid	s	singlet
NBS	*N*-bromosuccinimide	SHT	Shiga Toxin
NMP	*N*-methyl pyrrolidone	SLT	Shiga-Like Toxin
NMR	Nuclear Magnetic Resonance	SPR	Surface Plasmon Resonance
OD_{490}	optical density at 490 nm	*S. suis*	*Streptococcus suis*
PAK	*Pseudomonas aeruginosa* type K	t	triplet
PAO	*Pseudomonas aeruginosa* type O	*t*BDMS	*tert*-butyldimethyl silyl
PBS	phosphate buffered saline	TCA	trichloro acetyl
ph	phenyl	TFA	trifluoroacetic acid
Piv	pivaloyl	THF	tetrahydrofuran
ppm	parts per million	TLC	Thin Layer Chromatography
PT	Pertussis Toxin	TMSOTf	trimethyl silyl triflate
p-TsOH	*p*-toluenesulfonic acid	TOCSY	Total Correlation Spectroscopy
R_f	Retardation factor	Trt	trityl
rel. pot.	relative potency	WGA	Wheat Germ Agglutinin
r.t.	room temperature		

Summary

New methods in Carbohydrate Chemistry

The field of glycomics is still growing; novel chemical and enzymatic synthesis methods have been developed and also the methods for biological evaluation become more precise and allow screening in a high throughput fashion. Carbohydrates are also more and more of interest in drug design as they are important mediators in a whole range of biological processes. Because of the low affinity of carbohydrates for their receptors, multivalent ligand presentation was introduced. Multivalent compounds were shown to improve the affinity towards lectins, AB_5 toxins and bacteria. In this thesis both the design and synthesis of new glycodendrimers is described. Standard methods have been used to evaluate the binding properties of these new ligands. We also successfully introduced microarray technology with multivalent carbohydrate ligands.

Solid phase carbohydrate synthesis

Chapter 2 describes a new solid phase synthesis for oligosaccharides. We aimed at the development of a solid phase strategy which required only simple building blocks, in which differentiation between the sugar's hydroxyl function would be accomplished *on-bead*. A cysteine residue was initially used as linker and after loading the sugar on the resin, the sugar was deacetylated and ready for our new strategy. The classical selective C-6 protection was demonstrated. The 4,4-dimethoxy trityl group was chosen as the protecting group because of its mild cleavage conditions and visual properties. After selective tritylation the remaining hydroxyl functions were protected as benzoate esters and the trityl group was removed. Glycosylation was performed with a simple glycosyl donor. After cleavage from the resin the product was obtained. Unfortunately also a product from an intermolecular aglycon transfer was obtained, due to the nucleophilicity of the sulphur atom. Glycosylation conditions were varied but we could never exclude the formation of this side product so we changed our route towards a glutamine linked strategy. The C-6 strategy was repeated using this linker, and this time the desired product was isolated and repetition of the process led to a trisaccharide. This procedure was demonstrated but we were not able to extend the chemistry towards other protecting group strategies.

Synthesis and binding of novel multivalent galabiose ligands

Chapter 3 demonstrates an optimized method for the preparation of galabiose containing dendrimers. Galabiose dendrimers previously showed to be good inhibitors of *S. suis* bacterial adhesion. The novel strategy relies on the enzymatic degradation of polygalacturonic acid into dimer and trimer fragments. Chemical modification of these fragments led to two galabiose-based building blocks. Alkyne derived dendrimers were used for ligation using the "click" reaction. The galabiose dendrimers were tested for their inhibition capacity with a standard hemagglutination assay and showed comparable inhibition properties with previously prepared glycodendrimers. This new route is economically more attractive for the large scale preparation of galabiose dendrimers and derivatives compared to fully synthetic routes.

Inhibition of Cholera Toxin by multivalent GM1os ligands

Novel GM1os dendrimers are introduced in chapter 4. Azide functionalized GM1os and GM2os were available from enzymatic glycosylations steps. Mono- to octavalent dendrimers were outfitted with long spacer arms to span the GM1 binding sites in the pentameric CTB_5. The GM1 pentasaccharide and the dendrimers were coupled using the "click" reaction and the products were isolated after HPLC purification. Next to this GM1os dendrimers, we prepared a range of GM2os dendrimers. GM2os has a lower affinity for CTB_5 over the GM1os. Both series of dendrimers were tested for their inhibition capacity. The performed inhibition assay clearly revealed strong multivalent binding to CTB_5, with an at least 380,000-fold stronger binding for the octavalent GM1os conjugate compared to the monovalent GM1os derivative. As expected the GM2os series showed a lower affinity but the tetravalent GM2os dendrimer still surpassed the monovalent GM1os derivatives.

Inhibition of Cholera Toxin by multivalent galactose ligands

The GM1os dendrimers described in Chapter 4, showed a very large multivalency effect, nevertheless they may not be suitable for clinical use because of the expensive preparation of GM1os derivatives. Although galactose is a moderate ligand for CT, multivalency could be usefull for the design of high-affinity CT ligands. Chapter 5 describes the design and synthesis of a galactose building block which was easily prepared from cheap starting materials. Ligation to the previously used dendrimers gave galactose dendrimers. The tetra-

and octavalent dendrimers were able to surpass the GM1os in affinity for CTB_5. A series of divalent galactose dendrimers with increasing spacer lengths showed indeed that long spacers are required for CTB_5 affinity.

Microarray analysis with multivalent mannose ligands

Chapter 6 deals with the synthesis of mannose dendrimers which contain an anchoring amine functionality for immobilization on a chip surface. This dendrimers were immobilized on a porous aluminium oxide surface and a microarray fluorescent detection system was used to observe binding of lectins to the chip surface. Mannose binding lectins showed binding to the chip surface in time. ConA showed equal binding curves for mono- to octavalent dendrimers. The ConA binding curves were fitted and binding constants were determined. The microarray set up was also successfully applied in a competition experiment with a monovalent ligand for ConA in solution. An IC_{50} value for MeαMan was obtained. A different mannose binding lectin, GNA, showed a clear difference for binding to the dendrimers. While mono- and divalent dendrimers showed both poor binding, a large gain in affinity was observed for the tetra- and octavalent dendrimers.

Nederlandse Samenvatting

Nieuwe methoden in de Koolhydraat chemie

Het werkgebied van de glycomics is de laatste jaren flink gegroeid; nieuwe chemische en enzymatische methoden zijn ontwikkeld. Ook de methoden voor de biologische analyse zijn verbeterd en soms zelfs geschikt voor een hoge doorvoer screening (high throughput screening). Koolhydraten worden ook meer en meer gebruikt voor de ontwikkeling van nieuwe medicijnen en diagnostica aangezien ze in veel biologische processen een belangrijke rol spelen. Vanwege de lage affiniteit van koolhydraten voor de receptoren, werden multivalente structuren ontwikkeld om deze affiniteit te verhogen. Het is aangetoond dat multivalentie kan worden gebruikt voor verhoging van de affiniteit van koolhydraten voor lectines, AB_5 toxines en bacteriën. In dit proefschrift wordt ingegaan op het ontwerp en synthese van nieuwe glycodendrimeren. Standaardmethoden zijn gebruikt om de affiniteit van deze liganden te bepalen. Tevens is er een microarray technologie ontwikkeld voor de bindingsanalyse van multivalente liganden.

Vaste-drager koolhydraat synthese

Hoofdstuk 2 beschrijft een nieuwe methode voor de vaste-drager synthese van koolhydraten. Doel van het onderzoek was het ontwikkelen van een strategie waarbij alleen eenvoudige bouwstenen gebruikt worden. Het onderscheid maken tussen de verschillende hydroxyl groepen werd gedaan met de suiker gebonden aan de vaste drager. Als linker werd het aminozuur cysteine gebruikt en nadat de suiker aan de drager was gekoppeld, werd de suiker geheel ontschermd. De klassieke selectieve bescherming van de C-6 groep werd aangetoond. De 4,4-dimethoxy trityl groep werd gebruikt als beschermgroep vanwege de mild zure afsplitsings condities en de visuele eigenschappen. Na selectieve tritylering werden de overgebleven hydroxyl groepen beschermd als benzoyl ester en werd de trityl groep verwijderd. Voor de glycosylering werd een eenvoudige glycosyl donor gebruikt. Na het afsplitsen van de drager werd het gewenste product geïsoleerd, helaas werd er ook een bijproduct gevonden wat gevormd werd door een intramoleculaire aglycon overdracht veroorzaakt door het nucleofiele zwavel atoom. De glycosylerings condities zijn gevarieerd maar de vorming van dit bijproduct kon niet worden voorkomen en daarom werd de strategie aangepast en is glutamine zuur als linker gebruikt. De C-6 strategie werd met deze

linker toegepast en ditmaal werd het gewenste product geïsoleerd. De strategie werd herhaald en het overeenkomstige trisaccharide kon worden geïsoleerd. Ondanks dat is aangetoond dat de strategie werkt, kon deze methode niet worden toegepast met andere beschermgroepen.

Synthese en bindings evaluatie van nieuwe multivalente galabiose liganden

Hoofdstuk 3 beschrijft een geoptimaliseerde methode voor de synthese van galabiose dendrimeren. Galabiose dendrimeren blijken goede inhibitoren te zijn voor de bacteriële adhesie van *S. suis*. De nieuwe synthetische strategie is gebaseerd op de enzymatische degradatie van polygalacturon zuur tot dimeren en trimeren. Chemische modificaties aan het dimeer leidde tot het gewenste galabiose bouwsteen. Alkyn gefunctionaliseerde dendrimeren werden gebruikt voor ligatie met behulp van de "click" reactie. De galabiose dendrimeren werden getest voor hun inhibitie capaciteit met een standaard hemagglutinatie experiment en de resultaten waren vergelijkbaar met de eerder gesynthetiseerde dendrimeren. De hier ontwikkelde route is economische gezien zeer geschikt voor de productie van grotere hoeveelheden galabiose dendrimeren en andere galabiose derivaten in vergelijking met volledige synthetische routes.

Inhibitie van Cholera Toxine met multivalente GM1os liganden

Nieuwe GM1os dendrimeren werden geïntroduceerd in hoofdstuk 4. Azide gefunctionaliseerd GM1os en GM2os werden gesynthetiseerd volgens enzymatische glycosylerings procedures. Mono- tot octavalente dendrimeren werden voorzien van lange spacer armen om de GM1 binding sites in het CT-pentameer te kunnen overbruggen. Het GM1 pentasaccharide en de dendrimeren werden aan elkaar gekoppeld middels de "click" reactie en de producten werden gezuiverd met chromatografie. Er werd ook een serie GM2os dendrimeren gesynthetiseerd, het GM2os heeft een veel lagere affiniteit voor CTB_5 in vergelijking met GM1os. Beide series dendrimeren werden getest op hun inhibitie capaciteit. Uit de uitgevoerde experimenten bleek een duidelijk multivalentie effect voor binding aan CTB_5, de octavalente verbinding bond minstens 380,000-keer sterker in vergelijking met het monovalent GM1os. Zoals verwacht bonden de GM2os dendrimeren veel zwakker, al bond het tetravalent GM2os dendrimeer sterker dan het monovalente GM1os.

Inhibitie van Cholera Toxine met multivalente galactose liganden

De in hoofdstuk 4 beschreven GM1os dendrimeren gaven een groot multivalentie effect, deze verbindingen zijn echter niet gewenst voor klinisch gebruik vanwege de dure synthese van het GM1os bouwsteen. Ondanks dat galactose een matige affiniteit heeft voor CTB_5, kan multivalentie gebruikt worden voor de synthese van hoge affiniteits liganden voor CTB_5. Hoofdstuk 5 beschrijft het ontwerp en de synthese van een galactose bouwsteen. Koppeling van dit bouwsteen aan de eerder beschreven dendrimeren gaf galactose liganden waarvan de tetra- en octavalente dendrimeren een vergelijkbare inhibitie te zien gaven met monovalent GM1os. Een serie met divalente dendrimeren met verschillende arm lengtes toonde aan dat de juiste armlengte een belangrijke factor is voor het maken van sterk bindende liganden voor CTB_5.

Microarray analyse met multivalente mannose liganden

Hoofdstuk 6 behandeld de synthese van mannose dendrimeren met een centraal amine functionaliteit waarmee het dendrimeer verankerd kan worden aan een chip oppervlak. Deze dendrimeren werden op een poreus aluminium oxide oppervlak geïmmobiliseerd en een microarray fluorescentie detectie systeem werd gebruikt voor de detectie van lectine binding aan het chip oppervlak. Mannose bindende lectines bonden aan het chip oppervlak als functie van tijd. Het ConA lectine gaf vergelijkbare bindingscurves voor de mono- tot octavalente dendrimeren. Met behulp van deze curves zijn bindingsconstanten bepaald. De microarray technologie is ook gebruikt voor een competitie experiment met een monovalent ConA ligand in oplossing. Hieruit werd een IC_{50} waarde voor MeαMan bepaald. Een ander mannose bindend lectine, GNA, gaf een duidelijk ander bindingspatroon. De mono- en divalente dendrimeren lieten een matige binding zien, terwijl de tetra- en octavalente dendrimeren beide een sterke toename in affiniteit lieten zien.

Curriculum Vitae

Hilbert Branderhorst werd geboren op 24 augustus 1980 te Wijk en Aalburg. Het VWO diploma werd in 1998 behaald aan het Willem van Oranje College te Waalwijk, waarna in dat zelfde jaar werd begonnen met de studie Scheikunde aan de Universiteit Utrecht. Het propedeutisch examen werd behaald in 1999. Het hierop volgende doctoraal programma werd afgesloten in 2003 met een afstudeer project bij de vakgroep Medicinal Chemistry & Chemical Biology onder begeleiding van Dr. R. J. Pieters. Het afstudeer onderzoek omvatte metaal gekatalyseerde reacties aan diazoacetaat gefunctionaliseerde suikers met als doel nieuwe functionaliteiten aan koolhydraten te introduceren. In augustus 2003 trad de auteur in dienst bij de hierboven genoemde vakgroep als assistent in opleiding (AIO) onder begeleiding van Dr. R. J. Pieters en Prof. Dr. R. M. J. Liskamp. Het eerste doel was het ontwikkelen van een nieuwe vaste drager synthese voor oligosacchariden. Later werd er een tweede onderzoekslijn gestart; de synthese van glycodendrimeren en de biologische evaluatie van deze. Mondelinge presentaties met betrekking tot het onderzoek zijn gegeven op het 23^{e} International Carbohydrate Symposium te Whistler (Canada) en op het 14^{e} European Carbohydrate Symposium te Lübeck (Duitsland). De resultaten van het promotie onderzoek zijn in dit proefschrift beschreven. Sinds november 2007 is de auteur werkzaam als post doctoraal onderzoeker bij dezelfde vakgroep. Doel van het project is het synthetiseren van nieuwe selectieve liganden voor Galectine-1 en Galectine-3. Tevens worden er nieuwe multivalente liganden gesynthetiseerd om meer inzicht te krijgen in de multivalentie effecten die gevonden zijn met de nieuw ontwikkelde microarray technologie. Vanaf 1 januari 2009 zal de auteur in dienst treden als Organisch Chemicus bij Cambridge Major Laboratories Europe, gevestigd te Weert.

List of Publications

- H. M. Branderhorst, J. Kemmink, R. M. J. Liskamp, R. J. Pieters; Catalytic conversions of diazosugars; *Tetrahedron Lett.* **2002**, *43*, 9601-9603
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- A. V. Pukin, H. M. Branderhorst, C. Sisu, C. A. G. M. Weijers, M. Gilbert, R. M. J. Liskamp, G. M. Visser, H. Zuilhof, R. J. Pieters; Strong inhibition of Cholera Toxin by multivalent GM1 derivatives *ChemBioChem* **2007**, *8*, 1500-1503
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International Symposia

- H. M. Branderhorst, R. M. J. Liskamp, R. J. Pieters; Synthesis of a resin bound disaccharide by on-bead selective C-6 chemistry followed by glycosylation (oral presentation, abstract) 23th International Carbohydrate Symposium, 23-28 july 2006, Whistler, Canada.
- H. M. Branderhorst, A. V. Pukin, C. Sisu, C. A. G. M. Weijers, M. Gilbert, R. M. J. Liskamp, G. M. Visser, H. Zuilhof, R. J. Pieters; Strong inhibition of Cholera Toxin by synthetic GM1 dendrimers (oral presentation, abstract) 14th European Carbohydrate Symposium, 2-7 september 2007, Lübeck, Germany.

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