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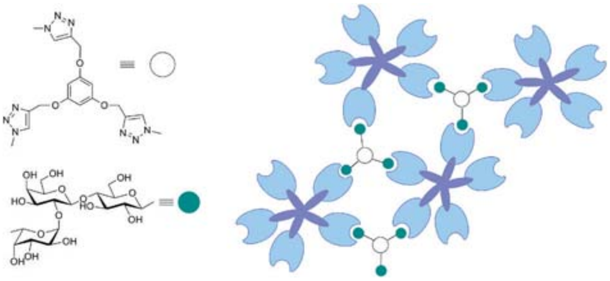
Guan-Nan Wang, Sabine André, Hans-Joachim Gabius and Paul V. Murphy*

Q3 The emerging functional versatility of cellular glycans makes research on the design of synthetic inhibitors a timely topic.



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PAPER

Bi- to tetravalent glycoclusters: synthesis, structure–activity profiles as lectin inhibitors and impact of combining both valency and headgroup tailoring on selectivity†

Q1

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The emerging functional versatility of cellular glycans makes research on the design of synthetic inhibitors a timely topic. In detail, the combination of ligand (or headgroup or contact site) structure with spatial parameters that depend on topological and geometrical factors underlies the physiological selectivity of glycan-protein (lectin) recognition. We herein tested a panel of bi-, tri- and tetravalent compounds against two plant agglutinins and adhesion/growth-regulatory lectins (galectins). In addition, we examined the impact of headgroup tailoring (converting lactose to 2′-fucosyllactose) in combination with valency increase in two assay types of increasing biorelevance (from solid-phase binding to cell binding). Compounds were prepared using copper-catalysed azide alkyne cycloaddition from peracetylated lactosyl or 2′-fucosyllactosyl azides. Significant inhibition was achieved for the plant toxin with a tetravalent compound. Different levels of sensitivity were noted for the three groups of the galectin family. The headgroup extension to 2′-fucosyllactose led to a selectivity gain, especially for the chimera-type galectin-3. Valency increase established discrimination against the homodimeric proteins, whereas the combination of valency with the headgroup extension led to discrimination against the tandem-repeat-type galectin-8 for chicken galectins but not human galectins-3 and -4. Thus, detailed structure–activity profiling of glycoclusters combined with suitably modifying the contact site for the targeted lectin will help minimize cross-reactivity among this class of closely related proteins.

Introduction

The steadily growing body of evidence on the physiological significance of protein (lectin)-glycan recognition gives reason to aim at lectin-directed rational drug design in order to block clinically unfavourable interactions, *e.g.* in inflammation, tumour progression or pathogen adhesion.[1] Clues to which parameters deserve special attention in the design of (bio)pharmaceuticals come from the delineation of the levels of affinity regulation of glycan binding to lectins.[2] The ligand structure (headgroup and aglycone) and the spatial presentation of the ligand in glycoclusters are two key features to be considered in attaining the overall objective. A third is the valency order (monovalent *vs.* bivalent *vs.* trivalent *etc.*). In considering spacing, geometry, topology and inter-ligand distances need to be taken into account. The elegant work on targeting membrane lectins with a sterically rigid presentation of carbohydrate-binding sites, especially the hepatic asialoglycoprotein receptor using a triantennary *N*-glycan or synthetic cluster glycosides with a matching spatial arrangement, laid the foundation for the concept of the glycoside cluster effect.[3] In that case, a numerical increase in valency from one to three reactive headgroups in a neoglycoconjugate led to an enhancement in affinity, mimicking the potency of the type I triantennary *N*-glycan.[4] Since the natural effector activity of lectins, presented in membranes or in solution, is based on binding to structurally and spatially suitable counter-receptors, devising an adequately tailored combination of these two parameters is considered helpful in addressing the challenge to design inhibitors with optimal potency. Given the wide variety of natural ways for lectin-site presentation[2] and the diversity of (bio)chemical scaffolds which can be used in glycocluster formation,[5] a clear study design will help to discern structure–activity relationships for medically relevant lectins. Following our initial reports on different types of bivalent lactosides,[6] we herein explore the effect of stepwise increases in valency, from mono- to tetravalency, as well as changing the structure of the headgroup from lactose to the histo-blood H-type structure – 2′-fucosyllactose (Chart 1). In detail, the monovalent control compounds that were investigated are 2′-fucosyllactose (FL) **1** and

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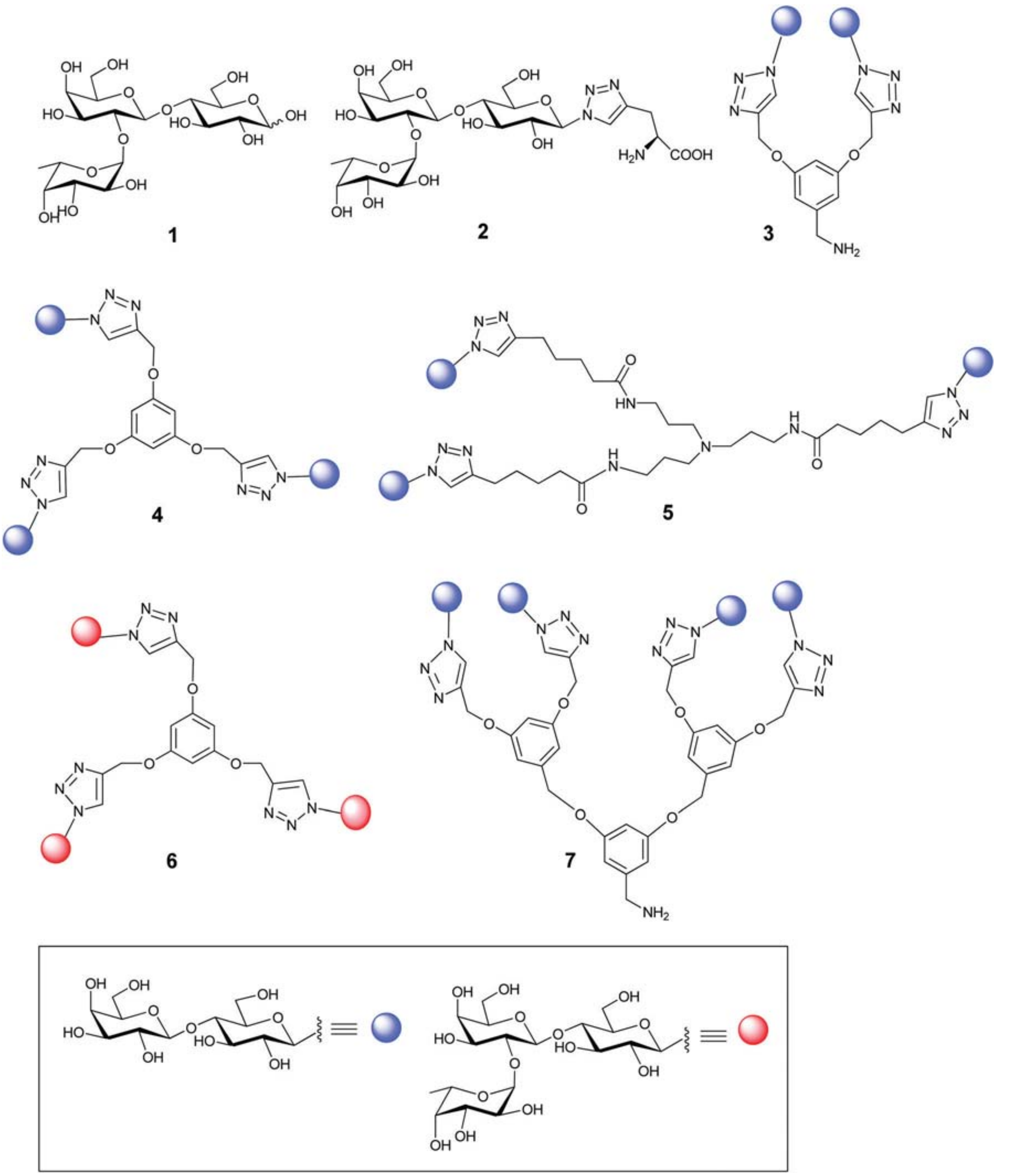


Chart 1 Structures of the synthetic compounds **1–7**.

a triazole conjugate **2**, together with lactose. The set of lactose-presenting di-, tri- and tetravalent glycoclusters **3–5** and **7** and the trivalent FL derivative **6**, which has the same core scaffold as **4**, complete the compound panel. The testing of this panel was performed in assays involving the same group of lectins in the quest to define the impact of the given two parameters on bioactivity.

The test panel of lectins investigated herein consists of a plant toxin (*Viscum album* L. agglutinin, VAA) and two types of β-sandwich-fold proteins, *i.e.* a leguminous lectin (*Erythrina crystagalli* agglutinin, ECA) and adhesion/growth-regulatory galectins. Thus, the synthetic compounds are tested for potency as anti-toxin (VAA) and for reactivity to lectins sharing the same fold but differing in positioning the lectin sites (ECA, galectins). We comprehensively studied all known members of the latter family from one organism, *i.e.* the five chicken galectins (CGs), to spot intrafamily differences and added work on two human galectins, *i.e.* Gal-3/-4, for comparison. These lectins' contact sites for carbohydrates are presented in three modes of topological display: proto-type (non-covalently associated homodimers: CG-1A/-B/-2), tandem-repeat-type (two different domains covalently linked by a peptide: CG-8, Gal-4) and chimera-type (a single carbohydrate recognition domain connected to a collagenase-sensitive stalk and an N-terminal section with two acceptors for serine phosphorylation: CG-3, Gal-3).[7] With regard to the carbohydrate the extension from lactose to the histo-blood H-type structure was expected to have little impact on VAA/ECA,[8] to slightly prefer CG-1B when compared to CG-1A[9] and to increase the affinity for CG-8,[10] Gal-3[11] and Gal-4.[12] The respective comparative measurements provide an instructive example for the influence of headgroup/valency tailoring on lectin affinity and selectivity. They were performed in two experimental set-ups. The first is a solid-phase assay, in which extent of lectin binding to a glycoprotein matrix (asialofetuin, ASF, which has up to nine *N*-acetyllactosamine termini on its bi- and triantennary *N*-glycans; they all can bind to VAA and galectins[13]) was determined. To increase biorelevance, that is to work with cells, an assay was subsequently utilised where blocking of lectin binding to the cell surface was assessed.

Results and discussion

Synthesis

The synthetic routes to the bi-, tri- and tetravalent alkyne precursors to **1–8** are shown in Scheme 1. The dialkyne **9** and tetraalkyne **11** were obtained *via* the reaction of alkyl bromides **8** and **10** with potassium phthalimide (PhthK).[14] 1,3,5-Tris(alkynyloxy)-benzene **13** was prepared from phloroglucinol **12** and propargyl bromide.[15] The trialkyne **16** was made from the coupling reaction of **14** and **15**.

Multivalent lactosides were all prepared from the lactose azide **17** (Scheme 2).[6a] Thus alkyne **9**, **11**, **13**, **16**, when reacted with **17** using copper(I)-catalysed azide alkyne cycloaddition (CuAAC)[16] reactions, gave the protected intermediates **18–21**. The CuAAC reactions were carried out using the *in situ* reduction of copper(II) sulphate by sodium ascorbate in aqueous solution using methanol as a co-solvent. As the number of alkynes increased, the completion of CuAAC reaction was found to be more difficult to achieve. Similar to the situation in the synthesis of compounds **19**, **20** and **21**, either ultrasonic radiation[17] or heating was required to accelerate the reactions. Both phthalimido groups and acetyl groups in **18** and **19** were removed using ethylenediamine in ethanol by heating at reflux, giving **3** and **7** after washing the solid product with small amount of methanol. The trivalent lactosides **20** and **21** afforded **4** and **5** after Zemplén deacetylation.

The synthesis of trisaccharide **24** was achieved *via* glycosidation with the fucosyl donor **22**[18] and acceptor **23** (Scheme 3). For the synthesis of **23** an approach originally described by Matta and co-workers was used.[19a] Two promoter systems NIS–TfOH (46%) and benzenesulfinylpiperidine (BSP)–Tf_2O (70%) were comparatively investigated for the glycosidation to give the trisaccharide **24**. In terms of reaction time and yield, the BSP–Tf_2O promoter system turned out to be substantially better for this glycosidation. The anomeric configuration of the glycosidic linkage between fucose and lactose residues was assigned based on the size of the coupling constant ($J_{1'',2''}$ = 3.3 Hz) in the ^{1}H NMR spectrum. The signal for C-1″ occurred at δ 95.2 ppm in the ^{13}C-NMR spectrum. Debenzoylation of **24** was carried out using methanolic sodium methoxide, hydrolysis of the acetonide groups using 60% acetic acid at 60 °C and subsequent catalytic hydrogenolysis provided FL **1**.[19,20] This trisaccharide was acetylated and the azide group was introduced using $SnCl_4$ and $TMSN_3$ to give **27** (Scheme 3). It is worth mentioning that the α-glycosidic linkage was sensitive to the Lewis acid if the benzyl groups were present on the fucose residue while trying to introduce the azide group to form a fucosyllactosyl azide. The use of $TMSN_3$-$SnCl_4$, 33% HBr in AcOH, and $BiBr_3$/TMSBr[21] all led to the cleavage of this fucosidic bond. In contrast, the fully acetylated FL was found to be more stable.

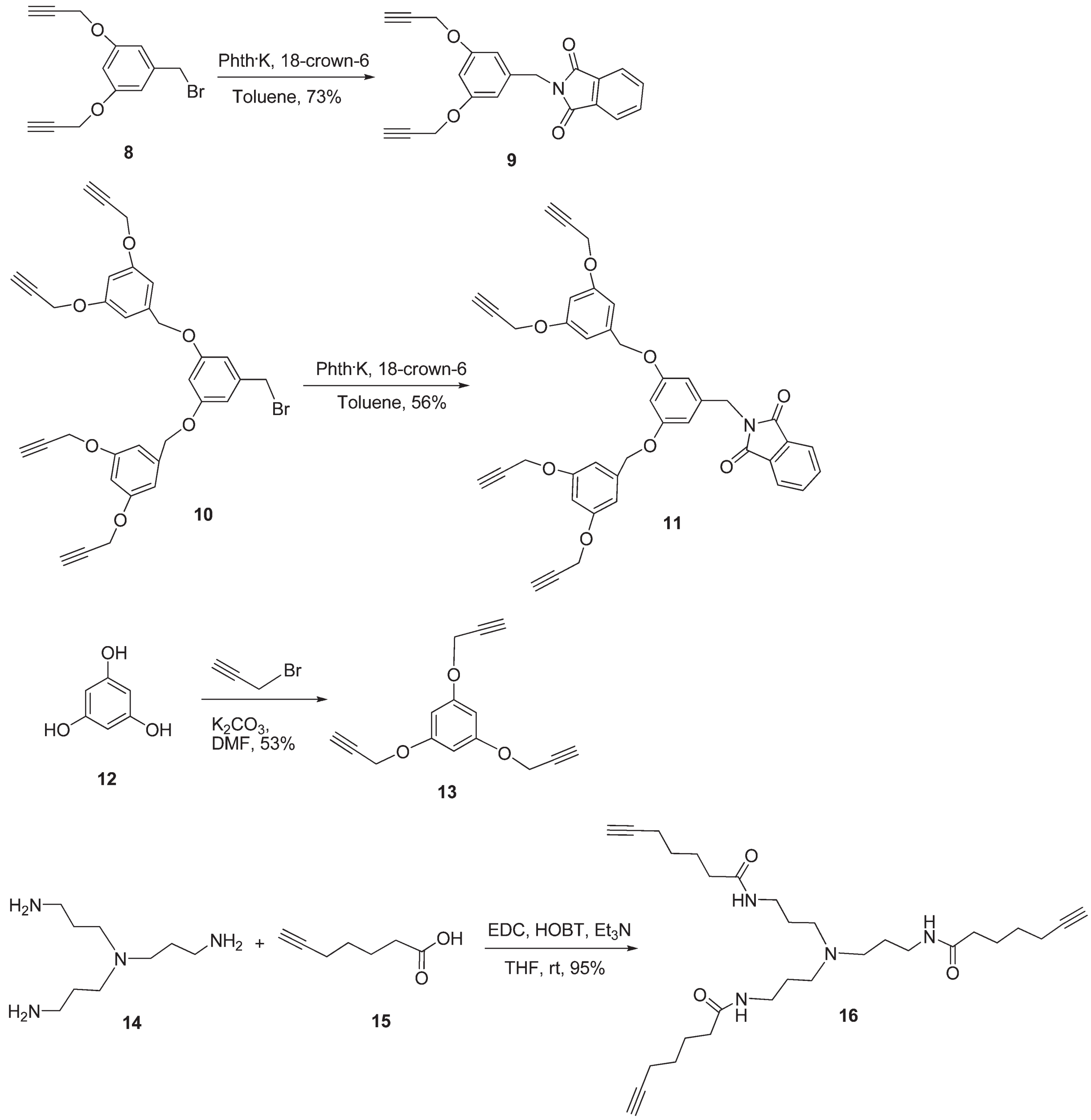
The CuAAC reaction between fucosyllactosyl azide **27** and Fmoc-protected L-propargylglycine,[22] which was followed by protecting group removal using initially piperidine and then methanolic sodium methoxide, provided the glycamino acid **2** (Scheme 4). The trivalent fucosyllactoside **6** was prepared *via* click reaction of 1,3,5-tris(alkynyloxy)benzene **13**[15] and fucosyllactosyl azide **27** and subsequent deacetylation. Having herewith completed the synthetic component of this work, insights into the spatial property of the maximal distance between sugar headgroups within each type of glycocluster were obtained by molecular modelling.

Molecular modeling

The assessment of the distance between the sugar headgroups in the synthetic glycoclusters was set as goal for this part of the study. As a common feature and not influenced by changes in the conformation of the terminal galactose unit, the anomeric center of the glucose moiety was selected as the reference point. Using the Maestro interface, adequate constraints were applied (distances between residues, angles) in an iterative fashion to generate extended conformations. While maintaining these constraints energy minimizations were performed using Macromodel (OPLSAA force field, gas phase). The resulting extended conformations are shown in Fig. 1. As expected, the distances between headgroups in **3** and **4** were rather similar at 13–15 Å. This goes well beyond the 5.9 Å/8.1 Å seen in fully extended or backfolded biantennary *N*-glycans, in which both branch-end sugars can bind with the tested lectins.[13,23] The long and mostly aliphatic spacer in **5** facilitates the lactose residues to attain an interligand distance limit of about 27 Å (Fig. 1), which is ∼5 Å more than that found in the core-disubstituted *N*-glycan with a backfolded and an extended antenna.[23] As depicted in Fig. 1, the glucose anomeric carbon atoms in **7** adopt a rectangular arrangement with the following set of distances: ∼14 Å and ∼18 Å along the sides and ∼22 Å on the diagonal. Of course, in all cases the inherent flexibility in the scaffolds would allow the positions of the headgroups to fluctuate in space and time and acquire conformers with lowered levels of spatial headgroup separation. The comparison to the biantennary *N*-glycans described confirms that there are no spatial restrictions which would impede headgroup reactivity with the lectins in the tested panel.

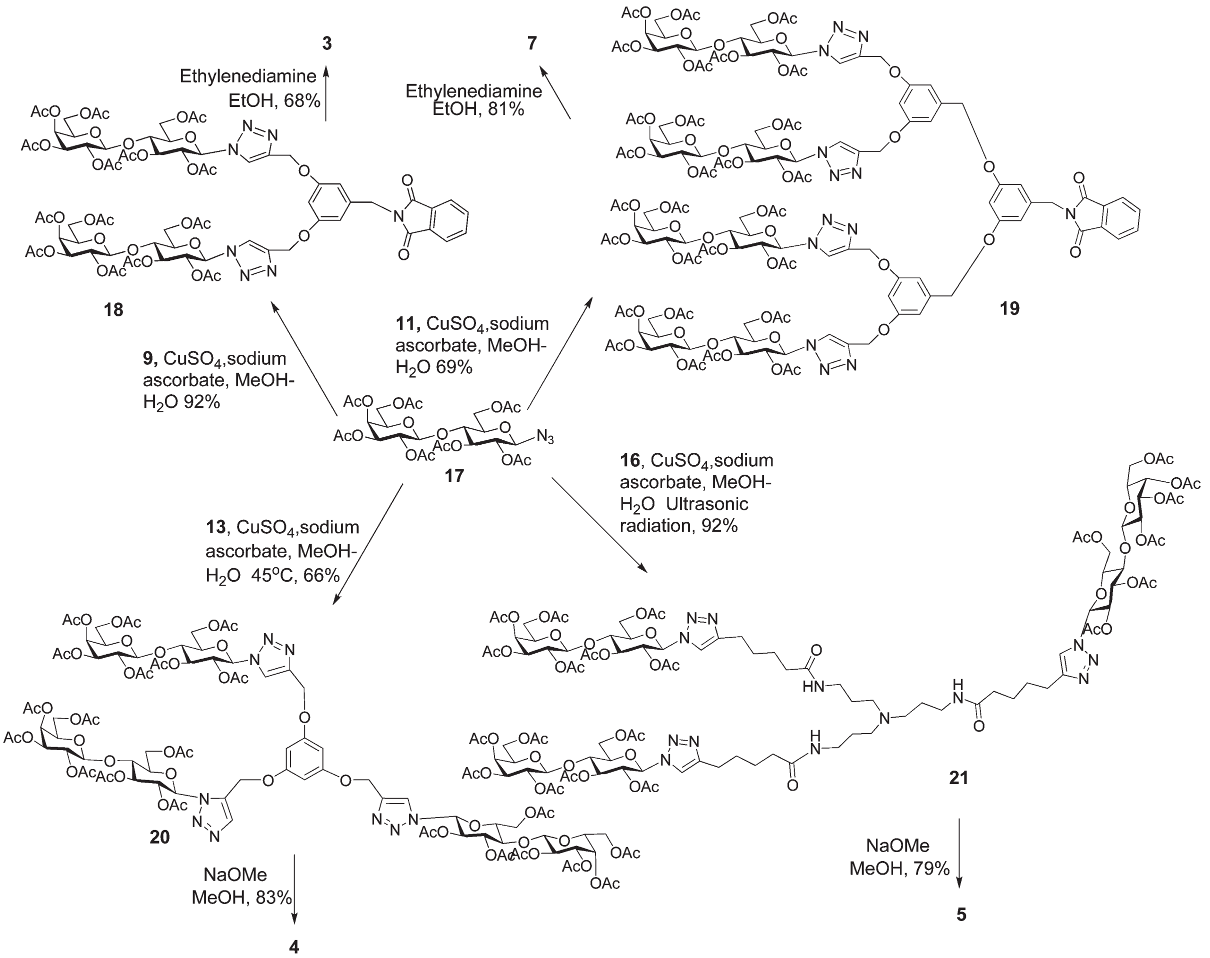
Assaying inhibitory properties on plant lectins

In principle, the solid-phase assay reflects the physiological situation, in which the lectin in solution can bind to surface-presented glycans. This binding can be disrupted by inhibitors. The glycoprotein asialofetuin was adsorbed to the plastic surface of



Scheme 1 Synthesis of alkyne precursors.

microtitre plate wells, and the evaluation of lectin-glycoprotein binding was in each case shown to be saturable and inhibitable by the cognate sugar lactose but not by mannose or glucose. In order to reach optimal sensitivity experimental conditions were defined so that the signal increase associated with increasing lectin concentration and consequently binding to the glycoprotein was in the linear and not saturated range. Titrations with the synthetic products as inhibitors at a constant lectin concentration established curves of decreasing signal intensity. The apparent inhibitory activity fulfilled the expectation raised by the molecular modelling. In order to compare the relative potencies, these curves enabled us to define the concentration of sugar presented by the compounds at which a 50% decrease in optical density (IC_{50}-value) was reached (for an example, please see Fig. 2). In accordance with previous affinity measurements[8] the trisaccharide FL **1** was not found to be more active than lactose (Table 1, see end of document). However, a major avidity increase occurred in the progression to tetravalency for the toxin (Fig. 2, Table 1). This held true for its hololectin constituted by dimers of the toxin (A) and lectin (B) subunits (in which the two accessible Tyr249 sites are separated by 87 Å) and also the isolated B-chain (with a distance between the Tyr249 and Trp38 sites of 62 Å[24]), all distances thus beyond the range coverable by the glycoclusters **3–7**. The Trp38 sites are only 15 Å apart but not fully accessible.[24] In contrast, the positioning of contact sites on opposing sides of the leguminous lectin ECA apparently precluded there being an enhancement for the tetravalent compound **7**. Previous experience with tetravalent glycoclusters attests that



Scheme 2 Synthesis of di- to tetravalent lactosides.

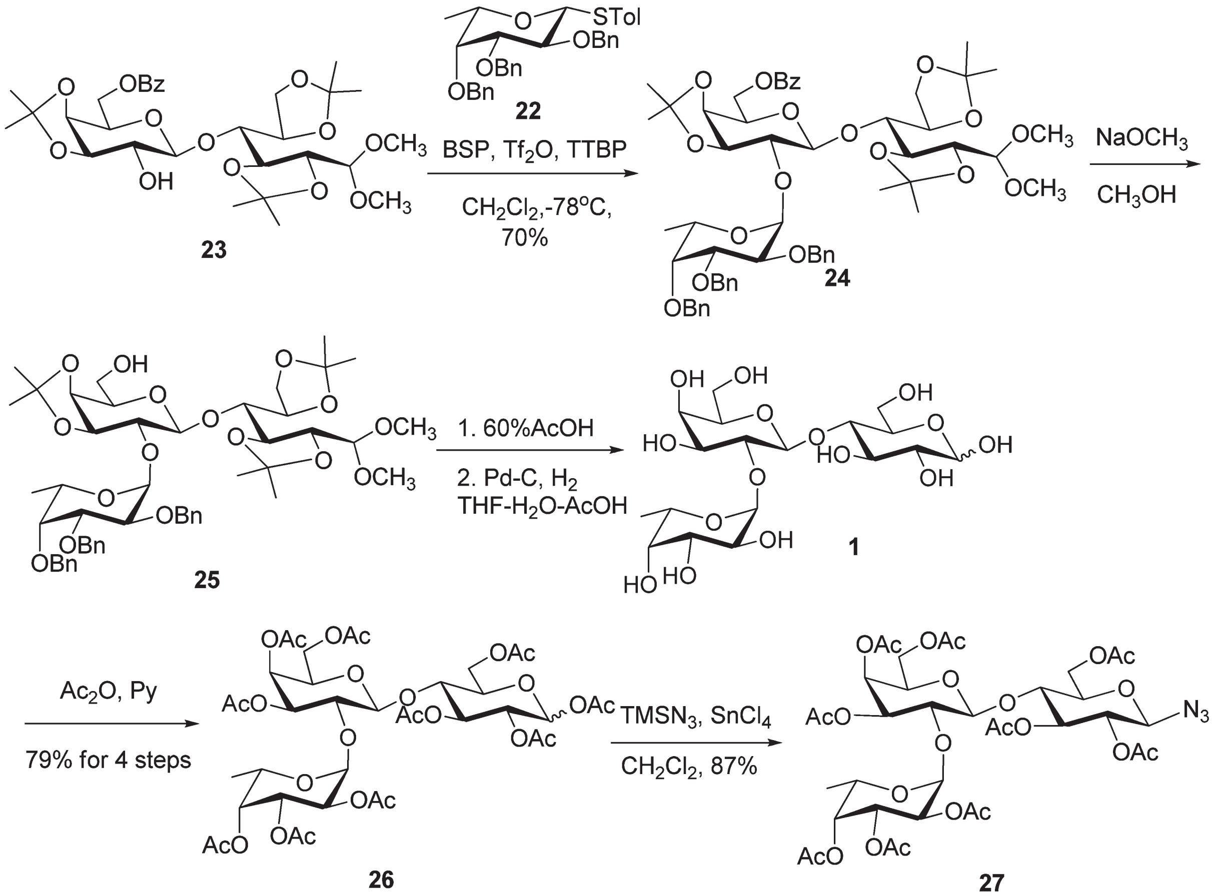
not just valency but the geometric mode of ligand presentation matters, giving both functionalized dendritic poly(amidoamine) and pentaerythritol-based compounds exceptional potencies.[25]

Since the type of glycan display on the matrix (*e.g.* branching mode of *N*-glycans) can have an influence on the inhibitory efficiency of glycoclusters,[25a,e] it was essential to confirm potency on a more relevant physiological level, *i.e.* by revealing the efficiency of compounds to protect cells from lectin binding. Thus, we performed cell assays with fluorescently labelled lectin.

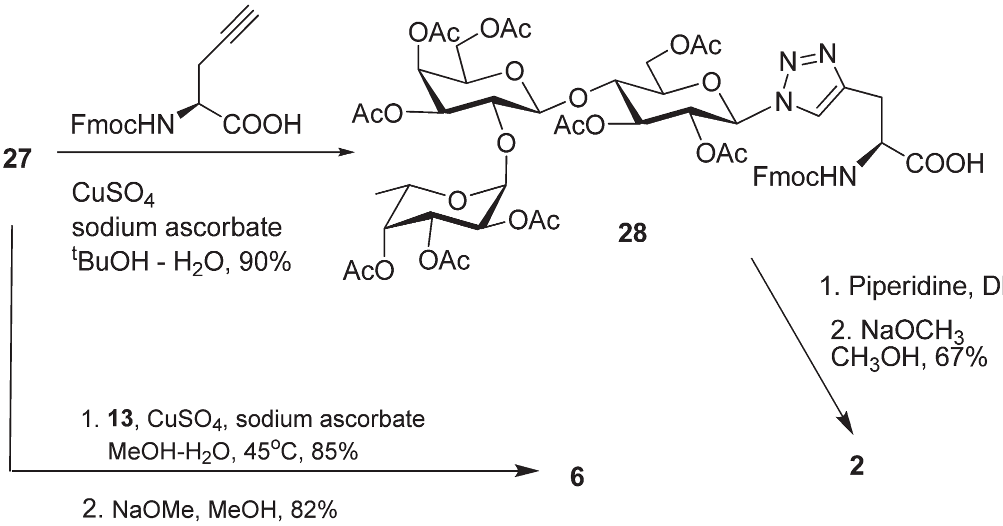
In this type of assay, the synthetic compounds compete with cell surface glycans for lectin binding. The extent of signal reduction (in terms of percentage of positive cells and mean fluorescence intensity) that results by blocking lectin binding was determined. Assays were routinely performed with aliquots of the same cell suspension, avoiding prolonged culture times and routinely running internal standards (0%/100%-values, inhibition with lactose). As in the case of the solid-phase system, dependence of signals on lectin concentration and presence of cognate sugar was first ascertained, as exemplarily shown for VAA (Fig. 3A and B). Compared to lactose the inhibitory capacity of the test compounds was in most cases only slightly improved (please see documented examples for the bivalent compound **3** and the trivalent compound **4** in Fig. 3C and D), in accord with the solid-phase-based data (Table 1). Tetravalent compound **7**, which reached approximately a 20-fold enhancement (Table 1), was the most potent inhibitor. While 20 mM lactose led to decreases to 50%/23.6 from the control level of 72%/89.3, the presence of 0.5 mM lactose in a glycocluster presentation reduced lectin binding to the cells to 50%/32.9 (Fig. 3B and D). Overall, correlation between the results of the two types of assays (solid phase *vs.* cell) was thus found. Having started with two plant lectins, we proceeded to measurements with the five CGs. They exhibit sequence variations in their carbohydrate recognition domains and cover the three modes of lectin-site presentation, an attractive model to address the issues on impact and headgroup extension and valency.

Assaying inhibitory properties on chicken galectins

The structure of the headgroup was clearly relevant among this group of lectins (Table 1). A lowered affinity with CG-1A had been indicated by frontal affinity chromatography.[11d] The same holds true for human and rat Gal-1.[11a,26] Occurrence of intrafamily differences between CGs was further underscored by slight enhancement of reactivity for CG-8[10] and reduced sensitivity to α1,2- or α1,3-substitutions seen for CG-1B relative to CG-1A.[9]



Scheme 3 Synthesis of FL and fucosyllactosyl azide **27**.



Scheme 4 Synthesis of fucosyllactosyl conjugates **2** and **6**.

The comprehensive profiling of lectin reactivity to **1–7** singled out the chimera-type lectin as the most responsive (Table 1). The CG-3 was especially reactive with FL and found to be susceptible to an increase in valency, with the highest affinity being observed towards **6** (Table 1). The proteolytic removal of the collagenase-sensitive stalk, which underlies galectin-3′s capacity to form stable aggregates in the presence of oligovalent ligands,[27] did not reduce the relative affinity to lactose but did impair the sensitivity to valency increase (comparing lactose and **1–7** for CG-3 and trCG-3). Tri- and tetravalency affected the tandem-repeat-type CG-8 and its separate domains differently, and the increase in length of the linker peptide from nine (CG-8S) to 28

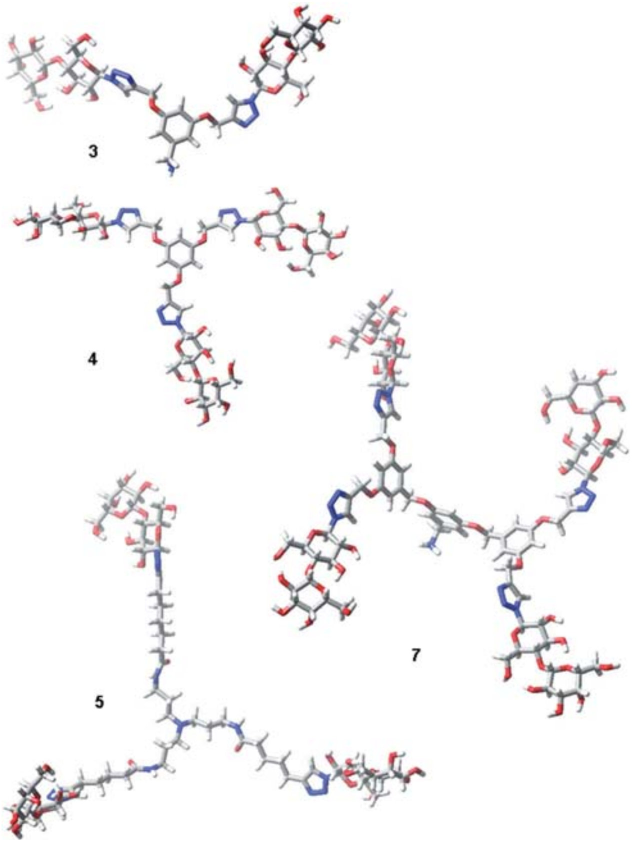


Fig. 1 Models of extended conformers of compounds **3–5** and **7**.

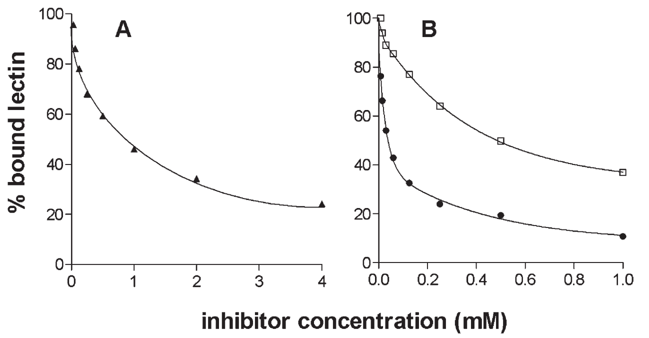


Fig. 2 Titration curves illustrating the course of inhibition of binding of biotinylated VAA (1.5 μg ml^{-1}) to asialofetuin in a solid-phase assay upon increasing the concentration of lactose (▲, A) as well as (B) trivalent compound **4** (□) and tetravalent compound **7** (●).

amino acids (CG-8L) appeared to be associated with a minor enhancement (Table 1). In terms of achieving selectivity, the modification to include fucose in the headgroup when combined with trivalency as seen in **6** led to the highest inhibition for the full-length CG-3, less so for CG-8 and its domains. Drawing on data for human Gal-3 affords a route to further enhancements. Since the fucose moiety in α1,2-linkage is only weakly involved in interactions to human Gal-3 relative to the additional α1,3-substitution in histo-blood group AB-determinants based on flexible ligand docking,[11f] a further elaboration to generate a

Table 1 IC_{50}-values of the mono- to tetravalent lactosides[a] and free lactose (Lac) for blocking binding of biotinylated plant and chicken lectins to surface-immobilized ASF (in mM)

Lectin inhibitor	VAA (1.5 μg ml^{-1})	VAA-B (0.5 μg ml^{-1})	ECA (0.2 μg ml^{-1})	CG-1A (3 μg ml^{-1})	CG-1B (1 μg ml^{-1})	CG-2 (2 μg ml^{-1})	CG-3 (0.5 μg ml^{-1})	trCG-3 (2.5 μg ml^{-1})	CG-8S (3 μg ml^{-1})	CG-8L (0.5 μg ml^{-1})	CG-8N (3 μg ml^{-1})	CG-8C (3 μg ml^{-1})
1	1.8 (0.4)	1.7 (0.6)	0.35 (1.4)	0.9 (0.4)	1.9 (0.9)	4.8 (1.3)	0.14 (3.6)	0.34 (3.5)	3.1 (1.2)	2.6 (1.2)	1.4 (1.1)	1.2 (1.6)
2	1.6 (0.5)	1.5 (0.7)	0.4 (1.3)	0.8 (0.5)	1.8 (1)	4.6 (1.3)	0.12 (4.2)	0.44 (2.7)	3.2 (1.2)	2.4 (1.3)	2.2 (0.7)	2.1 (0.9)
3	0.6 (1.3)	0.9 (1.1)	0.32 (1.6)	0.24 (1.7)	1.5 (1.2)	4.2 (1.4)	0.18 (2.8)	0.9 (1.3)	3.1 (1.2)	3.2 (0.9)	1.8 (0.9)	2.2 (0.9)
4	0.5 (1.6)	0.7 (1.4)	0.28 (1.8)	0.26 (1.5)	2.2 (0.8)	4.9 (1.2)	0.08 (6.3)	0.56 (2.1)	2.6 (1.5)	2.3 (1.3)	0.8 (2.0)	1.4 (1.4)
5	0.6 (1.3)	0.6 (1.7)	0.31 (1.6)	0.31 (1.3)	2.6 (0.7)	5.2 (1.2)	0.06 (8.3)	[b]	2.1 (1.8)	1.8 (1.7)	[b]	0.13 (15)
6	1.1 (0.7)	1.0 (1)	0.24 (2.1)	1.1 (0.4)	2.0 (0.9)	4.8 (1.3)	0.008 (63)	0.09 (13)	1.1 (3.5)	0.82 (3.7)	0.17 (9.4)	0.16 (12)
7	0.04 (20)	0.03 (33)	0.26 (1.9)	0.16 (2.5)	2.2 (0.8)	3.2 (1.9)	0.012 (42)	0.26 (4.6)	0.25 (15)	0.16 (19)	0.36 (4.4)	0.86 (2.2)
Lac	0.8 (1)	1 (1)	0.5 (1)	0.4 (1)	1.8 (1)	6 (1)	0.5 (1)	1.2 (1)	3.8 (1)	3 (1)	1.6 (1)	1.9 (1)

[a] For structures see Chart 1; titrations were performed using a fixed glycoprotein quantity for coating (0.5 μg per well) with eight concentrations of sugar in duplicates and up to five independent series, reaching an upper limit of 14.8% for the standard deviation (for exemplary titration curves, see Fig. 1); the concentration is always given for lactose, free in solution or conjugated to a scaffold. [b] Tendency for stimulation at concentrations above 1 mM; numbers in brackets denote relative potency.

tetrasaccharide rather than a trisaccharide headgroup will be conducive for affinity increase, shown calorimetrically to move ΔG from −16.4 kJ mol^{-1} for lactose and −19.15 kJ mol^{-1} for FL to −24.88 kJ mol^{-1} for the histo-blood group A-tetrasaccharide (at about 280 K).[28] In inhibition assays, the relative potency, with lactose set to 1, increased by a factor of 2.8 for FL and to 35 by the added α1,3-substitution.[12b] As noted above for the plant agglutinins, cell assays with the chicken galectins also corroborated the changes in inhibitory potency listed in Table 1. These experiments *e.g.* illustrated the relative efficiency of the trivalent **6** to block binding of trCG-3 (0.1 mM sugar presented by **6** yielded a decrease from 51%/32.9 (control) to 32%/15.0 compared to 45%/19.7 for 1 mM lactose; Fig. 4A–D) and the N-terminal domain of CG-8 (Fig. 4E and F). In order to ensure that these results from CG-3 can be extrapolated to the human orthologue we next ran experiments under identical conditions with human Gal-3. Because glycoprotein binding of the tandem-repeat-type Gal-4 had been reported to be sensitive to ligand presentation by cyclic scaffolds (calixarenes,[15d] cyclodecapeptides[29]), we performed respective experiments with this two-domain protein and its separate domains in parallel.

Assaying inhibitory properties on human galectins

The obtained data document interspecies maintenance of sensitivity for respective headgroup tailoring and valency increase in the case of the chimera-type protein (Table 2). The results are also in accord with the 2.8-fold affinity enhancement previously reported for FL in a similar inhibition assay.[12b] Binding of Gal-4 proved to be rather susceptible to the presence of the tetravalent compound **7**, being less so towards the trivalent compound, so that this structural design appears to hit tandem-repeat-type galectins (Gal-4, CG-8) as well as the chimera-type Gal-3 (Table 2). The strong inhibitory potency of **7** on cell binding, in comparison to the bi- and trivalent substances, is illustrated in Fig. 5, again correlating rather well with the results from the solid-phase assay. The monovalent association of the separate domains of Gal-4 to glycoprotein glycans could also be effectively blocked more strongly than in the case of the lectin domain of Gal-3 (Table 2). Of note, set in relation to the plant toxin, the case of dithiodigalactoside, which shows low-affinity binding, if at all, to human galectins, exemplifies the possibility for markedly different headgroup affinities despite presence of galactose and thus enabling the targeting of the toxin with a galactose-based compound while avoiding side effects that would result from binding to galectins.[30]

Conclusions

The molecular characterization of the counterreceptors for tissue lectins is unlocking the virtues of spatial parameters for generating the high-level selectivity found in nature. In fact, their local density can matter in different contexts up to the presentation of target sites in membrane microdomains.[2] The fundamental importance of this property, that is a particular spatial organization of carbohydrate recognition groups and not just their mere presence, has recently been proven for galectins-1 and -3 in relation to their high-affinity binding to ganglioside GM1 exposed on human neuroblastoma cells by perturbing the integrity of microdomains.[31] This switch-like impact on affinity, together with similar effects on the other levels given in Table 1, prompts the efforts to delineate detailed structure–activity profiles for glycoclusters.

Proceeding from our previous reports on bivalent presentation,[6] we herein delineate special sensitivity of the tandem-repeat and chimera-type galectins for the tested tri- and tetravalent compounds when compared to the group of homodimeric proteins. Synthetic α1,3-substitution had even been found to add to the discrimination between galectins-1 and -3 on the level of cell binding.[32] Evidently, the tested natural headgroup elaboration could enhance the respective potency for galectin-3. This result intimates the possibility of such tailoring to allow the attainment of affinity differences between the chimera- and tandem-repeat-type proteins and also within the latter group. Along this line, the identification of distinct natural docking sites for certain lectins, *e.g.* sulphated glycosphingolipids for galectin-4,[33] and of aglyconic extensions conferring selectivity gains such as the β-naphthyl sulfone[34] will help achieve stepwise progress. And here choosing the optimum geometry to match an increase in valency can come into play, because the comparison of inhibitory capacity of tetravalent clusters built with different scaffolds[25] (for scaffold development[35]) underlines the fact that geometry can matter markedly. Our results on CG-3/CG-8 and the combination of trivalency with headgroup tailoring encouraged further consideration of the feasibility of this proposal. The same strategy of changing the headgroup could be applied to the toxin, which tolerates α2,6-sialylation of lactose in sharp contrast to the galectins, hereby precluding cross-reactivity of an anti-toxin compound with the galectins. Giving direction to further work, the detailed analysis of lectin specificity will continue to provide inspirations for the design of the contact region. Equally important, comparative analysis within and between lectin families will be required to track down the most suited glycocluster design to attain optimal selectivity.

Experimental section

General experimental

Unless otherwise noted, all commercially available compounds were used as provided without further purification. Solvents for chromatography were technical grade. Petroleum ether 40–60 °C was used for column chromatography and thin layer chromatography (TLC). NMR spectra were recorded (25 °C) with 500 MHz spectrometer. The frequency is 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Data are reported in the following order: chemical shift (δ) in ppm; multiplicities are indicated s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet); coupling constants (J) are given in Hertz (Hz). Chemical shifts are reported relative to internal Me_4Si in $CDCl_3$ (d 0.0) or HOD for D_2O (d 4.72, 25 °C) for ^{1}H and Me_4Si in $CDCl_3$ (d 0.0) or $CDCl_3$ (d 77.0) for ^{13}C. ^{1}H NMR signals were assigned with the aid of COSY, ^{13}C NMR signals using DEPT, gHSQCAD and/or gHMBCAD. Low- and high-resolution mass spectra were in positive and/or negative mode as indicated in each case. TLC was performed on aluminium sheets precoated with silica gel and spots visualized by UV and charring with H_2SO_4–EtOH

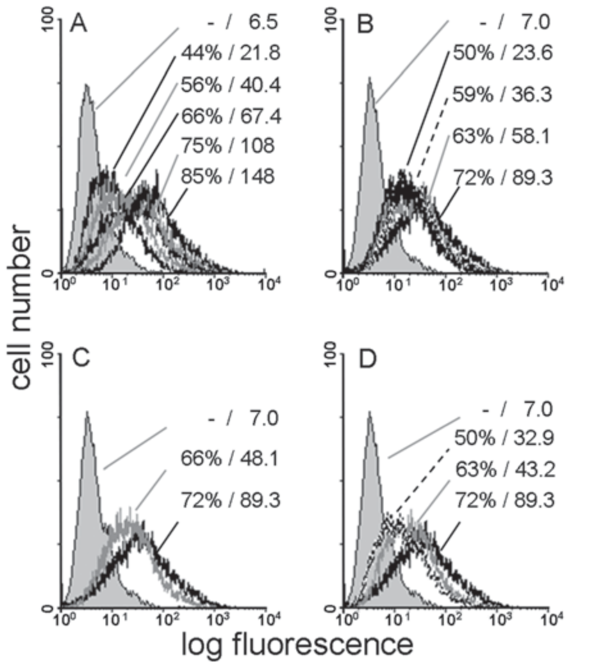


Fig. 3 Semilogarithmic representation of fluorescent surface staining of human B-lymphoblastoid cells (Croco II) by VAA. The control value (background) for the signal obtained by processing cells with the fluorescent indicator in the absence of lectin is given as grey-shaded curve area, the 100%-value (lectin-dependent staining without presence of inhibitor) as thick black line. Numbers for staining (percentage of positive cells/mean fluorescence intensity) are added to each panel in the order of listing compound/concentration (from top to bottom) here. All concentrations are given in terms of the sugar (free or conjugated to a scaffold). A: staining parameters measured with increasing concentrations of VAA from 1 μg ml^{-1} to 2 μg ml^{-1}, 4 μg ml^{-1}, 5 μg ml^{-1} and 6 μg ml^{-1}. B: inhibition of VAA staining (4 μg ml^{-1}) by lactose concentrations of 20 mM, 4 mM and 1 mM relative to the 100%-value in the absence of inhibitor. C: inhibition of staining by 1 mM lactose presented by trivalent compound **4** relative to the 100%-control. D: inhibition of staining by 0.5 mM lactose presented by tetravalent compound **7** and 2 mM lactose presented by bivalent compound **3**.

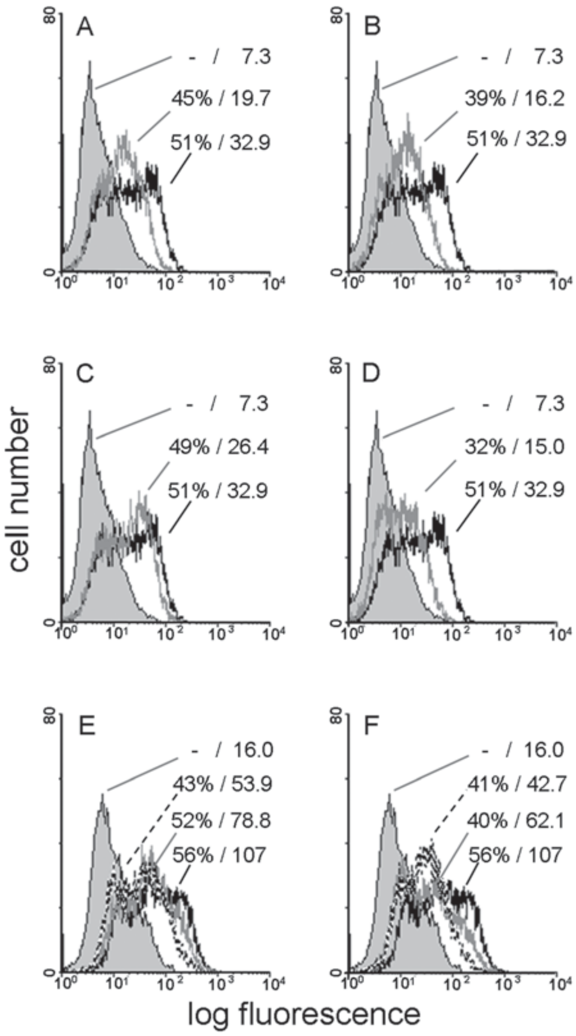


Fig. 4 Semilogarithmic representation of fluorescent surface staining of Chinese hamster ovary cells by proteolytically truncated CG-3 (A–D) and the N-domain of CG-8, *i.e.* CG-8N (E, F) (for further details, please see legend to Fig. 2). A–D: inhibition of staining with trCG-3 (2 μg ml^{-1}) by 1 mM lactose (A), 0.5 mM lactose presented by trivalent compound **4** (B), 0.5 mM lactose presented by trivalent compound **5** (C) and 0.1 M sugar presented by trivalent compound **6** (D). E, F: inhibition of staining with CG-8N (10 μg ml^{-1}) by 4 mM and 0.5 mM lactose (E) as well as by 0.5 mM 2′-fucosyllactose presented by trivalent compound **6** and 4 mM derivatized 2′-fucosyllactose (F).

(1 : 20), or cerium molybdate. Flash chromatography was carried out with silica gel 60 (0.040–0.630 mm) and using a stepwise solvent polarity gradient correlated with TLC mobility. CH_2Cl_2, MeOH, toluene and THF reaction solvents were used as obtained from a Pure Solv™ Solvent Purification System. Anhydrous DMF, pyridine, and EtOH were used as purchased.

2-(3,5-Bis(prop-2-ynyloxy)benzyl)isoindoline-1,3-dione 9

To a mixture of potassium phthalimide (408 mg, 2.2 mmol) and bromide **8** (500 mg, 1.8 mmol) in toluene (15 mL) was added 18-crown-6 (49 mg, 0.18 mmol). The mixture was heated at 100 °C for 6 h with stirring under N_2 and then water was added. The organic layer was separated and aqueous layer was extracted with CH_2Cl_2. The organic portions were combined, then dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. Silica gel chromatography (petroleum ether–EtOAc, gradient elution, 3 : 1 to 1 : 1) afforded **9** (454 mg, 73%) as an white amorphous solid (73%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (dd, J = 5.3, 3.0 Hz, 2H), 7.71 (dd, J = 5.3, 3.0 Hz, 2H), 6.67 (d, J = 1.9 Hz, 2H), 6.52 (s, 1H), 4.79 (s, 2H), 4.64 (d, J = 2.1 Hz, 4H), 2.49 (t, J = 2.1 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.9, 158.8, 138.7 (each C), 134.0 (CH), 132.1 (C), 123.4, 108.1, 101.6 (each CH), 78.2 (C), 75.7 (CH), 55.9 (CH_2), 41.5 (CH_2); HRMS-ESI: calcd for $C_{21}H_{16}NO_4[M + H]^+$, 346.1079; found, 346.1087.

2-(3,5-Bis(3,5-bis(prop-2-ynyloxy)benzyloxy)benzyl)isoindoline-1,3-dione 11

Compound **11** (56%) was prepared from bromide **10** as described in the preparation of **9** as white solid after column chromatography (petroleum ether–ethyl acetate, 6 : 1); ^{1}H NMR

Table 2 IC_{50}-values of the mono- to tetravalent lactosides[a] and free lactose (Lac) for blocking binding of biotinylated human galectins to surface-immobilized ASF (in mM)

Lectin inhibitor	Gal-3 (1 μg ml^{-1})	trGal-3 (10 μg ml^{-1})	Gal-4 (5 μg ml^{-1})	Gal-4N (5 μg ml^{-1})	Gal-4C (10 μg ml^{-1})
1	0.16 (2.5)	0.12 (2.5)	0.09 (2.8)	2.4 (1.3)	0.13 (3.1)
2	0.11 (3.6)	0.08 (3.8)	0.14 (1.8)	2.6 (1.2)	0.18 (2.2)
3	0.14 (2.9)	0.32 (1.1)	0.10 (2.5)	0.9 (3.3)	0.18 (2.2)
4	0.13 (3.1)	0.22 (1.4)	0.07 (3.6)	1.2 (2.5)	0.24 (1.7)
5	0.08 (5.0)	[b]	0.05 (5.0)	1.4 (2.1)	0.21 (1.9)
6	0.02 (20)	0.06 (5.0)	0.02 (13)	0.5 (6.0)	0.06 (6.7)
7	0.013 (31)	0.27 (1.1)	0.008 (31)	0.16 (19)	0.03 (13)
Lac	0.4 (1)	0.3 (1)	0.25 (1)	3 (1)	0.4 (1)

[a] For structures see Chart 1; titrations were performed using a fixed glycoprotein quantity for coating (0.5 μg per well) with eight concentrations of sugar in duplicates and up to three independent series, reaching an upper limit of 15.7% for the standard deviation (for exemplary titration curves, see Fig. 1); the concentration is always given for lactose, free in solution or conjugated to a scaffold. [b] Tendency for stimulation at concentrations above 1 mM, numbers in brackets denote relative potency.

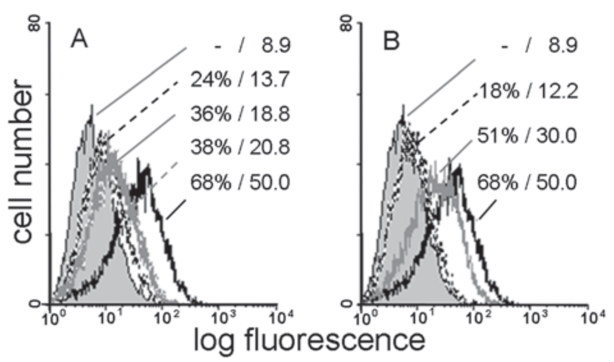


Fig. 5 Semilogarithmic representation of fluorescent surface staining of human pancreatic carcinoma cells (Capan-1), reconstituted for expression of the tumor suppressor p16^{INK4a}, by human galectin-4 (for further details, please see legend to Fig. 2). A, B: inhibition of staining with 10 μg ml^{-1} lectin by 0.5 mM lactose as well as by 0.05 mM lactose presented by the trivalent compound **5** and 0.1 mM lactose in bivalent compound **3** (A) as well as by 20 μM and 7.5 μM lactose presented by the tetravalent compound **7** (B).

(500 MHz, $CDCl_3$) δ 7.85 (dd, J = 5.4, 3.0 Hz, 2H), 7.72 (dd, J = 5.4, 3.0 Hz, 2H), 6.65 (dd, J = 6.1, 2.2 Hz, 6H), 6.54 (t, J = 2.2 Hz, 2H), 6.47 (t, J = 2.2 Hz, 1H), 4.96 (s, 4H), 4.76 (s, 2H), 4.67 (d, J = 2.4 Hz, 8H), 2.52 (t, J = 2.4 Hz, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.0, 159.9, 158.8, 139.3, 138.6 (each C), 134.0 (CH), 132.1 (C), 123.4, 107.6, 106.8, 101.9, 101.6 (each CH), 78.3 (C), 75.7 (CH), 69.8, 56.0, 41.6 (each CH_2); HRMS-ESI: calcd for $C_{41}H_{31}NO_8Na[M + Na]^+$, 688.1947; found, 688.1947.

N,*N′*,*N″*-(3,3′,3″-Nitrilotris(propane-3,1-diyl))trihept-6-ynamide 16

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl; 606 mg, 3.2 mmol) was added to a mixture of **15** (0.21 mL, 1.6 mmol), tris(3-aminopropyl)amine **14** (0.1 mL, 0.48 mmol), 1-hydroxybenzotriazole and triethylamine (442 μL, 3.2 mmol) in THF (25 mL) and the mixture was stirred at room temperature overnight. THF was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (150 mL) and washed with saturated $NaHCO_3$ and brine, dried (Na_2SO_4), filtered, and the solvent was removed under reduced pressure. Silica-gel chromatography (CH_2Cl_2–CH_3OH, gradient elution, 50 : 1 to 10 : 1) gave the title compound **16** as a white solid (234 mg, 95%); R_f 0.2 (CH_2Cl_2–CH_3OH, 10 : 1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.58 (t, J = 5.4 Hz, 3H, N*H*), 3.29 (q, J = 6.6 Hz, 6H), 2.41 (t, J = 6.5 Hz, 6H), 2.26–2.18 (m, 12H), 1.96 (t, J = 2.6 Hz, 3H), 1.79–1.71 (m, 6H), 1.64 (p, J = 6.5 Hz, 6H), 1.60–1.52 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.0 (C), 84.1 (C), 68.6 (CH), 51.4, 37.9, 36.0, 28.0, 26.9, 24.9, 18.2 (each CH_2); HRMS-ESI: calcd for $C_{30}H_{49}N_4O_3[M + H]^+$, 513.3805; found, 513.3808.

2-(3,5-Bis((1-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl)-methoxy)benzyl)isoindoline-1,3-dione 18

Compound **17** (154 mg, 0.23 mmol) was dissolved in CH_3OH–H_2O (5 : 1, 12 mL), then compound **9** (40 mg, 0.12 mmol), sodium ascorbate (9.2 mg in 1 mL H_2O, 0.047 mmol) and $CuSO_4$ (3.7 mg in 1 mL H_2O, 0.023 mmol) were subsequently added and the mixture was sonicated for 2 h, whereafter the solvent was removed and the residue was partitioned between CH_2Cl_2 (100 mL) and water (15 mL). The organic portion was washed by water (15 mL ×2), dried by Na_2SO_4 and concentrated. Silica gel chromatography (CH_2Cl_2–CH_3OH, gradient elution, 80 : 1 to 70 : 1 to 60 : 1) gave **18** as a white foam (178 mg, 92%); $[\alpha]_D^{20}$ −20.8 (c 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86 (dd, J = 5.4, 3.0 Hz, 2H), 7.80 (s, 2H), 7.73 (dd, J = 5.4, 3.0 Hz, 2H), 6.67 (d, J = 2.1 Hz, 2H), 6.52 (t, J = 2.0 Hz, 1H), 5.83 (d, J = 9.0 Hz, 2H, H-1), 5.42–5.40 (m, 4H), 5.37 (d, J = 2.9 Hz, 2H), 5.17–5.11 (m, 6H, H-2′, OCH_2), 4.98 (dd, J = 10.4, 3.4 Hz, 2H), 4.79 (s, 2H), 4.54 (d, J = 7.9 Hz, 2H, H-1′), 4.48 (d, J = 10.8 Hz, 2H), 4.17–4.08 (m, 6H), 4.00–3.94 (m, 2H), 3.95–3.88 (m, 4H), 2.17 (s, 6H), 2.10 (s, 6H), 2.08 (s, 6H), 2.06 (2s, 12H), 1.98 (s, 6H), 1.84 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3, 170.2, 170.1, 170.0, 169.5, 169.1 (2s), 168.0, 159.5, 144.5, 138.9 (each C), 134.1 (CH), 132.1 (C), 123.4, 121.3, 107.8, 101.13 (C-1′), 101.06, 85.6 (C-1), 75.9, 75.7, 72.6, 70.91, 70.86, 70.5, 69.0, 66.6 (each CH), 61.9, 61.8, 60.8, 41.5 (each CH_2), 20.8, 20.71, 20.67, 20.64, 20.63, 20.5, 20.2 (each CH_3); HRMS-ESI: calcd for $C_{73}H_{86}N_7O_{38}[M + H]^+$, 1668.5012; found, 1668.5001.

(3,5-Bis((1-(β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl)-methoxy)phenyl)methanamine 3

Compound **18** (100 mg, 60.0 μmol) and ethylenediamine (0.5 mL) in anhydrous ethanol (5 mL) was heated at reflux. After 6 h, the mixture was cooled to room temperature. The precipitate was filtered, washed with methanol (0.5 mL ×3) and dried under vacuum to give **3** as white solid (39 mg, 68%); $[\alpha]_D^{20}$ 8.8 (c 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 8.33 (s, 2H), 6.71 (s, 2H), 6.65 (s, 1H), 5.80 (d, J = 9.0 Hz, 2H, H-1), 5.28 (s, 4H), 4.52 (d, J = 7.8 Hz, 2H, H-1′), 4.07 (t, J = 9.0 Hz, 2H), 3.99–3.75 (m, 20H), 3.70 (dd, J = 10.0, 3.4 Hz, 2H), 3.59 (dd, J = 10.0, 7.8 Hz, 2H); ^{13}C NMR (125 MHz, D_2O) δ 158.6 (C), 144.7 (C), 143.5 (C), 124.4, 107.7, 102.8, 101.5, 87.2, 77.7, 77.3, 75.3, 74.5, 72.4, 71.9, 70.9, 68.5 (each CH), 61.3, 61.0, 59.7, 44.4 (each CH_2); HRMS-ESI: calcd for $C_{37}H_{56}N_7O_{22}$[M + H]$^+$, 950.3478; found, 950.3441.\

2-(3,5-Bis(3,5-bis((1-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl-methyloxy)-benzyloxy)-benzyl)-isoindoline-1,3-dione 19

Compound **17** (200 mg, 0.30 mmol) was dissolved in CH_3OH (8 mL), to which a solution of compound **11** (50 mg, 0.076 mmol) in CH_2Cl_2 (2 mL) was added. Then solutions of $CuSO_4$ (4.8 mg dissolved in 1 mL H_2O, 30.0 μmol) and sodium ascorbate (12 mg dissolved in 1 mL H_2O, 60.0 μmol) were subsequently added and the mixture was sonicated for 2 h, after which it was stirred at 40 °C overnight. Thereafter the solvent was removed and the residue was partitioned between CH_2Cl_2 (100 mL) and water (15 mL). The organic phase was washed by water (15 mL ×2), dried (Na_2SO_4) and the solvent was removed under reduced pressure. Silica gel chromatography (CH_2Cl_2–CH_3OH, gradient elution, 80 : 1 to 60 : 1 to 50 : 1) gave **19** as a white solid (172 mg, 69%); $[\alpha]_D^{20}$ −28.0 (c 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84 (dd, J = 5.4, 3.0 Hz, 2H), 7.82 (s, 4H), 7.71 (dd, J = 5.4, 3.0 Hz, 2H), 6.66–6.65 (m, 6H), 6.54 (s, 2H), 6.50 (s, 1H), 5.86 (d, J = 9.0 Hz, 4H, H-1), 5.46–5.39 (m, 8H), 5.37 (d, J = 3.3 Hz, 4H), 5.16 (s, 8H), 5.14 (dd, J = 10.4, 8.0 Hz, 4H), 4.99 (dd, J = 10.4, 3.4 Hz, 4H), 4.96 (s, 4H), 4.77 (s, 2H), 4.55 (d, J = 8.0 Hz, 4H, H-1′), 4.49 (d, J = 11.1 Hz, 4H), 4.18–4.08 (m, 12H), 4.02–3.89 (m, 12H), 2.17 (s, 12H), 2.10 (s, 12H), 2.07 (s, 12H), 2.063 (s, 12H), 2.061 (s, 12H), 1.98 (s, 12H), 1.85 (s, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3, 170.2, 170.1, 170.0, 169.5, 169.13, 169.07, 168.0, 159.9, 159.5, 144.6, 139.5, 138.6 (each C), 134.1 (CH), 132.0 (C), 123.4, 121.4, 107.6, 106.6, 101.4, 101.1, 85.6, 76.0, 75.7, 72.6, 70.9, 70.8, 70.5 (each CH), 69.8 (CH_2), 69.1 (CH), 66.6 (CH), 62.0, 61.8, 60.8, 41.6 (each CH_2), 20.8, 20.72, 20.67, 20.65, 20.6, 20.5, 20.2 (each CH_3); HRMS-ESI: calcd for $C_{145}H_{171}N_{13}O_{76}$[M + Cl]$^-$, 3344.9604; found, 3344.9629.

(3,5-Bis(3,5-bis((1-(β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl-methoxy)-benzyloxy)phenyl)methanamine 7

Compound **7** was prepared (81%, amorphous solid) from **19** as described for the preparation of compound **3**; $[\alpha]_D^{20}$ 6.0 (c 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 8.06 (s, 4H), 6.44–6.34 (m, 8H), 6.25 (s, 1H), 5.65 (d, J = 8.7 Hz, 4H, H-1), 4.81 (s, 8H), 4.62 (s, 4H), 4.47 (d, J = 7.4 Hz, 4H, H-1′), 3.98 (t, J = 7.4 Hz, 4H), 3.94 (d, J = 2.9 Hz, 4H), 3.92–3.68 (m, 32H), 3.67 (dd, J = 10.0, 2.9 Hz, 4H), 3.59 (dd, J = 7.4, 10.0 Hz, 4H), 3.45 (s, 2H); ^{13}C NMR (125 MHz, D_2O) δ 159.2, 158.8, 143.2, 139.4 (each C), 124.1, 106.7, 102.9, 100.8, 87.2, 77.6, 77.5, 75.3, 74.5, 72.5, 71.9, 70.9, 68.5 (each CH), 61.0, 60.8, 59.8, 44.2 (each CH_2); HRMS-ESI: calcd for $C_{81}H_{114}N_{13}O_{46}$[M + H]$^+$, 2004.6981; found, 2004.6969.

1,3,5-Tris((1-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl)-methoxy)benzene 20

Compound **17** (200 mg, 0.30 mmol) was dissolved in CH_3OH–H_2O (4 : 1, 15 mL), then 1,3,5-tris(alkynyloxy)benzene[15] **13** (17 mg, 0.10 mol), sodium ascorbate (24 mg dissolved in 1 mL H_2O, 0.12 mmol) and $CuSO_4$ (10 mg dissolved in 1 mL H_2O, 0.06 mmol) were subsequently added and the mixture was stirred at 45 °C overnight, after which the solvent was removed and the residue was partitioned between CH_2Cl_2 (50 mL) and water (15 mL). The organic phase was washed by water (15 mL ×2), dried (Na_2SO_4) and concentrated. Silica gel chromatography (CH_2Cl_2–CH_3OH, gradient elution, 80 : 1 to 60 : 1 to 55 : 1) gave **20** a white amorphous solid (148 mg, 66%); R_f 0.45 (CH_2Cl_2–CH_3OH, 20 : 1); $[\alpha]_D^{20}$ −22.8 (c 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.81 (s, 3H), 6.27 (s, 3H), 5.84 (d, J = 9.0 Hz, 3H, H-1), 5.44–5.38 (m, 6H), 5.37 (d, J = 3.3 Hz, 3H), 5.17–5.10 (m, 9H), 4.98 (dd, J = 10.4, 3.4 Hz, 3H), 4.54 (d, J = 7.9 Hz, 3H, H-1′), 4.49 (d, J = 10.9 Hz, 3H), 4.17–4.08 (m, 9H), 3.99–3.89 (m, 9H), 2.17 (s, 9H), 2.11 (s, 9H), 2.08 (s, 9H), 2.07 (s, 9H), 2.06 (s, 9H), 1.98 (s, 9H), 1.86 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.3, 170.2, 170.1, 170.0, 169.5, 169.13, 169.06, 160.0, 144.5 (each C), 121.3, 101.1 (CH-1′), 95.1, 85.6 (CH-1), 76.0, 75.6, 72.6, 70.90, 70.86, 70.5, 69.1, 66.6 (each CH), 62.0 (CH_2), 61.8 (CH_2), 60.8 (CH_2), 20.8, 20.71, 20.67, 20.64, 20.63, 20.5, 20.2 (each CH_3); HRMS-ESI: calcd for $C_{93}H_{118}N_9O_{54}$[M + H]$^+$, 2224.6764; found, 2224.6799.

1,3,5-Tris((1-(β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl) methoxy)benzene 4

Compound **20** (80 mg, 36.0 μmol) was dissolved in methanol (5 mL). A catalytic amount of NaOMe (0.1 mL of a 0.2 M solution in MeOH) was added to the solution and the resulting mixture was stirred for 1 h at room temperature. Amberlite IR-120 (plus) was added to neutralize (pH = 7), whereafter the resin was removed by filtration and washed with water. The solvent was removed and the residue was purified by BioGel P-2 gel filtration column to give **4** as an amorphous solid (40 mg, 83%); $[\alpha]_D^{20}$ 6.2 (c 0.9, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 8.32 (s, 3H), 6.43 (s, 3H), 5.81 (d, J = 9.0 Hz, 3H, H-1), 5.26 (s, 6H), 4.52 (d, J = 7.8 Hz, 3H, H-1′), 4.08 (t, J = 9.0 Hz, 3H), 3.96–3.95 (m, 6H), 3.90–3.83 (m, 12H), 3.81–3.76 (m, 9H), 3.70 (dd, J = 10.0, 3.4 Hz, 3H), 3.60 (dd, J = 10.0, 7.8 Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 159.4, 143.3 (each C), 124.3, 102.9, 95.8, 87.2, 77.6, 77.3, 75.3, 74.5, 72.4, 71.9, 70.9, 68.5 (each CH), 61.1, 61.0, 59.7 (each CH_2); HRMS-ESI: calcd for $C_{51}H_{75}N_9O_{33}Na$[M + Na]$^+$, 1364.4365; found, 1364.4332.

_N,N′,N″_-(3,3′,3″-Nitrilotris(propane-3,1-diyl))tris(5-(1-(2,3,4,6-tetra-_O_-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-_O_-acetyl-β-D-glucopyranosyl)-1_H_-1,2,3-triazol-4-yl) pentanamide 21

Compound **21** was prepared from lactoside **17** and compound **16** as described for the preparation of **20**, using ultrasonic radiation instead of heating. The title compound was obtained (92%) as an amorphous solid after chromatography (CH_2Cl_2–CH_3OH, gradient elution, 80 : 1 to 30 : 1); $[\alpha]_D^{20}$ −14.4 (c 1.0, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.53 (s, 3H), 7.16 (s, 3H, N*H*), 5.83 (d, *J* = 8.4 Hz, 3H, H-1), 5.44–5.34 (m, 6H), 5.37 (d, *J* = 3.1 Hz, 3H), 5.13 (dd, *J* = 10.2, 8.0 Hz, 3H), 4.99 (dd, *J* = 10.2, 3.3 Hz, 3H), 4.58 (d, *J* = 7.9 Hz, 3H, H-1′), 4.51 (d, *J* = 11.9 Hz, 3H), 4.18–4.08 (m, 9H), 4.05–3.86 (m, 9H), 3.31 (d, *J* = 4.8 Hz, 6H), 2.96 (m, 6H), 2.72 (t, *J* = 6.5 Hz, 6H), 2.25 (s, 6H), 2.17 (s, 9H), 2.11 (s, 9H), 2,074 (s, 9H), 2.066 (s, 9H), 2.06 (s, 9H), 1.98 (s, 9H), 1.89 (m, 6H), 1.86 (s, 9H), 1.69 (s, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.9, 170.4, 170.3, 170.1, 170.0, 169.5, 169.2, 169.1, 148.3 (each C), 119.6, 101.1 (CH-1′), 85.4 (CH-1), 75.9, 75.6, 72.7, 70.9, 70.8, 70.5, 69.1, 66.6 (each CH), 61.8, 60.8, 50.9, 36.6, 35.9, 28.6, 25.2, 25.0, 24.5 (each CH_2), 20.9, 20.74, 20.67, 20.65, 20.64, 20.5, 20.3 (each CH_3); HRMS-ESI: calcd for $C_{108}H_{154}N_{13}O_{54}$[M + H]$^+$, 2496.9704; found, 2496.9739.

_N,N′,N″_-(3,3′,3″-Nitrilotris(propane-3,1-diyl))tris(5-(1-(β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl)-1_H_-1,2,3-triazol-4-yl))-pentanamide 5

Compound **21** (100 mg, 40 μmol) was dissolved in methanol (5 mL) to which a catalytic amount of NaOMe (0.5 mL of a 0.2 M solution in MeOH) was added and the resulting solution was stirred for 4 h at room temperature. The solvent was removed and the residue was purified by BioGel P-2 gel filtration column to give **5** as an amorphous solid (50 mg, 79%); $[\alpha]_D^{20}$ −5.6 (c 1.0, H_2O); ^1H NMR (500 MHz, D_2O) δ 8.02 (s, 3H), 5.74 (d, *J* = 9.2 Hz, 3H, H-1), 4.53 (d, *J* = 8.0 Hz, 3H, H-1′), 4.04 (t, *J* = 9.2 Hz, 3H, H-2), 3.97–3.76 (m, 27H), 3.70 (dd, *J* = 9.6, 2.9 Hz, 3H), 3.60 (dd, *J* = 9.6, 8.0 Hz, 3H), 3.14 (t, *J* = 6.6 Hz, 6H), 2.74 (t, *J* = 6.8 Hz, 6H), 2.43–2.40 (m, 6H), 2.25 (t, *J* = 6.7 Hz, 6H), 1.65–1.57 (m, 18H); ^{13}C NMR (125 MHz, D_2O) δ 176.4, 148.3 (each C), 122.1 (CH), 102.8 (CH, C-1′), 87.1 (CH, C-1), 77.6, 77.3, 75.3, 74.5, 72.5, 71.9, 70.9, 68.5 (each CH), 61.0, 59.7, 50.3, 37.4, 35.3, 27.7, 24.9, 24.7, 24.1 (each CH_2); HRMS-ESI: calcd for $C_{66}H_{111}N_{13}O_{33}$Na[M + Na]$^+$, 1636.7305; found, 1636.7290.

_O_-(2,3,4-Tri-_O_-benzyl)-α-L-fucopyranosyl)-(1 → 2)-_O_-(6-_O_-benzoyl-3,4-_O_-isopropylidene -β-D-galactopyranosyl)-(1 → 4)-2,3:5,6-di-_O_-isopropylidene-D-glucose dimethyl acetal 24

A mixture of fucosyl donor **22**[18] (1.26 g, 2.34 mmol), disaccharide building block **23**[19] (1.30 g, 2.12 mmol), BSP (252 mg, 1.17 mmol) and 4 Å MS was stirred in dry CH_2Cl_2 (25 mL) at room temperature for 1 h under N_2. It was cooled to −78 °C, followed by the addition of Tf_2O (219 μL, 1.27 mmol) and 2,4,6-tri-*tert*-butylpyrimidine (TTBP, 598 mg, 2.34 mmol). Then the temperature was increased gradually from −78 °C to 0 °C within 2 h and the mixture was stirred at 0 °C for an additional period of 4 h. The reaction was quenched with triethylamine (3 mL) and diluted with CH_2Cl_2. The reaction mixture was filtered and washed sequentially with saturated $Na_2S_2O_3$, saturated $NaHCO_3$, H_2O, and brine, dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure. Chromatography (petroleum ether–EtOAc, gradient elution, 7 : 1 to 6 : 1) gave **24** as a white foam (1.63 g, 70%), R_f 0.5 (petroleum ether–EtOAc, 2.5 : 1). ^1H-NMR (500 MHz, $CDCl_3$): δ 8.04 (d, *J* = 7.5 Hz, 2H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.44 (t, *J* = 7.5 Hz, 2H), 7.40–7.24 (m, 15H), 5.59 (d, *J* = 3.3 Hz, 1H, H-1″), 4.98 (d, *J* = 11.6 Hz, 1H), 4.87 (d, *J* = 11.9 Hz, 1H), 4.75–4.73 (m, 3H), 4.70 (d, *J* = 8.0 Hz, 1H, H-1′), 4.65 (d, *J* = 11.6 Hz, 1H), 4.57–4.49 (m, 3H), 4.34 (d, *J* = 5.9 Hz, 1H, H-1), 4.25 (dd, *J* = 10.9, 5.9 Hz, 1H), 4.21 (t, *J* = 6.0 Hz, 1H), 4.16 (dd, *J* = 5.6, 2.0 Hz, 1H), 4.09–4.00 (m, 7H), 3.90 (dd, *J* = 8.3, 6.5 Hz, 1H), 3.75 (dd, *J* = 8.0, 6.7 Hz, 1H), 3.66 (d, *J* = 1.1 Hz, 1H), 3.36 (s, 3H), 3.34 (s, 3H), 1.49 (s, 3H), 1.43 (s, 3H), 1.37 (s, 6H), 1.33 (s, 3H), 1.27 (s, 3H), 1.11 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.3 (C), 139.3 (C), 139.1 (C), 138.9 (C), 133.1, 130.0, 129.7, 128.42, 128.35, 128.3, 128.13, 128.12, 127.6, 127.43, 127.40, 127.31, 127.27 (each CH), 110.3 (C), 110.0 (C), 108.6 (C), 105.1 (CH, C-1), 101.3 (CH, C-1′), 95.2 (CH, C-1″), 80.2, 79.2, 78.1, 77.7, 77.5, 76.5, 75.4, 75.0 (each CH), 74.8 (CH_2), 74.1 (CH), 73.8 (CH), 73.2 (CH_2), 72.7 (CH_2), 70.9 (CH), 66.5 (CH), 65.2 (CH_2), 63.8 (CH_2), 56.0, 52.9, 29.7, 27.8, 27.2, 26.9, 26.5, 25.2, 16.9 (each CH_3); HRMS-ESI: calcd for $C_{57}H_{72}O_{17}$Na[M + Na]$^+$, 1051.4667; found, 1051.4664.

_O_-(2,3,4-Tri-_O_-acetyl-α-L-fucopyranosyl)-(1 → 2)-_O_-(3,4,6-tri-_O_-acetyl-β-D-galactopyranosyl)-(1 → 4)-1,2,3,6-tetra-_O_-acetyl-α/β-D-glucopyranose 26[19b]

To compound **24** (2.12 g, 2.33 mmol) in anhydrous MeOH, NaOMe (0.5 mL of a 2 M solution in MeOH) was added and the resulting mixture was stirred for 1 h at room temperature. Amberlite IR-120 (plus) was added to neutralize (pH = 7), and the solvent was removed under reduced pressure to afford a colourless oil. This residue was dissolved in aq 60% acetic acid (30 mL) and heated for 6 h at 60 °C. The reaction mixture was diluted with toluene and the volatile components removed. Then to a solution of the residue in THF–H_2O–AcOH (4 : 2 : 1, 14 mL), 10% Pd–C (50 mg) was added. The suspension was stirred under an atmosphere of hydrogen for 2 days at ambient temperature. When the reaction was completed, the mixture was filtered over Celite and concentrated. Toluene (3 × 30 mL) was evaporated from the residue to remove the acetic acid and water. Then fucosyllactose **1**[19] was dissolved in pyridine–acetic anhydride (2 : 1, 30 mL) and then stirred overnight under an atmosphere of nitrogen at ambient temperature. The solvent was then removed and the residue was partitioned between CH_2Cl_2 (100 mL) and water (25 mL). The organic phase was washed with water (25 mL ×2), dried (Na_2SO_4) and the solvent was removed. Chromatography (petroleum ether–EtOAc, gradient elution, 4 : 1 to 3 : 1) gave **26** as a white foam (1.67 mg, 79%); R_f 0.35 (petroleum ether–EtOAc, 1.5 : 1), which were a mixture of anomers (β : α = 1 : 1); selected ^1H NMR (500 MHz, $CDCl_3$) data for the β anomer: δ 5.69 (d, *J* = 8.2 Hz, 1H, H-1), 5.37 (d, *J* = 3.4 Hz, 1H, H-1″), 5.20 (t, *J* = 9.5 Hz, 1H), 5.09 (dd, *J* = 9.7, 8.3 Hz, 1H); selected ^1H NMR data for the α anomer: δ 6.30 (d,

J = 3.7 Hz, 1H, H-1), 5.41 (t, J = 9.9 Hz, 1H), 5.37 (d, J = 3.5 Hz, 1H, H-1″), 5.05 (dd, J = 10.4, 3.7 Hz, 1H); overlapped ^{1}H NMR data for both β and α anomer: 5.31–5.27 (m, 4H), 5.18–5.12 (m, 2H), 5.00–4.96 (m, 4H), 4.50–4.38 (m, 6H), 4.27 (dd, J = 12.3, 5.3 Hz, 2H), 4.18–4.12 (m, 2H), 4.10–4.03 (m, 3H), 3.91–3.80 (m, 7H), 2.18–1.97 (19s, 60H), 1.24–1.21 (2d, J = 6.5 Hz, 6H); mixture ^{13}C NMR (125 MHz, $CDCl_3$) data for both α and β anomer: δ 171.1, 170.7, 170.65, 170.6, 170.5, 170.3 (2s), 170.1, 170.0 (2s), 169.8, 169.7, 169.6, 169.3, 168.8, 168.7 (each C), 100.2, 99.9, 95.7, 73.9, 73.8, 73.7, 73.4, 73.3, 71.7, 71.11, 71.09, 70.9, 70.8, 70.7, 70.3, 69.2, 69.1, 68.1, 68.0, 67.5, 67.3, 67.0, 65.0, 64.9 (each CH), 62.1, 61.9, 61.0, 60.8 (each CH_2), 21.1, 21.0, 20.9, 20.8, 20.7, 20.65, 20.6, 20.5, 15.6, 15.4 (each CH_3); selected ^{13}C NMR data for the α anomer: δ 89.0 (CH, C-1); Selected ^{13}C NMR data for the β-anomer: δ 91.5 (CH, C-1); HRMS-ESI: calcd for $C_{38}H_{52}N_3O_{25}Na$-[M + Na]$^+$, 931.2695; found, 931.2686.

O-(2,3,4-Tri-*O*-acetyl-α-L-fucopyranosyl)-(1 → 2)-*O*-(3,4,6-tri-*O*-acetyl-β-D-galactopyran osyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl azide 27

Compound **26** (1.35 g, 1.49 mmol) was dissolved in CH_2Cl_2 (30 mL, anhydrous) under an atmosphere of N_2. To this solution was added $TMSN_3$ (0.54 mL, 4.46 mmol) followed by the drop-wise addition of $SnCl_4$ (88 μL, 0.74 mmol). After 20 h, the solution was diluted with CH_2Cl_2, quenched by the addition of saturated $NaHCO_3$ solution (10 mL) and stirred for further 30 min. The biphasic solution was then filtered through celite and the organic layer extracted, washed with saturated $NaHCO_3$ (20 mL ×2), H_2O (20 mL ×2), dried (Na_2SO_4) and the solvent was removed under reduced pressure. Chromatography (petroleum ether–EtOAc = 3 : 1) gave **27** as a white foam (1.15 g, 87%), R_f 0.57 (petroleum ether–EtOAc, 1 : 1); ^{1}H NMR (500 MHz, $CDCl_3$) data for the β anomer: δ 5.38 (d, J = 3.0 Hz, 1H, H-1″), 5.30 (dd, J = 12.5, 3.0 Hz, 2H), 5.15–5.12 (m, 2H), 5.00–4.96 (m, 2H), 4.92 (t, J = 9.5 Hz, 1H), 4.64 (d, J = 9.0 Hz, 1H, H-1), 4.54 (d, J = 12.0 Hz, 1H), 4.41–4.37 (m, 2H), 4.29 (dd, J = 12.0, 6.0 Hz, 1H), 4.15 (dd, J = 11.1, 6.5 Hz, 1H), 4.08 (dd, J = 11.1, 7.0 Hz, 1H), 3.88–3.81 (m, 3H), 3.77–3.74 (m, 1H), 2.16 (2s, 6H), 2.12 (s, 3H), 2.09 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.00 (s, 3H), 1.98–1.97 (2s, 6H), 1.21 (d, J = 6.5 Hz, 3H); Selected ^{1}H NMR data for the α anomer: δ 5.35 (d, J = 3.0 Hz, 1H), 5.21 (t, J = 9.2 Hz, 1H), 5.10 (d, J = 8.0 Hz, 1H), 4.86 (t, J = 9.1 Hz, 1H), 4.63 (d, J = 8.7 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) for the β anomer: δ 170.7, 170.6 (2s), 170.3, 170.1, 170.0, 169.7, 169.3 (each C), 100.2, 95.5, 88.0, 75.2, 73.9, 73.4, 71.8, 71.3, 71.0, 70.8, 70.5, 68.0, 67.4, 67.0, 64.9 (each CH), 62.0 (CH_2), 60.8 (CH_2), 20.8, 20.7 (3s), 20.6 (3s), 15.6 (each CH_3); selected ^{13}C NMR data for the α anomer: δ 101.1, 87.7, 75.8, 74.8, 72.5, 71.0, 70.9, 70.8, 69.1, 66.6 (each CH), 61.7 (CH_2), 60.4 (CH_2), 14.2 (CH_3); HRMS-ESI: calcd for $C_{36}H_{49}N_3O_{23}Na$[M + Na]$^+$, 914.2655; found, 914.2621.

(*S*)-2-Amino-3-(1-(α-L-fucopyranosyl-(1 → 2)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl)propanoic acid 2

To a solution of glycoside **27** (106 mg, 0.12 mmol) and Fmoc-protected L-propargylglycine (41 mg, 0.12 mmol)[22] in *tert*-butanol (2 mL) and water (4 ml) was added a solution of $CuSO_4$ (0.04 M, 0.6 mL, 0.024 mmol) and sodium ascorbate (0.08 M, 0.6 mL) in H_2O. The mixture was stirred overnight, water (6 mL) was added, and the product was extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. Chromatography using (CH_2Cl_2–MeOH, 15 : 1 to 10 : 1) give the intermediate as a white foam 131 mg (90%), R_f 0.2 (CH_2Cl_2–MeOH, 15 : 1). The residue (131 mg, 0.11 mmol) was treated with 20% piperidine in DMF (v/v, 5 mL), then stirred for 20 min and solvent was finally removed under reduced pressure. The crude product was dissolved in MeOH (5 mL), and NaOMe (2 M, 0.05 mL) was added. The mixture was stirred for 1 h. The mixture was concentrated and passed through BioGel P-2 gel filtration column with water to give **2** (45 mg, 67%) as an amorphous solid; $[\alpha]_D^{20}$ −48.8 (c 0.5, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 8.06 (s, 1H), 5.75 (d, J = 9.2 Hz, 1H, H-1), 5.35 (d, J = 3.1 Hz, 1H, H-1″), 4.61 (d, J = 7.8 Hz, 1H, H-1′), 4.27 (q, J = 6.7 Hz, 1H, H-4″), 4.07 (t, J = 9.2 Hz, 1H, H-2), 4.01–3.97 (m, 2H), 3.93–3.90 (m, 2H), 3.87–3.718 (m, 10H), 3.67–3.64 (m, 1H), 3.16 (dd, J = 14.8, 5.2 Hz, 1H), 3.08 (dd, J = 14.8, 6.9 Hz, 1H), 1.28 (d, J = 6.6 Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 179.8 (C), 144.3 (C), 123.1, 100.2, 99.3, 87.3, 78.1, 76.2, 75.2, 74.9, 74.3, 73.5, 72.0, 71.6, 69.6, 69.1, 68.1, 66.9 (each CH), 61.1, 59.8 (each CH_2), 55.5 (CH), 29.6 (CH_2), 15.2 (CH_3). HRMS-ESI: calcd for $C_{23}H_{38}N_4O_{16}Na$ [M + Na]$^+$, 649.2181; found, 649.2176.

1,3,5-Tris((1-(α-L-fucopyranosyl-(1 → 2)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-benzene 6

Azide **27** (90 mg, 101 μmol) was dissolved in CH_3OH–H_2O (2 : 1, 15 mL), then **13**[15] (8.0 mg, 33.7 μmol), sodium ascorbate (4.0 mg dissolved in 1 mL H_2O, 20.2 μmol) and $CuSO_4$ (1.6 mg dissolved in 1 mL H_2O, 10.1 μmol) were subsequently added and the mixture was stirred at 45 °C overnight, after which the solvent was removed and the residue was participated by CH_2Cl_2 (50 mL) and water (15 mL). The organic phase was washed by water (15 mL × 2), dried by Na_2SO_4 and concentrated. Chromatography (CH_2Cl_2–CH_3OH, gradient elution, 80 : 1 to 70 : 1 to 60 : 1) gave the acetylated intermediate as a white foam (83 mg, 85%), R_f 0.55 (CH_2Cl_2–CH_3OH, 20 : 1). Removal of the protecting groups from this protected compound (39 mg, 0.013 mmol), as for the formation of **4**, gave **6**, after lyophilization, as a white solid (20 mg, 82%); $[\alpha]_D^{20}$ −50.5 (c 0.2, D_2O); ^{1}H-NMR (500 MHz, D_2O) δ 8.22 (s, 3H), 6.30 (s, 3H), 5.68 (d, J = 9.2 Hz, 3H, H-1), 5.25 (d, J = 3.1 Hz, 3H, H-1″), 5.11 (s, 6H), 4.50 (d, J = 7.8 Hz, 3H, H-1′), 4.16 (dd, J = 13.2, 6.5 Hz, 3H), 3.96 (t, J = 9.3 Hz, 3H), 3.90–3.61 (m, 42H), 1.17 (d, J = 6.5 Hz, 9H); ^{13}C NMR (125 MHz, D_2O) δ 159.4 (C), 143.5 (C), 124.4, 100.2, 99.3, 96.4, 87.3, 78.1, 76.3, 75.3, 74.9, 74.4, 73.5, 72.0, 71.6, 69.6, 69.1, 68.2, 66.9 (each CH), 61.3, 61.1, 59.8 (each CH_2), 15.3 (CH_3); HRMS-ESI: calcd for $C_{69}H_{105}N_9O_{45}Na$[M + Na]$^+$, 1802.6102; found, 1802.6108.

Molecular modelling

Structures were first built using Maestro version 6.0 (Schrodinger Inc., LLC, New York, USA). Constraints were then applied during an energy minimization (OPLSAA force field, gas phase, PRCG method to convergence) of each structure using Macromodel version 8.5 (Schrodinger Inc.) so as to generate an extended conformation for each structure. This approach enabled estimation of the maximum distances that could potentially be adopted between the lactose headgroups in **3**, **4**, **5** and **7**. In the case of **4** the angles defined by the three glucose anomeric carbon atoms were constrained at 60°, for **5** the angles between the glucose anomeric carbon atoms of two of the lactose residues and the nitrogen atom at the centre of the scaffold were constrained at 120°. In the case of **7** the angles between three of the glucose anomeric carbons were constrained to 90°. Distance constraints had to be applied between the glucose anomeric carbons in order to maximize the spacing between the headgroups. Torsions were adjusted using Maestro if necessary to help generate the extended structures. The final modeled structures obtained are shown in Fig. 1. The distances between the headgroups for these structures are given in the main text.

Lectin purifications and quality controls

Using extracts of dried plant material or bacterial pellets from recombinant production, affinity chromatography over lactosylated Sepharose 4B obtained by divinyl sulfone activation was performed as crucial step, following a standard procedure.[9,25d,36] The B-chain of the toxin was obtained after *in situ* cleavage of the disulfide bond linking the AB-chains by extensive treatment with β-mercaptoethanol, removal of the A-chain by column washes and covalent deactivation of the sulfhydryl group in the resin-bound B-chain by iodoacetamide treatment, trCG-3/Gal-3 by purifying the cloned product or collagenase treatment of the full-length protein.[37] One- and two-dimensional gel electrophoresis and gel filtration as well as haemagglutination assays were routinely performed to ensure purity, quaternary structure and activity.[25c,37] Biotinylation with the *N*-hydroxysuccinimide ester derivative (Sigma, Munich, Germany) under activity-preserving conditions followed a standard protocol, with incorporation yields measured by mass spectrometric analysis.[37b,c]

Inhibition assays

Microtiter plate wells were coated with ASF (0.5 µg per well in 50 µl phosphate-buffered saline) overnight at 4 °C and residual sites for binding protein were blocked with 100 µl buffer containing 1% (w/v) carbohydrate-free bovine serum albumin for 1 h at 37 °C. Following washing the series of steps comprising incubation with biotinylated lectin, washing, application of the streptavidin-peroxidase conjugate (0.5 µg ml^{-1}; Sigma), washing and signal development with the chromogenic substrates *o*-phenylenediamine (1 µg ml^{-1})/hydrogen peroxide (1 µl ml^{-1}) was completed with readings of the optical density at 490 nm as described.[6a,25a] Assays were routinely done in triplicates with up to five independent series, standard deviations for percentage of bound lectin as parameter not exceeding 16.2%.

Assays with the human B-lymphoblastoid/pancreatic carcinoma and the Chinese hamster ovary cell lines followed an optimized protocol ensuring interstudy comparison.[6,25c–f] Aliquots of cell suspensions at the same passage were routinely processed at least in duplicates, with at least three independent series, by washing to thoroughly remove serum components and then incubation with lectin-containing solution at 4 °C for 30 min. in the loading step, later with streptavidin-R-phycoerythrin (1 : 40; Sigma) in the labeling step. Controls included omission of the loading step to measure the level of lectin-independent background staining and application of non-cognate sugar to track down osmolarity effects. Following normalization of values based on the internal controls the standard deviations of measurements did not exceed 12.7%.

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References

1 (*a*) J. Holgersson, A. Gustafsson and S. Gaunitz, in *The Sugar Code. Fundamentals of Glycosciences*, ed. H.-J. Gabius, Wiley-VCH, Weinheim, 2009, pp. 279–300; (*b*) H. M. I. Osborn and A. Turkson, in *The Sugar Code. Fundamentals of Glycosciences*, ed. H.-J. Gabius, Wiley-VCH, Weinheim, 2009, pp. 469–483; (*c*) Y. M. Chabre, D. Giguère, B. Blanchard, J. Rodrigue, S. Rocheleau, M. Neault, S. Rauthu, A. Papadopoulos, A. A. Arnold, A. Imberty and R. Roy, *Chem.–Eur. J.*, 2011, **17**, 6545–6562; (*d*) H.-J. Gabius, S. André, J. Jiménez-Barbero, A. Romero and D. Solís, *Trends Biochem. Sci.*, 2011, **36**, 298–313.

2 H.-J. Gabius, in *The Sugar Code. Fundamentals of Glycosciences*, ed. H.-J. Gabius, Wiley-VCH, Weinheim, 2009, pp. 317–328.

3 (*a*) K. G. Rice, O. A. Weisz, T. Barthel, R. T. Lee and Y. C. Lee, *J. Biol. Chem.*, 1990, **265**, 18429–18434; (*b*) Y. C. Lee and R. T. Lee, in *Neoglycoconjugates: Preparation and Applications*, ed. Y. C. Lee and R. T. Lee, Academic Press, San Diego, 1994, pp. 23–50; (*c*) Y. C. Lee, *Trends Glycosci. Glycotechnol.*, 2010, **22**, 95–106.

4 (*a*) Y. C. Lee, R. R. Townsend, M. R. Hardy, J. Lönngren, J. Arnarp, M. Haraldsson and H. Lönn, *J. Biol. Chem.*, 1983, **258**, 199–202; (*b*) R. T. Lee and Y. C. Lee, *Glycoconjugate J.*, 1987, **4**, 317–328; (*c*) H. F. Lodish, *Trends Biochem. Sci.*, 1991, **16**, 374–377; (*d*) Y. C. Lee, *FASEB J.*, 1992, **6**, 3193–3200; (*e*) S. André, S. Kojima, G. Gundel, R. Russwurm, X. Schratt, C. Unverzagt and H.-J. Gabius, *Biochim. Biophys. Acta, Gen. Subj.*, 2006, **1760**, 768–782.

5 (*a*) Y. M. Chabre and R. Roy, in *The Sugar Code. Fundamentals of Glycosciences*, ed. H.-J. Gabius, Wiley-VCH, Weinheim, 2009, pp. 53–70; (*b*) Y. M. Chabre and R. Roy, *Adv. Carbohydr. Chem. Biochem.*, 2010, **63**, 165–393.

6 (*a*) R. Leyden, T. Velasco-Torrijos, S. André, S. Gouin, H.-J. Gabius and P. V. Murphy, *J. Org. Chem.*, 2009, **74**, 9010–9026; (*b*) S. André, D. V. Jarikote, D. Yan, L. Vincenz, G.-N. Wang, H. Kaltner, P. V. Murphy and H.-J. Gabius, *Bioorg. Med. Chem. Lett.*, 2012, **22**, 313–318.

7 (*a*) K.-I. Kasai and J. Hirabayashi, *J. Biochem.*, 1996, **119**, 1–8; (*b*) D. N. W. Cooper, *Biochim. Biophys. Acta, Gen. Subj.*, 2002, **1572**, 209–231; (*c*) H. Kaltner and H.-J. Gabius, *Histol. Histopathol.*, 2012, **27**, 397–416.

8 (*a*) O. E. Galanina, H. Kaltner, N. V. Bovin and H.-J. Gabius, *J. Mol. Recognit.*, 1997, **10**, 139–147; (*b*) C. Svensson, S. Teneberg, C. L. Nilsson, A. Kjellberg, F. P. Schwarz, N. Sharon and U. Krengel, *J. Mol. Biol.*, 2002, **321**, 69–83.

9 A. M. Wu, T. Singh, J.-H. Liu, M. Krzeminski, R. Russwurm, H.-C. Siebert, A. M. J. J. Bonvin, S. André and H.-J. Gabius, *Glycobiology*, 2007, **17**, 165–184.

10 O. A. Vokhmyanina, R. M. Rapoport, I. M. Ryzhov, E. Y. Korchagina, G. V. Pazynina, V. V. Severov, H. Kaltner, S. André, H.-J. Gabius and N. V. Bovin, *Biochemistry*, 2011, **76**, 1185–1192.

11 (*a*) C. P. Sparrow, H. Leffler and S. H. Barondes, *J. Biol. Chem.*, 1987, **262**, 7383–7390; (*b*) S. Sato and R. C. Hughes, *J. Biol. Chem.*, 1992, **267**, 6983–6990; (*c*) T. Feizi, J. C. Solomon, C.-T. Yuen, K. C. G. Jeng, L. G. Frigeri, D. K. Hsu and F.-T. Liu, *Biochemistry*, 1994, **33**, 6342–6349; (*d*) J. Hirabayashi, T. Hashidate, Y. Arata, N. Nishi, T. Nakamura, M. Hirashima, T. Urashima, T. Oka, M. Futai, W. E. G. Müller, F. Yagi and K.-i. Kasai, *Biochim. Biophys. Acta, Gen. Subj.*, 2002, **1572**, 232–254; (*e*) S. R. Stowell, C. M. Arthur, P. Mehta, K. A. Slanina, O. Blixt, H. Leffler, D. F. Smith and R. D. Cummings, *J. Biol. Chem.*, 2008, **283**, 10109–10123; (*f*) M. Krzeminski, T. Singh, S. André, M. Lensch, A. M. Wu, A. M. J. J. Bonvin and H.-J. Gabius, *Biochim. Biophys. Acta, Gen. Subj.*, 2011, **1810**, 150–161.

12 (*a*) Y. Oda, J. Herrmann, M. A. Gitt, C. W. Turck, A. L. Burlingame, S. H. Barondes and H. Leffler, *J. Biol. Chem.*, 1993, **268**, 5929–5939; (*b*) H. Ideo, A. Seko, T. Ohkura, K. L. Matta and K. Yamashita, *Glycobiology*, 2002, **12**, 199–208; (*c*) A. M. Wu, J. H. Wu, J.-H. Liu, T. Singh, S. André, H. Kaltner and H.-J. Gabius, *Biochimie*, 2004, **86**, 317–326.

13 (*a*) D. Gupta, H. Kaltner, X. Dong, H.-J. Gabius and C. F. Brewer, *Glycobiology*, 1996, **6**, 843–849; (*b*) T. K. Dam, H.-J. Gabius, S. André, H. Kaltner, M. Lensch and C. F. Brewer, *Biochemistry*, 2005, **44**, 12564–12571.

14 K. Soai, A. Ookawa and K. Kato, *Bull. Chem. Soc. Jpn.*, 1982, **55**, 1671–1672.

15 J. D. Xie, L. H. Hu, W. F. Shi, X. X. Deng, Z. Q. Cao and Q. S. Shen, *Polym. Int.*, 2008, **57**, 965–974.

16 (*a*) H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004–2021; (*b*) F. Pérez-Balderas, M. Ortega-Muñoz, J. Morales-Sanfrutos, F. Hernández-Mateo, F. G. Calvo-Flores, J. A. Calvo-Asín, J. Isac-García and F. Santoyo-González, *Org. Lett.*, 2003, **5**, 1951–1954; (*c*) F. Morvan, A. Meyer, A. Jochum, C. Sabin, Y. Chevolot, A. Imberty, J. P. Praly, J. J. Vasseur, E. Souteyrand and S. Vidal, *Bioconjugate Chem.*, 2007, **18**, 1637–1643; (*d*) M. Meldal and C. W. Tornoe, *Chem. Rev.*, 2008, **108**, 2952–3015; (*e*) S. André, T. Velasco-Torrijos, R. Leyden, S. Gouin, M. Tosin, P. V. Murphy and H.-J. Gabius, *Org. Biomol. Chem.*, 2009, **7**, 4715–4725; (*f*) V. Aragao-Leoneti, V. L. Campo, S. A. Gomes, R. A. Field and I. Carvalho, *Tetrahedron*, 2010, **66**, 9475–9492; (*g*) V. L. Campo, I. Carvalho, C. H. T. P. Da Silva, S. Schenkman, L. Hill, S. A. Nepogodiev and R. A. Field, *Chem. Sci.*, 2010, **1**, 507–514; (*h*) A. Papadopolos, T. C. Shiao and R. Roy, *Mol. Pharmaceutics*, 2012, **9**, 394–403.

17 (*a*) G. Cravotto, V. V. Fokin, D. Garella, A. Binello, L. Boffa and A. Barge, *J. Comb. Chem.*, **12**, 13–15; (*b*) P. Cintas, A. Barge, S. Tagliapietra, L. Boffa and G. Cravotto, *Nat. Protocols.*, 2010, **5**, 607–616.

18 Z. Wang, L. Y. Zhou, K. El-Boubbou, X. S. Ye and X. F. Huang, *J. Org. Chem.*, 2007, **72**, 6409–6420.

19 (*a*) S. A. Abbas, J. J. Barlow and K. L. Matta, *Carbohydr. Res.*, 1981, **88**, 51–60; (*b*) M. Izumi, O. Tsuruta, S. Harayama and H. Hashimoto, *J. Org. Chem.*, 1997, **62**, 992–998.

20 A. J. Ott, M. Brackhagen, A. Davtyan, B. Nolting, H. Boye, A. Schoknecht and C. Vogel, *J. Carbohydr. Chem.*, 2001, **20**, 611–636.

21 J. L. Montero, J. Y. Winum, A. Leydet, M. Kamal, A. A. Pavia and J. P. Roque, *Carbohydr. Res.*, 1997, **297**, 175–180.

22 B. H. M. Kuijpers, S. Groothuys, A. C. Soede, P. Laverman, O. C. Boerman, F. L. van Delft and F. P. J. T. Rutjes, *Bioconjugate Chem.*, 2007, **18**, 1847–1854.

23 (*a*) S. André, T. Kožár, R. Schuberth, C. Unverzagt, S. Kojima and H.-J. Gabius, *Biochemistry*, 2007, **46**, 6984–6995; (*b*) S. André, T. Kožár, S. Kojima, C. Unverzagt and H.-J. Gabius, *Biol. Chem.*, 2009, **390**, 557–565.

24 (*a*) R. Mikeska, R. Wacker, R. Arni, T. P. Singh, A. Mikhailov, A. Gabdoulkhakov, W. Voelter and C. Betzel, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.*, 2005, **F61**, 17–25; (*b*) M. Jiménez, S. André, H.-C. Siebert, H.-J. Gabius and D. Solís, *Glycobiology*, 2006, **16**, 926–937.

25 (*a*) S. André, P. J. Ortega, M. A. Perez, R. Roy and H.-J. Gabius, *Glycobiology*, 1999, **9**, 1253–1261; (*b*) S. André, B. Liu, H.-J. Gabius and R. Roy, *Org. Biomol. Chem.*, 2003, **1**, 3909–3916; (*c*) S. André, H. Kaltner, T. Furuike, S.-I. Nishimura and H.-J. Gabius, *Bioconjugate Chem.*, 2004, **15**, 87–98; (*d*) S. André, F. Sansone, H. Kaltner, A. Casnati, J. Kopitz, H.-J. Gabius and R. Ungaro, *ChemBioChem.*, 2008, **9**, 1649–1661; (*e*) S. André, D. Specker, N. V. Bovin, M. Lensch, H. Kaltner, H.-J. Gabius and V. Wittmann, *Bioconjugate Chem.*, 2009, **20**, 1716–1728; (*f*) S. André, M. Lahmann, H.-J. Gabius and S. Oscarson, *Mol. Pharmacol.*, 2010, **7**, 2270–2279.

26 H. Leffler and S. H. Barondes, *J. Biol. Chem.*, 1986, **261**, 10119–10126.

27 N. Ahmad, H.-J. Gabius, S. André, H. Kaltner, S. Sabesan, R. Roy, B. Liu, F. Macaluso and C. F. Brewer, *J. Biol. Chem.*, 2004, **279**, 10841–10847.

28 K. Bachhawat-Sikder, C. J. Thomas and A. Surolia, *FEBS Lett.*, 2001, **500**, 75–79.

29 S. André, O. Renaudet, I. Bossu, P. Dumy and H.-J. Gabius, *J. Pept. Sci.*, 2011, **17**, 427–437.

30 S. Martín-Santamaría, S. André, E. Buzamet, R. Caraballo, G. Fernández-Cureses, M. Morando, J. P. Ribeiro, K. Ramírez-Gualito, B. de Pascual-Teresa, F. J. Cañada, M. Menéndez, O. Ramström, J. Jiménez-Barbero, D. Solís and H.-J. Gabius, *Org. Biomol. Chem.*, 2011, **9**, 5445–5455.

31 J. Kopitz, M. Bergmann and H.-J. Gabius, *IUBMB Life*, 2010, **62**, 624–68.

32 S. André, C. Grandjean, F. M. Gautier, S. Bernardi, F. Sansone, H.-J. Gabius and R. Ungaro, *Chem. Commun.*, 2011, **47**, 6126–6128.

33 (*a*) D. Delacour, V. Gouyer, J.-P. Zanetta, H. Drobecq, E. Leteurtre, G. Grard, O. Moureau-Hannedouche, E. Maes, A. Pons, S. André, A. Le Bisic, H.-J. Gabius, A. Manninen, K. Simons and G. Huet, *J. Cell Biol.*, 2005, **169**, 491–501; (*b*) H. Ideo, A. Seko and K. Yamashita, *J. Biol. Chem.*, 2005, **280**, 4730–4737.

34 S. André, D. Gigùere, T. K. Dam, C. F. Brewer, R. Roy and H.-J. Gabius, *New J. Chem.*, 2010, **34**, 2229–2240.

35 For some earlier work on scaffold development see: (*a*) M. Tosin, S. G. Gouin and P. V. Murphy, *Org. Lett.*, 2005, **7**, 211–214; (*b*) M. Tosin and P. V. Murphy, *J. Org. Chem.*, 2005, **70**, 4107–4117; (*c*) T. Velasco-Torrijos and P. V. Murphy, *Tetrahedron: Asymmetry.*, 2005, **16**, 261–272; (*d*) T. Velasco-Torrijos and P. V. Murphy, *Org. Lett.*, 2004, **5**, 3961–3964.

36 (*a*) H.-J. Gabius, *Anal. Biochem.*, 1990, **189**, 91–94; (*b*) H. Kaltner, D. Solís, J. Kopitz, M. Lensch, M. Lohr, J. C. Manning, M. Mürnseer, M. Schnölzer, S. André, J. L. Sáiz and H.-J. Gabius, *Biochem. J.*, 2008, **409**, 591–599; (*c*) H. Kaltner, D. Solís, S. André, M. Lensch, J. C. Manning, M. Mürnseer, J. L. Sáiz and H.-J. Gabius, *Biochemistry*, 2009, **48**, 4403–4416.

37 (*a*) H.-J. Gabius, R. Engelhardt, S. Rehm and F. Cramer, *J. Natl. Cancer Inst.*, 1984, **73**, 1349–1357; (*b*) H.-J. Gabius, F. Darro, M. Remmelink, S. André, J. Kopitz, A. Danguy, S. Gabius, I. Salmon and R. Kiss, *Cancer Invest.*, 2001, **19**, 114–126; (*c*) D. Kübler, C.-W. Hung, T. K. Dam, J. Kopitz, S. André, H. Kaltner, M. Lohr, J. C. Manning, L. He, H. Wang, A. Middelberg, C. F. Brewer, J. Reed, W.-D. Lehmann and H.-J. Gabius, *Biochim. Biophys. Acta*, 2008, **1780**, 716–722; (*d*) H. Kaltner, D. Kübler, L. López-Merino, M. Lohr, J. C. Manning, M. Lensch, J. Seidler, W.-D. Lehmann, S. André, D. Solís and H.-J. Gabius, *Anat. Rec.*, 2011, **294**, 427–444.