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First demonstration of differential inhibition of lectin binding by synthetic tri- and tetravalent glycoclusters from cross-coupling of rigidified 2-propynyl lactoside

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The interplay of mammalian lectins such as galectins with cellular glycoconjugates is intimately involved in crucial reaction pathways including tumor cell adhesion, migration or growth regulation. These clinically relevant functions explain the interest in designing glycoclusters with potent activity to interfere with lectin binding. In view of the perspective for medical applications the following objective arises: to correlate topological factors of ligand display most favorably to reactivity against endogenous lectins. To date, plant agglutinins have commonly been used as models. Properly addressing this issue we first prepared di- to tetravalent clusters from 2-propynyl lactoside under mild oxidative homocoupling conditions and using the Sonogashira palladium-catalyzed cross-coupling reaction with triiodobenzene or pentaerythritol cores. These products were tested for bioactivity in a competitive solid-phase assay using different labeled sugar receptors as probes, *i.e.* the β-trefoil mistletoe lectin, the natural lactoside-binding immunoglobulin G fraction from human serum and three mammalian galectins from two subgroups. The lactose headgroups in the derivatives retained ligand properties. Differences in inhibitory capacity were marked between the galectins. In contrast to homodimeric proto-type galectins-1 and -7 significant inhibition of galectin-3 binding with a 7-fold increase in relative potency was observed for the trivalent compound. In comparison, the binding of the β-trefoil mistletoe agglutinin was reduced best by tetravalent substances. The result for galectin-3 was independently confirmed by haemagglutination and cytofluorometric cell binding assays. These data underline the feasibility of galectin-type target selectivity by compound design despite using an identical headgroup (lactose) in synthesis.

Introduction

The coining of terms such as *sugar code* or *glycomics* reflects the growing realization of how well glycans of natural glycoconjugates are suited for biological information storage and transfer. Their unrivalled potential for building isomers including formation of branched structures, the versatile template-free but intimately controlled production of glycan determinants by a complex enzymatic machinery with regulation of expression of distinct epitopes on different levels, and the strategic positioning of the final products on cell surfaces readily accessible for intermolecular interactions make a strong case for carbohydrates as code words.[1] That glycan diversity and placement are matched by occurrence of various families of lectins epitomizes the concept of information transfer by productive protein–carbohydrate interactions.[2] Naturally, insights into lectin functionality will be the basis for devising medical applications, for example to target drugs exploiting sugar signals as postal code, to visualize lectin activity in relation to disease, for example in tumor diagnosis, or to block lectin binding, thereby interfering with glycan routing, unwanted cell adhesion, tissue invasion or infections.[3] With this aim in mind synthetic efforts have been directed at defining scaffolds for multivalent display of carbohydrate ligands.[4]

A major lesson taught by studies in this area is that multivalency will not necessarily lead to marked increases of biological activity of the carrier-immobilized lectin ligands. The well-ordered positioning of binding sites in the membrane engaged by endocytic C-type lectins,[2a,2f,3a] a blueprint for complementarity design of neoglycoconjugates, is thus a particularly favorable case. In fact, plant lectins such as concanavalin A or *Erythrina cristagalli* agglutinin were instrumental in revealing the limits to naive expectations that increases in ligand density would automatically trigger a cluster effect.[5] When thus accomplishing a new synthetic approach to glycoclusters, it is essential to thoroughly examine their potency in inhibition assays. Given the medical perspective noted above, plant lectins should still be regarded as models, with, eventually, endogenous lectins involved in disease-associated processes entering the test panel. Due to their involvement in tumor progression and metastasis formation, as well as frequent correlation of expression to patients' prognosis, the ligand-crosslinking members of the galectin family of β-galactoside-specific lectins afford suitable test objects.[6] Additionally, the different described modes of presentation of the carbohydrate recognition domains may even gain access to galectin-type selective reagents. Initial work with starburst and wedge-like glycodendrimers has underscored the potential of this concept.[7] This study extends work along this line.

Having recently developed a strategy to design tri- and tetravalent glycoclusters using palladium cross-coupling and Sonogashira reactions and triiodobenzene or pentaerythritol cores,[8] we herein describe the preparation of lactoside-bearing glycotope bio-isosteres with assumed reactivity to lectins. Next, we demonstrate the validity of this assumption. To figure out whether and to what extent the glycoclusters can react with carbohydrate-binding proteins we tested, following the reasoning given above, three galectins (the homodimeric proto-type galectins-1 and -7 and the chimera-type galectin-3 monomeric in solution and forming aggregates when surface immobilized[9]). To add further modes of presentation of target sites to testing the bioactive ligands we ran assays with the β-trefoil mistletoe lectin (*Viscum album* agglutinin, VAA) and a natural β-galactoside-specific immunoglobulin G fraction from human serum in parallel. The capacity of the new bi- to tetravalent lactose clusters to inhibit binding of these proteins to a matrix

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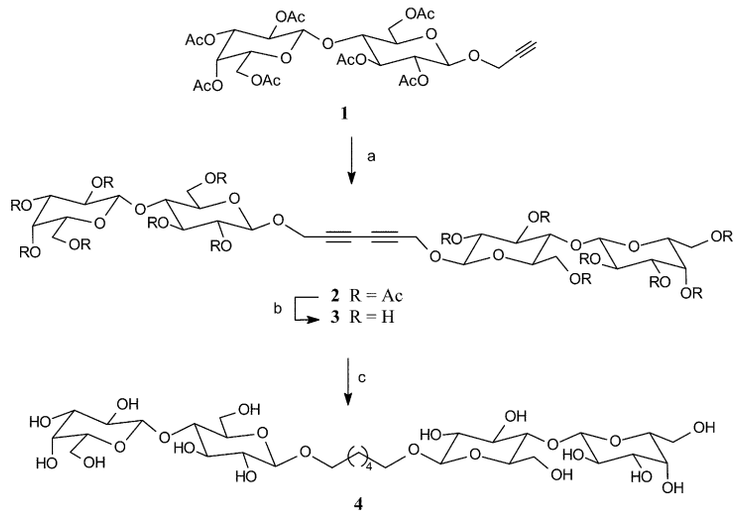
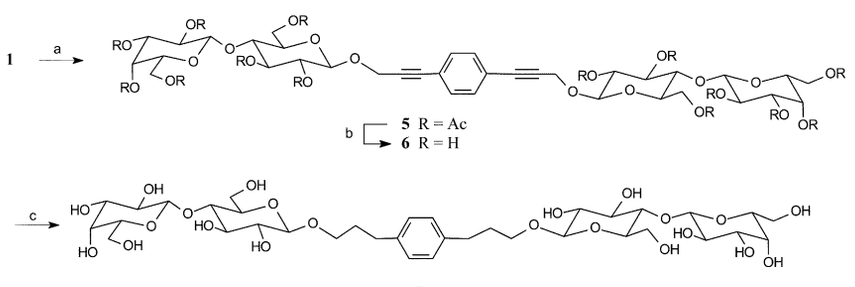


Scheme 1 *Reactions and conditions:* a) $(Ph_3P)_2PdCl_2$, CuI, NEt_3, DMF, rt, 3h (95%); b) NaOMe, MeOH, rt, 24 h, 95%; c) H_2, 10% Pd–C, MeOH, 5 h, 97%.



Scheme 2 *Reactions and conditions:* a) 1,4-Diiodobenzene, $(Ph_3P)_2PdCl_2$, CuI, NEt_3, DMF, rt, 3 h (90%); b) NaOMe, MeOH, rt, 24 h, 95%; c) H_2, 10% Pd–C, MeOH, 5 h, 95%.

established by surface-immobilized lactosylated neoglycoprotein, a structural mimic of a cell surface, was comparatively assessed. That glycoclusters effective in blocking galectin binding to the model matrix were also able to interfere with cell surface interactions was finally demonstrated in haemagglutination and cytofluorometric analysis using tumor cells. We reveal a clear preference of cluster design for the plant lectin and galectin-3.

Results and discussion

Synthesis

Starting with 2-propynyl lactoside **1** we prepared a series of di-, tri, and tetravalent compounds (cpd) to initiate testing of their bioactivity. In detail, 2-propynyl lactoside **1** was treated under mild oxidative homocoupling conditions to provide dimer **2** in 95% yield using dichlorobis(triphenylphosphine)palladium(II) ($(Ph_3P)_2PdCl_2$), copper(I) iodide (NEt_3 : DMF, 1 : 1, v/v, 25 °C) (Scheme 1). The choice of the catalysts was not critical since other palladium catalysts such as tetrakis(triphenylphosphine)-palladium(0) ($(Ph_3P)_4Pd$) or tris(dibenzylideneacetone)-dipalladium (0) ($Pd_2(dba)_3$) were equally effective. Zemplén de-*O*-acetylation of dimer **2** (NaOMe, MeOH) produced freely water-soluble di-lactoside **3** in 95% yield. Hydrogenation of **3** with 10% palladium on charcoal gave dimer **4** in quantitative yield.

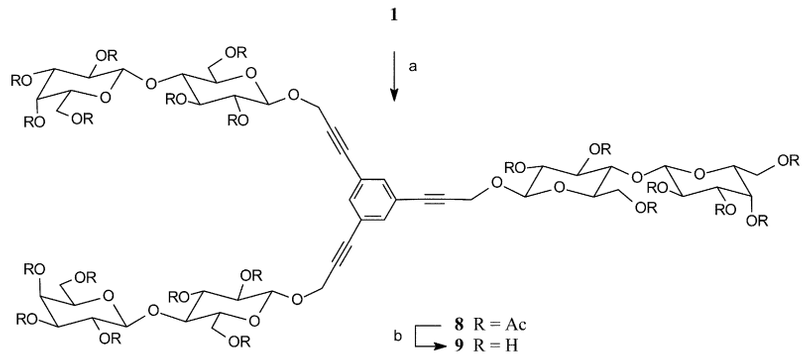
All the next cross-coupling reactions were done using the general protocol described above for **2** ($(Ph_3P)_2PdCl_2$, DMF : Et_3N (1 : 1. v/v), CuI, rt, 3–5 h). The simultaneous cross-coupling of 2-propynyl lactoside **1** to 1,4-diiodobenzene (Scheme 2), 1,3,5-triiodobenzene (Scheme 3), and to pentaerythritol tetrakis(*m*- and *p*-iodobenzyl)ether **10** and **11** (Scheme 4) afforded an efficacious entry into this family of carbohydrate clusters **5** (dimer 90%), **8** (trimer 80%), and tetramers **12** (*meta*) and **14** (*para*) in 78 and 75% yields, respectively. Complete de-*O*-acetylation of these clusters was uneventful, resulting in freely water-soluble clusters **6**,**9**,**13** and **15** in more then 90% yields. The solubility of these lactosylated clusters is in striking contrast to those observed for the corresponding β-D-galactoside clusters.[8a] Thus, the extension of the sugar part improves this physicochemical parameter and could also be exploited to tailor lectin-specific headgroups, if bioactivity of the compounds will be proven.

Tetramers **13** and **15** could not be hydrogenated due to the benzylic nature of the core pentaerythritol moieties which would be cleaved under such conditions. However, catalytic hydrogenation of dimer **6** under standard conditions (H_2, 10% Pd–C, MeOH) gave dimer **7** in 95% yield which possesses a slightly more flexible arm between the lactoside residues in comparison to homodimer **6**.

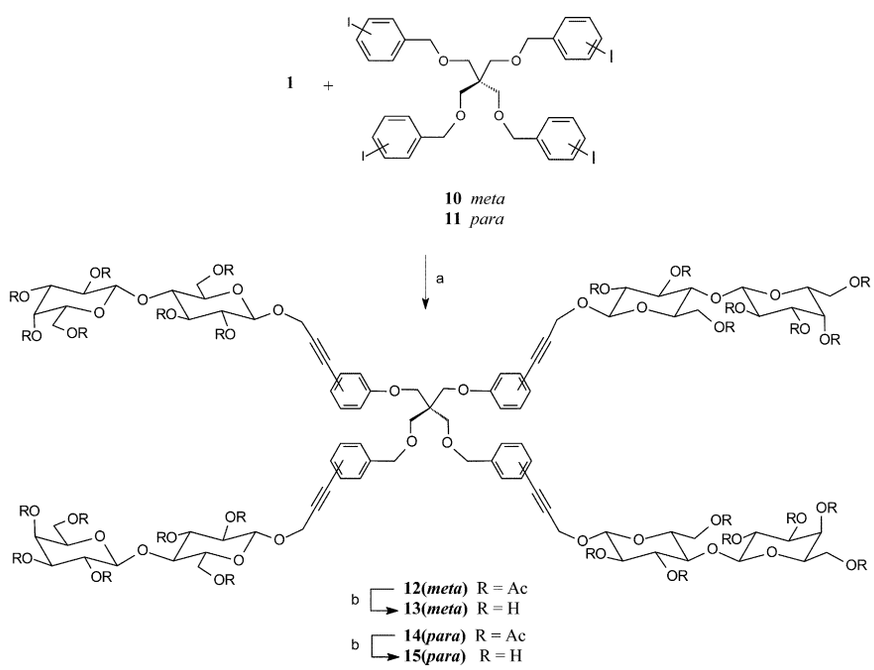
Having finished the synthesis of the test compounds and their analysis, we next addressed the question as to whether the derivatized sugar parts are still bioactive. For this purpose, we used the experimental setting of a solid-phase lectin-binding assay where these compounds were introduced as competitive inhibitors.

Biochemical and cell biological assays

The binding of the different sugar receptors to the matrix-immobilized neoglycoprotein was consistently dependent on ligand presentation on the surface and sensitive to the presence of lactose. If bioactivity of the synthetic compounds' headgroups was maintained, they should thus also interfere with protein–carbohydrate interaction responsible for signal generation in this assay. Indeed, this assumption was experimentally verified. Therefore, it was possible to assess the concentration which reduced the initial signal by 50% (IC_{50}), as shown exemplarily for the mistletoe lectin in Fig. 1. Stepwise increases



Scheme 3 *Reactions and conditions:* a) 1,3,5-Triiodobenzene, $(Ph_3P)_2PdCl_2$, CuI, NEt_3, DMF, rt, 5 h (81%); b) NaOMe, MeOH, rt, 24 h, 92%.



Scheme 4 *Reactions and conditions:* a) $(Ph_3P)_2PdCl_2$, CuI, NEt_3, DMF, rt, 5 h (78% *meta*, 75% *para*); b) NaOMe, MeOH, rt, 24 h, (quant).

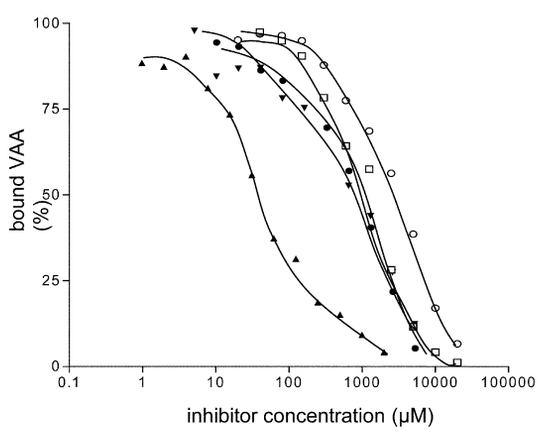


Fig. 1 Inhibition curves of binding of biotinylated *Viscum album* L. (VAA) agglutinin to surface-immobilized lactose maxiclusters using galactose (○), lactose (□), cpd-**3** (●), cpd-**4** (▼) and cpd-**15** (▲) in an exemplary series; for compound structures, please see illustrations in schemes.

of the inhibitor concentration reduced lectin binding to the surface-presented ligands, and the different curves reflect that glycoclusters can well surpass the inhibitory capacity of lactose. Running these assays systematically for the different sugar receptors led to the data compilation in Table 1. Of note, binding of the two homodimeric proto-type galectins-1 and -7 was not influenced effectively by the presence of these di- to tetravalent compounds, precluding the determination of accurate IC_{50}-values. Only the tetravalent compound **15** for galectin-1 and compounds **4**,**9**,**13** and **15** for galectin-7 harbored inhibitory capacity in the range of free lactose, establishing cases of negative correlation between carrier-dependent presentation of lactose and inhibitory efficiency. As seen in Table 1, the chimera-type galectin-3, however, reacted with the compounds rather well except for the divalent lactosides linked by an aromatic spacer. The strongest cluster effect was seen with the trivalent compound, a result shown for this murine, and also obtained for human, galectin-3. Independently, we determined the comparative inhibitory potency of these compounds in haemagglutination. These assays confirmed that compound **9** was the strongest inhibitor, again clearly surpassing free lactose. It should be noted that no evidence for formation of aggregates by visible precipitation was obtained under either of the experimental conditions.

Compared to the galectins the tetravalent compounds were more effective inhibitors in the case of the plant lectin VAA, while the *meta*-derivative **13** was also a good inhibitor for the natural immunoglobulin G fraction. The previous observation that the topological aspects of ligand display (*e.g.* presentation of bi-, tri, and tetraantennary N-glycans) modulate lectin affinity and inhibitory capacity of glycoclusters [7c,10] calls for a confirmation of the effectiveness of the trivalent compound to interfere differentially with galectin binding. For this purpose, we performed binding assays with tumor cells *in vitro*, a system with increased clinical relevance relative to the solid-phase assays. As shown in the upper panel of Fig. 2, differential activity of the trivalent compound **9** to impair galectin-3 but not

Table 1 Determination of the IC_{50}-values and the relative inhibitory potency of lactose-bearing clusters with limited spacer flexibility in a solid-phase assay with surface-immobilized lactosylated BSA and different types of sugar receptor in solution[a]

	Matrix	0.2 μg LacBSA		0.25 μg LacBSA		0.05 μg LacBSA	
	probe	VAA (1.5 μg ml^{-1})		Galectin-3 (20 μg ml^{-1})		IgG($\alpha^-\beta^+$) (2 μg ml^{-1})	
Inhibitor	Lac-content/molecule	IC_{50} (μM)	Relative potency	IC_{50} (μM)	Relative potency	IC_{50} (μM)	Relative potency
D-galactose	1	4000	0.5	5000	0.14	—[b]	—
Lactose	1	2000	1	700	1	3000	1
3	2	950	2.1 (1.1)	165	4.2 (2.1)	1058	2.8 (1.4)
4	2	653	3.1 (1.6)	163	4.3 (2.1)	2610	1.1 (0.55)
6	2	4315	0.5 (0.25)	—[c]	—	431	7.0 (3.5)
7	2	1900	1.1 (0.55)	—[c]	—	570	5.3 (2.6)
9	3	825	2.4 (0.8)	30.8	22.7 (7.6)	1650	1.8 (0.6)
13	4	40	50 (12.5)	125	5.6 (1.4)	199	15.1 (3.8)
15	4	40	50 (12.5)	62.3	11.2 (2.8)	1992 μM = 31% inh.	—

[a] Compound numbers denote substances as given in the Experimental Section and reaction Schemes. Numbers in parentheses express the relative potency of each lactose unit in the synthetic derivatives compared to lactose. The results are based on at least four independent experimental series presenting average values with standard deviations up to 14%. [b] Not inhibitory up to 200 mM. [c] Not inhibitory up to 4.8 mM.

Fig. 2 Cytofluorometric analysis of binding of galectins to tumor cell surfaces (a, b: murine monocyte/macrophage line J774.A1; c, d: human ovary adenocarcinoma line NIH-OVCAR 3) in an exemplary series testing the effect of the absence or presence of glyco-inhibitors (the molar concentration of lactose in the cell suspension was deliberately kept constant regardless of the nature of the test substance in each assay series). a: Level of background staining (—), staining with galectin-1 (——, 25 μg ml^{-1}) and staining with galectin-1 (25 μg ml^{-1}) in the presence of 0.5 mg ml^{-1} cpd-**9** (grey——); b: level of background staining (—), staining with galectin-3 (——, 20 μg ml^{-1}) and staining with galectin-3 (20 μg ml^{-1}) in the presence of 0.5 mg ml^{-1} cpd-**9** (grey——); c: level of background staining (—), staining with galectin-3 (——, 20 μg ml^{-1}) and staining with galectin-3 in the presence of 1.25 mM lactose (grey——); d: level of background staining (—), staining with galectin-3 (——, 20 μg ml^{-1}) and staining with galectin-3 in the presence of 0.5 mg ml^{-1} cpd-**9** (1.25 mM in lactose content) (grey——).

galectin-1 binding to a tumor cell surface was recorded. Next, we monitored the effects of this glycocluster relative to lactose used at increased molarity to reach equal total ligand concentrations and observed strong effects of the inhibitors (Fig. 2, bottom panel). Thus, the trivalent compound **9** (an illustration of a low-energy conformation of compounds **9** and **15**, respectively, is presented in Fig. 3) is a potent inhibitor of galectin-3 binding to native cell surfaces with the same differential activity against homodimeric galectins noted in the solid-phase assay.

Conclusion

Our study has proven that the lactoside headgroups in these glycoclusters retained their ligand activity. In fact, the tetravalent products obtained from the *m*- and *p*-iodobenzyl precursors showed a remarkable enhancement of inhibitory capacity on binding of the mistletoe lectin relative to free lactose. That an extrapolation of these data for a plant lectin to any mammalian lectin is not valid is clearly demonstrated by the respective experimental series. The emerging prominent role of galectins in tumor biology involved in cell growth, migration, invasion and metastasis formation has guided the decision to scrutinise members of this family. Also, different modes of target-site presentation and reports on antagonistic activities of galectins-1 and -3 give reasons for aiming at the development of target-selective glycoclusters.[9] Indeed, the trivalent design brought about selectivity for the only known chimera-type galectin under these experimental conditions. In view of the documented effect of ligand topology on inhibitory activity of

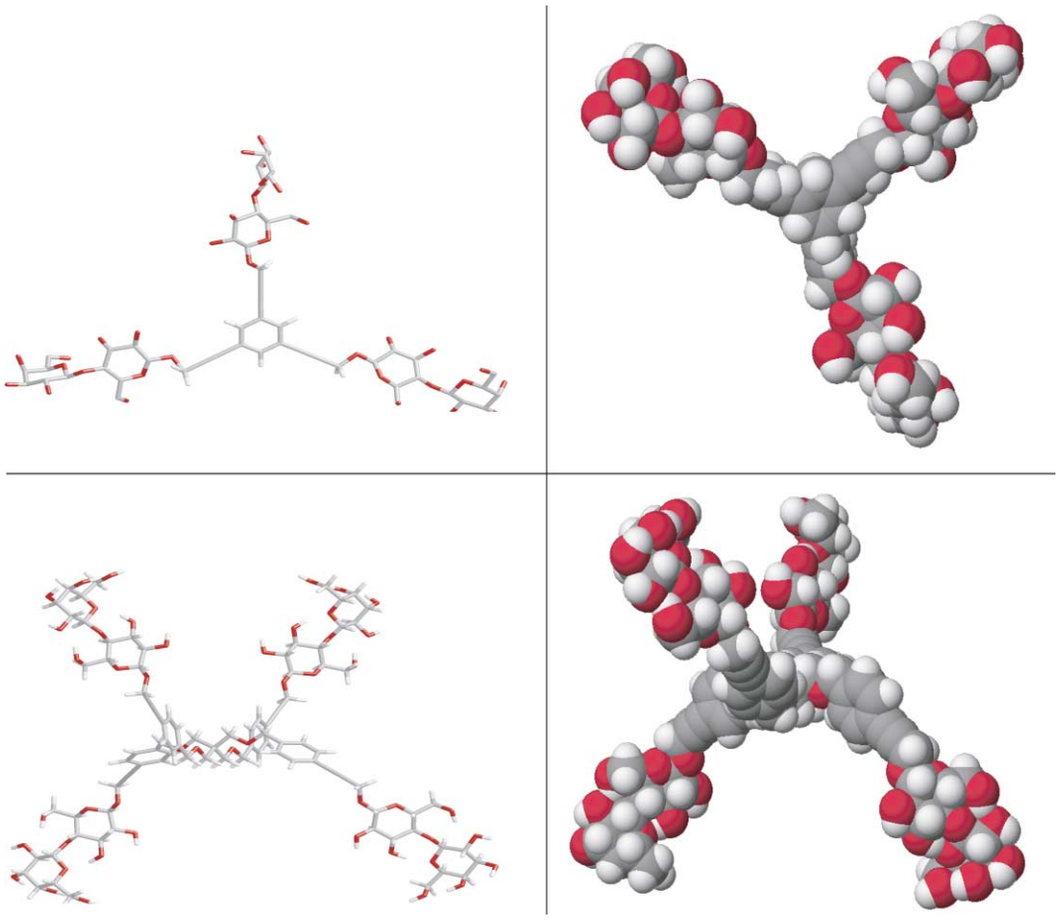


Fig. 3 Schematic representations of trimer **9** (upper panel: stick left, CPK right) and tetramer **15** (lower panel: stick left, CPK right) in one of their low-energy (MM3) extended conformations. The lactose portion was adapted from the X-ray data on the galectin-7/lactose complex (PDB 4GAL). Illustrations were rendered from SymApps software (BioRad) for the stick models and with CAChe 6.0 software from Fujitsu for the CPK models.

multivalent probes[7] further studies with bi- to tetraantennary N-glycans in addition to the tested maxiclusters are warranted. At any rate, the reported analyses by haemagglutination and cytofluorometry support the interpretation that the trivalent compound has preference for blocking galectin-3 binding. Having thus shown that this type of trivalency can distinguish between proto- and chimera-type galectins, further activity and/or selectivity enhancements might be feasible by tailoring the ligand structure and conformation and by hydrophobic tagging.[11] In more general terms, this work – together with previous studies on starburst and wedge-like glycodendrimers[7] – proves the potential of the concept to view glycoclusters as effective blocking reagents against clinically interesting endogenous lectins. Further efforts to interfere with antibody binding, for example in xenotransplantation, appear to be likewise rewarding.

Experimental

Synthetic procedures

All the chemicals were purchased from Aldrich Chemicals and used without further purification. Instrumentation description follows that described previously.[8a]

1,6-Bis-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1–4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxy]-2,4-hexadiyne (2). Prop-2-ynyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1–4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**1**)[12] (67.4 mg, 0.10 mmol) was dissolved in a solution of 10 mL of DMF and TEA (1 : 1) to which were added $Pd(PPh_3)_2Cl_2$ (3.6 mg, 5 mol%) and CuI (3.8 mg, 20 mmol%). After stirring for 3 h at rt, the solvents were removed under reduced pressure. The brownish residue was purified by silica gel column chromatography using hexane : ethyl acetate (2 : 3) to provide **3** as a white solid in 95% yield. $[\alpha]_D$ −22.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.31 (2H, bd, *J* = 3.4, H-4′), 5.19 (2H, t, *J* = 9.3, H-3), 5.07 (2H, dd, *J* = 8.0, 10.3, H-2′), 4.91 (2H, dd, *J* = 3.4, 10.3, H-3′), 4.88 (2H, dd, *J* = 8.0, 9.4, H-2), 4.66 (2H, d, *J* = 8.0, H-1), 4.49 (2H, dd, *J* = 1.6, 12.0, H-6a), 4.45 (2H, d, *J* = 8.0 H-1′), 4.39 (2H, s, H-1″), 4.02–4.11 (6H, m, H-6b and H-6′), 3.84 (2H, t, *J* = 7.0, H-5′), 3.79 (2H, t, *J* = 9.5, H-4), 3.61–3.64 (2H, m, H-5), 2.12, 2.10, 2.03, 2.02, 2.01, 1.93 (42H, 6s, C*H*$_3$CO); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.3, 170.1, 170.0, 169.7, 169.6 and 169.0 (CH_3CO), 101.0 (C-1′), 98.2 (C-1), 76.0 (C-4), 74.2 (alkynyl), 72.8 (C-5), 72.6 (C-3), 71.2 (C-2), 70.9 (C-3′), 70.8 (alkynyl), 70.7 (C-5′), 69.0 (C-2′), 66.6 (C-4′), 61.7 (C-6), 60.8 (C-6′), 56.3 (C-1″), 20.8, 20.7, 20.6, 20.5 and 20.4 (CH_3CO); ESI-MS calcd for $C_{58}H_{74}O_{36}$ + (NH_4^+): 1364.4; found: 1364.3.

De-*O*-acetylation of 3,6,9,13 and 15 under Zemplén conditions. The fully protected homodimer **2** (68 mg, 0.05 mmol) was suspended in methanol (15 mL), to which was added a catalytic amount of sodium methoxide. The solution was stirred at room temperature for 24 h. After neutralization of sodium methoxide with Amberlite resin (120 H^+), the solution was filtered. Removal of methanol under reduced pressure gave **3** (95% yield). Compounds **6**, **9**, **13**, and **15** were prepared in the same manner in 95%, 92%, 90%, and 91% yields, respectively.

1,6-Bis-[(β-D-galactopyranosyl)-(1–4)-β-D-glucopyranosyloxy]-2,4-hexadiyne (3). $[\alpha]_D$ −20.8 (*c* 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 4.70 (2H, *J* = 8.0, d, H-1), 4.63 (4H, s, H-1″), 4.49 (2H, d, *J* = 7.8, H-1′), 4.03 (2H, dd, *J* = 1.7, 11.8, H-6a), 3.98 (2H, d, *J* = 3.2, H-4′), 3.96 (2H, dd, *J* = 6.9, 12.0, dd, H-1a″), 3.81–3.88 (8H, m, H-5′, H-6b, H-6,), 3.67–3.78 (8H,

m,H-3, H-3′, H-4, H-5), 3.59 (2H, dd, J = 7.8, 9.9, H-2′), 3.40 (2H, t, J = 8.3, H-2); ^{13}C NMR (125.7 MHz, D_2O) δ 102.5 (C-1′), 100.1 (C-1), 77.8, 74.9 (C-5′), 74.4 (C-5), 73.9, 72.4, 72.1, 72.0, 70.5 (C-2′), 69.9 (alkynyl), 68.1 (C-4′), 60.5 (C-6′), 59.5 (C-6), 56.6 (C-1″); ESI-MS calcd for $C_{30}H_{46}O_{22}$ + (Na^+): 781.1; found: 781.2.

1,6-Bis-[(β-D-galactopyranosyl)-(1–4)-β-D-glucopyranosyloxy]-hexane (4). Homodimer **3** (39 mg, 0.05 mmol) was dissolved in methanol (10 mL) to which was added a catalytic amount of 10% Pd–C. The mixture was stirred at rt for 5 h. After filtration, the filtrate was concentrated to dryness under reduced pressure to provide **4** (97% yield). $[a]_D$ −21.5 (c 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 4.53 (2H, J = 8.0, d, H-1), 4.50 (2H, J = 7.8, d, H-1′), 4.02 (2H, d, J = 12.0, H-6a), 3.97 (2H, bd, J = 3.2, H-4′), 3.96 (2H, dd, J = 6.9, 12.0, H-1a″), 3.63–3.90 (18H, m, H-3, H-3′, H-4, H-5, H-5′, H-6b, H-6, H-1b″), 3.59 (2H, dd, J = 7.8, 9.9, H-2′), 3.45 (2H, t, J = 8.3, H-2), 1.69 (4H, m, H-2″), 1.44 (4H, bs, H-3″); ^{13}C NMR (125.7 MHz, D_2O) δ 102.5 (C-1′), 101.5 (C-1), 78.0, 74.9, 74.3, 74.0, 72.4, 72.1, 71.3, 70.5, 70.3, 70.1 (C-2), 69.9, 68.1 (C-2′), 67.9 (C-1″), 60.5 (C-6′), 59.7 (C-6), 28.1 (C-2″), 24.3(C-3″); ESI-MS calcd for $C_{30}H_{54}O_{22}$ + (NH_4^+): 784.3; found: 784.2.

General procedure for the cross-coupling reactions

1,4-Diiodobenzene (16.6 mg, 0.05 mmol) was dissolved in 10 mL of DMF and TEA (1 : 1), to which were added $Pd(PPh_3)_2Cl_2$ (3.6 mg, 5 mol%), lactoside **1** (80.9 mg, 0.12 mmol, 2.2 eq.) and CuI (3.8 mg, 20 mmol%). The solution was stirred at rt for 5 h under a nitrogen atmosphere. The solvent and triethylamine were removed under reduced pressure. The residue was purified by silica gel column chromatography using hexane and ethyl acetate (2 : 3) to give **5** (90% yield). The identical procedure was used toward the synthesis of clusters **8**,**12** and **14** in yields of 81%, 78% and 75%, respectively.

1,4-Phenylenedi-2-propyne-3,1-diyl bis[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1–4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside] (5). $[a]_D$ −31.0 (c 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 (4H, s, aromatic), 5.31 (2H, bd, J = 2.9, H-4′), 5.21 (2H, t, J = 9.2, H-3), 5.07 (2H, dd, J = 7.9, 10.3, H-2′), 4.91 (2H, dd, J = 3.5, 10.3, H-3′), 4.90 (2H, dd, J = 7.9, 9.4, H-2), 4.76 (2H, d, J = 7.9, H-1), 4.54 (2H, d, J = 16.1, H-1a″), 4.50 (2H, d, J = 16.1, H-1b″), 4.44–4.48 (4H, m, H-6a and H-1′), 4.02–4.11 (6H, m, H-6b and H-6′), 3.84 (2H, t, J = 6.8, H-5′), 3.80 (2H, t, J = 9.6, H-4), 3.61–3.64 (2H, m, H-5), 2.11, 2.08, 2.03, 2.02, 2.01, 2.00, 1.99 and 1.93 (42H, 7s, CH_3CO); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.3, 170.2, 170.1, 170.0, 169.7, 169.6 and 169.0 (CH_3CO), 131.6 and 122.5 (aromatic), 101.0 (C-1′), 98.2 (C-1), 86.4 and 85.4 (alkynyl), 76.0 (C-4), 72.8 (C-5), 72.7 (C-3), 71.4 (C-2), 70.9 (C-3′), 70.7 (C-5′), 69.1 (C-2′), 66.6 (C-4′), 61.8 (C-6), 60.8 (C-6′), 56.7 (C-1″), 20.8, 20.7, 20.6, 20.5 and 20.4 (CH_3CO); ESI-MS calcd for $C_{64}H_{78}O_{36}$ + (Na^+): 1445.3; found: 1445.2.

1,4-Phenylenedi-2-propyne-3,1-diyl bis[(β-D-galactopyranosyl)-(1–4)-β-D-glucopyranoside] (6). This compound was obtained under the Zemplén conditions described above for **3:** $[a]_D$ −7.0 (c 1.0, DMSO); ^{1}H NMR (500 MHz, DMSO) δ 7.30 (4H, s, aromatic), 4.64 (2H, d, J = 15.9, H-1a″), 4.53 (2H, d, J = 15.9, H-1b″), 4.38 (2H, d, J = 7.9, H-1), 4.17 (2H, d, J = 7.1, H-1′), 3.26–3.79 (22H, m, H-2′, H-3, H-3′, H-4, H-4′, H-5, H-5′, H-6, H-6′), 3.03–3.08 (2H, t, J = 8.3, H-2); ^{13}C NMR (125.7 MHz, DMSO) δ 131.7 and 122.2 (aromatic), 103.8 (C-1′), 100.9 (C-1), 87.7 and 85.0 (alkynyl), 80.6, 75.5, 75.0, 74.9, 73.2, 73.0, 70.5, 68.1, 60.5 (C-6′), 60.3 (C-6), 55.8 (C-1″); ESI-MS calcd for $C_{36}H_{50}O_{22}$ + (NH_4^+): 852.3; found: 852.2.

1,4-Bis-[[[(β-D-galactopyranosyl)-(1–4)-β-D-glucopyranosyl]-oxy]propyl]benzene (7). Hydrogenation of homodimer **6** (42 mg, 0.05 mmol) was done as above for **4** to provide compound **7** in 95% yield. $[a]_D$ −6.0 (c 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 7.30 (4H, s, aromatic), 4.50 (4H, d, J = 7.8, H-1 and H-1′), 3.94–4.06 (6H, m, H-4′, H-1a″, H-6a), 3.58–3.86 (24H, m, H-2′, H-3, H-3′, H-4, H-4′, H-5, H-5′, H-6b, H-6′, H-1″), 3.37 (2H, t, J = 8.3, H-2), 2.74 (4H, t, J = 7.4, H-3″), 1.97 (4H, t, J = 6.8, H-2″); ^{13}C NMR (125.7 MHz, D_2O) δ 139.3 and 128.2 (aromatic), 102.5 (C-1′), 102.4 (C-1), 78.0, 74.9, 74.3, 74.0, 72.4, 72.1, 70.5, 69.2 (C-1″), 68.1, 59.7 (C-6′), 59.4 (C-6), 30.3 and 30.1 (C-2″ and C-3″); ESI-MS calcd for $C_{36}H_{58}O_{22}$ + (Na^+): 865.3; found: 865.2.

Trimer 8. $[a]_D$ −22.0 (c 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42 (3H, s, aromatic), 5.31 (3H, dd, J = 0.9, 3.4, H-4′), 5.20 (3H, t, J = 9.3, H-3), 5.07 (3H, dd, J = 7.9, 10.4, H-2′), 4.91 (3H, dd, J = 3.4, 10.4, H-3′), 4.90 (3H, dd, J = 7.9, 9.4, H-2), 4.73 (3H, d, J = 7.9, H-1), 4.55 (3H, d, J = 16.1, H-1a″), 4.50 (3H, d, J = 16.1, H-1b″), 4.44–4.48 (6H, m, H-6a and H-1′), 4.02–4.11 (9H, m, H-6b and H-6′), 3.85 (3H, t, J = 7.1, H-5′), 3.80 (3H, t, J = 9.2, H-4), 3.61–3.66 (3H, m, H-5), 2.11, 2.08, 2.03, 2.02, 2.01, 2.00, 1.99 and 1.93 (63H, 7s, CH_3CO); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.7, 170.5, 170.4, 170.1, 170.0, 169.4 (CH_3CO), 135.1 and 123.5 (aromatic), 101.4 (C-1′), 98.6 (C-1), 85.5 and 85.3 (alkynyl), 76.4 (C-4), 73.1 (C-5), 73.0 (C-3), 71.7 (C-2), 71.3 (C-3′), 71.0 (C-5′), 69.4 (C-2′), 66.9 (C-4′), 62.1 (C-6), 61.1 (C-6′), 56.9 (C-1″), 21.2, 21.1, 21.0 and 20.8 (CH_3CO); ESI-MS calcd for $C_{93}H_{114}O_{54}$ + (NH_4^+): 2112.6; found: 2112.4.

Deprotected trimer 9. This compound was obtained as described above for **3:** $[a]_D$ −24.8 (c 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 7.62 (3H, s, aromatic), 4.73–4.78 (9H, m, H-1, H-1″), 4.53 (3H, J = 7.8, d, H-1′), 4.03 (3H, bd, J = 11.2, H-6a), 4.00 (3H, bd, J = 3.2, H-4′), 3.73–3.92 (24H, m, H-3, H-3′, H-4, H-4′, H-5′, H-6b, H-6′), 3.67 (3H, m, H-5), 3.63 (3H, dd, J = 7.8 and 9.5, H-2′), 3.47 (3H, m, H-2); ^{13}C NMR (125.7 MHz, D_2O) δ 134.4 and 122.3 (aromatic), 102.5 (C-1′), 100.3 (C-1), 85.4 and 84.6 (alkynyl), 77.8, 74.9, 74.4, 73.9, 72.2 (C-2), 72.1, 70.5 (C-2′), 68.1 (C-4′), 60.1 (C-6′), 59.6 (C-6), 56.7 (C-1″); ESI-MS calcd for $C_{51}H_{72}O_{33}$ + (NH_4^+): 1230.4; found: 1230.4.

Tetramer (*meta*) 12. This compound was obtained by the cross-coupling of **1** and tetraiodide **10:** $[a]_D$ −24.5 (c 1.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.17–7.41 (16H, m, aromatic), 5.29 (4H, bd, J = 3.1, H-4′), 5.18 (4H, t, J = 9.3, H-3), 5.05 (4H, dd, J = 7.9, 10.4, H-2′), 4.91 (4H, dd, J = 3.2, 10.3, H-3′), 4.90 (4H, dd, J = 7.9, 9.4, H-2), 4.73 (4H, d, J = 7.9, H-1), 4.51 (4H, d, J = 16.0, H-1a″), 4.50 (4H, d, J = 16.0, H-1b″), 4.44–4.48 (8H, m, H-6a and H-1′), 4.40 (8H, s, benzyl), 4.02–4.11 (12H, m, H-6b and H-6′), 3.83 (4H, t, J = 6.7, H-5′), 3.78 (4H, t, J = 9.6, H-4), 3.61–3.64 (4H, m, H-5), 3.49 (8H, s, C(CH_2OR)$_4$), 2.10, 2.05, 2.00, 1.99, 1.98, 1.96 and 1.91 (84H, 7s, CH_3CO); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.2, 170.0, 169.9, 169.7, 169.6 and 168.9 (CH_3CO), 139.0, 130.7, 130.3, 128.3, 127.6 and 121.9 (aromatic), 100.9 (C-1′), 98.1 (C-1), 86.9 and 83.3 (alkynyl), 76.0 (C-4), 72.7 (C-5 and C-3), 72.6 (benzylic), 71.4 and 70.9 (C-3′ and C-2), 70.6 (C-5′), 69.2 (C(CH_2OR)$_4$), 69.0 (C-2′), 66.5 (C-4′), 61.8 (C-6), 60.7 (C-6′), 56.7 (C-1″), 45.6 ($C(CH_2OR)_4$), 20.7, 20.6, 20.5 and 20.4 (CH_3CO); ESI-MS calcd for $C_{149}H_{180}O_{76}$ + ($2NH_4^+$): 1610.5; found: 1610.5.

Deprotected *meta*-tetramer 13. This compound was obtained as described above for **3:** $[a]_D$ −7.2 (c 1.0, H_2O), ^{1}H NMR (500 MHz, D_2O at 60 °C) δ 7.47–7.78 (16H, m, aromatic), 4.98–5.10 (12H, m, H-1, H-1″), 4.92 (4H, d, J = 7.4, H-1′), 4.65 (8H, bs, PhCH_2), 4.44 (4H, bs, H-4′), 4.06–4.36 (36H, m, H-2′, H-3, H-3′, H-4, H-5′, H-6, H-6′), 3.93 (4H, bs, H-5), 3.88 (4H, 8, H-2), 3.47 (4H, m, H-2), 3.29 (8H, bs, C(CH_2OR)$_4$); ^{13}C NMR (125.7 MHz, D_2O) δ 138.7, 131.0, 130.2, 128.5, 127.6 and 121.9

(aromatic), 103.1 (C-1′), 101.1 (C-1), 86.8 and 85.0 (alkynyl), 78.7, 75.3, 74.8 (C-5), 74.5, 72.8, 72.3 (PhCH$_2$), 71.0, 68.8 (C(CH$_2$OR)$_4$), 68.6 (C-4′), 61.0 (C-6′), 60.4 (C-6), 57.2 (C-1″), 45.9 (C(CH$_2$OR)$_4$); ESI-MS calcd for $C_{93}H_{124}O_{48}$ + (Na^+): 2031.7; found: 2031.4.

Tetramer (*para*) 14. This compound was obtained by the Sonogashira coupling of **1** and tetraiodide **11** as described above**:** $[\alpha]_D$ −22.7 (*c* 1.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 (8H, d, J = 8.1, aromatic), 7.19 (8H, d, J = 8.1, aromatic), 5.30 (4H, bd, J = 2.9, H-4′), 5.19 (4H, t, J = 9.3, H-3), 5.06 (4H, dd, J = 7.8, 10.4, H-2′), 4.91 (4H, dd, J = 3.4, 10.4, H-3′), 4.89 (4H, dd, J = 7.9, 9.4, H-2), 4.76 (4H, d, J = 7.9, H-1), 4.53 (4H, d, J = 16.0, H-1a″), 4.50 (4H, d, J = 16.0, H-1b″), 4.48 (4H, m, H-6a), 4.46 (4H, J = 7.8, d, H-1′), 4.40 (8H, s, benzyl), 4.02–4.11 (12H, m, H-6b and H-6′), 3.84 (4H, t, J = 6.7, H-5′), 3.80 (4H, t, J = 9.6, H-4), 3.60–3.65 (4H, m, H-5), 3.52 (8H, s, C(CH$_2$OR)$_4$), 2.10, 2.07, 2.01, 2.00, 1.99, 1.98 and 1.91 (84H, 7s, CH$_3$CO); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.2, 170.0, 169.9, 169.7, 169.6 and 168.9 (CH$_3$CO), 139.5, 131.6, 127.0 and 121.0 (aromatic), 100.9 (C-1′), 98.1 (C-1), 86.9 and 83.3 (alkynyl), 76.0 (C-4), 72.7 (C-5 and C-3), 72.6 (benzylic), 71.4 and 70.9 (C-3′ and C-2), 70.6 (C-5′), 69.2 (C(CH$_2$OR)$_4$), 69.1 (C-2′), 66.6 (C-4′), 61.8 (C-6), 60.7 (C-6′), 56.8 (C-1″), 45.6 (C(CH$_2$OR)$_4$), 20.7, 20.6, 20.5 and 20.4 (CH$_3$CO); ESI-MS calcd for $C_{149}H_{180}O_{76}$ + ($2NH_4^+$): 1610.5; found: 1610.7.

Deprotected *para*-tetramer 15. This compound was obtained as described above for **3:** $[\alpha]_D$ 60.0 (*c* 0.5, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 7.31 (8H, bs, aromatic), 7.00 (8H, bs, aromatic), 4.62–4.78 (12H, m, H-1, H-1″), 4.52 (4H, J = 7.1, d, H-1′), 4.01 ((8H, bs, PhCH$_2$), 3.63–3.91 (44H, m, H-2′, H-3, H-3′, H-4, H-4′, H-5, H-5′, H-6, H-6′), 3.47 (4H, m, H-2), 3.29 (8H, bs, C(CH$_2$OR)$_4$); ^{13}C NMR (125.7 MHz, D_2O) δ 138.5, 131.3, 126.8 and 120.6 (aromatic), 102.5 (C-1′), 100.6 (C-1), 86.3 and 84.5 (alkynyl), 77.8, 74.9, 74.2, 74.0, 72.2, 71.7 (PhCH$_2$), 70.5 (C-2′), 68.1, 65.5, 60.6 (C-6′), 59.7 (C-6), 56.7 (C-1″), 45.9 (C(CH$_2$OR)$_4$); ESI-MS calcd for $C_{93}H_{124}O_{48}$ + (Na^+): 2031.7; found: 2031.5.

Biochemical and cell biological procedures

The five different types of lactoside-binding proteins (human galectins-1, -3, and -7, murine galectin-3, the IgG-fraction from human serum and the mistletoe lectin) were purified under optimized conditions with affinity chromatography on lactosylated Sepharose 4B, derived from divinyl sulfone activation,[13] as the crucial step, and purity was ascertained by gel filtration and one- and two-dimensional gel electrophoretic analysis as described.[7] Following their biotinylation under activity-preserving conditions and rigorous controls to exclude any loss of activity the solid-phase assay with bovine serum albumin exposing 24–28 *p*-isothiocyanatophenyl lactoside moieties as ligand-bearing matrix was performed as described.[7] Experimental conditions to obtain optimal responses (*i.e.* concentration of neoglycoprotein in the coating step and of labeled lectin) were defined in systematic experimental series. The individual parameters are listed in Table 1 for each case. Inhibition of haemagglutination and lectin binding to tumor cell surfaces using the commercially available murine monocyte/macrophage line J774A.1 and the human ovary adenocarcinoma line NIH-OVCAR 3 (American Type Culture Collection, Rockville, MD, USA) was measured as described.[7c,9a,14] Trypsin-treated and glutaraldehyde-fixed rabbit erythrocytes were tested in solution containing 2-fold serial dilutions of standards with the compounds and free lactose to determine the minimal inhibitory concentration. Cytofluorometric analyses used at least three separate cell batches independently. Identical molar concentrations of lactose (free or as constituent of the compounds) were assayed in direct comparison. To allow valid comparison individual experimental series were always performed with aliquots of the same cell batch on the same day (for further experimental details, please see legend to Fig. 2).

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