ARTICLE

OBC
www.rsc.org/obc

Rigidified multivalent lactose molecules and their interactions with mammalian galectins: a route to selective inhibitors

Ioannis Vrasidas,[a] Sabine André,[b] Paola Valentini,[a] Corina Böck,[b] Martin Lensch,[b] Herbert Kaltner,[b] Rob M. J. Liskamp,[a] Hans-J. Gabius[b] and Roland J. Pieters*[a]

[a] *Department of Medicinal Chemistry, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, NL-3508 TB Utrecht, The Netherlands*

[b] *Institut für Physiologische Chemie, Tierärztliche Fakultät, Ludwig-Maximilians-Universität München, Veterinärstr. 13, D-80539 München, Germany*

Received 2nd November 2002, Accepted 9th January 2003
First published as an Advance Article on the web 7th February 2003

New and rigid multivalent lactose molecules were prepared. The structures contain lactose-2-aminothiazoline units at the periphery that were formed from a cyclisation of the thiourea sulphur onto the triple bond of the spacer. The lactosides were evaluated as inhibitors against lectin binding in a solid phase inhibition assay. In this assay the glycoprotein asialofetuin was immobilized onto the surface of microtiter plate wells, mimicking cell surface presentation, while mammalian galectins-1, -3 or -5 were in solution. Between the three galectins, the folding pattern and sequence are closely related but the topology of presentation of the carbohydrate recognition domains differs. Strong multivalency effects were observed for the tetravalent lactoside in the inhibition of galectin-3 binding with enhancements of almost 4300-fold compared to lactose. Remarkable selectivity was obtained in the inhibition since relative potencies of the tetravalent lactoside with the proto type galectins-1 and -5 did not exceed a factor of 143 relative to lactose. The binding of the lactosides to galectin-3 was also studied by fluorescence spectroscopy with all components in solution. These studies showed no multivalency effects in the inherent binding affinities.

Introduction

Throughout nature protein(lectin)–carbohydrate interactions play a variety of important roles.[1] With the continuous appearance of more and more discoveries, for example in the role of lectins as cell adhesion molecules, it is becoming increasingly clear that interference with these interactions has great potential in medicine.[2] One hurdle to be overcome on the way to therapeutics is the weak millimolar affinity typically encountered between monovalent carbohydrates and their receptor proteins. Looking at biological systems several research groups have taken the lesson to heart that higher affinities can be attained by linking sugars together to produce multivalent systems that can exhibit greatly improved affinities. The area of multivalency has been extensively reviewed[3] and while the benefits are universally accepted it is often not yet clear what the origin of the enhanced affinities is. In some cases the chelate effect and favorable entropic contributions are probably involved[4] while in others aggregation into a lattice-like network plays a role.[5]

Despite the fact that the causes of multivalency effects are largely unknown it is worthwhile, for the long-term goal of optimising increases in binding affinities, to make variations in the spacers between linked carbohydrate units. To this end we have started work to make rigidified versions of previously reported lactose dendrimers.[6] We report herein on the synthesis of these rigidified multivalent lactose systems and their biological evaluation in solid phase binding assays of three mammalian galectins. We deliberately focused on this lectin class instead of commonly studied plant agglutinins to add an immediate medical perspective. Galectins are animal Ca^{2+} independent carbohydrate binding proteins with a β-galactoside specificity, that share a jelly roll like folding pattern.[7] They are involved in cell–cell and cell–matrix adhesion, cell migration and growth regulation with relevance to inflammation and tumor spread. Because of their involvement in these important processes the galectins are attractive target proteins for synthetic ligand design.[2d,8] For multivalency effects the topological display of the binding sites is of interest. The proto type and tandem repeat type family are both divalent cross-linkers, whereas galectin-3 is established by the carbohydrate recogniton domain linked to a stalk section.[7] The orientation of the binding sites in galectins is such that effective chelation by a spaced divalent carbohydrate is not likely.[9] Many of the biological effects of galectins are believed to be due to their ability to act as cross-linkers.[10] Our efforts towards rigidification of the spacers should be viewed as affecting the cross-linking, rather than aiming at chelation of the binding sites. The effects of rigidification are more often than not disadvantageous, but when the proper geometries can be created large benefits can be expected. Notably, the topological factor, properly exploited, might lead to galectin-type selective compounds. In this respect, comparative analysis of proto type galectins (in our study, galectins-1 and -5) against galectin-3 is a point of interest. In fact, galectin-3 is known to be a monomer in solution but aggregates when bound to surface ligands and does so with positive cooperativity.[11] Thus, we performed this analysis with the aim of finding out whether strong and selective inhibition of a distinct galectin could be detected. Such a selectivity might find various uses since galectins have a characteristic profile of biomedical functions. One example was found in a colon model, where liver metastasis was correlated with galectin-3 expression. Another was discovered in a neuroblastoma model, where galectin-1 induced non-classical apoptosis of the tumor cells akin to mechanisms of spontaneous regression while galectin-3 blocks this effect. A third example can be the observation that in glioblastoma cells galectin-1 is a major factor for the invasive behavior.[12] As noted above, the topology of the multivalent carbohydrate–lectin binding is assumed to be crucial for important signalling phenomena.

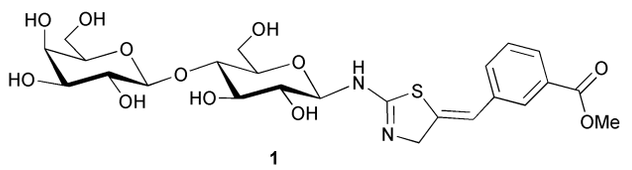
Results and discussion

Synthesis

We previously prepared and tested lactose dendrimers based on a flexible 3,5-di-(2-aminoethoxy)benzoic acid branching unit and evaluated them up to the third generation (octavalent)

DOI: 10.1039/b210923a

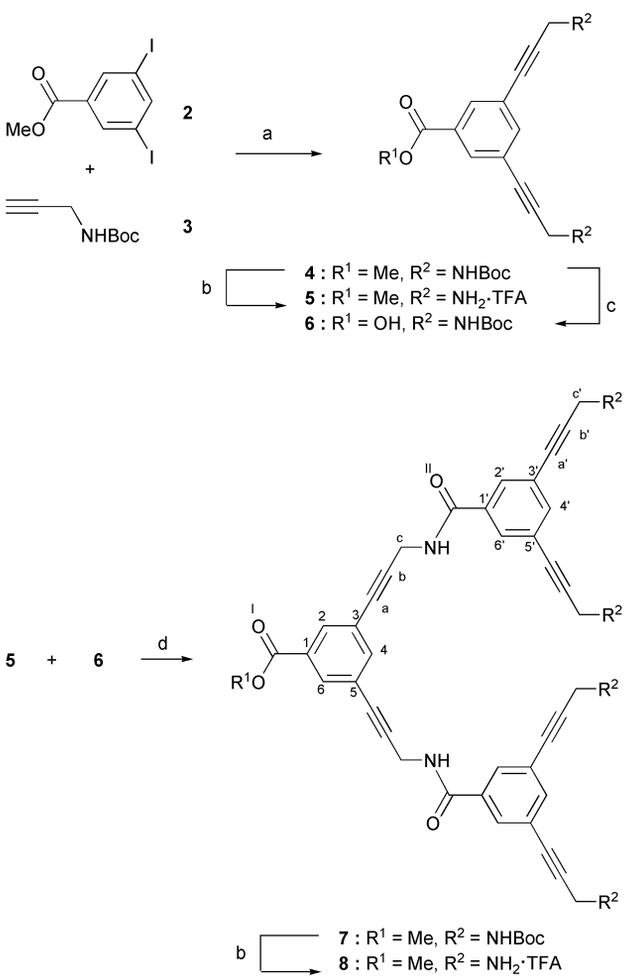
in inhibition studies with galectins and non-homologous galactose specific carbohydrate binding proteins.[13] We then started to introduce the more rigid propargylic amine spacers into the dendritic branching unit. In the case of a monovalent version, as we showed recently,[14] reaction of lactose isothiocyanate led to an initially formed thiourea-linked sugar, which underwent a facile conversion to a 2-aminothiazoline moiety. This was due to attack of the thiourea sulfur onto the triple bond and resulted in compound **1** (Fig. 1). The structure of this compound was identified by several two-dimensional NMR techniques, and the compound turned out to be a remarkably strong binder of the cholera toxin B-subunit.



1

Fig. 1 Structure of monovalent lactoside **1**.

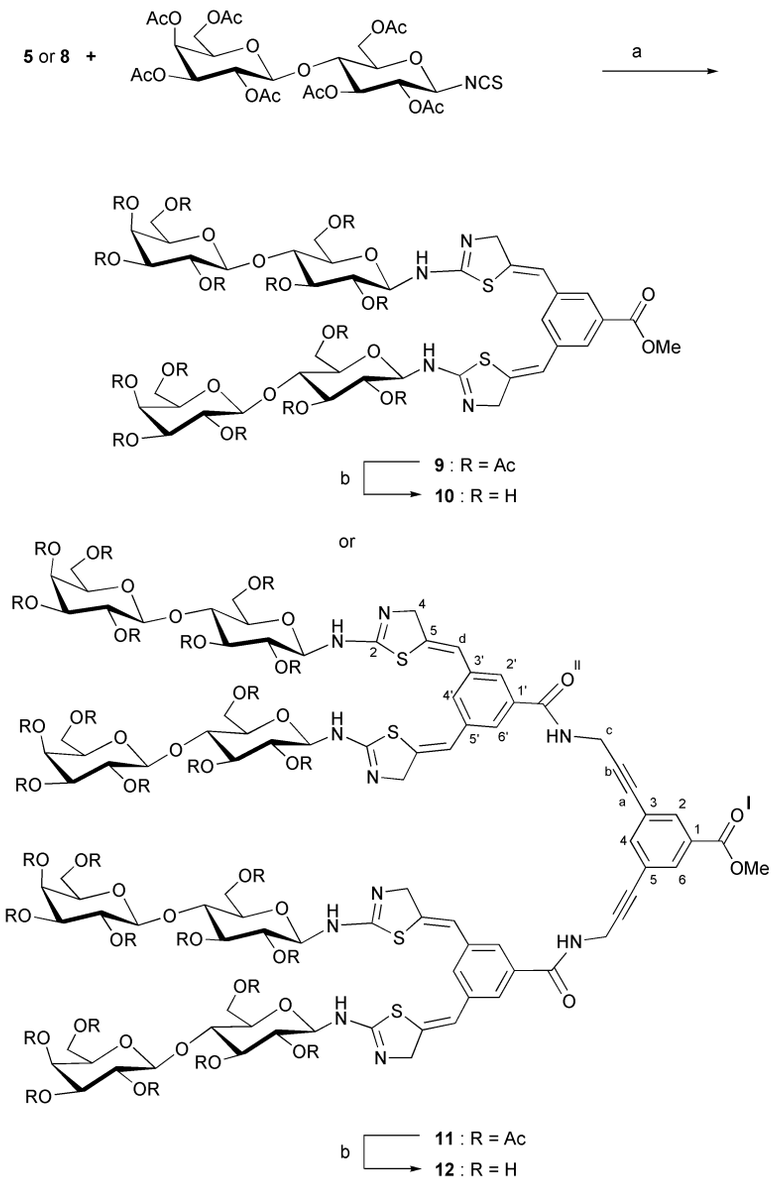
The extension of this chemistry to di- and tetravalent systems (Scheme 1) started with diiodide **2**. This compound was reacted with Boc-protected propargylamine **3** under modified Sonogashira reaction conditions,[15] and yielded the fully protected branching unit **4**. Part of this material was exposed to a CH_2Cl_2–TFA 5 : 1 mixture to yield the *N*-deprotected compound **5**. Another part of **4** was exposed to basic conditions in order to hydrolyse the methyl ester, which led to carboxylic acid **6**. The two differently protected building blocks **5** and **6** were coupled by means of the BOP coupling reagent[16] and gave the protected scaffold **7**, which after treatment with TFA yielded the tetraamino compound **8**, in its poly TFA salt form. The stage was now set for the coupling of the di- and tetraamine salts **5** and **8** to acetylated lactose β-isothiocyanate (Scheme 2). The coupling of **5** was performed in CH_2Cl_2 in the presence of iPr$_2$NEt. In order to facilitate the cyclisations[14] of the intermediate to the 2-aminothiazoline rings, the crude product was exposed to acetic acid in CH_2Cl_2. The desired product **9** was isolated by column chromatography from a mixture of two other byproducts. These two compounds were identified by mass spectrometry as oxidation products of **9** containing one or two additional oxygen atoms, presumably attached to sulfur of the 2-aminothazoline ring. In a similar way **8** was treated with lactose β-isothiocyanate in acetonitrile and iPr$_2$NEt to yield, after work up and chromatographic purification, the tetravalent lactose derivative **11**. Characterization of **9** and **11** was difficult by ^{1}H-NMR spectroscopy due to the large broadening of the signals, consistent with the reduced mobility of the rigid arms linking the sugars. However, crucial signals in the ^{1}H-NMR spectra at δ 6.6 ppm representing the bridging olefinic protons were observed for both compounds. Characteristic signals of the 2-aminothiazoline moiety of **9** and both the 2-aminothiazoline unit and the triple bonds of **11** could clearly be identified in the ^{13}C-NMR spectra. Further confirmation of the structure assignment came from the use of HSQC and HMBC techniques and gave correlations also observed for **1**.[14] ESI mass spectra from **9** and **11** showed the $[M + 2H]^{2+}$ (100%) ion at m/z = 799.95 and $[M + 3H]^{3+}$ (100%) ion at m/z = 1125.40, respectively, along with some galactose cleavage as observed before in the mass spectra.[6] Both divalent **9** and tetravalent **11** were deprotected with base using the same conditions that were used to protect the flexible analogues **14** and **15** (see next section).[6] The NMR spectra of **10** and **12** in D_2O were even broader than at the protected stage. In the ^{13}C-NMR spectra only signals of the galactose units were visible, indicating this to be the most mobile part of the molecule. Such a selective broadening has also been observed in lactose polymers.[9] However, ESI mass spectra showed the correct masses for **10** and **12** and indicated complete deprotection. Again, the $[M + 2H]^{2+}$ (100%) and $[M + 3H]^{3+}$ (100%) ions for **10** and **12** respectively were observed. From our experiments with **1** we knew that the basic deprotection conditions do not affect the heterocyclic ring.[14]



Scheme 1 *Reaction conditions*: a) Pd(o), CuI, NEt_3, CH_3CN, 2 d (65%); b) TFA, CH_2Cl_2, 20 min (quant.); c) Tesser's base, 14 h (quant.); d) BOP, iPr$_2$NEt, CH_3CN, 14 h (71%).

Assays

The inhibitory potency of the prepared lactose-containing compounds was determined with the use of a solid phase assay. In this assay the biotin-labeled lectins were allowed to bind to the carbohydrate moieties of a glycoprotein coated onto the surface of microtiter plate wells (the matrix) in competition with our lactosides.[10a,13] This design deliberately resembles properties of a model cell surface. The inhibition assay was performed with the galectins-1, -3, and -5. The comparative analysis of the homologous members of the galectin family required that their purity had to be ascertained rigorously. Thus, we examined their biochemical characteristics, *i.e.* molecular weight and isoelectric point, after purification by two dimensional gel electrophoretic analysis. As shown in Fig. 2, the three galectin preparations were free of contamination. The molecular weights calculated from their electrophoretic mobilities were close to the theoretical values based on the amino acid sequence. Occurrence of shifts of about 0.5–1 kDa from theoretical to experimental data, as seen for galectin-5, are known from other galectins, for example the chicken liver galectin CG-16 is referred to as a 16 kDa protein based on electrophoretic mobility despite its molecular mass of 14,976 Da.[17] Presence of isoelectric variants was only observed for bovine galectin-1. In this case, bovine heart and spleen



Scheme 2 *Reaction conditions*: a) first *i*Pr_2NEt, CH_2Cl_2, then AcOH, CH_2Cl_2, 14 h (65%) for **9**; *i*Pr_2NEt, CH_3CN, 14 h (33%) for **11**; b) NaOMe, MeOH, 2 h (70%) for **10**; dioxane, MeOH, 1 M NaOH, 3 h (94%) for **12**.

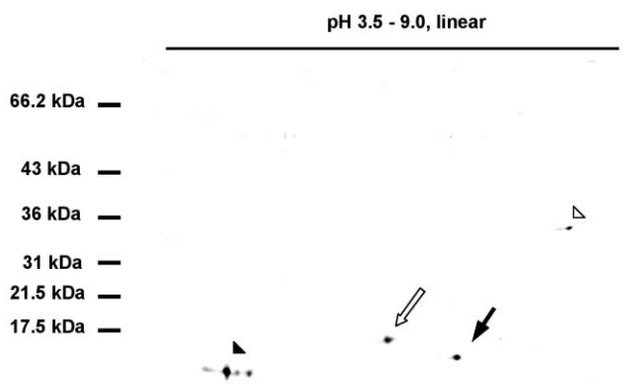


Fig. 2 Two dimensional gel electrophoresis analysis after silver staining of the three tested galectins showing purity in the cases of bovine galectin-1 (black arrowhead), rat galectin-5 (open arrow), and mouse galectin-3 (open arrowhead). Human galectin-7 (black arrow) was added as a further control to underscore the resolving power of the method. Occurrence of isoelectric variants was detected for bovine galectin-1.

galectin-1 obtained by chromatography on ASF-Sepharose had already shown pI heterogeneity in the detected range.[18,19] The matrix coated on the microtiter well was the glycoprotein asialofetuin (ASF) which presents a heterogeneous glycan population. It contains three *N*-glycosylation sites with complex type triantennary chains either with an *N*-acetyllactosamine terminus (74%) or with the Galβ1,3GlcNAc isomer in the outer Manα1,3-arm (9%) and a biantennary chain (17%). These *N*-glycan branches are known to be potent ligands for galectins in cross-linking experiments.[20] Three *O*-linked chains, present as well, primarily with the Galβ1,3GalNAcα-disaccharide,[20] also contribute to ASF binding.[21]

In the assay the following inhibitory carbohydrates were evaluated: galactose, lactose, ASF and compounds **1**, **10** and **12**. IC_{50} values are listed in Table 1. From the table it can be seen that for homodimeric galectin-1, a potent agent of haemagglutination, no significant multivalency effect was observed, except for ASF. For the other proto type galectin, galectin-5, however, a gradual increase in potency was detected while going from **1** to **10** to **12**. The tetravalent lactoside **12** showed a 21-fold increase in potency per lactose when compared to the most relevant monovalent reference: compound **1**. Large multivalency effects, even better than for ASF, were observed in the experiments with galectin-3, with enhancements, relative to lactose, of almost 4300-fold for **12** and an IC_{50} of 70 nM. In comparison to **1** the multivalency effect of **12** is a factor of 75 per monovalent unit while for **10** this number is 53.

Table 1 Determination of the IC_{50} values and the relative inhibitory capacity (relative potency, rel. pot.)[a] of lactosides and a glycoprotein (ASF) in a solid phase assay with surface immobilized ASF and three different labeled galectins in solution

Matrix probe:		1 μg ASF galectin-1 (10 μg ml^{-1})		1 μg ASF galectin-3 (30 μg ml^{-1})		1 μg ASF galectin-5 (15 μg ml^{-1})	
Inhibitor	Lactose units per molecule	IC_{50}/μM	Rel. pot.	IC_{50}/μM	Rel. pot.	IC_{50}/μM	Rel. pot.
Galactose	1	70000	0.06	400	0.75	7000	0.04
Lactose	1	4000	1	300	1	300	1
ASF	9	1.2	3200 (354)	0.08	3750 (417)	0.34	882 (98.0)
1	1	436	9.2 (9.2)	21	14 (14)	174.5	1.7 (1.7)
10	2	151	26.5 (13.3)	0.2	1500 (750)	15.1	19.9 (9.9)
12	4	178	22.5 (5.6)	0.07	4286 (1071)	2.1	143 (35.7)

[a] The numbers in parentheses express the relative potency of each lactose unit in the more than univalent inhibitor compared to carrier-free lactose.

Table 2 Effect of lactosides in the solid phase inhibition assay with galectin-3 (see Table 1) compared with the apparent K_d's of the same lactosides to galectin-3 as determined by a fluorescence binding assay[a]

		Solid phase inhibition assay matrix: 1 μg ASF		Fluorescence titration	
Carbohydrate	Lactose units per molecule	IC_{50}/μM	rel. pot.	K_d/μM	rel. pot.
Lactose	1	300	1	618	1
13	1	n.d.	—	162	4 (4)
14	2	260	1.2 (0.6)	79	8 (4)
15	4	23	13 (3.3)	31	20 (5)
1	1	21	14 (14)	50	12 (12)
10	2	0.2	1500 (750)	30	21 (10)
12	4	0.07	4286 (1071)	14	44 (11)

[a] The numbers in parentheses express the relative potency of each lactose unit in the more than univalent inhibitor compared to carrier-free lactose.

In order to investigate in more detail whether or not these multivalency phenomena were a direct consequence of enhanced affinity for the galectin-3 monomers in solution, we performed fluorescence titration studies. Galectin-3 contains a single conserved tryptophan unit in the binding site whose fluorescence spectrum is affected by ligand binding.[22] Fig. 3 shows an example of a titration experiment. Besides the rigidified compounds we also tested their more flexible counterparts **13**–**15** (Fig. 4).[6,13] In the solid phase assay **14** and **15** were not particularly effective inhibitors. Table 2 shows all compounds as evaluated in both solid phase inhibition tests (IC_{50}) and fluorescence titrations (K_d). The large differences in efficacy between **14** and **15** *versus* **10** and **12** in the solid phase inhibition studies is the most striking observation. In the fluorescence assay both spacered monovalent lactosides **13** and **1** showed improved binding relative to lactose, 4-fold and 12-fold respectively. For **1** this was also the case in the solid phase assay, where a 14-fold enhancement relative to lactose was seen. In the fluorescence titrations no multivalency effects were seen. In the flexible spacer series all three compounds **13**–**15** exhibited a relative potency per lactose of around 4. In the rigid spacer series all three compounds **1**, **10** and **12** were roughly equally potent on a per lactose basis: about 11-fold more potent than lactose. In order to verify whether or not these results were influenced by a different concentration of galectin-3 used in both assays (0.86 μM (30 μg mL^{-1}) in the solid phase inhibition assay and 0.57 μM in the fluorescence titration) we repeated the fluorescence titration of **12** with a galectin-3 concentration of 1.5 μM. In the fluorescence titration this had no significant effect as at both galectin-3 concentrations K_d's were comparable.

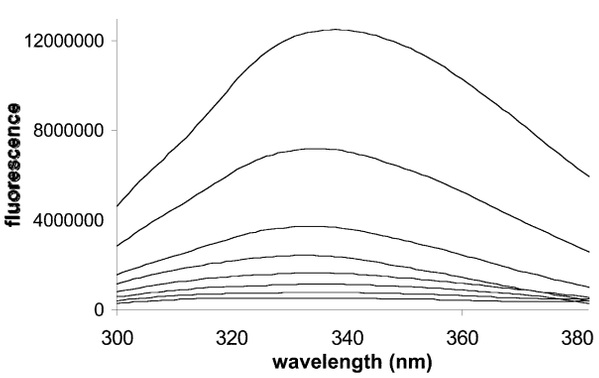


Fig. 3 Fluorescence titration of murine galectin-3 by **12**.

Conclusion

In the present paper a pronounced effect of spacer rigidification of a glycodendrimer on its selectivity for lectin binding was demonstrated. Equally important, a striking difference between multivalency effects observed in a common solid phase ELISA type inhibition assay, a model for cell surface binding, and a direct binding assay in solution using fluorescence spectroscopy was observed. For the rigid tetravalent **12** an apparent K_d for galectin-3 of 14 μM was determined. However, **12** led to a 50% inhibition of galectin-3 binding to matrix ASF at a concentration of 70 nM, a concentration at which very little of this compound is associated to galectin-3 in solution according to the fluorescence binding assay. In the literature reports have appeared that emphasise that the type of assay can be an important factor in the size of the multivalency effects measured. Not all assays measure the same phenomena.[3g,5b] Toone *et al.* and Brewer *et al.* have demonstrated this with the binding of mannose dendrimers to the tetrameric plant lectin ConA and noted sizeable effects they attributed to aggregation phenomena.[5c,23] Another illustration of the discrepancy between assays in different set-ups was recently shown with a divalent ligand binding to the Shiga-like toxin of pathogenic *E. coli.* A mass spectrometric analysis gave only a 6-fold multivalency enhancement, while an enzyme-linked immunosorbent assay showed a 40-fold enhancement.[24] The explanation of the enhanced inhibitory power of **12** for galectin-3 likely results from the aggregation behaviour of the chimeric galectin-3 on a

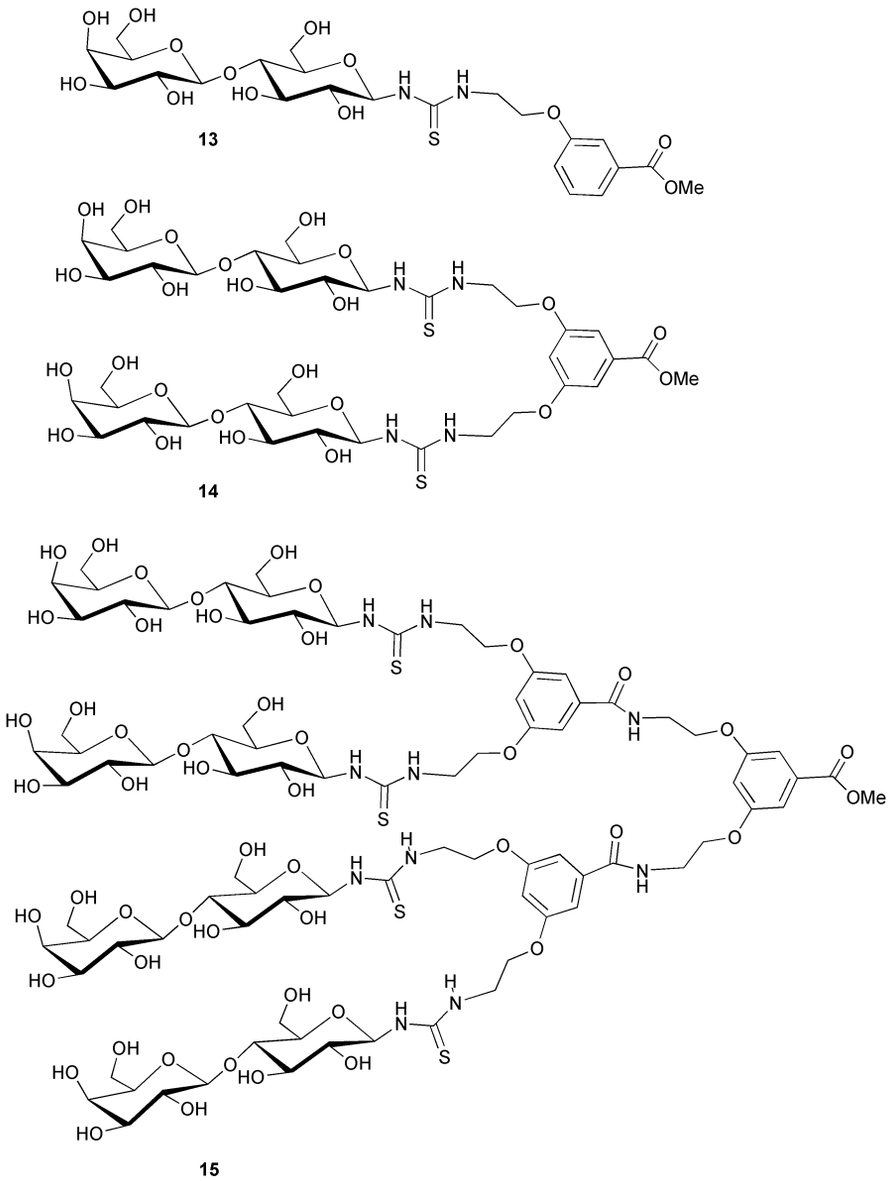


Fig. 4 Structures of lactosides **13**–**14**.

surface presented ligand. Corroborating previous work on positive cooperativity of laminin binding by rat galectin-3,[11b] Hughes *et al.*[25] have concluded from careful analysis of SPR studies with hamster galectin-3 that initially the carbohydrate recognition domain (CRD) of the galectin binds to the carbohydrate but that this is followed by a recruitment of additional lectin molecules from free solution forming lectin aggregates on the surface *via* protein–protein interactions. This recruitment is primarily mediated by contacts between the *N*-terminal collagen like domains of galectin-3. Fittingly, presence of this domain is essential for positive cooperativity and potent biomedical effects.[11b,12b,25] It could explain that despite the fact that only a small portion of **12** was bound to the CRD (as indicated by fluorescence) at 70 nM, a large portion of galectin-3 was made unavailable for firmly binding to the ASF matrix by additional aggregation. Observations made by mass spectrometry are consistent with this scenario. Free galectin-3 is present as a monomer,[26] while the addition of equimolar (10 μM) amounts of a multivalent lactose derivative leads to a total disappearance of the monomer signal, presumably due to the fact that large aggregates are formed.[27] The fact that the galectin-3 in the solid phase assay was biotinylated and non-biotinylated in the fluorescence assay can not be fully excluded as the cause of the discrepancy between the assays, however biotinylation was performed in the presence of a saturating lactose concentration. Moreover, studies with a related lectin showed no notable differences between biotinylated and non-biotinylated forms.[35] Additional studies will have to shed more light on galectin-3 behaviour in the presence of multivalent carbohydrates, which is relevant to its biological activities. Regarding the capacity of cells to express more than one galectin,[28] our studies showed that selective blocking of one particular galectin is possible with synthetic compounds exposing the same head group. It is a salient finding of this study that this topological parameter already engendered a marked selectivtiy. It can further be increased by exploiting subtle inter-galectin and inter-lectin family differences in ligand specificity.[8,29] Such selective compounds may prove to be valuable research tools in further determining the biological roles of the galectins and may also provide an entry into drug development, a promising perspective especially for galectin-3 and its role in promoting metastasis and angiogenesis.[30]

Experimental

General remarks

Chemicals were obtained from commercial sources and used without any further purification unless stated otherwise. The solvents CH_2Cl_2, MeOH and dioxane were purchased from

Biosolve, the Netherlands. The solvents CH_2Cl_2 and MeOH were stored on molecular sieves (4 Å and 3 Å respectively). The base iPr_2NEt was distilled from ninhydrin and KOH. All the solvents used for the Sonogashira reaction were dried with molecular sieves and degassed using nitrogen and the reaction was performed under an argon stream in the dark. Column chromatography was performed on Merck Kieselgel 60 (40–63 μm). For neutralization Dowex 50 × 8 (H^+-form; 20–50 mesh) purchased from Fluka, was used. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were recorded on a Varian G-300 spectrometer. Chemical shifts are reported in ppm relative to $CHCl_3$ (δ = 7.26 and δ = 77.0 respectively) or DMSO-d_6 (δ^1H = 2.49) or to CD_3OD (δ ^{13}C = 49). Coupling constants (J) are given in Hz. Assignments were given and the numbering schemes of the compounds can be seen in both Scheme 1 and Scheme 2. In addition to that the carbons of the 2-aminothiazoline ring carry the index 'h' as a subscript to indicate this fact. Electrospray ionization (ESI) mass spectrometry was carried out with a Shimadzu LCMS QP-8000 single quadrupole benchtop mass spectrometer (m/z range < 2000), coupled with a QP-8000 data system. Elemental analyses were carried out at Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany). For the fluorescence measurements a Fluorolog-3 FL 3–21 spectrofluorometer was used, connected to a PC with the program Datamax for Windows™ 2.00.

Galectins and solid phase inhibition assay

The three galactoside specific lectins were purified from aqueous extracts (as material sources, we used a bovine heart obtained from a local slaughterhouse in the case of galectin-1 and bacteria within recombinant production in the cases of galectins-3 and -5) by affinity chromatography on lactosylated Sepharose 4B, derived from ligand coupling to divinyl sulfone activated resin, as the crucial step.[31] For recombinant expression of murine galectin-3 we used the plasmid prCBP35, kindly provided by J. L. Wang (East Lansing, MI, USA), and *E. coli* JA221 cells, as described previously.[13] The cDNA of rat galectin-5 was cloned from total RNA of rat kidney isolated using an RNAeasy kit (Qiagen, Hilden, Germany) and was subjected to reverse transcription using 200 U Superscript™II RnaseH$^-$ Reverse Transcriptase (Invitrogen/Life Technologies, Karlsruhe, Germany). PCR amplification was directed by the sense primer 5′-CATGCCATGGCTTCCTTCAGCACCGAGA-3′ with an internal *NcoI* restriction site (underlined) and the antisense primer 5′-CGGCATGGTAGGTCTCCACGTGTGTT-3′ with an internal *BamHI* restriction site (underlined) based on the published sequence of this galectin.[32] The introduction of the described *NcoI* cleavage site led to a Ser2–Ala2 (S2A) substitution in the protein. Digestion with *NcoI*/*BamHI* of the amplicon was followed by its insertion into the procaryotic expression vector pQE60, and recombinant production was carried out in TB (terrific broth) medium (Roth, Karlsruhe, Germany) at 30 °C following induction with 0.5 mM isopropyl-β-D-thiogalactoside using *E. coli* M15-[pREP4] cells from Qiagen (Hilden, Germany). The isolated lectins were checked for purity and molecular characteristics by two dimensional gel electrophoresis using the IPGphor™ isoelectric focusing system and the Hoefer SE600 standard vertical unit (16 × 18 cm) with Immobiline™ dry strips (pH 3–10, linear) from Amersham Biosciences (Freiburg, Germany). The silver staining kit for proteins from this supplier and computer assisted analysis of the staining profile using a commercial set of standards as internal controls for calibration enabled calculation of molecular weights and isoelectric points. Biotinylation of the lectins was carried out using the *N*-hydroxysuccinimide ester derivative of biotin under activity preserving conditions.[33,34] After being prepared as described previously,[13] ASF was further subjected to β-elimination by treatment with 0.2 M NaOH for 4 h at 45 °C to remove the three *O*-glycan disaccharides. The solid phase assays on a matrix established by the glycoprotein, which resembles a cell surface, were performed and quantitatively evaluated, as described previously.[13,34] In brief, 1 μg asialofetuin in 50 μl 20 mM phosphate-buffered saline (pH 7.2) was coated for 12 h at 4 °C to the surface of each well in an ELISA plate. Following blocking of remaining protein-binding sites with buffer containing 1% bovine serum albumin and thorough washing a solution containing the galectin and a glycodendrimer which had been preincubated for 30 min at room temperature was transferred to the wells. Probing of the extent of specifically bound marker by streptavidin peroxidase and chromogenic substrate followed after the incubation with the galectin containing solution for 1 h at 37 °C and washing steps to remove any traces of galectin completely. The assay conditions had been selected on the basis of a prior experimental series defining an optimal carbohydrate dependent response in this system.

3,5-Bis(3-*tert*-butoxycarbonylamino-prop-1-ynyl)benzoic acid methyl ester (4)

To a suspension of 3,5-diiodobenzoic acid methyl ester **2** (7.25 g, 18.7 mmol), tetrakis(triphenylphosphine)palladium(0) (2.16 g, 1.87 mmol) and copper(I) iodide (356 mg, 1.87 mmol) in acetonitrile (80 mL) was added simultaneously a solution of prop-2-ynylcarbamic acid *tert*-butyl ester **3** (9.26 g, 56.1 mmol, 94% pure) in acetonitrile (10 mL) and Et_3N (13 mL, 93.5 mmol). The reaction mixture was stirred at room temperature. After 14 h a homogeneous solution was obtained but according to the TLC (EtOAc–hexanes 1 : 1) the reaction was not completed. After stirring for an additional 24 h a precipitate appeared and complete conversion of **2** was observed. The precipitate was filtered off and washed with cold acetonitrile and hexanes (−20 °C) to yield 5.4 g (65%) of a colourless solid. (Found: C 65.04; H 6.75; N 6.26. Calc. for $C_{24}H_{30}N_2O_6$: C 65.14; H 6.83; N 6.33); δ_H(300 MHz; $CDCl_3$) 8.00 (2 H, d, J 1.8 2/6-H), 7.60 (1 H, s, 4-H), 4.78 (2 H, br, NH), 4.15 (4 H, d, J 5.1, CH_2), 3.92 (3 H, s, CH_3O), 1.47 (18 H, s, CH_3^{Boc}); δ_C(75.4 MHz; $CDCl_3$) 165.5 (C=O^I), 155.3 (C=O^{Boc}), 138.3 (4-C), 132.2 (2/6-C), 130.4 (1-C), 123.4 (3/5-C), 87.2 (C^b), 81.0 (C^a), 80.0 (C^{Boc}), 52.3 (CH_3O), 30.9 (CH_2), 28.2 (CH_3^{Boc}); m/z (ESI) 465.15 $(M + Na)^+$.

TFA-salt of 3,5-bis(3-aminoprop-1-ynyl)benzoic acid methyl ester (5)

To a solution of **4** (500 mg, 1.1 mmol) in CH_2Cl_2 (10 mL) trifluoroacetic acid (TFA) (2 mL) was added and the reaction mixture stirred at room temperature for 20 minutes. After the solvent was removed *in vacuo* and the TFA co-evaporated with CH_2Cl_2 the product was obtained as an oil in quantitative yield. δ_H(300 MHz; D_2O) 7.93 (2 H, d, J 1.1, 2/6-H), 7.65 (1 H, s, 4-H), 3.89 (4 H, s, CH_2), 3.75 (3 H, s, CH_3O); δ_C(75.4 MHz; D_2O) 170.2 (C=O^I), 141.7 (4-C), 135.9 (2/6-C), 133.3 (1-C), 125.0 (3/5-C), 87.3 (C^b), 84.4 (C^a), 55.8 (CH_3O), 32.4 (CH_2).

3,5-Bis(3-*tert*-butoxycarbonylaminoprop-1-ynyl)benzoic acid (6)

Compound **4** (2 g, 4.5 mmol) was dissolved in a mixture of dioxane (119 mL) and methanol (42.5 mL). To that solution 4 M NaOH (8.5 mL) were added. After stirring at room temperature for 14 h, the pH was adjusted to approximately 2 with 1 M $KHSO_4$ and the solvents were evaporated *in vacuo*. To the residue CH_2Cl_2 (100 mL) was added and the solution was washed with brine (2 × 75 mL) and dried (Na_2SO_4). Acid **6** was isolated as a white powder in quantitative yield. δ_H(300 MHz; $CDCl_3$) 8.05 (2 H, br, 2/6-H), 7.60 (1 H, s, 4-H), 4.85 (2 H, br, NH), 4.15 (4 H, br, CH_2), 1.48 (18 H, s, CH_3^{Boc}); m/z (ESI) 339.40 $(M - 2CH{=}C(CH_3)_2 + Na)^+$ 395.37 $(M - CH{=}C(CH_3)_2 + Na)^+$ 451.52 $(M + Na, 100\%)^+$.

Protected tetraamine (7)

iPr_2NEt (118 μL, 675 μmol) was added to a suspension of **5** (42.3 mg, 90 μmol), **6** (75 mg, 175 μmol) and BOP (88 mg, 198 μmol) in dry acetonitrile (5 mL) to give a homogeneous solution. The reaction mixture was stirred at room temperature for 14 h and the obtained precipitate was filtered and washed with ice cold acetonitrile yielding **7** (68 mg, 71%) as a white solid. (Found: C 67.86; H 6.27; N 7.83; Calc. for $C_{60}H_{66}N_6O_{12}$: C 67.78; H 6.26; N 7.90); δ_H(300 MHz; DMSO-d_6) 9.23 (2 H, br, NHCO), 7.89 (6 H, s, 2/6-H, 2′/6′-H), 7.72 (s, 1H, 4-H), 7.53 (2 H, s, 4′-H), 7.40 (4 H, br, N*H*Boc), 4.32 (4 H, d, *J* 4.8, CH_2), 3.99 (8 H, d, *J* 5.4, CH_2′), 3.85 (3 H, s, CH_3O), 1.38 (36 H, s, CH_3^{Boc}). δ_C(75.4 MHz; 43% CD_3OD–CD_3COCD_3,) 166.4 ($C{=}O^{I}$), 166.0 ($C{=}O^{II}$), 156.9 ($C{=}O^{Boc}$), 138.9, 137.5, 135.7, 132.7, 132.0, 130.9 (1-C + 2/6-C + 4-C + 1′-C + 2′/6′-C + 4′-C), 124.9 (3/5-C + 3′/5′-C), 89.2, 88.5 (C^b + $C^{b'}$), 81.0, 80.9 (C^a + $C^{a'}$), 79.8 (C^{Boc}), 52.8 (CH_3O), 31.0 (C^c), 28.5 (CH_3^{Boc}).

Tetra-TFA-salt 8

Boc deprotection of compound **7** (60 mg, 56 μmol) was performed using the procedure described for **5** to afford **8** as a colorless oil in quantitative yield. Compound **8** was used without any further purification and characterization for the synthesis of **11**.

Protected divalent lactose (9)

A suspension of **5** (219 mg, 466 μmol) and Lac(OAc)$_7$NCS (920 mg, 1.4 mmol) in dry CH_2Cl_2 (5 mL) was treated with *i*Pr_2NEt (245 μL, 1.4 mmol) and stirred at room temperature overnight. The reaction mixture was washed with 1 M $KHSO_4$ (twice), 0.5 M NaOH (twice) and brine, dried (Na_2SO_4) and concentrated to dryness. The residue was redissolved in 6 mL CH_2Cl_2 and was treated with acetic acid (7 mL) at room temperature for 14 h. The crude mixture was purified by column chromatography (4% MeOH–CH_2Cl_2) and afforded product **9** in 65% (446 mg) yield. δ_C(75.4 MHz; 4% AcOH–$CDCl_3$) 170.8, 170.6, 170.4, 170.2, 170.1, 169.9, 169.3 (all $C{=}O^{OAc}$), 167.6 (2-C_h), 166.1 (C=O), 133.2 (5-C_h), 136.3, 131.1, 126.8 (1-C + 2/6-C + 3/5-C + 4-C), 120.2 (C^d), 100.6 (Gal–C_{anom}), 83.9 (Glc–C_{anom}), 75.5, 74.1, 72.7, 70.9, 70.8, 69.0, 66.7 (all Lac–CH), 61.9, 60.7 (2 × Lac–CH_2), 59.8 (4-C_h), 52.4 (CH_3O), 20.3, 20.2, 20.1, 20.0, 19.9 (all CH_3^{OAc}); *m*/*z* (ESI) 655.75 (M − Gal + 2Na)$^{2+}$, 778.60 (M − CH_2CO + 2H)$^{2+}$, 799.95 (M + 2H, 100%)$^{2+}$.

Divalent lactose (10)

NaOMe (30 wt% solution in MeOH, 46 μL, 238 μmol) was added to a solution of **9** (27 mg, 17 μmol) in dry MeOH (1.5 mL). The resulting suspension was agitated at room temperature for 2 h. The reaction mixture was neutralized with Dowex resin. After addition of water (1 mL) and removal of the resin by filtration, the solvents were evaporated *in vacuo*. The material was dissolved and lyophilized from H_2O to yield 12 mg (70%) of **10** as a yellowish solid. *m*/*z* (ESI) 505.15 (M + 2H, 100%)$^{2+}$, 1010.35 (M + H)$^+$.

Protected tetravalent lactose (11)

To a suspension of **8** (63 mg, 56 μmol) in acetonitrile (5 mL), LacNCS (152 mg, 224 μmol) was added and the reaction mixture treated at room temperature with at least 5 equiv. *i*Pr_2NEt until a basic (pH 8) homogeneous solution was obtained. After stirring at room temperature for 14 h the mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 and washed with 1 M $KHSO_4$ (twice), 0.5 M NaOH (twice) and brine, dried (Na_2SO_4) and concentrated to dryness. Compound **11** was isolated in 33% yield (62 mg) after purification by column chromatography (3% MeOH–7% acetone–CH_2Cl_2). δ_C(75.4 MHz; $CDCl_3$) 170.6, 170.3, 170.2, 170.1, 169.7, 169.2 (all $C{=}O^{OAc}$), 166.9, 166.6 ($C{=}O^{I}$ + $C{=}O^{II}$), 158.3 (2-C_h), 140.3, 138.6, 137.0, 134.3, 132.3, 130.5, 126.1, 125.2, 123.1 (br, all i-C + 5-C_h), 117.1 (C^d), 100.9 (Gal–C_{anom}), 86.8, 82.5–81.3 (C^a + C^b + Gal–C_{anom}), 75.8, 74.5–73.5, 71.0, 70.9, 70.2, 70.0, 68.9, 68.5, 68.0, 66.4 (all Lac–CH), 61.8, 60.6 (2 × Lac–CH_2), 60.5–59.5 (4-C_h), 52.4 (CH_3O), 30.4 (C^c), 20.9, 20.8, 20.7, 20.6, 20.5 (all CH_3^{OAc}); *m*/*z* (ESI) 772.85 (M − Gal + 2H + 2Na)$^{4+}$, 844.00 (M + 4H)$^{4+}$, 919.55 (M − 2Gal + H + 2Na)$^{3+}$, 1014.95 (M − Gal − CH_2CO + H + 2Na)$^{3+}$, 1029.50 55 (M − Gal + H + 2Na)$^{3+}$, 1125.40 (M + 3H, 100%)$^{3+}$, 1110.95 (M − CH_2CO + 3H)$^{3+}$, 1542.65 (M + 2Na)$^{2+}$, 1687.10 (M + 2H)$^{2+}$.

Tetravalent lactose (12)

Aqueous NaOH (1 M, 0.095 mL) was added to a solution of **11** (55 mg, 16.3 μmol) in dioxane (1.33 mL) and MeOH (0.475 mL). The resulting suspension was agitated at room temperature for 3 h. A homogeneous solution was obtained after neutralization with Dowex resin. After removal of the resin by filtration the solvents were evaporated *in vacuo*. The material was dissolved and lyophilized from H_2O to yield **12** as a white solid (33.7 mg, 94%). *m*/*z* (ESI) 625.35 (M − 2Gal + 3H)$^{3+}$, 678.80 (M − Gal + 3H)$^{3+}$, 732.8 (M + 3H, 100%)$^{3+}$, 1017.90 (M − Gal + 2H)$^{2+}$, 1099.00 (M + 2H)$^{2+}$.

Fluorescence titrations

A solution (1000 μL) of galectin-3 (0.57 μM) in PBS buffer (Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, pH 7.2, 0.15 M NaCl) was placed in a cuvette (1 × 1 cm). The fluorescence spectrum was recorded between 300 nm and 500 nm, using an irradiation wavelength of 282 nm, and the temperature was maintained at 25 °C. Incremental additions (> 10 datapoints) of a solution containing the lactose (derivative) and galectin-3 (0.57 μM) were added until at least 75% saturation was achieved. The fluorescence spectra were recorded by scans of 1 nm sec^{-1} and the data were stored. An additional spectrum of a reference cuvette with the same amount of the ligand but no galectin-3 was taken for each datapoint. These spectra were subtracted from those in which galectin-3 was present to correct for some inherent ligand fluorescence. The samples were left to incubate for about 5 min after each addition before the measurements were started. Fluorescence data at 350 nm, 340 nm and 335 nm for lactose derivatives **1**, **10** and **12** respectively were used to determine the apparent dissociation constant by fitting them to a one-site binding model.

Acknowledgements

The excellent technical assistance by B. Hofer and L. Mantel and the generous financial support of the Royal Netherlands Academy of Arts and Sciences (KNAW), the EC program for high level conferences (BIOPATH) and the Wilhelm Sander-Stiftung (Munich, Germany) are gratefully acknowledged.

References

1 (*a*) S. J. Williams and G. J. Davies, *Trends Biotechnol.*, 2001, **19**, 356–362; (*b*) H.-J. Gabius, S. André, H. Kaltner and H.-C. Siebert, *Biochim. Biophys. Acta*, 2002, **1572**, 165–177.

2 (*a*) H. Kaltner and B. Stierstorfer, *Acta Anat.*, 1998, **161**, 162–179; (*b*) E. E. Simanek, G. J. McGarvey, J. A. Jablonowski and C.-H. Wong, *Chem. Rev.*, 1998, **98**, 833–862; (*c*) J. C. McAuliffe and O. Hindsgaul, *Frontiers Mol. Biol.*, 2000, **30**, 249–285; (*d*) H. Rüdiger, H.-C. Siebert, D. Solís, J. Jiménez-Barbero, A. Romero, C.-W. von der Lieth, T. Diaz-Mauriño and H.-J. Gabius, *Curr. Med. Chem.*, 2000, **7**, 389–416.

3 (*a*) Y. C. Lee, *FASEB J.*, 1992, **6**, 3193–3200; (*b*) N. V. Bovin and H.-J. Gabius, *Chem. Soc. Rev.*, 1995, **24**, 413–421; (*c*) R. Roy, *Curr. Opin. Struct. Biol.*, 1996, **6**, 692–702; (*d*) M. Mammen, S.-K. Chio and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794; (*e*) L. L. Kiessling, L. E. Strong and J. E. Gestwicki, *Ann. Rep. Med.*

Chem., 2000, **35**, 321–330; (*f*) H.-J. Gabius, *Anat. Histol. Embryol.*, 2001, **30**, 3–31; (*g*) T. K. Dam and C. F. Brewer, *Chem. Rev.*, 2002, **102**, 387–430; (*h*) J. J. Lundquist and E. J. Toone, *Chem. Rev.*, 2002, **102**, 555–578; (*i*) T. K. Lindhorst, *Top. Curr. Chem.*, 2002, **218**, 201–235.

4 P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Arnstrong, H. Ling, N. S. Pannu, R. J. Read and D. R. Bundle, *Nature*, 2000, **403**, 669–672.

5 (*a*) S. M. Dimick, S. C. Powell, S. A. McMahon, D. N. Moothoo, J. H. Naismith and E. J. Toone, *J. Am. Chem. Soc.*, 1999, **121**, 10286–10296; (*b*) T. K. Dam, R. Roy, S. K. Das, S. Oscarson and C. F. Brewer, *J. Biol. Chem.*, 2000, **275**, 14223–14230; (*c*) T. K. Dam, R. Roy, D. Pagé and C. F. Brewer, *Biochemistry*, 2002, **41**, 1351–1358.

6 I. Vrasidas, N. J. de Mol, R. M. J. Liskamp and R. J. Pieters, *Eur. J. Org. Chem.*, 2001, 4685–4692.

7 (*a*) H.-J. Gabius, *Eur. J. Biochem.*, 1997, **243**, 543–576; (*b*) H. Lis and N. Sharon, *Chem. Rev.*, 1998, **98**, 637–674; (*c*) R. Loris, *Biochim. Biophys. Acta*, 2002, **1572**, 198–208; (*d*) G. A. Rabinovich, N. Rubinstein and M. A. Toscano, *Biochim. Biophys. Acta*, 2002, **1572**, 274–284.

8 P. Sörme, Y. Qian, P.-G. Nyholm, H. Leffler and U. J. Nilsson, *ChemBioChem*, 2002, **3**, 183–189 and references cited therein.

9 See for a study with galectin-3 and lactose polymers: N. L. Pohl and L. L. Kiessling, *Synthesis*, 1999, 1515–1519.

10 (*a*) S. André, P. J. C. Ortega, M. A. Perez, R. Roy and H.-J. Gabius, *Glycobiology*, 1999, **9**, 1253–1261; (*b*) C. F. Brewer, *Biochim. Biophys. Acta*, 2002, **1572**, 255–262.

11 (*a*) L. G. Frigeri, M. W. Robertson and F.-T. Liu, *J. Biol. Chem.*, 1990, **265**, 20763–20769; (*b*) S. M. Massa, D. N. W. Cooper, H. Leffler and S. H. Barondes, *Biochemistry*, 1993, **32**, 260–267.

12 (*a*) R. S. Bresalier, N. Mazurek, L. R. Sternberg, J. C. Byrd, C. K. Yunker, P. Nangia-Makker and A. Raz, *Gastroenterology*, 1998, **115**, 287–296; (*b*) J. Kopitz, C. von Reitzenstein, S. André, H. Kaltner, J. Uhl, V. Ehemann, M. Cantz and H.-J. Gabius, *J. Biol. Chem.*, 2001, **276**, 35917–35923; (*c*) I. Camby, N. Belot, F. Lefranc, N. Sadeghi, Y. de Launoit, H. Kaltner, S. Musette, F. Darro, A. Danguy, I. Salmon, H.-J. Gabius and R. Kiss, *J. Neuropathol. Exp. Neurol.*, 2002, **61**, 585–596.

13 S. André, R. J. Pieters, I. Vrasidas, H. Kaltner, I. Kuwabara, F.-T. Liu, R. M. J. Liskamp and H.-J. Gabius, *ChemBioChem*, 2001, **2**, 822–830.

14 I. Vrasidas, J. Kemmink, R. M. J. Liskamp and R. J. Pieters, *Org. Lett.*, 2002, **4**, 1807–1808.

15 K. Sonogashira, Y. Tohda and N. Hagihara, *Tetrahedron Lett.*, 1975, **16**, 4467–4470.

16 BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate.

17 M. Schneller, S. André, J. Cihak, H. Kaltner, H. Merkle, G. J. Rademaker, J. Haverkamp, J. Thomas-Oates, U. Lösch and H.-J. Gabius, *Cell. Immunol.*, 1995, **166**, 35–43.

18 A. De Waard, S. Hickman and S. Kornfeld, *J. Biol. Chem.*, 1976, **251**, 7581–7587.

19 H. Ahmed, N. E. Fink, J. Pohl and G. R. Vasta, *J. Biochem.*, 1996, **120**, 1007–1019.

20 D. Gupta, H. Kaltner, X. Dong, H.-J. Gabius and C. F. Brewer, *Glycobiology*, 1996, **6**, 843–849.

21 In order to decipher whether the three *O*-glycan disaccharides could also serve as ligands under these experimental conditions, we removed them chemically by β-elimination and performed solid phase assays to determine the contribution of the *O*-glycans to the overall binding. In the solid phase assays, binding to ASF was reduced for the lectins. In detail, by about 30% in the cases of galectins-1, -3 and -5.

22 (*a*) S. Jia and J. L. Wang, *J. Biol. Chem.*, 1988, **263**, 6009–6011; (*b*) J. Seetharaman, A. Kanigsberg, R. Slaaby, H. Leffler, S. H. Barondes and J. M. Rini, *J. Biol. Chem.*, 1998, **273**, 13047–13052.

23 J. B. Corbell, J. L. Lundquist and E. J. Toone, *Tetrahedron: Asymmetry*, 2000, **11**, 95–111.

24 E. N. Kitova, P. I. Kitov, D. R. Bundle and J. S. Klassen, *Glycobiology*, 2001, **11**, 605–611.

25 E. A. M. Barboni, S. Bawumia and R. C. Hughes, *Glycoconjugate J.*, 1999, **16**, 365–373.

26 J. Kopitz, S. André, C. von Reitzenstein, K. Versluis, H. Kaltner, R. J. Pieters, K. Wasano, I. Kuwabara, F.-T. Liu, M. Cantz, A. J. R. Heck and H.-J. Gabius, submitted.

27 K. Versluis, A. J. R. Heck, unpublished observation.

28 (*a*) H. Lahm, S. André, A. Hoeflich, J. R. Fischer, B. Sordat, H. Kaltner, E. Wolf and H.-J. Gabius, *J. Cancer Res. Clin. Oncol.*, 2001, **127**, 375–386; (*b*) A. Danguy, I. Camby and R. Kiss, *Biochim. Biophys. Acta*, 2002, **1572**, 285–293; (*c*) U. Wollina, T. Graefe, S. Feldpappe, S. André, K. Wasano, H. Kaltner, Y. Zick and H.-J. Gabius, *J. Cancer Res. Clin. Oncol.*, 2002, **128**, 103–110.

29 (*a*) D. Solís, A. Romero, H. Kaltner, H.-J. Gabius and T. Diaz-Mauriño, *J. Biol. Chem.*, 1996, **271**, 12744–12748; (*b*) S. André, B. Frisch, H. Kaltner, D. L. Desouza, F. Schuber and H.-J. Gabius, *Pharm. Res.*, 2000, **17**, 985–990; (*c*) N. Ahmad, H.-J. Gabius, H. Kaltner, S. André, I. Kuwabara, F.-T. Liu, S. Oscarson, T. Norberg and C. F. Brewer, *Can. J. Chem.*, 2002, **80**, 1096–1104; (*d*) A. M. Wu, J. H. Wu, M.-S. Tsai, J.-H. Liu, S. André, K. Wasano, H. Kaltner and H.-J. Gabius, *Biochem. J.*, 2002, **367**, 653–664.

30 P. Nangia-Makker, J. Conklin, V. Hogan and A. Raz, *Trends Mol. Med.*, 2002, **8**, 187–192.

31 H.-J. Gabius, *Anal. Biochem.*, 1990, **189**, 91–94.

32 M. A. Gitt, M. F. Wiser, H. Leffler, J. Herrmann, Y.-R. Xia, S. M. Massa, D. N. W. Cooper, A. J. Lusis and S. H. Barondes, *J. Biol. Chem.*, 1995, **270**, 5032–5038.

33 H.-J. Gabius, B. Wosgien, U. Brinck and A. Schauer, *Pathol. Res. Pract.*, 1991, **187**, 839–847.

34 (*a*) S. André, C. Unverzagt, S. Kojima, X. Dong, C. Fink, K. Kayser and H.-J. Gabius, *Bioconjugate Chem.*, 1997, **8**, 845–855; (*b*) C. Unverzagt, S. André, J. Seifert, S. Kojima, C. Fink, G. Srikrishna, H. H. Freeze, K. Kayser and H.-J. Gabius, *J. Med. Chem.*, 2002, **45**, 478–491.

35 O. E. Galanina, H. Kaltner, L. S. Khraltsova, N. V. Bovin and H.-J. Gabius, *J. Mol. Recognit.*, 1997, **10**, 139–147.