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# Combining carbohydrate substitutions at bioinspired positions with multivalent presentation towards optimising lectin inhibitors: case study with calixarenes†‡

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**Carbohydrate derivatisation and glycocluster formation are both known to enhance avidity for lectin binding. Using a plant toxin and human adhesion/growth-regulatory lectins (inter- and intrafamily comparisons) the effect of their combination is examined. In detail, aromatic substituents were introduced at the 2-*N* or 3′-positions of *N*-acetyllactosamine and the products conjugated to two types of calix[*n*]arenes (*n* = 4, 6) *via* thiourea-linker chemistry.**

The growing realisation of the high-density coding capacity of glycans gives reason to devise routes for medical applications.[1] Thus, sugar receptors (lectins), which translate the carbohydrate-encoded message into cellular responses,[1,2] become targets for drug development. Custom-made inhibitors would protect cells from harmful lectin activities.[3] This concept can be turned into compounds by teaming up carbohydrate and supramolecular chemistry as follows: (i) tailoring the carbohydrate for best-possible contact complementarity to the lectin and (ii) acquiring lectin-matching multivalent presentation using suitable conjugation chemistry and scaffold selection. This perspective defines the aim of our study, *i.e.* to determine the effects of exploiting changes in a core unit together with clustered presentation. We used members of the family of human adhesion/growth-regulatory galectins[2] and a potent plant toxin akin to ricin (*Viscum album* agglutinin, VAA) as models.

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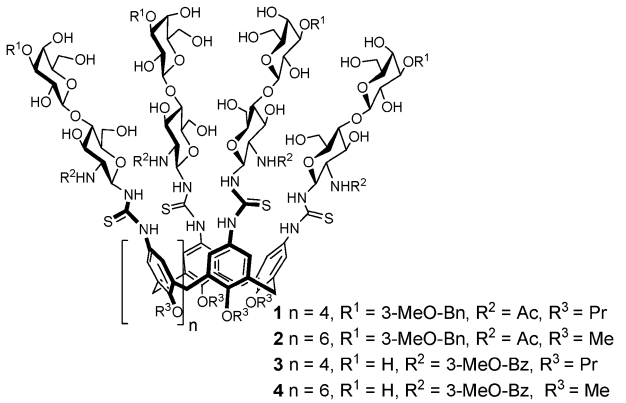
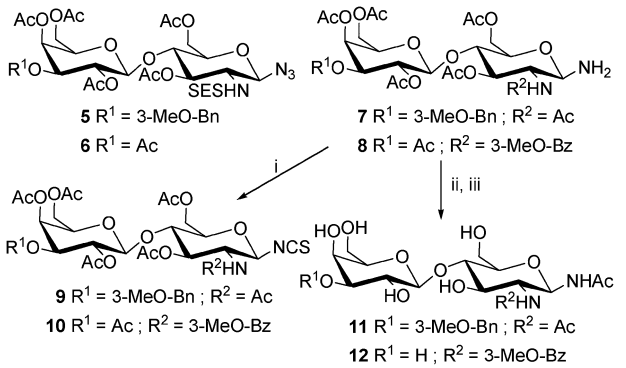
Galectins share affinity to *N*-acetyllactosamine (Lac*N*Ac) but exhibit differing degrees of reactivity for the histo-blood group A epitope with its 3′-extension.[4] Analyses by crystallography and flexible docking provided a detailed structural rationalization for this preference in the case of human galectin-3 and prompted synthetic work to capitalize on it.[5] However, cross-reactivity of such derivatives between the chimera-type galectin-3 and at least the tandem-repeat-type galectins-4 and -9 was delineated. In addition to this bioinspired site for exchange of a hexopyranose by an aromatic moiety the 2-*N*-position of Lac*N*Ac, held in its place by three hydrogen bonds with the 3-hydroxyl group,[5a,c] is a target. Aromatic substitution at this point has attracted interest as source for affinity generation.[6] Due to its impact on solubility aromatic bisubstitution is impeded, as is introduction of bulky substituents.

In contrast to galectins, the plant toxin is less permissive for substitutions at these sites, therefore furnishing an inherent negative control.[5b,7] Having on this ground reached the decision on where to enter structural additions, the previously proven compatibility of the linker chemistry with lectin activity could be applied.[8] We selected the *cone* calix[4]arene and the conformationally mobile calix[6]arene as scaffolds to obtain clusters **1–4** (for structures, please see Scheme 1) owing to their graded activity profiles to galectins-1, -3 and -4 [with $IC_{50}$-values (inhibitor concentration to reach 50%-level of signal reduction) of lactoside clusters of 10 mM/0.6 mM for galectin-1, 0.2 mM/0.4 mM for galectin-3, 20 μM/5 μM for galectin-4].[8] By testing the toxin and five human galectins against the free Lac*N*Ac derivatives and the glycoclusters in two assays of increasing biorelevance the following question is answered: will combination of ligand substitution with valency improve target properties?

A modified Danishefsky's iodosulfonamidation/azidation process,[9] accompanied or not by a tin-mediated, regioselective arylation carried out on D-lactal derivatives, led to the key 1-azido-2-sulfonamido intermediates **5** and **6**, as precursors of 2-amido-1-isothiocyanato lactopyranoses (Scheme 2, see ESI for full details‡). Acylation, removal of the sulfonamide protecting groups followed by selective reduction of the azides produced amines **7** and **8**, which were not isolated, but turned into the corresponding isothiocyanates **9** and **10**, or trapped




**Scheme 1** Structures of the calixarene glycoclusters based on 2/3′-substitutions in the Lac*N*Ac core.




**Scheme 2** Preparation of isothiocyanates and reference ligands. *Reagents and conditions*: (i) thiophosgene (2 equiv.), $CaCO_3$ (3 equiv.), THF, 0 °C to r.t., overnight or 1,1′-thiocarbonyldiimidazole (1.5 equiv.), $CH_2Cl_2$, r.t., 6 h; (ii) $Ac_2O$ (3 equiv.), $Et_3N$ (2 equiv.), $CH_2Cl_2$, 0 °C to r.t., 2 h; (iii) MeONa, MeOH, r.t., 2 h.

with excess acetic anhydride and deprotected under Zemplén conditions to provide monovalent reference compounds **11** and **12**. Isothiocyanates **9** and **10** were then conjugated at the upper rim of amino-alkoxycalix[4/6]arenes followed by deprotection,[8] to yield the glycoclusters **1–4** (Scheme 1). These glycoclusters, along with the monovalent controls, established the test panel. Physiologically, lectin functionality rests on binding to multivalent cellular glycoconjugates so that a matrix of a glycoprotein (here asialofetuin with up to nine terminal Lac*N*Ac units reactive with these lectins[10]) is an adequate model for testing.[8] It served as reactant for the labelled lectins, and the inhibitory capacity of the compounds was determined by co-incubation of increasing amounts with the lectin in solution, as described in detail previously.[8]

As exemplarily shown for galectin-3 (Fig. 1 in ESI‡), titrations revealed successively increasing extent of inhibition. They enabled the determination of $IC_{50}$-values. Used as control with predictive value, the tested substitutions were not favorable for VAA, as expected, although especially hexavalent presentation was still capable to establish some activity (Table 1). On the other hand, the inhibitory capacity was markedly increased by both types of substitution for galectin-3 (Fig. 1 in ESI‡) and also galectin-9, less for galectin-4 (Table 1). The chimera-type galectin-3 maintained its characteristic sensitivity for *cone*-type tetravalent presentation (Table 1),

**Table 1** $IC_{50}$-values of the free sugar derivatives and the sugar-bearing calix[4/6]arenes on binding for lectins to the glycoprotein[a]

| Type of inhibitor | VAA (0.5 μg ml$^{-1}$) | Galectin-3 (3 μg ml$^{-1}$) | Galectin-4 (5 μg ml$^{-1}$) | Galectin-9 (10 μg ml$^{-1}$) |
|---|---|---|---|---|
| Lactose | 300 | 2500 | 1200 | 3000 |
| **11** | 4800 | 250 | 800 | 900 |
| **12** | 800 | 360 | 1250 | 300 |
| **1** | 2800 | 0.15 | 2.2 | 2.8 |
| **2** | 420 | 0.4 | 0.8 | 1.4 |
| **3** | 200 | 1.3 | 2.5 | 2.0 |
| **4** | 45 | 1.5 | 1.2 | 0.8 |

[a] Coating with constant amount of 0.5 μg asialofetuin per well; assays to determine the $IC_{50}$-value given in μM sugar (please see Fig. 1 in ESI for example‡) were routinely performed in triplicates for up to five independent series with standard deviations not exceeding 14.2%; for structures of the glycoclusters, please see Scheme 1.

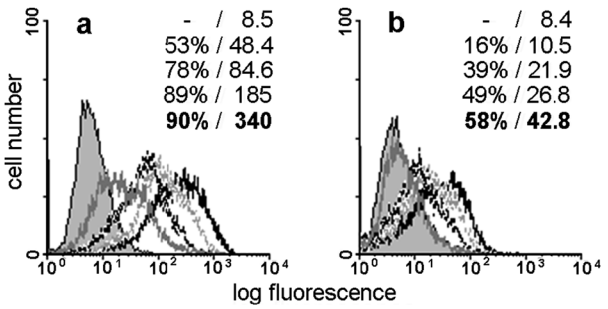



**Fig. 1** Inhibition of lectin binding to cells by the test compounds: the background control given as gray area, 100% -value as solid black line; data on percentage of positive cells/mean fluorescence intensity; data in each panel in the order of compound listing from bottom to top. (a, b) Galectin-4 (20 μg ml$^{-1}$) in the presence of 0.5 mM **12**, 10 μM **3** and 10 μM **4** (a), 0.5 mM **11**, 10 μM **1** and 10 μM **2** (b) for tumor-suppressor p16$^{INK4a}$-expressing pancreatic carcinoma Capan-1 cells. Assays were routinely performed in duplicates with up to five independent series on aliquots of cell suspensions of the same or next passage with standard deviations not exceeding 13.7%.

noted previously.[8] An about 10-fold increase in inhibition ensued from 3′-substitution relative to lactose, and tetravalency lowered the $IC_{50}$-value to 0.15 μM (in sugar), relative to 200 μM reached for lactose-bearing *cone*-4-type calixarene.[8] Both synthetic processes thus substantially add up to a significant gain in lectin-blocking capacity.

Whether the level of intrafamily selectivity is increased is answered by assays using galectins-1 and -8. Parallel testing disclosed rather weak inhibitory activity of the test panel with $IC_{50}$-values at least above 2 mM (not shown).

These results thus encouraged to proceed to examine these compounds in an *in vitro* system, *i.e.* challenging them with the task to protect cells from lectin binding. Cell surface glycans present a wide array of terminal galactosides along with particular spatial arrangements such as microdomains.

A recent study on galectins had proven their significance to form high-affinity contact sites for galectins-1 and -3.[11] Routinely, sugar-dependent lectin binding was ascertained and working concentrations for lectins in the linear range of signal intensity were set for comparative analysis, as described for initial calixarene testing previously.[8]

To interpret the scans presented in Fig. 1 (and Fig. 2 in ESI‡) the binding of a labelled lectin to cells will shift the signal from the position of the control curve (in gray) to the right. An inhibitor will then interfere with lectin association (measured in percentage of positive cells and mean fluorescence intensity), moving the fluorescence profile back into the direction of the control. This principle can now readily be applied to the scans. In full accord with Table 1, the monovalent derivative **12** was not inhibitory at this tested concentration on VAA, whereas lactose and the two glycoclusters **3** and **4** reduced lectin binding with increasing potency (Fig. 2a in ESI‡). As noted previously,[8] the *cone*-4-type presentation showed increased reactivity relative to the hexavalent compound in this system. This was further underscored by testing a different cell line, *i.e.* Chinese hamster ovary (CHO) cells with defective α2,3-sialylation (Lec2 mutant[12]) (not shown). The tetravalent glycocluster **1** proved most active against galectin-3 (Fig. 2b in ESI‡). In addition to the colon carcinoma cells, CHO cells with normal and reduced levels of α2,3-sialylation and decreased β1,6-branching of *N*–glycans (wild-type cells; Lec2/Lec4 mutant cells[12]) as well as pancreatic carcinoma cells, in which galectin-3 can act as competitive inhibitor against galectin-1 which induces anoikis under the control of the tumor suppressor $p16^{INK4a}$,[13] were processed, yielding comparable results. These data provide further evidence for the *cone*-4-type calixarene as suitable scaffold to hit galectin-3, irrespective of changes in the glycomic profile. Of note, galectin-1 binding was not affected at 10 μM sugar concentration by this calixarene (not shown), and galectin-4 remained more sensitive to the hexa- than the tetravalent glycoclusters (Fig. 1). The same tendency with rather similar quantitative data was observed for galectin-9, with optimal activity for the 2-*N*-substituted hexavalent **4** (not shown), in full accord with the data from the solid-phase assays (Table 1).

Overall, our results illustrate the benefit to combine core derivatisation with conjugation of the biomimetic product to distinct scaffolds for multivalent display. Practically, the documented interference of galectin-3 with the pro-anoikis effector of galectin-1 as *e.g.* in pancreatic cancer *in vitro*,[13] can be precluded by using such a selective blocking reagent, *i.e.* *cone*-4-type calixarene with 3′(or 2-*N*-)-substituted Lac*N*Ac. Further tailoring the nature of the substituent to fully match the galectin's individual microenvironment in the contact site, comprising Arg144, His158, Asn160, Lys176 and Trp181,[5a,c] affords the possibility for iterative improvements. Using bivalent glycophanes with/without conformational flexibility the attained selectivity increases could then be exploited to affect galectin-3 and its proteolytically processed form differently.[14] 2′-Substitution and starburst glycodendrimers will be worthy of testing VAA,[7,15] and the sulfatide headgroup is a commendable candidate for galectin-4.[4c,16] In general terms, the presented strategy to amalgamate carbohydrate and supramolecular chemistry can have relevance beyond the particular lectins tested in this study.

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