Glycosylated asterisks are among the most potent low valency inducers of Concanavalin A aggregation†

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A new class of sulfurated, semi-rigid, radial and low-valent glycosylated asterisk ligands with potential dual function as ligand and probe has some of the highest inhibition potencies of Con A-induced hemagglutination, by using a cross-linking mechanism of Con A which amplifies the enhancement to near nanomolar concentrations with the α-D-mannose asterisk.

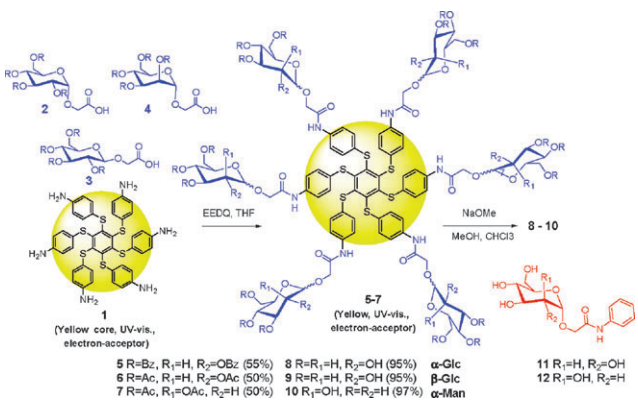
Protein–carbohydrate interactions mediate a wide range of intercellular communication processes, including cellular adhesion.[1] Although those interactions are often generated by individual weak association constants of limited specificity,[2] the assembly of multiple carbohydrate–protein complexes offers, through polyvalency, the avidity and selectivity necessary for their biological function.[3]

The structure of the molecular scaffold, the mechanism and effectiveness of the inhibition remains a central issue for understanding multivalent interactions. Many glycoclusters,[4] glycodendrimers[5] and polyglycomers[6] have been prepared in order to delineate the structure/activity relationships of the scaffold.[3,6a,7]

Our contribution here relies on the concept of a spherical-shaped scaffold with a dual function as a ligand and as a probe, combined with the need for semi-rigidity and amplification of inhibition through Con A/ligand auto-assemblies under kinetic conditions. The asterisks were selected as scaffolds/probes based on their synthetic, physical, and conformational properties. They are well-defined and have demonstrated potentially useful properties (redox potentials, UV/Vis absorption, conductivity, *etc.*)[8] which could facilitate detection by colorimetric, electrochemical and spectroscopic methods. They usually exhibit a conformational preference for an alternating up and down pattern of the thiophenyl groups above and below the plane of the central benzene ring,[8] which potentially could be used to pre-orient epitopes into particular patterns.

In practice, we synthesized and evaluated a new class of semi-rigid ligands based on a hexa-amino persulfurated benzene asterisk **1**,[9] decorated with α-glucose, β-glucose and α-mannose epitopes. The hexa-amino asterisk **1** was prepared on a multi-gram scale, by coupling of sodium 4-acetamidothiophenolate with C_6Cl_6 in DMI and deprotection in concentrated hydrochloric acid (93% overall yield; >12 g). Coupling of the asterisk **1** to the glycoconjugate acids **2**,[10] **3**,[11] and **4**[12] of α-glucose, β-glucose and α-mannose, respectively, was achieved using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as a coupling agent in yields ranging from 50 to 55% (Scheme 1). The deprotection of the glycosylated asterisks **5**–**7** proceeded smoothly under Zemplén conditions to afford compounds **8**–**10** (Scheme 1).

A biochemical evaluation of the glyco-asterisks **8**–**10** was investigated with Concanavalin A (*Canavalia ensiformis*, Con A).[13] This lectin interacts with cell surfaces by a process implicating multivalent complexes with non-reducing α-glucose and α-mannose residues. The inhibition of hemagglutination with sugar-based ligands in the presence of Con A has been widely used as a reference in carbohydrate–protein interaction studies, and was thus chosen for this first evaluation. In order to assess the activity of the glyco-asterisks, we compared their ability to inhibit agglutination of rabbit erythrocytes by Con A with that of their monovalent analogues, Me-α-Glc, Me-α-Man, and the aniline amides, *N*-phenyl-2-(α-D-Glc)acetamide **11** and *N*-phenyl-2-(α-D-Man)acetamide



Scheme 1

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Table 1 Hemagglutination inhibition assays for **8**–**10** with Con A

Ligand	IC_{50}[a]/mM	β[b]	β/N[c]
Me-α-D-glucopyranoside	4.0	1	1
N-Phenyl-2-(α-D-glucopyranosyloxy)acetamide **11**	9.9	0.4	0.4
8 (α-Glc)	0.011	364	61
9 (β-Glc)	NI at 11.7	—	—
Me-α-D-mannopyranoside	2.0	1	1
N-Phenyl-2-(α-D-mannopyranosyloxy)acetamide **12**	2.5	0.8	0.8
10 (α-Man)	8.9×10^{-5}	22 472	3750

[a] Each value represents the average of at least three assays. [b] Enhancement values $\beta = IC_{50}$(monomer)/IC_{50}(cluster). [c] Relative enhancement per sugar unit β/N.

12, and as a negative control, the hexavalent β-glucose asterisk **9**, whose sugar moiety is not recognized by the lectin (Table 1).

After careful blank control experiments and several reproduceable assays, the α-glucose and α-mannose asterisks **8** and **10** showed a strongly enhanced inhibition in both cases relative to their monovalent analogues. The α-glucose asterisk **8** inhibited hemagglutination at 11 μM, which corresponds to a 60-fold enhancement per sugar relative to Me-α-glucoside. On the other hand, the α-mannose asterisk **10** showed one of the best reported inhibitory potency at a minimum concentration of 89 nM, and hence greater than 3750-fold increase in relative activity per sugar compared to Me-α-mannoside. Two control experiments indicated that non-specific interactions of the scaffold were not responsible for the inhibition effect: the low enhancement values (β) for the aniline amides exclude subsite assisted binding of the sugar by the amide or aromatic group;[14] the β-glucose asterisk **9**, the most structurally analogous compound available, did not show any inhibition even at 11.7 mM concentration. The high enhancement of inhibition by **8** and **10**, in comparison to their monovalent analogues, indicates a powerful cross-linking ability.

In addition, there is a 60-fold amplification of the *selectivity* between the mannose and glucose asterisks relative to the monovalent compounds. While in the monovalent case, Me-α-Man inhibits at only a two-fold lower concentration than Me-α-D-glucoside, the mannose asterisk **10** inhibits at a 120-fold lower concentration than its glucose analogue **8**. A 100-fold selectivity has rarely been reported, except in the case of polyvalent Man/Glc polyglycomer scaffolds.[15]

In a nutshell, the α-*O*-mannosylated asterisk **10** has one of the highest reported enhancements (β = 22 472) and selectities (IC_{50} **8**/IC_{50} **10** = 120) to date for inhibition of hemagglutination induced by Con A, amongst discrete low valency ligands.

A visual turbidity test showed a rapid precipitation of Con A in the presence of an equimolar amount of the asterisk.[16] This result suggests that the semi-rigid architecture of the asterisk plays an important role in inducing a strong cross-linking of Con A.[6a,17] Dynamic light scattering (DLS) experiments[16] confirmed a strong aggregating effect on Con A by **10** at concentrations slightly above the nanomolar range. Fig. 1 shows a series of solutions containing Con A (17 μM) and **10** at different concentrations (0, 50, 100, 150, 200 and 400 nM). Without **10** a distribution centered around a hydrodynamic radius of 3 nm (red curve) was observed. A 100 nM concentration of the asterisk **10** induced a dramatic shift to nearly

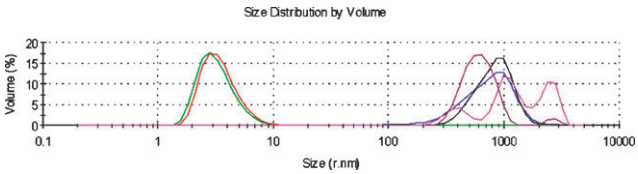

Fig. 1 DLS experiments on α-Man asterisk **10** with Con A. (a) Red: Con A 17 μM in 450 μl of HEPES 20 mM, pH 7.2, 150 mM NaCl, 2 μM $CaCl_2$, 2 μM $MnCl_2$: solution **A**. (b) Green: **A** + 50 nM of **10**; (c) Blue: **A** + 100 nM of **10**. (d) Black: **A** + 150 nM of **10**. (e) Purple: **A** + 200 nM of **10**. (f) Pink: **A** + 400 nM of **10**.

exclusively an aggregated species with a hydrodynamic radius near 850 nm (blue curve). This process is mannose-dependent and is partially reversible. A high concentration of Me-α-Man is required to offset the effect of the asterisk. Adding increasing concentrations of Me-α-Man (0, 1, 10, 500, 1000 μM) to a series of solutions containing Con A (17 μM) and **10** (0.5 μM) showed reversion of the aggregation only above 1 mM of Me-α-Man. It is noteworthy that identical solutions mixed in a different order did not give the same final result, even after several hours. These results suggest that, rather than an increased avidity for Con A, the unusual potency of the asterisks is due to an exceptionally efficient, kinetically controlled aggregation process that strongly amplifies the effect of these low valency ligands.

In conclusion, we have successfully validated several new concepts with the asterisk scaffolds and Con A. We synthesized a new class of circular, semi-rigid glycosylated sulfurated asterisks which have some of the highest inhibition potencies of Con A-induced hemagglutination, in addition to their potentially useful optical and electrophysical properties. A complete, rapid, and sugar-dependent aggregation of Con A at such low concentrations (89 nM) has rarely been reported with low-valency clusters.[18] DLS data indicated a cross-linking mechanism capable of aggregating Con A near the nanomolar range for the α-mannose asterisk. This activity can be attributed to a powerful cross-linking effect of the asterisk, which sequesters the lectin in a macromolecular assembly,[7] resulting in an amplification of the inhibition. It seems unusually favorable, compared to other systems of similar valency and our current hypothesis is based on the ability of semi-rigid structures to optimally adjust and properly present the sugar epitopes to the receptor for the *rapid* formation of a crosslinked macromolecular complex.[17] In an encouraging result, we have observed similar potencies with

more biologically relevant lectins, using similar asterisks, and the results will be reported in due course.

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