JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Subscriber access provided by Grand Valley State | University

Article

Topological effects and binding modes operating with multivalent imino-sugar-based glycoclusters and mannosidases

Yoan Brissonnet, Carmen Ortiz Mellet, Sandrine Morandat, M. Isabel Garcia-Moreno, David Deniaud, Susan E. Matthews, Sebastien Vidal, Sergej Sestak, Karim El Kirat, and Sébastien Gemayel Gouin

J. Am. Chem. Soc., **Just Accepted Manuscript** • Publication Date (Web): 13 Nov 2013

Downloaded from http://pubs.acs.org on November 14, 2013

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

ACS Publications
High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036
Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Topological effects and binding modes operating with multivalent iminosugar-based glycoclusters and mannosidases

Yoan Brissonnet,[†] Carmen Ortiz Mellet,[‡] Sandrine Morandat,[├] M. Isabel Garcia Moreno,[‡] David Deniaud,[†] Susan E. Matthews,[∀] Sébastien Vidal,[I] Sergej Šesták,[II] Karim El Kirat[‖] and Sébastien G. Gouin.*[†]

[†] LUNAM Université, CEISAM, Chimie Et Interdisciplinarité, Synthèse, Analyse, Modélisation, UMR CNRS 6230, UFR des Sciences et des Techniques, 2, rue de la Houssinière, BP 92208, 44322 NANTES Cedex 3, France.

[‡] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, C/ Profesor García González nº 1, 41012 Sevilla, Spain,

[├] Dr. S. Morandat, Laboratoire de Génie Enzymatique et Cellulaire, FRE-CNRS 3580, Université de Technologie de Compiègne (UTC), BP 20529, 60205 Compiègne Cedex, France,

[∀] Dr S.E. Matthews School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom,

I Dr. S. Vidal Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, Laboratoire de Chimie Organique 2 Glycochimie, UMR 5246, CNRS, Université Claude Bernard Lyon 1, F-69622 Villeurbanne, France,

II Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dúbravska cesta 9, 845 38 Bratislava, Slovakia,

[‖] Dr. K. El Kirat, Laboratoire de Biomécanique et Bioingénierie, UMR-CNRS 7338, Université de Technologie de Compiègne (UTC), BP 20529, 60205 Compiègne Cedex, France.

KEYWORDS. Iminosugars, Multivalency, Glycosidases, Atomic Force Microscopy, Binding mode, Click Chemistry, Glycoclusters.

ABSTRACT: Multivalent iminosugars have been recently explored for glycosidase inhibition. Affinity enhancements due to multivalency have been reported for specific targets, which are particularly appealing when a gain in enzyme selectivity is achieved, but raise the question of the binding mode operating with this new class of inhibitors. Here we describe the development of a set of tetra- and octavalent iminosugar probes with specific topologies and an assessment of their binding affinities towards a panel of glycosidases including the Jack Bean α-mannosidase (JBαMan) and the biologically relevant class II α-mannosidases from *Drosophila melanogaster* belonging to glycohydrolase family 38, namely Golgi α-mannosidase GMIIb (GM) and lysosomal α-mannosidase LManII (LM). Very different inhibitory profiles were observed for compounds with identical valencies, indicating that the spatial distribution of the iminosugars is critical to fine-tune the enzymatic inhibitory activity. Compared to the monovalent reference, the best multivalent compound showed a dramatic 800-fold improvement in the inhibitory potency for JBαMan, which is outstanding for just a tetravalent ligand. The compound was also shown to increase both the inhibitory activity and the selectivity for GM over LM. This suggests that multivalency could be an alternative strategy in developing therapeutic GM inhibitors not affecting the lysosomal mannosidases. Dynamic light scattering experiments and atomic force microscopy performed with co-incubated solutions of the compounds with JBαMan shed light on the multivalent binding mode. The multivalent compounds were shown to promote the formation of JBαMan aggregates with different sizes and shapes. The dimeric nature of the JBαMan allows such intermolecular cross-linking mechanisms to occur.

INTRODUCTION. Synthetic glycoclusters presenting multiple copies of a sugar epitope have been extensively investigated to inhibit carbohydrate binding proteins called lectins.[1] Logarithmic affinity enhancements compared to monovalent references are often observed with fine-tuned glycoclusters. These affinity improvements termed "multivalent or glycoside cluster effects",[2] can be rationalized by a limited number of binding mechanisms occurring independently or simultaneously.[3] The highest multivalent effects have been reported when the tethered epitopes of a glycocluster embrace at least two binding sites of a multimeric lectin (chelate binding mode).[4] When the linkers are too short for such chelation, organized and stabilized glycocluster-lectin networks can be formed. Both interactions require a multimeric presentation of the receptors, a situation generally encountered with lectins.

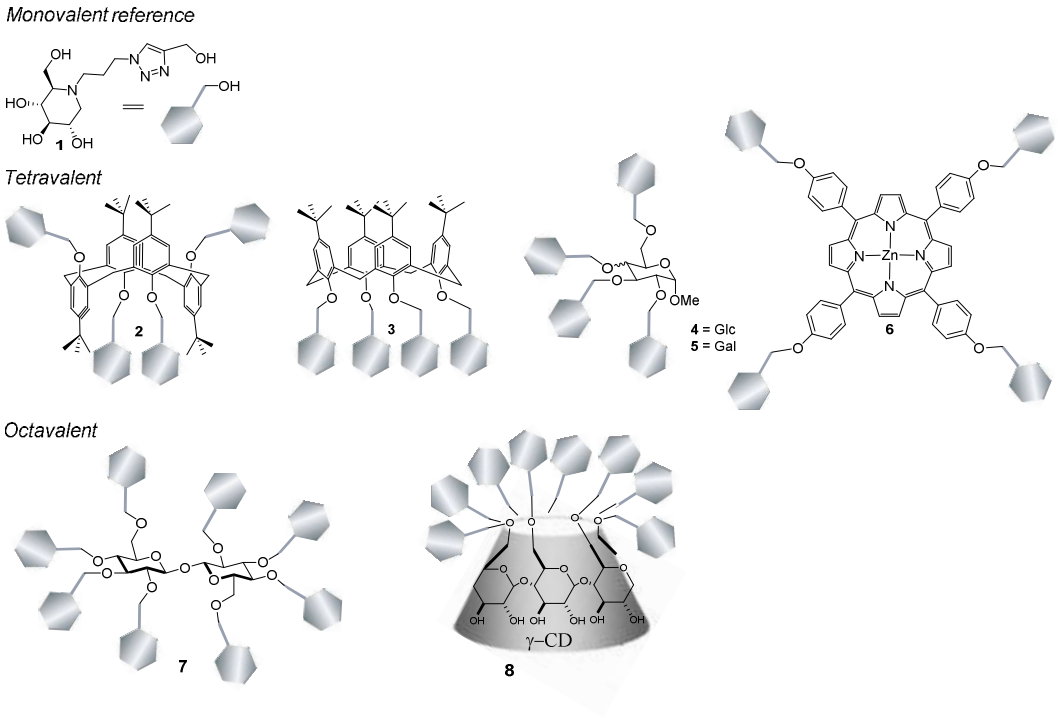
In stark contrast, multivalency was largely disregarded as a means to inhibit carbohydrate processing enzymes, until recently. Only a few examples of multivalent glycosidase

inhibitors were reported in the literature.[5] This can be explained by the synthetic hurdles in obtaining acceptable quantities of inhibitors for multivalent grafting, and by the generally monovalent nature of glycosidases, which are not prone to chelate or aggregative processes. Nevertheless, significant affinity enhancements may also be expected with multivalent conjugates interacting at a single protein binding site. The locally high concentration of the tethered ligands in close proximity to the binding site may favour a rebinding mechanism and prevent complex dissociation, as previously observed with lectins.[6] These considerations stimulated us to study multivalency in the context of glycosidase inhibition. In previous work, we designed mono-, di- and trivalent deoxynojyrimycin (DNJ) analogues (a broad glycosidase inhibitor) grafted onto ethylene-glycol scaffolds by click chemistry and assessed the inhibitory activities of our compounds toward a panel of glycosidases. We observed different inhibitory profiles depending on the enzymes, and showed the first significant multivalent effect on a glycosidase (Jack Bean-α-D-mannosidase = JBαMan).[7] These results were further supported by others with click DNJ-based dodecavalent fullerenes showing high multivalent effects (up to three orders of magnitude) on the same enzyme.[8] Multivalent effects were also reported on biologically relevant enzymes such as glucosylceramide synthase and the bacterial heptosyltransferase WaaC, with implications for Gaucher diseases[9] and bacterial infections,[10] respectively. Together, these results highlight the great potential for multivalent inhibition of glycosidase enzymes and pave the way to potential biomedical applications.

Surprisingly, positive responses to multivalency are only observed with certain enzymes, which is very beneficial for selectivity improvements, but remains intriguing. Insights into the multivalent binding mode operating should give clues about such selectivity and may enable prediction of the efficiency of multivalent inhibitors for new targets. In the present work, we wished to assess the multivalent binding mode(s) operating with JBαMan, for which the first and the highest multivalent effect has been described. JBαMan is a class II-α-mannosidase, a group of glycoside hydrolases of considerable interest. Golgi α-mannosidase (GM) and lysosomal α-mannosidases (LM) are two other members of this family (GH38) with closely related protein sequences. Inhibition of the former enzyme by the indolizidine alkaloid swainsonine, has been shown to reduce tumor growth and cell metastasis[11] while a deficiency in the latter is observed in the inherited disorder mannosidosis. There is a significant interest in developing selective GM inhibitors over LM, to prevent un-wanted mannosidosis syndromes during cancer chemotherapies.

Based on structural analogies, JBαMan is considered a good model for these two glycosidases, and studying multivalent effects on JBαMan to develop a new enzymatic mode of inhibition is therefore of particular interest. For this purpose, we designed a series of mono-, tetra- and octavalent DNJ probes 1-8 based on porphyrin, calix[4]arene, glucose, galactose, trehalose and γ-cyclodextrin scaffolds (Chart 1).

Chart 1. Structure of the multivalent glycoclusters incorporating 4 to 8 DNJ moieties.



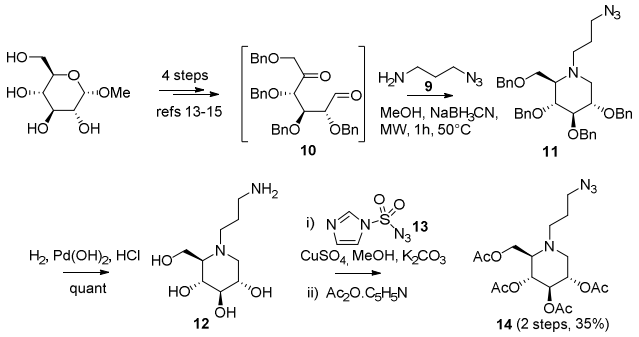
The different scaffolds selected give specific spatial orientations of the tethered DNJ, and may thus impact the inhibition. The inhibitory activities of the compounds were tested on a range of commercially available glycosidases including JBαMan. The two compounds showing the highest inhibition of JBαMan were also tested against GM and LM to evaluate the potential of this multivalent approach in anti-cancer therapy.

Dynamic Light Scattering experiments (DLS) and Atomic Force Microscopy (AFM) images shed light on the binding mode operating with the multivalent iminosugars and JBαMan.

Synthesis.

Benzoylated azido-functionalized DNJ 11[7] was obtained from commercially available methyl-α-D-glucopyranoside as we previously described (Scheme 1).

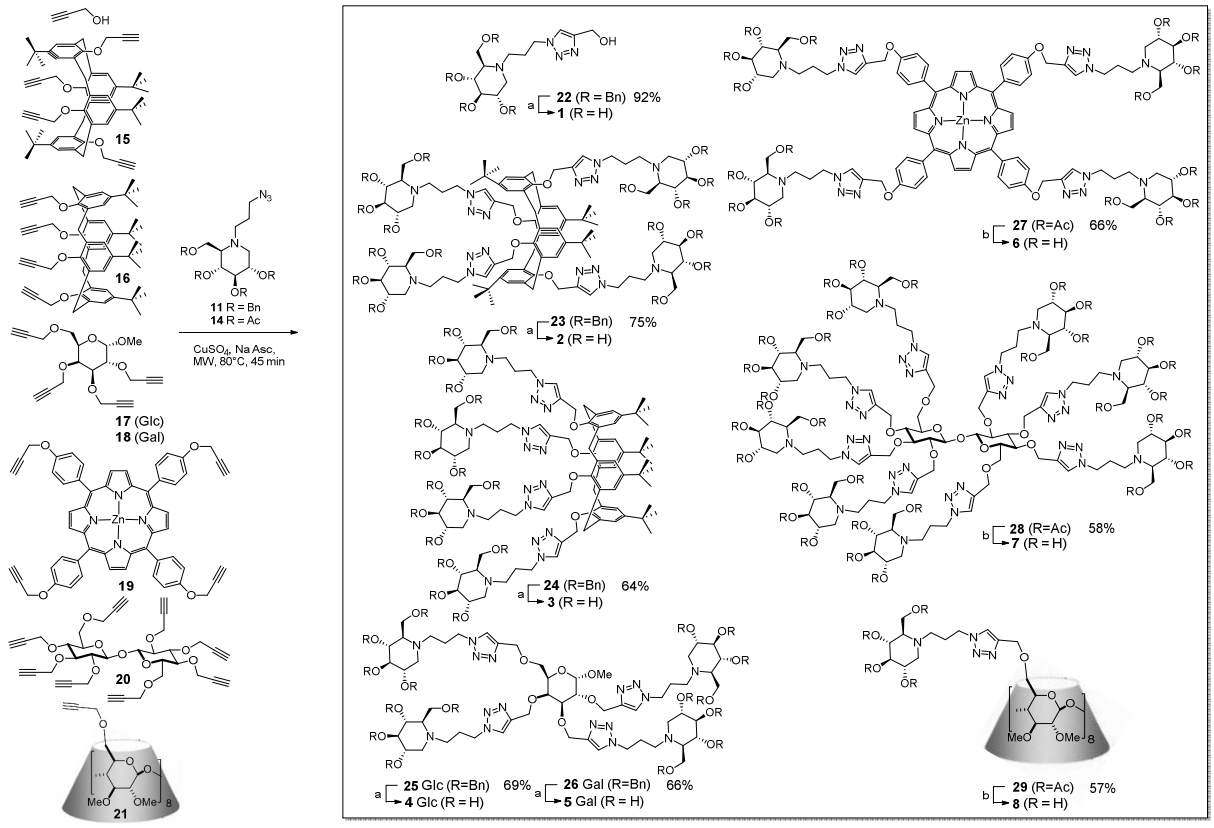
Scheme 1. Synthesis of the acetylated azido-functionalized DNJ intermediate **14**.



This procedure requires a critical double reductive amination step involving 3-azidopropylamine 9[13] and the 1,5-dicarbonylated compound 10[14]. Deprotection of the benzyl groups was problematic on octavalent and porphyrin-based compounds. Thus, we synthesized a second N-azidopropyl-DNJ synthon 14 with acetate protecting groups. The double reductive amination developed for the cyclization of 11 was not applicable to compound 14 due to the rather labile esters under these conditions. Thus, we chose to obtain compound 14 directly from 11. Initial attempts to remove the benzyl groups selectively with BCl_3 failed and gave a complex mixture of products.[15] Azides and benzyl groups of 11 were then reduced with Pearlman's catalyst in acidic media.[16] The azide group was recovered with a copper-catalyzed diazo-transfer reaction performed with imidazole-1-sulfonyl azide 13,[17] a safer reagent than the generally used triflyl azide.[18] The crude product was acetylated in a mixture of pyridine and acetic anhydride to give 14.

Propargylated scaffolds 15[19], 16[20], 17,[21] 18, 20, 21 (Scheme 2) were obtained in yields ranging from 46 to 90% with propargyl bromide, sodium hydride and the corresponding alcohols in DMF (see Supporting Information). 19 was obtained as previously described.[22]

Scheme 2. Structure of the eight glycoclusters obtained by CuAAC



Yields are indicated for the cyclization-deprotection steps. Conditions: (a) $Pd(OH)_2$, MeOH-1M aq HCl; r.t., 48 h; (b) Amberlite resin IRN 78, MeOH-H_2O, r.t., 14 h.

The protected azidopropyl-DNJ 11 or 14 was tethered to the propargylated scaffolds 15-21 with microwave-assisted copper-catalyzed azide-alkyne cyclization (CuAAC). The compounds were irradiated in the presence of copper sulfate and sodium ascorbate in a mixture of dioxane and water.

The exclusive formation of the 1,4-cycloadducts was observed in all cases as shown by the large Δ(δC-4–δC-5) val-

ues (>20 ppm) recorded by 13C NMR spectroscopy for the different structures.[23] The 1,3-dipolar cycloaddition was initially performed with 11 and propargyl alcohol to form the monovalent reference 1 after hydrogenolysis of the benzyl groups of 22 with Pearlman's catalyst in acidic media. Deprotection of the benzyl groups by such protocols was shown to be problematic with octavalent and porphyrin-based scaffolds and incomplete on the octavalent iminosugars. This problem was overcome with the corresponding acetylated analogues 22, 23, 27-29 that were easily deprotected using basic anion exchange resin Amberlite IRN-78. Compounds 1-9 were obtained with fair to good yields (57-92%) for the cycloaddition-deprotection sequence depending on the difficulty of purification using silica gel column chromatography.

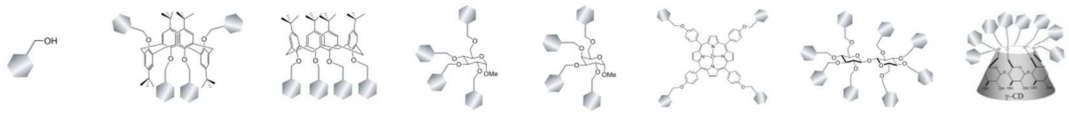
Inhibition studies on glycosidases. Variation of the spatial ligand presentation on glycoclusters was previously shown to affect the binding profiles for specific lectins.[24] Multivalent glycoconjugates with identical valencies were shown to discriminate between lectins with closely related sequences.[25] The set of multivalent DNJ 2-6 and 7,8 based on different chemical architectures but with identical valencies are interesting tools to investigate if such topological effects are also of importance for inhibition. In particular, the alternate 2 and cone calix[4]arene 3 are isomers with distinct spatial presentations of the DNJ epitopes.[26,27] The two sets of carbohydrate-based ligands 4-5 and 7,8 also possess rather similar scaffolds but should provide distinct presentations of the DNJ ligands.

The inhibition constants (Ki) for the monovalent reference 1, the tetravalent compounds 2-6 and the octavalents 7,8 are presented in Table 1.

In agreement with previous studies,[7-9] different inhibitory profiles were observed depending on the glycosidase targets. This further supports the hypothesis that multivalency cannot be considered a general strategy for glycosidase inhibition as it is for lectins since not all enzymes were inhibited by these multivalent inhibitors.[27]

Significant affinity enhancements were observed for JBαMan and multivalent ligands 2-8. In order to quantify better the level of the multivalent inhibitions observed, the relative inhibitory potencies (RIP) and affinity enhancement per DNJ motif (AED) are presented in Table 2.

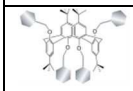
Table 1. Glycosidases inhibitory activities (*Ki*, μM) for compounds 1-8.



	1	2	3	4	5	6	7	8
β-galactosidase (bovine liver)	n.i.	58 ± 6	38 ± 4	n.i.	64 ± 6	52 ± 5	71 ± 9	191 ± 19
β-galactosidase (E. coli)	85 ± 9	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
α-galactosidase (green coffee)	55 ± 6	36 ± 4	24 ± 2	228 ± 23	24 ± 2	18 ± 1	1.7 ± 0.2	40 ± 4
β-glucosidase (almonds pH 7.3)	233 ± 24	729 ± 87	n.i.	311 ± 36	283 ± 28	n.i.	281 ± 27	177 ± 18
α-glucosidase (baker's yeast)	n.i.	45 ± 4	585 ± 59	n.i.	n.i.	n.i.	243	n.i.
α-mannosidase (jack bean)	400 ± 48	20 ± 2	1.5 ± 0.2	76 ± 8	41 ± 4	0.5 ± 0.05	21 ± 2	23 ± 2
β-mannosidase (helix pomatia)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
amyloglucosidase (Aspergillus niger)	45 ± 4	11	43 ± 5	11 ± 1	2.3 ± 0.2	24 ± 2	4.6 ± 0.5	8 ± 0.8

n.i. = no inhibition observed at 2 mM concentration of the inhibitor. Most of the compounds displayed significant multivalent effects relative to **1** on α-mannosidase from Jack Bean (grey cells)

Table 2. Affinity enhancements observed with glycoclusters 2-8 on the α-mannosidase from jack bean.

	Compound	Valency	α-mannosidase	
			RIP[a]	AED[b]
	1	1	1	1
	2	4	20	5
	3	4	267	67
	4	4	5	1.2
	5	4	10	2.5
	6	4	800	200
	7	8	19	2.4
	8	8	17	2.1

[a] RIP = Relative Inhibitory Potency calculated by dividing Ki of reference 1 by the Ki of the corresponding multivalent compound. [b] AED = Affinity Enhancement per DNJ motifs calculated by dividing RIP values by the compound valency. AED values > 1 are indicative of a positive multivalent effect. The highest multivalent effect observed on JBαMan is presented in the grey cells.

RIP values are calculated by dividing the Ki of the monovalent reference 1 by the Ki of the multi-DNJ and gives the affinity enhancement per molecule. The RIP values were divided by the ligand valency to assess strictly the affinity gain per DNJ motif (positive multivalent effect for AED>1). All the compounds showed positive multivalent effects on JBαMan. The cone calix[4]arene 3 showed a strong multivalent effect on JBαMan (RIP = 267) but was surpassed by 6 (RIP = 800) for which a dramatic AED gain of 200 was observed. This tetravalent porphyrin-based compound displayed a higher enhancement per DNJ motif than a previously described dodecavalent DNJ-based fullerene (AED = 179),[8] which is remarkable for such a low-valency ligand.

JBαMan is a zinc metalloenzyme which can be significantly inactivated by micromolar concentrations of copper.[29] We therefore ascertained that the strong affinity observed with the multivalent DNJ was not due to the presence of residual copper from the CuAAC step. The residual concentration of free copper was estimated with a colorimetric test kit (excluding colored compound 6).[30] In each case, the residual amount of copper ranged from 50 to 150 ppm. As an example, for the second most potent compound 3 (Ki = 1.5 μM, Cu<150ppm) this corresponds to a sub-nanomolar concentration of copper (Cu<0.2 nM) during the enzymatic assay, well below the micromolar concentration required for JBαMan inhibition. The glycosidase inhibitory activities observed for the compounds are therefore not related to or affected by the low residual amount of copper.

The octavalent γ-cyclodextrin-based compound 8 only displayed a limited multivalent effect (AED = 2.1) which was surprising considering that β-CD analogues with closely related sequences and spatial distribution of the epitopes were previously shown to be potent inhibitors of the same enzyme.[31] The latter differed in valency (7 or 14-valent) and displayed more carbon atoms in the *N*-alkyl chain (*N*-hexyl or *N*-nonyl DNJ). Subtle differences in ligand presentations, or non-specific positive interactions of the β-CD scaffold with the mannosidase may explain the strong differences in inhibitory activities among these compounds.

Importantly, topological effects were shown here to influence strongly the selectivity and inhibition for specific glycosidases. This is well illustrated with isomeric alternate 2 and cone calix[4]arene 3 displaying significantly different AED values on JBαMan of 5 and 67, respectively.

These results clearly highlight the importance of the scaffold selection in fine-tuning the epitope presentation in order to increase multivalent glycosidase inhibition. The fact that compounds with the highest valencies are not necessarily the most potent inhibitors may be similar to the situation encountered with lectins, where plateaus of inhibition are often observed when the valency of inhibitors is further increased.[32]

Inhibition studies on GM and LM. Potent GM inhibitors such as the indolizidine alkaloid swainsonine have a strong potential in cancer therapy by blocking *N*-glycan biosynthesis. However, their development is greatly hampered by un-wanted coinhibition of the closely related mannosidase LM, resulting in symptoms of α-mannosidosis.

In view of the results obtained on JBαMan, we wanted to evaluate here if the multivalent strategy could be a means to increase the affinity and selectivity for GM over LM. For this reason we used human homologues of *Drosophila melanogaster* Golgi (GMIIb, labeled here GM) and lysosomal (LManII, labeled here LM) α-mannosidases as model enzymes. The recombinant enzymes were expressed in *Pichia pastoris*, processed and assayed as already described.[33] Briefly, concentrated and dialyzed induced culture medium was used as an enzyme in the assay with p-nitrophenyl-α-D-mannopyranoside (pNP-Man) as a substrate. Liberated p-nitrophenol was measured spectrophotometrically.

The tetravalent inhibitors **3** and **6** showing the highest inhibition of JBαMan and the monovalent references **1** were assessed on GM and LM (Table 3). Interestingly, an affinity enhancement due to valency was observed with **6**. Although the effect on GM was moderate compared to the inhibition observed on JBαMan, the results show that multivalent iminosugars may be considered to increase affinity for this pharmaceutical target. Compound **6** was also selective for GM with no inhibition observed on LM at the high concentration of 1 mM. At present, the reason for such selectivity is unclear. A specific residue in LM may sterically prevent the approach of the large scaffolds of **3** and **6**. Alternatively, LM may also respond to multivalency but at a smaller level than GM and the associated increased inhibitory activity may remain undetectable (below 1 mM).

Compound **3** did not show any significant GM and LM inhibition which is surprising in view of the inhibitory activity observed on JBαMan. It seems that the multivalent iminosugars are able to discriminate glycosidases from the same family with high homology.

Table 3. Mannosidase inhibitory activities (*Ki*, μM) for compounds 1, 3, 6.

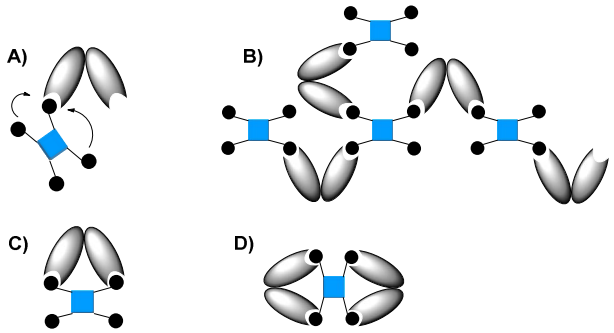
Compound	GM (Ki/ μM)	LM (Ki/ μM)
1	362 ± 88	n.i.
3	n.i.	n.i.
6	24 ± 10	n.i.

n.i. = no inhibition observed at 1 mM concentration of the inhibitor.

Binding mode operating with Jack Bean α-mannosidase. The fact that multivalent effects only occur with a limited number of glycosidases is of great interest in enzyme selectivity but raises the question of the binding mode(s) operating. It is of particular importance to answer this question in order to rationalize the future development of inhibitors and potentially predict possible multivalent effects with specific targets.

We wanted to assign the binding mode operating with JBαMan for which the highest multivalent effects are reported. JBαMan is a 230 kDa heterodimer with two subunits of around 49 and 66 kDa. The larger sub-unit was postulated as the catalytically competent active site.[34] Interestingly, a recent study suggested that each unit of 115 kDa (49 + 66) was catalytically active in the dimeric structure.[35] Based on these considerations, four different multivalent binding modes can be proposed to explain the affinity enhancement observed with the multivalent iminosugars. These specific interactions (Figure 1) are frequently observed with carbohydrate binding proteins.[1a] In the "bind and jump" process (Figure 1A),[6a, 36, 37] the high local concentration of the DNJ ligands in close proximity to the binding site favour a recapture mechanism on the adjacent ligands. Although strong affinity enhancements have been reported with lectin and polymeric structures by this mechanism,[38] size-controlled glycoclusters of low valency generally do not exceed a 10-fold increase in affinity compared to monovalent ligands. It is therefore unlikely that the strong multivalent effect observed with 6 could be solely due to this recapture mechanism. Aggregative processes (Figure 1B) can be expected with the tetravalent ligands because of the dimeric nature of the enzyme. Different types of ligand-lectin network have been previously described, with moderate to high stabilizing effects.[39] Chelate and aggregative chelate (Figures 1C, 1D) binding modes may be operating if the distance between the tethered iminosugars can embrace both catalytic sites of the divalent glycosidase. The highest multivalent effects reported on lectins with glycoconjugates occurred through chelate interactions.[4]

Figure 1. Illustration of the possible binding modes operating with a tetravalent ligand and a divalent protein



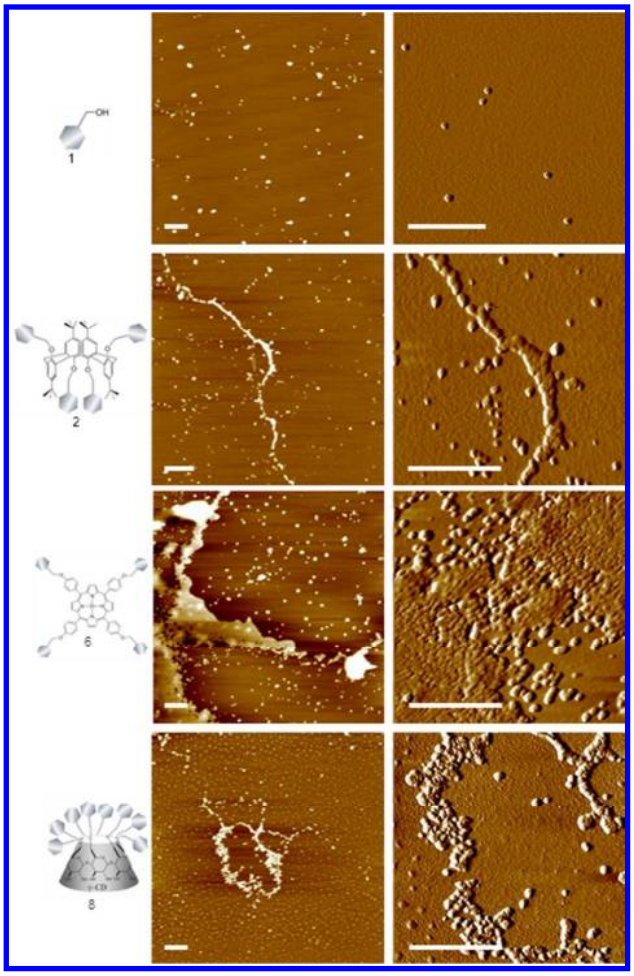
A) Recapture mechanism favored by the high local concentration of ligands; B) Formation of ligand-protein networks; C) Chelate binding mode; D) Aggregative chelate.

Atomic Force Microscopy experiments were performed to shed light on the binding mode operating with the multivalent iminosugars and JBαMan. AFM is a powerful tool for measuring and imaging interactions in biological samples. This technique provides nanoscale views of various important biological processes and structures such as receptor/ligand interactions, cell functions, microbial activity, lipid membrane properties and protein conformation/assemblies. Experiments were performed in triplicate and similar images were recorded for a given compound. AFM imaging of JBαMan alone (See Figure S7 in Supp Mat) or co-incubated with the monovalent reference 1 gave isolated spots at the mica surface matching the size of the JBαMan (height of 5.1±0.3 nm). In stark contrast, the interaction between JBαMan and the multivalent compounds 2-8 produced nano-assemblies (Figure 2 and Figures S8 and S9).

This clearly illustrates that the multivalent iminosugars 2-8 have the ability to aggregate JBαMan. The stabilization effect observed with the multivalent iminosugars may therefore be related to the formation of the stabilized supramolecular aggregates, in analogy to that previously described for lectin-saccharide networks.[38]

Remarkably, aggregates with different sizes and shapes were observed in AFM images depending on the compounds. Alternate calix[4]arene 2 formed rectilinear structures with JBαMan (Figure 2B). The specific presentation of the DNJ in 2, which are paired on each sides of the rigid calixarene scaffold, seems to favour the formation of rectilinear structures compared to more packed aggregates. It is worth mentioning that supramolecular assemblies of similar shapes were previously observed with a bacterial lectin and a tetragalactosylated glycocluster based on the same 1,3-alternate calix[4]arene scaffold.[40]

Figure 2: AFM height (left) and deflection (right) images of JBαMan interacting with mannose multivalent ligands (z= 10 nm in height images; scale bars = 500 nm).



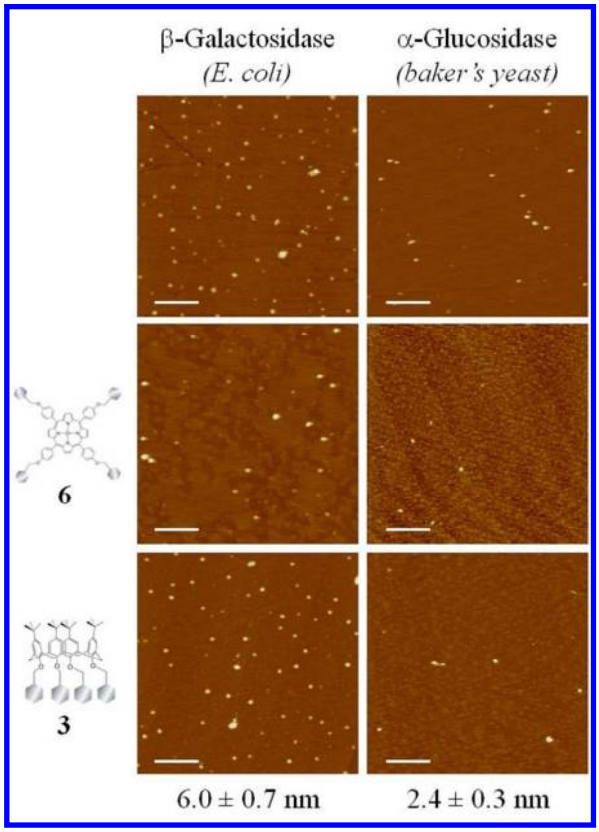
Significantly different networks were observed with the other multivalent DNJ glycoclusters. Compounds 3, 4, 5 or 8 formed large ring-shaped structures while the porphyrin-based compound 6, displaying the highest inhibitory activity, formed much larger aggregates with JBαMan (Figure 2, Figures S8 and S9).

Although the supramolecular ring-shape adopted by most of the compounds is difficult to explain by molecular level considerations, the very large 6-JBαMan aggregates formed may be due, at least in part, to the well-known tendency of porphyrin to self-assemble in water through intermolecular π-π stacking interactions.[41] Aggregates of 6 with enhanced valency could play the role of a template facilitating the formation of the larger JBαMan aggregates observed by AFM. Self-assembling multivalent ligands have been previously reported as powerful tools to target bacterial lectins.[42] This assumption is supported by AFM images of 6 alone showing small supramolecular structures with a thickness of 0.8 to 1 nm (Figure S10) and by DLS experiments (Figure S12, pick around 1 nm). It should be noted that aggregates in Figure 2 have a thickness matching the protein size (5 nm) and are therefore unambiguously ascribed to JBαMan and not to the ligands alone.

AFM analyses were also performed with different 6/JBαMan ratios. The AFM images showed that the size of aggregates increases with growing 6/JBαMan ratio (Figure S11). This confirms that the ratio influence the size of structures found in AFM images, and further supports the thesis that JBαMan aggregates are promoted by the multivalent iminosugars.

Next, we wanted to investigate if aggregative processes are also observed with glycosidases that do not respond to multivalency. The Alpha-glucosidase (αGlu) and beta-galactosidase (βGal) were selected and imaged with or without 3 and 6 (Figure 3). Aggregates were never observed and co-incubated solutions showed particles matching the size of αGlu or βGal alone. These results reinforce the hypothesis that the increased inhibitory activity observed with JBαMan is induced by the formation of large aggregates. This also potentially explains the inversion of the αGlu / JBαMan selectivity observed with the monovalent 1 and the multivalent DNJ inhibitors (Table 1). Despite its preference for the monovalent DNJ motif, αGlu is less inhibited by the multi-DNJ because the enzyme does not respond to multivalency and does not form the large aggregates observed with JBαMan.

Figure 3. Control AFM images of beta-galactosidase and alpha-glucosidase incubated with mannose tetravalent ligands.



(z=10 nm in height images ; scale bar is 500 nm). The mean size of particles is indicated below images. According to these images, these two enzymes cannot form complexes with mannose-based ligands.

The formation of DNJ glycoclusters-JBαMan aggregates was further explored in solution by DLS experiments (Table 4, Figure S12). DLS was used to determine the hydrodynamic diameter of JBαMan alone or in complex with monova-

lent compound 1 and tetravalent compounds 3 and 6, both showing the highest inhibitory activity with the enzyme. A hydrodynamic diameter of 10 nm was observed for JBαMan alone. Co-incubation with the monovalent reference 1 led to a similar particle-size distribution. In stark contrast, large aggregates of more than 500 nm were formed in the presence of the tetravalent iminosugars 3 or 6. These results are in good agreement with the AFM studies and further confirm the potency of the tetravalent iminosugars to form cross-linked complexes with JBαMan.

Table 4. Hydrodynamic diameters of JBαMan in the absence or presence of iminosugars.

JBαMan alone	10.4 ± 2.2
JBαMan/**1**	9.9 ± 2.5
JBαMan/**3**	663.3 ± 128.9
JBαMan/**6**	731.9 ± 161.9
β-galactosidase (*E. coli*) /**6**	7.4 ± 1.4
α-glucosidase (baker's yeast) /**6**	3.3 ± 0.7

Results expressed in nm correspond to the mean of three independent samples. The JBαMan/ligand ratio was 1/10. The size of ligands without proteins was less than 1 nm and control proteins (αGlu or βGal) did not yield aggregates in solution with the ligands.

Conclusion

After our first proof of the concept of the multivalent inhibition of JBαMan,[7] we wished to gain more insight into the binding mode operating with this enzyme. The different affinities and inhibitory profiles observed here with compounds of identical valencies, clearly highlight that the spatial presentation of the DNJ epitopes modulates the strength of the multivalent effects to a large extent. A careful selection of the scaffold is therefore essential to provide an adequate spatial distribution of the DNJ ligands. The octavalent compounds with the highest valencies were not the strongest inhibitors, and this further suggests that designing multivalent iminosugars with fine-tuned spatial distribution of the DNJ is much more relevant than an ever-increasing valency. The porphyrin-based compound 6, displayed a high JBαMan selectivity, with a dramatic 800-fold improvement compared to the monovalent reference, which is unprecedented for a tetravalent ligand. Insights into the multivalent binding mode operating are also provided here. DLS experiments indicate that large JBαMan aggregates are formed in the presence of the tetravalent compounds 3 and 6 but not with the monovalent 1. AFM images and DLS measurements also clearly show that the multivalent compounds are able to aggregate dimeric JBαMan, but not αGlu or βGal which did not respond to multivalency in the inhibitory assay, giving a rational basis for the multivalent effect seen with JBαMan. Interestingly, different patterns are observed depending on the multivalent inhibitors, and this may well explain the difference in inhibition among the compounds. Furthermore, JBαMan is a class II-α-mannosidase of the GH 38 family, a group of glycoside hydrolases of considerable interest, including GM and LM. Inhibitory activities measured with 6 on the pharmaceutical target GM showed an increased affinity compared to the monovalent reference 1 with a high selectivity over LM. This finding is of particular interest considering the importance of selectively inhibiting GM over LM in cancer chemotherapy. Studies are currently in progress in our laboratories to evaluate the potential of new multivalent iminosugars for selective GM inhibition.

Supporting Information.
Experimental procedures, AFM images, and spectral data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: Sebastien.gouin@univ-nantes.fr

ACKNOWLEDGMENT

This work was carried out with financial support from the Centre National de la Recherche Scientifique, the Ministère Délégué à l'Enseignement Supérieur et à la Recherche. Dr F. Albrieux, C. Duchamp and N. Henriques from the Centre Commun de Spectrometrie de Masse (University of Lyon) are gratefully acknowledged for mass spectrometry analyses. We thank Prof. I. B. H. Wilson (Universität für Bodenkultur, Wien, Austria) for generously providing the GMIIb plasmid.

REFERENCES

(1) For an overview see : (a) D. Deniaud, K. Julienne, S.G. Gouin, *Org. Biomol. Chem.* **2011**, *9*, 966-979. (b) R. J. Pieters, *Med. Res. Rev.*, **2007**, *27*, 796-816. (b) R. J. Pieters, *Org. Biomol. Chem.* **2009**, *7*, 2013-2025. (c) A. Imberty, Y.M. Chabre, R. Roy, *Chem. Eur. J.* **2008**, *14*, 7490-7499. (d)Y. M. Chabre, R. Roy, *Adv. Carbohydr. Chem. Biochem.* **2010**, *63*, 165-393. (e) A. Dondoni, A. Marra, *Chem. Rev.* **2010**, *110*, 4949-4977. (f) Martínez, Á.; Ortiz-Mellet, C.; García-Fernández J.M. *Chem. Soc. Rev.* **2013**, ***42***, 4746-4773. (g) A. Bernardi, J. Jiménez-Barbero, A. Casnati, C. De Castro, T. Darbre, F. Fieschi, J. Finne, H. Funken, K-E. Jaeger, M. Lahmann, T. K. Lindhorst, M. Marradi, P. Messner, A. Molinaro, P. V. Murphy, C. Nativi, S. Oscarson, S. Penadés, F. Peri, Roland J. Pieters, Olivier Renaudet, Jean-Louis Reymond, Barbara Richichi, Javier Rojo, Francesco Sansone, C. Schäffer, W. B. Turnbull, T. Velasco-Torrijos, S. Vidal, S. Vincent, T. Wennekes, H. Zuilhof, A. Imberty *Chem. Soc. Rev.*, **2013**, **42**, 4709-4727.

(2) (a) Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321-327. For a review see: (b) Lundquist, J.J.; Toone, E.J. *Chem. Rev.* **2002**, *102*, 555-578.

(3) For a review see: Mammen, M.; Choi, S.K.; Whitesides, G.M. *Angew. Chem. Int. Ed.* **1998**, *37*, 2754-2794.

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

(5) (a) A. Lohse, K.B. Jensen, K. Lundgren, M. Bols, *Bioorg. Med. Chem.* **1999**, *7*, 1965-1971. (b) B.A. John, C.R. Johnson, *Tetrahedron Lett.*, **1998**, *39*, 749-752. (c) I. McCort, M. Sanière, Y. Le Merrer, *Tetrahedron* **2003**, *59*, 2693-2700.

(6) (a) J. M. Benito, M. Gómez-García, C. Ortiz-Mellet, I. Baussanne, J. Defaye, J.M. García-Fernandez, *J. Am. Chem. Soc.* **2004**, *126*, 10355-10363. (b) M.S. Quesenberry, R.T. Lee, Y.C. Lee, *Biochemistry*, **1997**, *36*, 2724-2732. (c) K. Dam, T.A. Gerken, B. S. Kavada, K. S. Nascimento, T.R. Moura, F. C. Brewer, *J. Biol. Chem.*, **2007**, *282*, 28256-28263. (d) S.G. Gouin, E. Vanquelef, J.M. García-Fernández, C. Ortiz-Mellet, F-Y. Dupradeau, J. Kovensky, J. Org. Chem. **2007**, *72*, 9032-9045. (e) S.G. Gouin, J.M. García Fernández, E. Vanquelef, F-Y. Dupradeau, M. Ortegaz-Munoz, H. Leffler, J. Kovensky, ChemBioChem. **2010**, *11*, 1430-1442.

(7) J. Diot, I. García-Moreno, S.G Gouin, C. Ortiz-Mellet, K. Haupt, J. Kovensky, *Org. Biomol. Chem.* **2009**, *7*, 357-363.Barone, V.; Polimeno, A. *Chem. Soc. Rev.* **2007**, *36*, 1724-1731.

(8) P. Compain, C. Decroocq, J. Iehl, M. Holler, D. Hazelard, T. Mena Barragán, C. Ortiz-Mellet, J-F. Nierengarten, *Angew. Chem. Int. Ed.* **2010**, *49*, 5753-5756.

(9) C. Decroocq, D. Rodríguez-Lucena, K. Ikeda, N. Asano, P. Compain, *ChemBioChem*, **2012**, *13*, 661-664.

(10) M. Durka, K. Buffet, J. Iehl, M. Holler, J-F. Nierengarten, S. P. Vincent, *Chem. Eur. J.* **2012**, *18*, 641-651.

(11) (a) J.W. Dennis, K. Koch, S. Yousefi, I. Van der Elst, *Cancer. Res*, **1990**, *50*, 1867-1872. (b) P.E. Goss, C.L. Reid, D. Bailey, J.W. Dennis, *Clin. Cancer Res.* **1997**, *3*, 1077-1086.

(12) P.E. Goss, M.A. Baker, J.P. Carver, J.W. Dennis, *Clin. Cancer Res.* **1995**, 1, 935-944

(13) Y. Ju, D. Kumar, R.S. Varma, *J. Org. Chem.* **2006**, *71*, 6697.

(14) C.R.R. Matos, R.S.C. Lopes, C. Cristobal Lopes, *Synthesis*, **1999**, *4*, 571.

(15) A. Dondoni, A. Marra, *J. Org. Chem.* **2006**, *71*, 7546-7557.

(16) J. Diot, I. G. Moreno, G. Twigg, C. Ortiz-Mellet, K. Haupt, T. D. Butters, J. Kovensky, S. G. Gouin, *J. Org. Chem.* **2011**, *76*, 7757-7768.

(17) E.D. Goddard-Borger, R. V. Stick, *Org. Lett.* **2007**, *9*, 3797-3800.

(18) P.B. Alper, S.-C. Hung, C.-H. Wong, *Tetrahedron Lett.* **1996**, *34*, 6029-6032

(19) M.J. Chetcuti, A. M. J. Devoille, A. B. Othman, R. Souane, P. Thuery, J. Vicens, *Dalton Trans.* **2009**, 2999-3008.

(20) E.H. Ryu, Y. Zhao, *Org. Lett.* **2005**, *7*, 1035-1037.

(21) F. Perez-Balderas, J. Morales-Sanfrutos, F. Hernandez-Mateo, J. Isac-García, F. Santoyo-Gonzalez, *Eur. J. Org. Chem* **2009**, 2441-2453.

(22) (a) S. Cecioni, S. Faure, U. Darbost, I. Bonnamour, H. Parrot-Lopez, O. Roy, C. Taillefumier, M. Wimmerova, J.-P. Praly, A. Imberty, S.Vidal, *Chem. Eur. J.* **2011**, *17*, 2146-2159. (b) Y. Liu, C.-F. Ke, H.-Y. Zhang, J. Cui, F. Ding, *J. Am. Chem. Soc.* **2008**, *130*, 600-605. (c) Y. Chen, H. Vedala, G. P. Kotchey, A. Audfray, S. Cecioni, A. Imberty, S. Vidal, A. Star *ACS Nano* **2012**, *6*, 760-770. (d) H. Vedala, Y. Chen, S. Cecioni, A. Imberty, S. Vidal, A. Star *Nano Lett.* **2011**, *1*, 170-175.

(23) N.A. Rodios, *J. Heterocycl. Chem.* **1984**, *21*, 1169-1173.

(24) For an example see: S. André, F. Sansone, H. Kaltner, A. Casnati, J. Kopitz, H-J. Gabius, R. Ungaro, *ChemBioChem*, **2008**, *9*, 1649-1661.

(25) (a) I. Vrasidas, S. André, P. Valentini, C. Böck, M. Lensch, H. Kaltner, R.M.J. Liskamp, H-J. Gabius, R.J. Pieters, *Org. Biomol. Chem.*, **2003**, *1*, 803-810. (b) S. André, B. Liu, H-J. Gabius, R. Roy, *Org. Biomol. Chem.* **2003**, *1*, 3909-3916.

(26) S. Cecioni, R. Lalor, B. Blanchard, J-P. Praly, A. Imberty, S. E. Matthews, S. Vidal, *Chem. Eur. J.* **2009**, *15*, 13232-13240.

(27) For other imino-sugars ligands based on calixarene cores see: (a) F. Cardona, G. Isoldi, F. Sansone, A. Casnati, A. Goti, *J. Org. Chem.* **2012**, *77*, 6980-6988. (b) M. Marradi, S. Cicchi, F. Sansone, A. Casnati, A. Goti, *Beilstein J. Org. Chem.* **2012**, *8*, 951-957.

(28) T. Wennekes, R. J.B.H.N. van den Berg, K. M. Bonger, W. E. Donker-Koopman, A. Ghisaidoobe, G. A. van der Marel, A. Strijland, J. M.F.G. Aerts, H. S. Overkleeft *Tetrahedron: Asymmetry*, **2009**, *20*, 836-846.

(29) S. M. Snaith, *Biochem. J.* **1975**, *147*, 83-90.

(30) We used the Quantofic® Copper test stick, for a recent example in the context of CuAAC see: A. Baron, Y. Blériot, M. Sollogoub, B. Vauzeilles, *Org. Biomol. Chem.*, **2008**, *6*, 1898-1901.

(31) C. Decroocq, D. Rodríguez-Lucena, V. Russo, T. Mena Barragán, C. Ortiz-Mellet, P. Compain, *Chem. Eur. J.* **2011**, *17*, 13825-13831.

(32) (a) M. Kanai, K. H. Mortell, L. L. Kiessling, *J. Am. Chem. Soc.* **1997**, *119*, 9931-9932. (b) P.R. Ashton, E.F. Hounsell, N. Jayaraman, T.M. Nilsen, N. Spencer, J.F. Stoddart, M. Young, *J. Org. Chem.* **1998**, *63*, 3429-3437. (c) D. Pagé, R. Roy, *Bioconjugate Chem.* **1997**, *8*, 714-723.

(33) I. Nemčovičová, S. Šesták, D. Rendić, M. Plšková, J. Mucha, I. B. H.Wilson, *Glycoconj. J.* **2013**, DOI 10.1007/s10719-013-9495-5.

(34) S. Howards, S. He, S.G. Withers, *Biochem J.* **1998**, *273*, 2067-2072.

(35) A. Kumar, S. M. Gaikwad, *Int. J. Biol. Macromol.* **2011**, *49*, 1066-1071.

(36) M. Almant, A. Mastouri, L. Gallego-Yerga, J.M. García-Fernández, C. Ortiz-Mellet, J. Ko-

vensky, S. Morandat, K. El Kirat, S.G. Gouin, *Chem. Eur. J.* **2013**, 19, 729-736.
(37) T. K. Dam, T. A. Gerken, C. F. Brewer, *Biochemistry*, **2009**, *48*, 3822-3827.
(38) T. K. Dam, T. A. Gerken, B. S. Kavada, K. S. Nascimento, T. R.Moura and F. C. Brewer, *J. Biol. Chem.*, **2007**, *282*, 28256-28263.
(39) (a) J.C. Sacchettini, L.G. Baum, F.C. Brewer, *Biochemistry*, **2001**, *40*, 3009-3015. (b) F.C. Brewer, M.C. Micelli, L.G. Baum, *Curr. Opin. Struct. Biol.* **2002**, *12*, 616-623.
(40) D. Sicard, S. Cecioni, M. Iazykov, Y. Chevolot, S. E. Matthews, J-P. Praly, E. Souteyrand, A. Imberty, S. Vidal, M. Phaner-Goutorbe, *Chem. Commun.* **2011**, *47*, 9483-9485.
(41) T. Kiba, H. Suzuki, K. Hosokawa, H. Kobayashi, S. Baba, T. Kakuchi, S-i. Sato, *J. Phys. Chem. B,* **2009**, *113,* 11560-11563.
(42) M.K. Muller, M. Brunsveld, *Angew. Chem. Int. Ed*, **2009**, *48*, 2921-2924.

GRAPHICAL ABSTRACT