▌ Chemical Biology

Iminosugar-Cyclopeptoid Conjugates Raise Multivalent Effect in Glycosidase Inhibition at Unprecedented High Levels

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Abstract: A series of cyclopeptoid-based iminosugar clusters has been evaluated to finely probe the ligand content-dependent increase in α-mannosidase inhibition. This study led to the largest binding enhancement ever reported for an enzyme inhibitor (up to 4700-fold on a valency-corrected basis), which represents a substantial advance over the multivalent glycosidase inhibitors previously reported. Electron microscopy imaging and analytical data support, for the best multivalent effects, the formation of a strong chelate complex in which two mannosidase molecules are cross-linked by one inhibitor.

Multivalency is intuitively recognized by chemists as an appealing strategy for the design of ligands displaying high binding specificity for their receptors.[1] Emblematic examples are found in cells where many key intercellular recognitions events are mediated by multisite carbohydrate-binding proteins (lectins), despite the fact that carbohydrate–lectin interactions are individually weak.[2] The over-amplification of binding affinity beyond that expected from a concentration increase is referred to as the cluster or multivalent effect.[3] Inspired by Nature, chemists have synthesized a large variety of glycoclusters exhibiting impressive affinity enhancements over the corresponding monovalent lectin ligands.[2,4] Much less effort has been directed so far towards the design of multivalent inhibitors of carbohydrate-processing enzymes despite their therapeutic relevance. Enzymes appear a priori to be less suited for multivalent design since they usually display a single substrate binding site, rendering theoretically impossible chelation mechanisms at the origin of the largest multivalent effects reported for proteins.[2b, 5] Very recently, the field has experienced a major breakthrough with the discovery of the first strong multivalent effects in glycosidase inhibition observed with iminosugar clusters such as **1** bearing up to 21 DNJ (1-deoxynojirimycin) ligands (Figure 1).[6–10]

One of the next, forward-looking steps is to explore the maximum level of affinity enhancement achievable through inhibitory multivalent effects. Beyond breaking new records, identifying such limits, as well as the existence of a plateau[11] in the valency-dependent affinity increase, is expected to shed light on the molecular basis of this recently discovered effect. Our strategy to tackle these objectives was based on iminosugar clusters constructed on large cyclopeptoid cores with incrementally increasing valencies. The challenge of synthesizing large multivalent iminosugars was addressed using a highly convergent strategy based on Cu^{I}-catalyzed azide–alkyne cycloaddition reactions (CuAAC). In this approach, the initial valency of the polyalkyne scaffolds is tripled by the direct grafting of azido-armed trivalent iminosugar dendrons (Scheme 1).[10] Cyclopeptoids[12] appear as ideal tools to both push and precisely probe the limits of multivalent effects.[12, 13] In contrast to most scaffolds, they indeed offer synthetic flexibility, allowing the access to clusters with finely incremented valencies.[14, 15] Another challenge associated with high-valency systems is that steric crowding of neighboring ligands may limit the multivalent glycosidase binding.[10, 11, 16] This effect was expected to be overcome by the fact that, in the designed cyclopeptoid-based clusters, the scaffold size grows with the valency (Scheme 1).

To systematically increase valency in six ligand increments, azido-armed trivalent iminosugar **4** was attached by CuAAC onto a series of cyclopeptoids **5** bearing 6 to 16 propargyl groups (Scheme 1). These scaffolds were efficiently prepared

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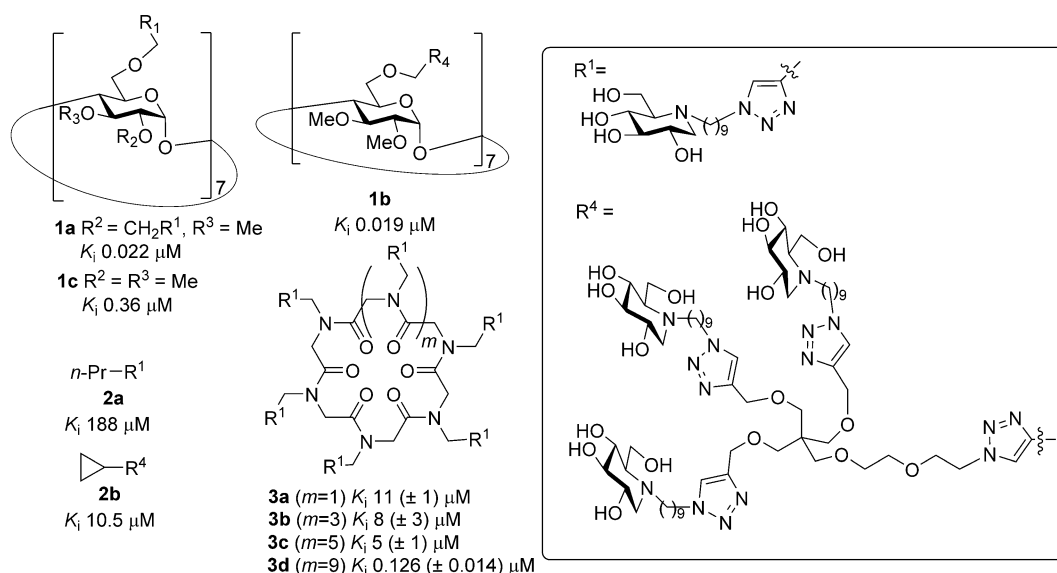
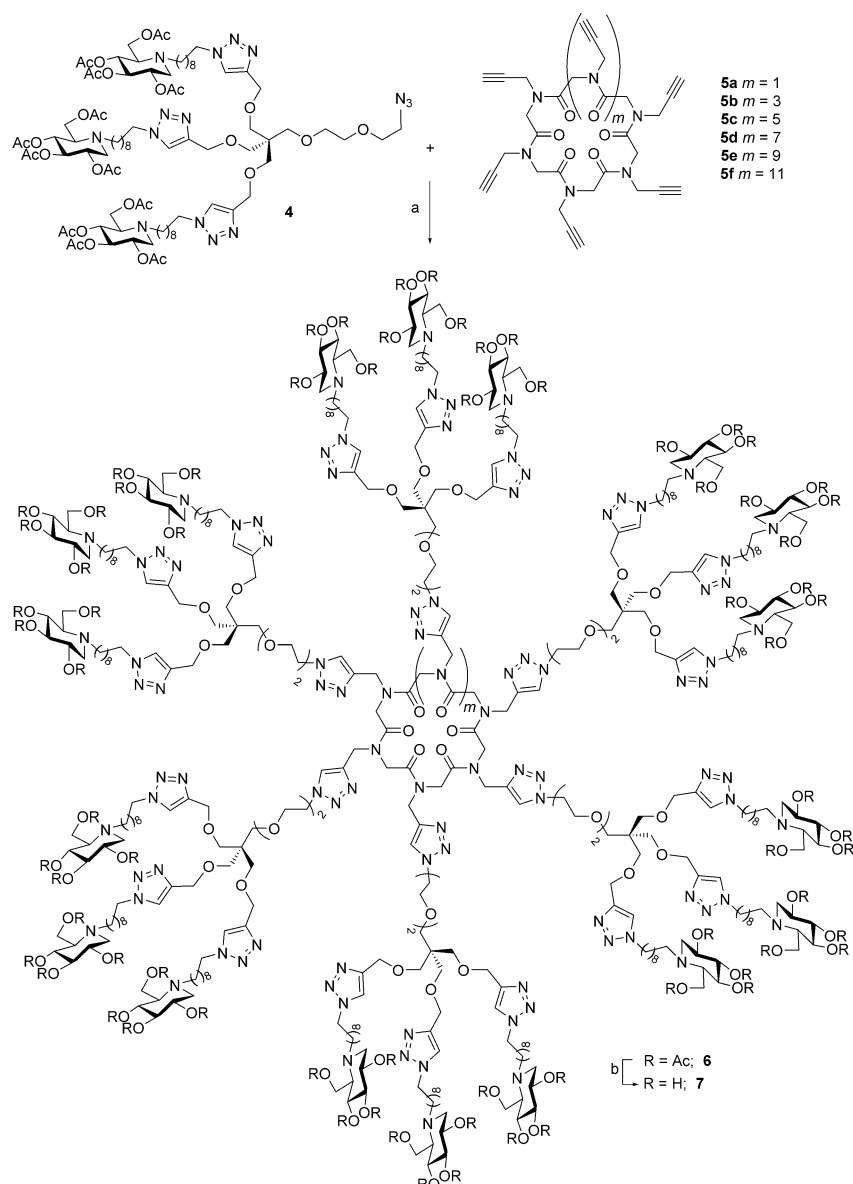**Figure 1.** Multi- and monovalent inhibitors of JBα-man.

by way of iterative submonomer solid-phase synthesis followed by cyclization in high dilution conditions, leading to ones of the largest cyclopeptoids prepared by head-to-tail coupling strategy (Scheme S3 in the Supporting Information).[13,17] The decisive microwave-assisted CuAAC reaction was found to proceed smoothly to provide the desired 18- to 48-valent peracetylated DNJ clusters **6** in 32 to 77% yields which were deprotected by using basic anion exchange resin. These compounds were evaluated as inhibitors of Jack bean α-mannosidase (JBα-man), the glycosidase which has shown the largest responses to multivalent inhibitor presentation to date.[8] The corresponding inhibition constants (K_i) as well as relative inhibition potency (rp) over monovalent control **2a** and valency-corrected rp (rp/n) are collected in Table 1. Due to the structure of neoglycoclusters **7** based on identical trivalent iminosugar subunits, the inhibition potency of trivalent model **2b**, obtained by CuAAC coupling between **4** and ethynylcyclopropane, has been determined (Scheme S1 in the Supporting Information and Table 1).

In a previous study,[13] we have shown that 6- to 10-valent cyclopeptoid-based DNJ clusters **3a–c** (Figure 1) displayed modest inhibitory multivalent effects ($rp/n \approx 3$–4). The addition of eight DNJ units, from **3c** to **7a**, led to a dramatic improvement in binding enhancements to reach a rp/n value of 74. To determine more precisely from which valency significant multivalent effects start to occur, 14-valent iminosugar **3d** was prepared (Scheme S2 in the Supporting Information). The submicromolar K_i value for α-mannosidase inhibition obtained for **3d** showed that a take-off in the multivalent effect could be obtained by the addition of only four DNJ units, from 10 to 14 (Table 1). Remarkably, the rp/n values measured for valency from 6 to 36 were found to fit an exponential curve with an astonishing high correlation (Figure S1 in the Supporting Information). The 36-valent cluster **7d** was found to display an outstanding relative inhibition potency of about 171000-fold, far

Table 1. Relative inhibition potencies of iminosugar clusters and inhibitory activities (K_i [μM]) against JBα-man.[6,7,10,13]

Cpd	DNJ unit	K_i[a]	rp[b]	rp/n[c]
2a[6]	1	188[6]	–	–
2b	3	10.5 ± 1.5	18	6
1b[10]	21	0.019 ± 0.003[10]	9895	471
3c[13]	10	5 ± 1[13]	38	3.8
3d	14	0.126 ± 0.014	1492	107
7a	18	0.142 ± 0.017	1324	74
7b	24	0.037 ± 0.012	5081	212
7c	30	0.0099 ± 0.0028	18990	633
7d	**36**	**0.0011 ± 0.0004**	**170909**	**4747**
7e	42	0.0015 ± 0.0003	125333	2984
7f	48	0.0011 ± 0.00007	170909	3560

[a] Means ± SD obtained from experiments performed in triplicate.
[b] Relative inhibition potency = K_i (monovalent reference **2a**)/K_i(cluster).
[c] $rp/n = rp$/number of DNJ units.

from the ones previously observed for clusters **1**—the previous standards in the field (4750-fold vs. 610-fold in a valency-corrected basis).[7,8,10] Beyond glycosidase inhibition, cluster **7d**[18] provides, to our knowledge, the largest binding enhancements ever reported for an enzyme inhibitor.[19,20] Further increasing the number of DNJ ligands (from 36 to 48) did not enhance the inhibitory potency of iminosugar clusters **7**, leading clearly to a plateau in terms of K_i values. To obtain insights into the binding modes underlying the outstandingly large multivalent effect observed, a mechanistic study using three different complementary techniques was performed. The interactions of JBα-man with **7d** were first analyzed using electron microscopy (EM) with negative staining (Figure 2). JBα-man is a high-molecular-weight metalloenzyme organized into a homodimer (LH)$_2$ of active heterodimers (LH for light and heavy chains) of approximately 66 (H) and about 44 kDa (L), respectively.[21] In

Scheme 1. Reagents and conditions: synthesis of DNJ clusters **7**: a) CuSO$_4$·5H$_2$O cat., sodium ascorbate, DMF/H$_2$O (5:1), MW, 80°C or 90°C; **6a** (m=1) 75%; **6b** (m=3) 73%; **6c** (m=5) 53%; **6d** (m=7) 70%; **6e** (m=9) 77%; **6f** (m=11) 32%. b) Amberlite (OH$^-$), MeOH/H$_2$O (1:1), 40°C; **7a** (m=1) 88%; **7b** (m=3) 77%; **7c** (m=5) quant.; **7d** (m=7) 57%; **7e** (m=9) 81%; **7f** (m=11) 85%.

the case of the mannosidase alone, the image showed clear clusters of approximately 10×20 nm (Figure 2, left, light-colored frame) matching the size of one homodimer (LH)$_2$.

For the complex of JBα-man with **7d**, several different arrangements are seen, including some approximately 20×20 nm in size that may correspond to the interaction of one inhibitor with two tetramers (LH)$_2$ (Figure 2, right, dark-colored frame). This hypothesis was confirmed by analytical ultracentrifugation sedimentation velocity (AUC-SV) experiments (Figure 3).[22]

For JBα-man alone, the main sedimenting species is observed at 9.3 S, corresponding to the tetramer (LH)$_2$ with a molar mass estimate of 210 kDa (see the Supporting information).[23] The sedimentation coefficient distribution c(s) showed also a minor species with a sedimentation coefficient value of
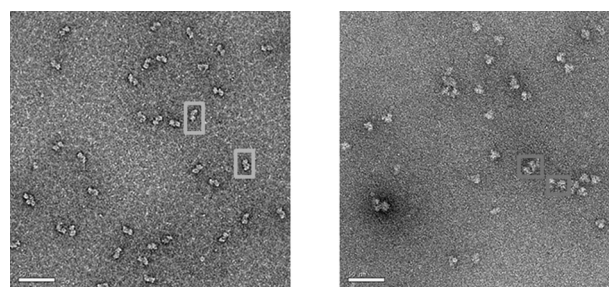

Figure 2. EM pictures of JBα-man (left) and JBα-man interacting with **7d** (right); scale bars = 50 nm.

13.4 S which may correspond to the association of two JBα-man molecules in a 2×(LH)$_2$ complex. The binding of JBα-man to **7d** created a broader sedimentation coefficient distribution
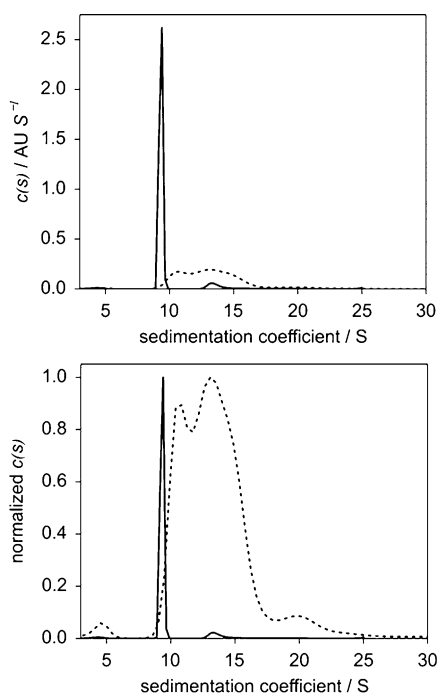
Figure 3. The top panel shows the sedimentation coefficient distribution plot for JBα-man (solid line) and for the JBα-man:**7d** complex (dotted line). The bottom panel shows the same distributions normalized to a maximum of 1.0.

exhibiting maxima at about 14 S, suggesting the reversible formation of a 2:1 tetramer(LH)$_2$–inhibitor complex in dynamic equilibrium with other species. AUC-SV control experiments

were also performed with a system displaying a small multivalent effect (DNJ cluster **3b**, $rp/n \sim 3$) or for which the binding stoichiometry had been previously determined to be close to one by isothermal titration calorimetry (**1c**).[24] In both cases, the major species observed corresponded to the (LH)$_2$–inhibitor 1:1 complex whereas for the larger cluster **7a**, with a rp/n value of 74, the equilibrium is clearly in favor of the 2:1 (LH)$_2$–inhibitor complex (Figure S3 in the Supporting Information). Native electrospray mass spectrometry (ESI-MS) was performed to further confirm the stoichiometry of the noncovalent macromolecular enzyme–inhibitor complex (Figure 4).[25] MS obtained on the native enzyme before and after the inhibitor addition showed the presence of three different multicharged ions patterns (Figure 4). When JBα-man is alone in the solution studied (Figure 4a), the molecular masses measured correspond to the primal heterodimer LH ($M_W = 121.3$ kDa), the tetramer (LH)$_2$ ($M_W = 242.3$ kDa) and the association of two tetramers (LH)$_2$ ($M_W = 484.9$ kDa), respectively (see the Supporting information). After addition of the inhibitor **7d** ($M_W = 17.4$ kDa, Figure 4b), both enzyme–inhibitor complexes are observed with a 17 kDa shift ($M_W = 259.9$ kDa for the 1:1 complex and $M_W = 501.9$ kDa for the 2:1 complex).

Based on the corroborating results obtained with three different techniques, the outstanding multivalent effects observed may be rationalized by the formation of a strong chelate complex in which the inhibitor cross-links two tetramers (LH)$_2$ (Figure 5). Such a complex would involve reversible active-site specific interactions but also protein–protein interactions between two tetramers. Increasing the valency and the global size of the scaffold shifts the equilibrium of the different
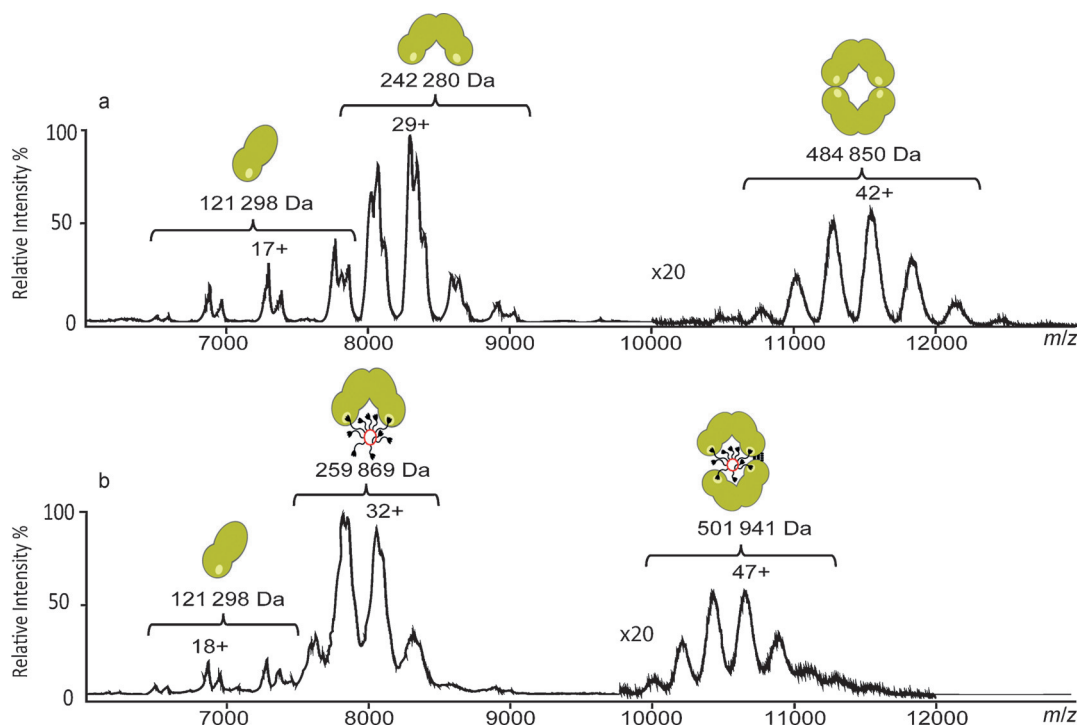

Figure 4. Native ESI-MS spectra for: a) JBα-man, and b) JBα-man interacting with **7d**. Multicharged ions patterns are labeled with the corresponding enzyme and enzyme–inhibitor complexes.
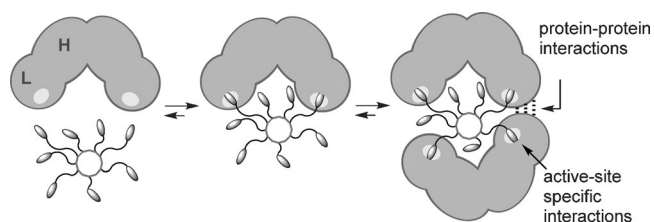
species in solution towards the 2:1 tetramer–inhibitor complex even in the presence of an excess of inhibitor showing that the 2:1 sandwich may be thermodynamically favored. The implications of these findings for the design of multivalent inhibitors and the identification from the protein data bank of other multimeric glycosidases responsive to multivalency are currently under investigation in our laboratories.

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