**Chemical Biology**

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


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 multise site carbohydrate-binding proteins (lec- tins), 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 glycoconjugates 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 multiva lent 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 experiences 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...
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). The decisive microwave-assisted CuAAC reaction was found to proceed smoothly to provide the desired 18- to 48-valent penta-ling strategy (Scheme S3 in the Supporting Information). \[13, 17\]

One of the largest cyclopeptoids prepared by head-to-tail coupling in high dilution conditions, leading 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).

Table 1. Relative inhibition potencies of iminosugar clusters and inhibitory activities (\(K_i\) [\(\mu M\)]) against \(\alpha\)-mannosidase (\(\alpha\)-man)–man. \[6, 7, 10, 13\]

<table>
<thead>
<tr>
<th>Cpd</th>
<th>DNUnit</th>
<th>(K_i) (\mu M)</th>
<th>(rp) (n)</th>
<th>(rp/n) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2a)</td>
<td>1</td>
<td>188 (\pm) 4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(2b)</td>
<td>3</td>
<td>10.5 (\pm) 1.5</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>(1b)</td>
<td>21</td>
<td>0.019 (\pm) 0.003 (\pm) 4</td>
<td>9895</td>
<td>471</td>
</tr>
<tr>
<td>(3c)</td>
<td>10</td>
<td>5 (\pm) 1 (\pm) 13</td>
<td>38</td>
<td>3.8</td>
</tr>
<tr>
<td>(3d)</td>
<td>14</td>
<td>0.126 (\pm) 0.014</td>
<td>1492</td>
<td>107</td>
</tr>
<tr>
<td>(7a)</td>
<td>18</td>
<td>0.142 (\pm) 0.017</td>
<td>1324</td>
<td>74</td>
</tr>
<tr>
<td>(7b)</td>
<td>24</td>
<td>0.037 (\pm) 0.012</td>
<td>5081</td>
<td>212</td>
</tr>
<tr>
<td>(7c)</td>
<td>30</td>
<td>0.0099 (\pm) 0.0028</td>
<td>18990</td>
<td>633</td>
</tr>
<tr>
<td>(7d)</td>
<td>36</td>
<td>0.0011 (\pm) 0.0004</td>
<td>170909</td>
<td>4747</td>
</tr>
<tr>
<td>(7e)</td>
<td>42</td>
<td>0.0015 (\pm) 0.0003</td>
<td>125333</td>
<td>2984</td>
</tr>
<tr>
<td>(7f)</td>
<td>48</td>
<td>0.0011 (\pm) 0.0007</td>
<td>170909</td>
<td>3560</td>
</tr>
</tbody>
</table>
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 J término 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 J término 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 13.4 S which may correspond to the association of two J término man molecules in a 2 × (LH)$_2$ complex. The binding of J término man to 7d created a broader sedimentation coefficient distribution.

Scheme 1. Reagents and conditions: synthesis of DNJ clusters 7: a) CuSO$_4·$5H$_2$O cat., sodium ascorbate, DMF/H$_2$O (5:1), MW, 80 °C or 90 °C; 6a (m = 1) 75%; 6b (m = 3) 73%; 6c (m = 5) 53%; 6d (m = 7) 70%; 6e (m = 9) 97%; 6f (m = 11) 32%. b) Amberlite (OH$^-$), MeOH/H$_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%.
exhibiting maxima at about 14 S, suggesting the reversible formation of a 2:1 tetramer (LH)\textsubscript{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, \textit{rp/n} = 3) or for which the binding stoichiometry had been previously determined to be close to one by isothermal titration calorimetry (1c). In both cases, the major species observed corresponded to the (LH)\textsubscript{2}–inhibitor 1:1 complex whereas for the larger cluster 7a, with a \textit{rp/n} value of 74, the equilibrium is clearly in favor of the 2:1 (LH)\textsubscript{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). MS obtained on the native enzyme before and after the inhibitor addition showed the presence of three different multicharged ions patterns (Figure 4). When JB\textalpha-man is alone in the solution studied (Figure 4a), the molecular masses measured correspond to the primal heterodimer LH (\textit{M}_{\text{w}}=121.3 \text{ kDa}), the tetramer (LH)\textsubscript{2} (\textit{M}_{\text{w}}=242.3 \text{ kDa}) and the association of two tetraters (LH)\textsubscript{2} (\textit{M}_{\text{w}}=484.9 \text{ kDa}), respectively (see the Supporting information). After addition of the inhibitor 7d (\textit{M}_{\text{w}}=17.4 \text{ kDa}, Figure 4b), both enzyme–inhibitor complexes are observed with a 17 kDa shift (\textit{M}_{\text{w}}=259.9 \text{ kDa for the 1:1 complex and } \textit{M}_{\text{w}}=501.9 \text{ 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)\textsubscript{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 3](image1.png)

**Figure 3.** The top panel shows the sedimentation coefficient distribution plot for JB\textalpha-man (solid line) and for the JB\textalpha-man:7d complex (dotted line). The bottom panel shows the same distributions normalized to a maximum of 1.0.

![Figure 4](image2.png)

**Figure 4.** Native ESI-MS spectra for: a) JB\textalpha-man, and b) JB\textalpha-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.

Acknowledgements

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Keywords: glycopeptides · glycosidases · iminosugars · inhibitors · multivalency

[18] A low content of residual copper ions (60 ppm) was measured in 7d by inductively coupled plasma atomic emission spectroscopy.
[19] Moroder et al. reported that divalent ligands bridging simultaneously two catalytic sites of human L-tryptase were up to 68 000-fold more potent than the corresponding monovalent inhibitor: N. Schaschke, G. Matschiner, F. Zettl, U. Marquardt, A. Bergner, W. Bode, C. P. Sommerhoff, L. Moroder, Chem. Biol. 2001, 8, 313–327.
[23] The molar mass distribution (m) allow to obtain molar mass estimates of the sedimenting species frequently within 10% of the correct value: see J. Darn, P. Schuck, Methods Enzymol. 2004, 384, 185–212.

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Breaking sweet records! Cyclopeptoid-based iminosugar clusters (see figure) show the largest multivalent effects ever reported in glycosidase inhibition (up to 4700-fold on a valency-corrected basis). Electron microscopy imaging and analytical data support the formation of sandwich-type complexes in which two mannosidase molecules are cross-linked by one inhibitor.