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Polymeric Iminosugars Improve the Activity of Carbohydrate-Processing Enzymes

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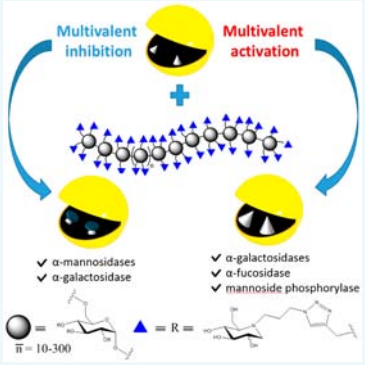
Supporting Information

ABSTRACT: Multivalent iminosugars have recently emerged as powerful tools to inhibit the activities of specific glycosidases. In this work, biocompatible dextrans were coated with iminosugars to form linear and ramified polymers with unprecedently high valencies (from 20 to 900) to probe the evolution of the multivalent inhibition as a function of ligand valency. This study led to the discovery that polyvalent iminosugars can also significantly enhance, not only inhibit, the enzymatic activity of specific glycoside-hydrolase, as observed on two galactosidases, a fucosidase, and a bacterial mannoside phosphorylase for which an impressive 70-fold activation was even reached. The concept of glycosidase activation is largely unexplored, with a unique recent example of small-molecules activators of a bacterial O-GlcNAc hydrolase. The possibility of using these polymers as "artificial enzyme effectors" may therefore open up new perspectives in therapeutics and biocatalysis.



INTRODUCTION

Multivalency is a well-established and powerful approach to develop strong and selective inhibitors of carbohydrate-binding proteins called lectins.[1−3] The so-called "glycoclusters", in which ligands are displayed in multiple copies on a synthetic core, mimic the glycocalyx displayed at the surface of cells and can lead to strong affinity enhancements compared to monovalent references. The simplicity and efficiency of the concept has led many groups to choose the multivalent way rather than improving the potency of a monovalent lead in a "lock and key" approach.

Until recently, the multivalent approach has been most successful with lectins, with only a few studies reported on carbohydrate-processing proteins. A striking example in the field was the development of multi- and polyvalent inhibitors of influenza neuraminidase, which showed outstanding ability to inhibit influenza virus replication in vivo.[4,5] To estimate the potential of the multivalent approach in glycosidase inhibition, we previously designed a set of mono-, di-, and trivalent inhibitors of the deoxynojirimycin (DNJ) iminosugar, a broad specificity glycosidase inhibitor.[6] The inhibitory activity of these compounds measured on nine glycosidases demonstrated that the multivalent strategy cannot be considered a general mode of inhibition of these enzymes as it is with lectins. However, a small but significant multivalent effect was detected for the first time on Jack bean α-mannosidase (JbMan). Much lower inhibitory activities and higher multivalent effects were then observed on the same enzyme with DNJ compounds of higher valencies.[7,8] Interestingly, multivalent effects were also seen on therapeutically relevant carbohydrate-processing enzymes including (i) β-glucocerebrosidase for which the deficiency in hydrolyzing glucosylceramide can lead to Gaucher disease,[9] the most common form of lysosomal storage disorders, (ii) the bacterial heptosyl-transferase WaaC incorporating heptosyl subunits in the outer membrane of Gram-negative bacteria,[10] (iii) human liver glycogen phosphorylase, a target for the treatment of noninsulin dependent diabetes mellitus,[11] and (iv) ManIIb as a representative of Golgi α-mannosidase II, a potential target of anticancer therapy.[12] Multivalent iminosugars were also shown to be able to correct the cell-deficiency of

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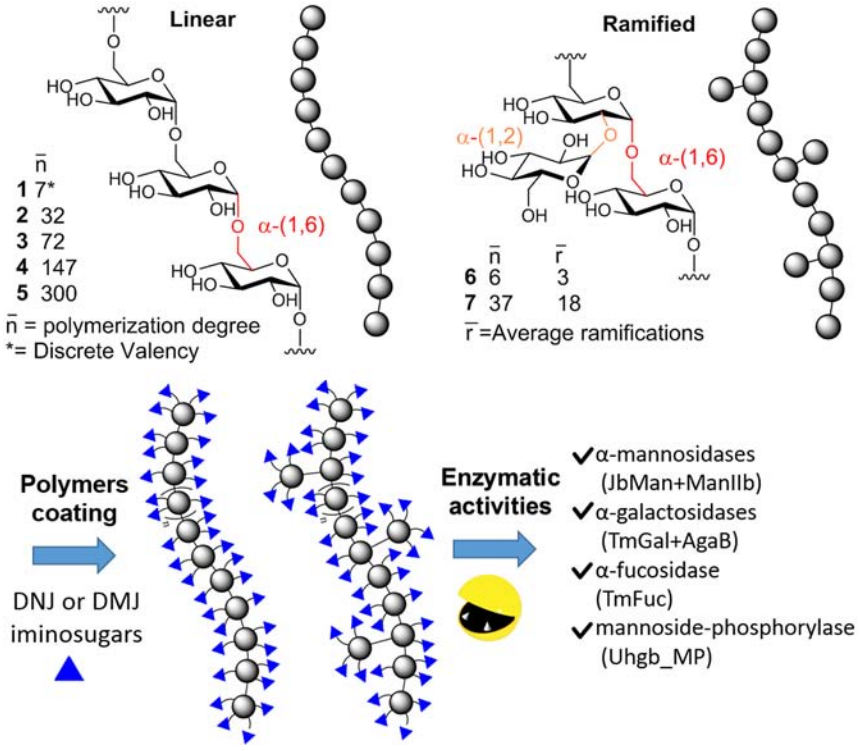

Figure 1. Schematic representation of the work. Linear (**1**−**5**) and ramified (**6**,**7**) dextran scaffolds were coated with DNJ and DMJ iminosugars. The effect on the enzymatic activity of the resulting biopolymers was assessed against six glycoside-processing enzymes.

a transmembrane regulator causing cystic fibrosis.[13] Taken together, these results highlight that the multivalent approach in inhibiting carbohydrate-processing enzymes is a rapidly expanding field,[14,15] with real pharmaceutical potential. The fact that all targeted enzymes do not respond positively to multivalency limits the potential applications, but is very beneficial in terms of selectivity.

Although the possibility of improving inhibitory activities is now well established, the crucial question of the multivalent binding mode(s) operating with this class of molecules is far from being answered. We,[12] and others,[16,17] addressed this question using the JbMan model and iminosugar or sp^2-iminosugar conjugates,[18] showing that the corresponding multivalent displays could promote the formation of large aggregates[12] and interact in enzyme subsites,[16] respectively. Recently, the importance of the density of iminosugar ligands was also studied with micellar glycopeptides.[19]

In this work, we were interested in studying the potential limit of the multivalent effect in the inhibition of glycoside-processing enzymes. As glycoclusters designed for lectins often show a plateau of affinity after exceeding a discrete valency, it is of interest to determine whether the same interaction profile also occurs with the enzymes targeted in this study. Importantly, this led to the discovery that polymeric iminosugars can also function as significant activators of specific glycoside hydrolases.

■ RESULTS AND DISCUSSION

Hydrophilic polymers, with an unprecedentedly high average number of DNJ or deoxymannojirimycin (DMJ) iminosugars ranging from 20 to 900, were designed from commercial dextrans. Inhibitory activities were assessed on a panel of five retaining glycosidases from the GH 29 (α-L-fucosidase from *Thermotoga maritima*, TmFuc), GH 36 (α-D-galactosidases from *T. maritima*, TmGal, and *Geobacillus stearothermophilus*, AgaB), and GH38 families (α-D-mannosidases from *Canavalia ensiformis*, JbMan, and *Drosophilia melanogaster*, ManIIb) and an inverting *N*-glycan phosphorylase (Uhgb_MP) belonging to the GH130 family. Multivalent effects were quantified as a function of the valency of the ligands for the model JbMan, the anticancer target Golgi α-mannosidase (ManIIb), and for TmGal.

We selected various commercial dextrans products of different molecular weights for the design of the polymeric iminosugars. Dextrans are highly hydrophilic and nontoxic scaffolds. They are homogeneous polymers of glucosyl units composed of approximately 95% α-(1,6) linkages.[20,21] The three available hydroxyls of each glucose unit can be functionalized and further loaded by iminosugars leading to highly dense DNJ and DMJ-coated dextrans (DNJ-DEX and DMJ-DEX). As the spatial presentation of DNJ in low valency ligands is a critical factor for enzyme affinity and selectivity,[12] the set of linear dextrans was completed with two ramified analogues bearing ∼33% of α-1,2 linked glucosyl units (Figure 1).

To produce scaffolds **6** and **7**, linear α-1,6 dextrans from *Leuconostoc mesenteroides* NRRL B-512F were incubated with sucrose and the α-1,2 branching sucrase, GBD-CD2, a truncated enzyme derived from the DSR-E dextransucrase from *L. mesenteroides* NRRL B-1299.[22] Their MW mass was calculated from the size of their backbone and their degree of branching, evaluated by NMR. Purification was achieved by preparative ion exchange followed by C18 chromatography.[23]

Dextrans were first functionalized by propargylic groups following a previously described protocol for arabinogalactans using a biphasic mixture of solubilized dextrans in an aqueous basic solution and propargyl bromide in toluene.[24] A catalytic amount of triethylbenzylammonium chloride (TEBAC) was

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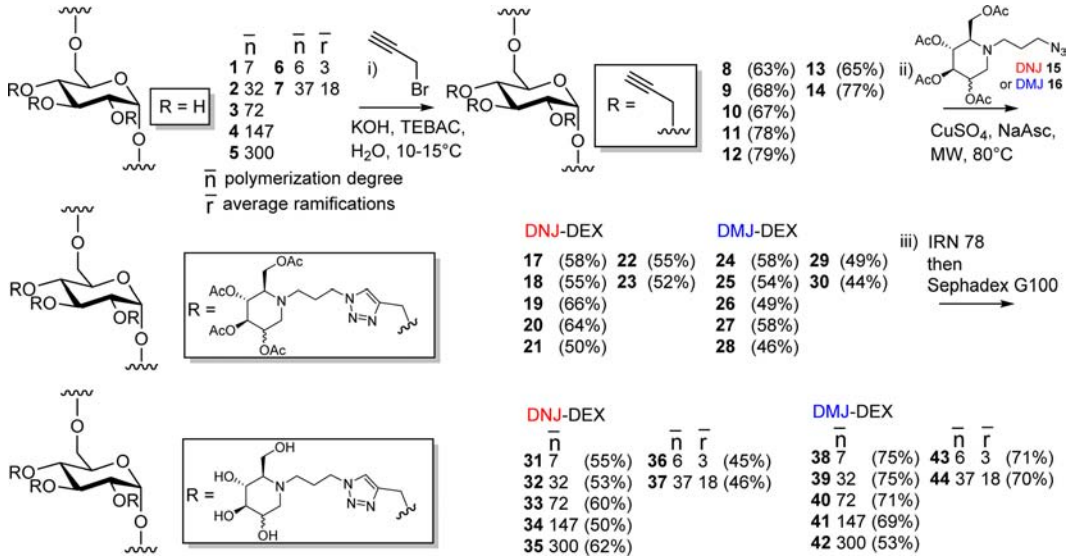
Scheme 1. Chemical Synthesis of the Linear and Ramified Ultravalent DNJ-DEX 31−37 and DMJ-DEX 38−44



shown to improve significantly the reaction yields of the linear **8−12** and ramified **13−14** alkynyl-dextrans (Scheme 1). Relative integration of the ^{1}H NMR signals for the sugar anomeric and propargylic protons indicated a full propargylation of the dextrans. This was further confirmed by infrared spectra showing the disappearance of the large 2700−3600 cm^{-1}(υ_{O-H}) band and new characteristic ($\upsilon_{\equiv C-H}$) and ($\upsilon_{C\equiv C}$) bands at around 3290 and 2120 cm^{-1}, respectively.

Azidopropyl-DMJ **16** was obtained in nine steps (Figure S1) using a similar procedure to that described for the DNJ analogue **15**.[12] Both iminosugars were grafted onto alkynyl-dextrans **8−14** by a microwave-assisted copper-catalyzed azide−alkyne cycloaddition to give multivalent iminosugars **17−30**. The complete conversion of the alkynes was evidenced by ^{1}H and ^{13}C NMR and the unique formation of the 1,4 cycloadduct was shown by the large $\Delta(\delta C\text{-}4-\delta C\text{-}5)$ values (>20 ppm).[25] Hydroxyl groups were deprotected with the basic Amberlite resin IRN 78 and the crude dextrans **31−44** were purified by size-exclusion chromatography on a Sephadex G100 column.

In addition, adequate monovalent references were synthesized to assess the level of potential multivalent effects (Figure 2). A tetravalent derivative based on a similar scaffold was also designed and included in the assays (Figure 2).

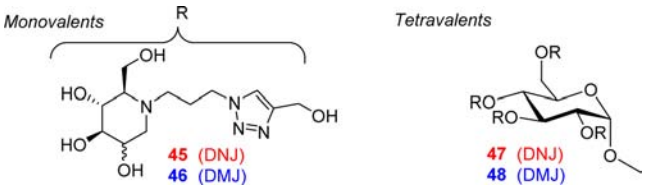


Figure 2. Structure of the monovalent DNJ **45** and DMJ **46** references and the tetravalent DNJ **47** and DMJ **48**.

Inhibitory activities were first evaluated on JbMan, for which the first and highest multivalent effects were reported with iminosugars.[6−8]

The inhibitory activities (K_{ic} values) reported in Table 1 are expressed in total concentration of iminosugars (valency-corrected) and not in polymer molecules concentrations (K_i values), for a fair assessment of the multivalent effect. As an example, the best compound **42**, with an average DMJ valency of 902, displayed a K_i value of 1.5 nM and a valency-corrected K_{ic} value of 1.4 μM (1.5 × 902) (Table 1). We also calculated the β value, a factor frequently used to quantify the multivalent effect observed with a glycocluster against a specific lectin. Similarly, the β factor represents here the improved inhibitory potency per iminosugar, and is calculated by dividing the K_i value of monovalent references **45** or **46** by the K_{ic} value of the ultravalent DNJ-DEX or DMJ-DEX, respectively. As an example, the β factor for **36** is calculated as follows (394:25 = 16).

Table 1. Inhibitory Activities against JbMan[a]

$\tilde{V}_{Im}$	DNJ-Cpd	K_{ic} (μM)	β	DMJ-Cpd	K_{ic} (μM)	β
1	45	394 ±28	1	46	167 ±17	1
4	47	196 ±64	2.0	48	132 ±48	1
23	31	6.0 ±1.5	66	38	2 ±0.5	79
29	36	25 ±1.8	16	43	4 ±1.2	40
98	32	5.7 ±0.3	69	39	2.2 ±0.1	74
167	37	32 ±1.8	12	44	3.4 ±0.1	49
218	33	12 ±2.4	34	40	2.0 ±0.4	83
443	34	26 ±3.6	15	41	2.3 ±0.3	71
902	35	11 ±2.6	36	42	1.4 ±0.1	121

[a]$\tilde{V}_{im}$ = average valency of iminosugars. K_{ic} = inhibitory activity (μM) calculated per iminosugar, average of three measurements. β = affinity enhancement for each iminosugar calculated by dividing K_i values of the adequate monovalent reference (**45** for DNJ compounds and **46** for DMJ) by the valency-corrected K_{ic} value of the ultravalent compound. Ramified compounds in gray cells.

The results showed that a significant multivalent effect occurred for the whole set of DNJ- and DMJ-DEX. In all cases, the linear DMJ-DEX were better inhibitors than their DNJ

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analogues and showed higher multivalent effects in the valency range considered (Figure 3). Ramified polymers **36**, **37**, **43**, and

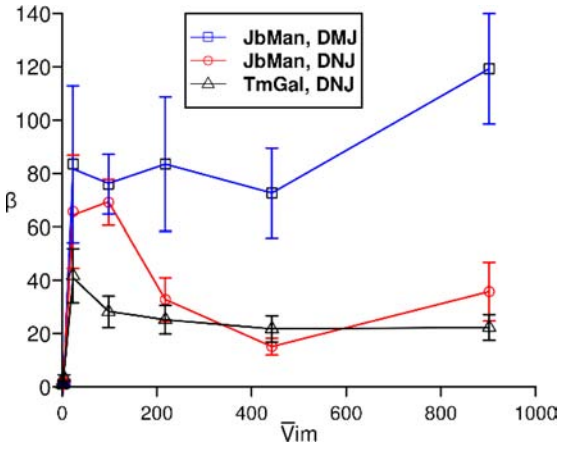

Figure 3. Evolution of the multivalent inhibitory effect β in function of the average iminosugar valency.

44 showed significantly lower β values than the linear ones, indicating the detrimental role of the spatial presentation of the ligands. β values rapidly reached a peak for valencies around 20 (Figure 3), then a subsequent decrease was observed for the DNJ-DEX series. The increased β value of +65% for the longest DMJ-DEX **42** compared to **38** can be seen as moderate considering the high valency gap between these two compounds (from 23 to 902 DMJ units). The maximal β value of 121 observed here for **42** can also be compared with published β values of the most potent ligands, with lower and discrete valencies, such as a tetravalent DNJ based porphyrine (β = 200),[12] and a tetradecavalent DNJ-based cyclodextrin (β = 610).[8] The higher β values obtained with these low valency compounds and the moderate improvement observed here at high DNJ or DMJ loading suggest that focusing on discrete compounds with adequate iminosugar topologies is more relevant for JbMan inhibition than overincreasing the ligand valency.

The *Drosophilia melanogaster* enzyme homologous to the human ManIIb was used for the assay. Results obtained with DMJ-DEX are presented in Table 2. They confirm that ManIIb is a potentially interesting target for multivalent inhibition.[12] However, this class of compounds only showed a significant multivalent effect at the highest valencies.

DNJ-DEX and DMJ-DEX were then assessed against TmGal which is a monomeric type I α-galactosidase belonging to the GH36 family. The DMJ derivatives did not show significant inhibition in preliminary assays and were not considered further. K_{ic} and β values for DNJ-DEX are reported in Table 2 and Figure 3. Although the whole set of DNJ compounds were competitive binders against JbMan, an uncompetitive binding mode prevailed with TmGal, as evidenced by the Lineweaver–Burk representations (Figures S8, S9).

Interestingly, the DNJ-DEX **31**–**35** showed significant multivalent effects against TmGal (Table 2), which is therefore a newly identified glycosidase target sensitive to multivalency. Consistent with the observations made with JbMan, the ramified compounds **36** and **37** showed much lower β values than linear DNJ-DEX. The inhibition profile of DNJ-DEX was also very similar to that observed on JbMan, with a peak at low valency, followed by a plateau of inhibition (Figure 3).

Table 2. Inhibitory Activities against ManIIb and TmGal[a]

	ManIIb			TmGal		
$\tilde{V}_{Im}$	DMJ Cpd	K_{ic} (μM)	β	DNJ Cpd	K_{ic} (μM)	β
1	46	435 ±68	1	45	960 ±150	1
4	48	62 ±11	1.8	47	276 ±44	3.5
23	38	306 ±84	1.4	31	23 ±2.0	42
29	43	--	--	36	45 ±3.6	21
98	39	321 ±96	1.4	32	34 ±1.8	28
167	44	141 ±48	3.1	37	159 ±27	6.0
218	40	--	--	33	38 ±2.1	25
443	41	66 ±12	6.6	34	44 ±3.0	22
902	42	51 ±18	8.5	35	43 ±2.6	22

[a] $\tilde{V}_{im}$ = average valency of iminosugars. K_{ic} = inhibitory activity (μM) calculated per iminosugar, average of three measurements. β = affinity enhancement for each iminosugar calculated by dividing K_i values of the adequate monovalent reference (**45** for DNJ compounds and **46** for DMJ) by the valency-corrected K_{ic} value of the polyvalent compound. Ramified compounds in gray cells. – = not evaluated.

Interestingly, the tetravalent **47** and ultravalent **31**–**35** showed dramatically different behavior at low concentrations (below 8 μM), switching from being inhibitors to significant activators of the enzymatic activity (Figure S12). This activation phenomenon was not observed with the monovalent DNJ derivatives. A similar scenario, but at a lower level, was observed with the whole set of DNJ-DEX and DMJ-DEX **31**–**42** against TmFuc, which belongs to the GH29 family and forms a hexameric complex in solution.[26] In comparison, the monovalent derivatives **45** and **46** showed only a slight inhibition.

This enhancing effect was more prevalent with our next target, the α-galactosidase B from *G. stearothermophilus* (AgaB). This enzyme is a tetrameric α-galactosidase belonging to the GH36 family. High activity enhancements were observed with the whole set of ultravalent compounds, with tetravalent DNJ **47** showing a specific behavior being either an enzyme activator or an inhibitor depending on the concentration.

To quantify the enhancing or inhibitory effect better, we calculated the variation in the initial turnover number (k_{cat}), which represents the number of substrate molecules converted to product by AgaB per unit of time. The activation effect of different concentrations of iminosugars on the activity of AgaB was tested at a fixed concentration of *p*NP-Gal as substrate (Figure 4), where F represents the relative activity compared to the rate in the absence of iminosugar (activation if F > 100%, inhibition if F < 100%).

The F values obtained for tetravalent **47** and **48** and ramified compounds **37** and **44** with ∼167 DNJ and DMJ units, respectively, are presented in Figure 4. The results clearly showed different inhibition and activation profiles depending on both the valency and nature of the iminosugar. Tetravalent DNJ-based **47** activated AgaB below 80 μM but switched to being an inhibitor at higher concentration, consistent with what was observed against TmGal.

The DMJ analogue **48** showed a pure AgaB activation with an F value of 140% at 250 μM. Ultravalent compounds **37** and **44** were potent activators in the concentration range tested. **37**

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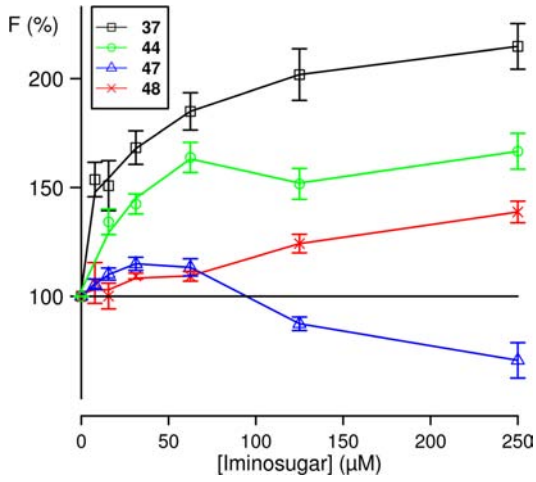

Figure 4. Activation of AgaB promoted by compounds **37**, **44**, **47**, and **48**. Average of three measurements per value.

was the most potent of the series, reaching 220% of the initial AgaB activity. Such an activation phenomenon was never reported with multivalent iminosugars.

Changing the concentration of a specific ultravalent compound raises the unexpected possibility of fine-tuning the enzymatic activity. This is possible because some ultravalent compounds have a specific activation/inhibition profile depending on the concentration as illustrated by compound **37** in Figure 5. For example, AgaB and TmGal would be activated in

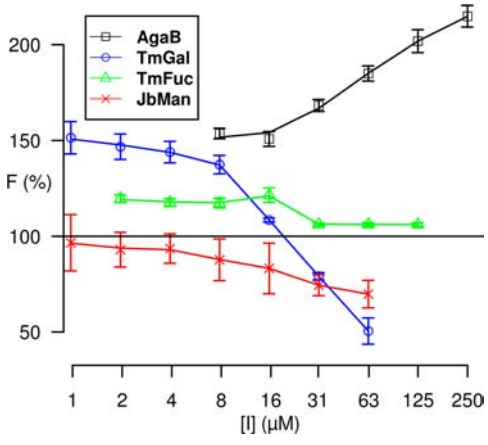

Figure 5. Activation or inhibition of AgaB, TmGal, TmFuc, and JbMan, promoted by compound **37**, average of three measurements per value.

the presence of low concentrations of **37** (8 μM and below). In contrast, higher concentrations of **37** (20 μM and above) will activate AgaB while inhibiting TmGal. As AgaB and TmGal are two enzymes from the same family (GH36), these results illustrate that ultravalent iminosugars are potential modulators able to discriminate between related glycosidases.

The iminosugars were then tested on another biologically relevant target, the mannoside-phosphorylase (EC 2.4.1.-) Uhgb_MP. Belonging to the GH130 family, this enzyme produced by an uncultivated *Bacteroides* human gut bacterium, is involved in *N*-glycan degradation.[27] Uhgb_MP and its homologues are encoded by highly abundant genes in the gut microbiome, especially that of patients suffering from inflammatory bowel diseases. They could thus participate in the alteration of the intestinal barrier, through human *N*-glycan degradation. **44** was a very strong activator of Uhgb_MP up to 500 μM. A 70-fold activation was even reached at this concentration when phosphorolysis activity was quantified by using *p*NP-β-D-mannopyranose as substrate (Figure 6). At 3

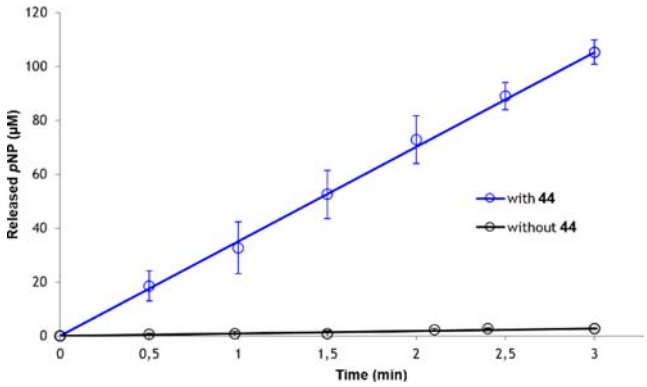

Figure 6. Activation of Uhgb_MP promoted by compound **44** at 500 μM, average of three measurements per value.

mM **44**, an inhibition was observed, due to enzyme aggregation and precipitation, which was clearly visible in the reaction medium. Uhgb_MP aggregation was confirmed by DLS analysis, which revealed particles increasing in size with increasing **44** concentration. At 500 μM particle size of 1990 nm was observed (Figure S13).

CONCLUSION

In summary, we have developed a set of polymeric DNJ and DMJ iminosugars to explore the multivalent effect on the inhibition of carbohydrate-processing enzymes of various structures and functions, at high iminosugar valencies ranging from 20 to 900.

DNJ- or DMJ-based polymers were shown to reach high β-values rapidly at low valencies against JbMan, the most studied glycosidase target for multivalency, and TmGal, a new glycosidase identified here as sensitive to multivalency, which extend the multivalent concept to the inhibition of α-galactosidases. At higher valencies, a drop in β-values was observed, followed at best by a moderate increase, or by a plateau of inhibition. These results suggest a threshold limit in the multivalent enzyme inhibition, as often observed with lectins. However, it should be noted that the multivalent effect limits observed here apply strictly only to dextran conjugates and may not be extrapolated to other iminosugar-coated polymers with different spatial presentation of the ligands.

Importantly, the polymeric DNJ and DMJ were shown to promote enzyme activity significantly on several targets. High activity enhancements were observed on a galactosidase (AgaB, F = 220%) and a mannoside-phosphorylase (Uhgb_MP, ×70-fold). We will next investigate the mechanism(s) leading to the enhanced enzymatic activity to decipher if DNJ- and DMJ-DEX are potential allosteric effectors or if a trans-glycosylation (or reverse-phosphorylation for Uhgb_MP) process occurs. Enzyme activations have been previously described with cyclodextrin-conjugates stabilizing the protein conformation through multiple hydrogen bonds and electrostatic interactions.[28] We do not favor this hypothesis here, as no signs of self-decreasing catalytic efficiency were seen during the experiments.

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Glycosidase activation remain an unexplored area and as far as we know this work is only the second example describing glycoside hydrolase activators. Small-molecule effectors of a bacterial glycoside hydrolase (O-GlcNAc hydrolase) have been recently published.[29] The fact that multivalent iminosugars can function as "artificial enzyme effectors" is an interesting finding that may open up new biological perspectives in therapy and biocatalysis where fine-tuning glycoside-hydrolase activity is required.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis conditions, protocols, inhibitory activities. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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