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# Synthesis of Mannosylated Glycodendrimers and Evaluation against BC2L-A Lectin from *Burkholderia Cenocepacia*

Carlo Pifferi<sup>+</sup>,<sup>[a]</sup> David Goyard<sup>+</sup>,<sup>[a]</sup> Emilie Gillon,<sup>[b]</sup> Anne Imberty,<sup>\*,[b]</sup> and Olivier Renaudet<sup>\*,[a, c]</sup>

Chronic colonization of lungs by opportunist bacteria is the major cause of mortality for cystic fibrosis patients. Among these pathogens, *Burkholderia cenocepacia* is responsible for cepacia syndrome, a deadly exacerbation of infection that is the main cause of poor outcomes of lung transplantation. This bacterium contains three soluble carbohydrate-binding proteins, including the *B. cenocepacia* lectin A (BC2L-A), which is proposed to bind to oligomannose-type *N*-glycan structures to

adhere to host tissues. In this work, several mannosylated glycoclusters and glycodendrimers with valencies ranging from four to 24 were prepared and their interactions with BC2L-A were thermodynamically characterized by isothermal titration calorimetry. The results show that a 24-valent structure binds to BC2L-A at nanomolar concentration, which makes this compound the highest affinity monodisperse ligand for this lectin.

## Introduction

Specific, reversible carbohydrate recognition by lectins is implicated in many normal and pathological processes from cell recognition and communication to pathogen invasion and tumor metastasis.<sup>[1,2]</sup> For instance, bacterial or viral infections are initiated by highly specific, multivalent recognition mechanisms between glycans displayed at the host cell surface and lectins expressed by these pathogens.<sup>[3–5]</sup> Despite the high specificity of lectins toward their carbohydrate ligands, these interactions are typically weak, with affinities in the low millimolar to high micromolar range. In order to circumvent this potential problem, lectins often adopt multimeric architectures, presenting several sites that bind clusters of carbohydrate ligands. The resulting interaction, being a combination of simultaneous binding events, is significantly stronger and is known as the “glycoside cluster effect”.<sup>[6–8]</sup> Capitalizing on this effect, it was recently demonstrated that synthetic molecules that can com-

pete with natural ligands of these proteins can potentially prevent cell adhesion and biofilm formation.<sup>[9–15]</sup> Therapeutic effects have indeed been observed with fucose- and galactose-based structures against *Pseudomonas aeruginosa*, which highlights the potency of this new lectin-directed anti-adhesion strategy against pathogenic bacteria.<sup>[16,17]</sup>

The opportunistic bacterium *Burkholderia cenocepacia* is responsible for numerous nosocomial infections affecting mainly cystic fibrosis and immunocompromised patients.<sup>[18]</sup> Treatment of *B. cenocepacia* infections is difficult owing to widespread multidrug resistance<sup>[19]</sup> and its ability to form biofilms. New strategies for inhibiting the formation of *P. aeruginosa* biofilms have been developed recently; these target the binding sites of LecA and LecB lectins.<sup>[5,20–23]</sup> LecB-like genes have been identified in several Gram-negative bacteria, including *B. cenocepacia*,<sup>[24]</sup> which produces three related proteins: BC2L-A (BCAM0186), BC2L-B (BCMA0184) and BC2L-C (BCMA0185). We have focused our study on BC2L-A, which has been structurally characterized in detail.<sup>[24–26]</sup> BC2L-A assembles as a dimer of 13.8 kDa monomers with a  $\beta$ -sheet core structure and a dependence on two  $\text{Ca}^{2+}$  ions for binding to carbohydrate targets. Whereas LecB is tetrameric and shows a high affinity for L-fucose, BC2L-A is a dimer and binds poorly to this ligand ( $K_d = 2.3 \text{ mM}$ ) but has a high affinity toward methyl  $\alpha$ -D-mannopyranoside ( $K_d = 2.7 \text{ }\mu\text{M}$ ). BC2L-A also binds also to L-glycero-D-mannoheptose,<sup>[26]</sup> a major constituent of bacterial lipopolysaccharide, with a strict diastereoselectivity for (6S)-mannoheptose.<sup>[27]</sup>

Only a few studies have been conducted to evaluate the affinity of multivalent structures toward BC2L-A. For example, Laimegnere et al. have tested a trimannoside as well as two dimannoside analogues separated by either a flexible or rigid spacer. They have measured a  $K_d$  value of 220 nM for their best-binding compound.<sup>[25]</sup> More recently, Faure et al. have

[a] C. Pifferi,<sup>+</sup> Dr. D. Goyard,<sup>+</sup> Prof. Dr. O. Renaudet  
Université Grenoble Alpes  
CNRS, DCM UMR 5250  
38000 Grenoble (France)  
E-mail: olivier.renaudet@univ-grenoble-alpes.fr

[b] E. Gillon, Dr. A. Imberty  
CERMAV, UPR5301, CNRS  
and Université Grenoble Alpes  
601 rue de la Chimie, BP 53, 38041 Grenoble (France)  
E-mail: anne.imberty@cermav.cnrs.fr

[c] Prof. Dr. O. Renaudet  
Institut Universitaire de France  
103 boulevard Saint-Michel, 75005 Paris (France)

[<sup>+</sup>] These authors contributed equally to this research.

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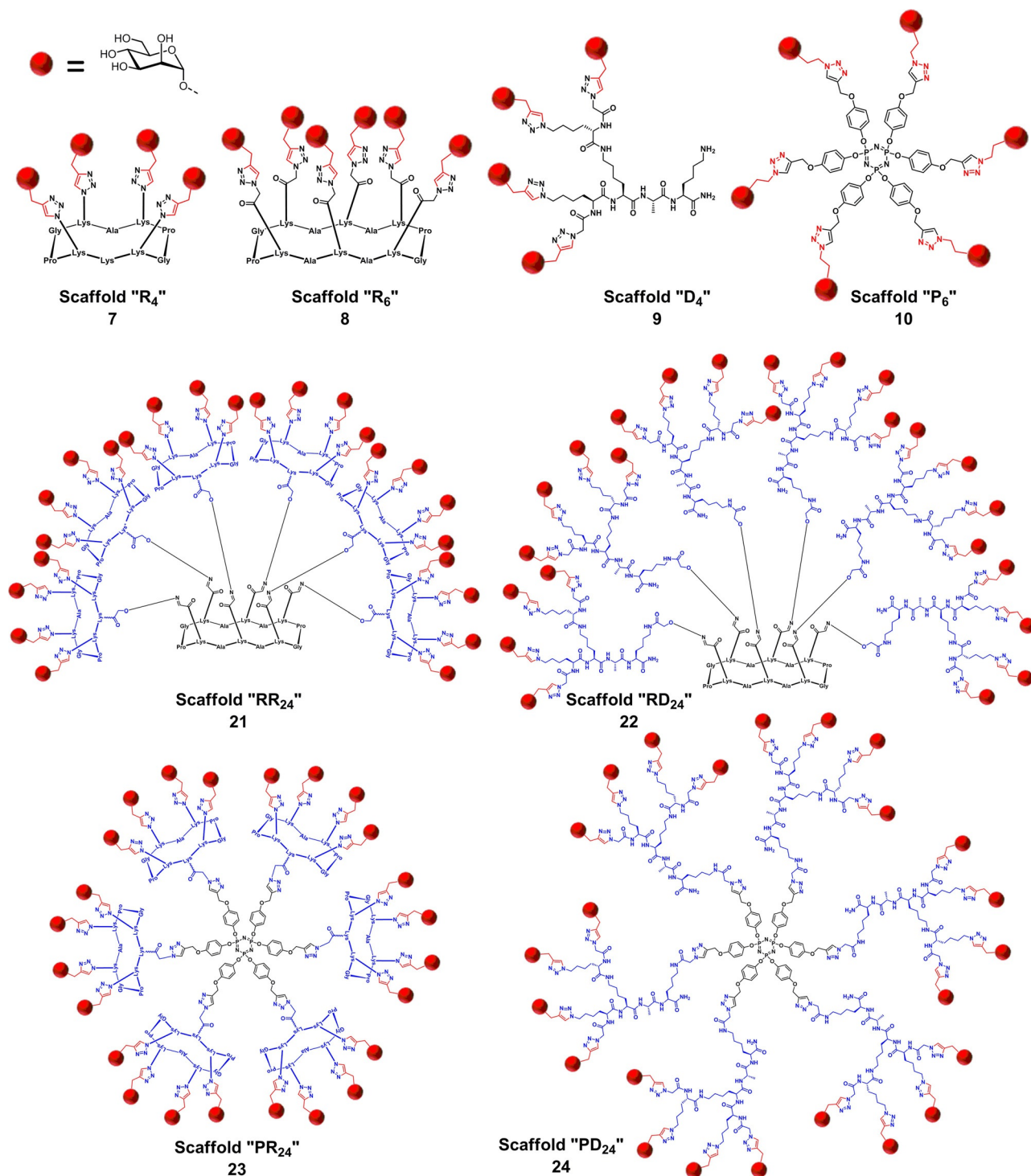
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prepared tetravalent thiomannoside clusters, the most potent of which having a  $K_d$  value of 204 nM.<sup>[28]</sup> Higher valency architectures were obtained with mannosylated gold glyco-nanoparticles and a  $K_d$  value of 19 nM was measured for a construct with an average valency of 46.<sup>[29]</sup> In this present study, we report the synthesis of a new series of glycoclusters and glyco-dendrimers presenting mannose residues with valencies of four, six and 24 in order to evaluate the influence of valency

and tridimensional architecture on the binding affinity with BC2L-A (Figure 1).

## Results and Discussion

First, regioselectively addressable functionalized template (RAFT) cyclopeptides<sup>[30–33]</sup> containing four (scaffold  $R_4$ ) or six (scaffold  $R_6$ ) lysines oriented out of the upper domain of the



**Figure 1.** Structures of tetra-, hexa- and tetracosavalent glycoclusters. The scaffolds on which they are constructed are indicated above the compound number.

scaffold and one more lysine pointing toward the other side (scaffold  $R_4$  only) were synthesized (Figure 1). In addition, we prepared a flexible lysine-based dendron ( $D_4$ ) and a hexavalent cyclotriphosphazene-based core ( $P_6$ ; Figure 1).<sup>[34]</sup>

The latter  $P_6$  platform has been shown by X-ray crystallography to adopt a conformation in which three substituents point toward the upper face of the planar phosphazene and three point downward.<sup>[35,36]</sup> With these platforms in hand, different combinations were prepared to provide tetracosavalent (24-valent) glycodendrimers (Figure 1). The hexavalent cyclopeptide  $R_6$  was first functionalized with the tetravalent cyclopeptide  $R_4$  (to give  $RR_{24}$ ) and with the lysine-based dendron  $D_4$  ( $RD_{24}$ ). Then the phosphazene core  $P_6$  was conjugated with either the tetravalent cyclopeptide  $R_4$  (to give  $PR_{24}$ ) or the lysine-based dendron  $D_4$  ( $PD_{24}$ ). The resulting glyoclusters and glycodendrimers were characterized and evaluated by isothermal titration calorimetry (ITC) for their binding to BC2L-A.

### Synthesis of the multivalent glycosylated structures

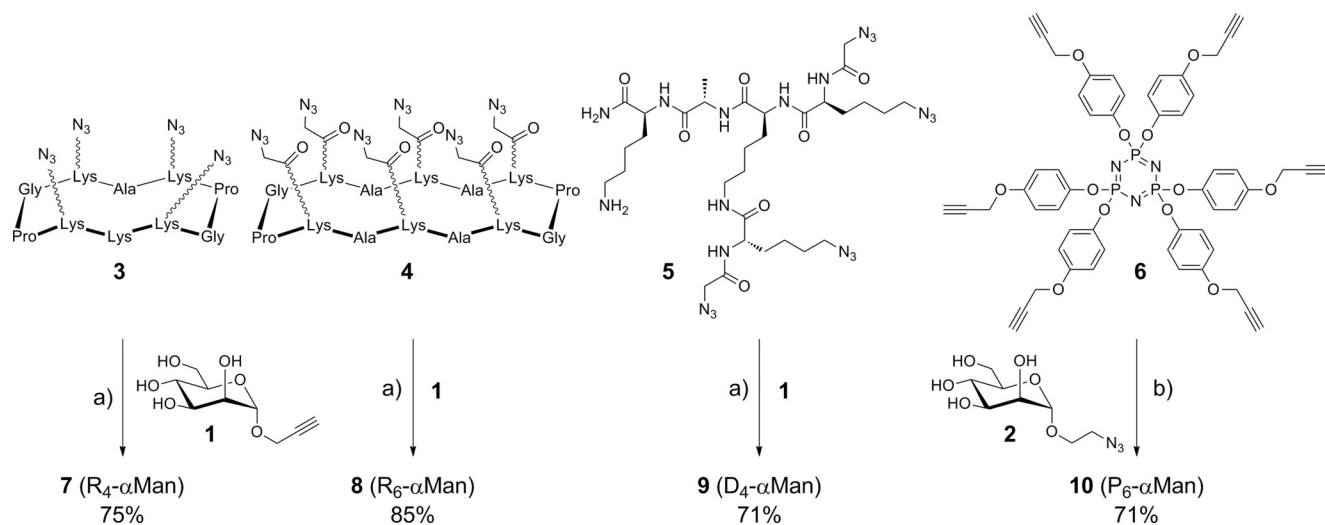
Tetra- and hexavalent mannosylated glyoclusters were synthesized by following a highly efficient copper-catalyzed azide-alkyne cycloaddition (CuAAC) protocol.<sup>[37]</sup> This strategy required the preparation of propargyl and azidoethyl mannoses that were obtained by treatment of peracetylated mannanopyranose with propargyl alcohol and 2-bromoethanol, respectively. Lewis-acid-promoted glycosidation with these alcohols afforded the desired  $\alpha$ -mannosides stereoselectively in 90% yield.<sup>[38]</sup> Propargylated compound **1** was obtained after Zemplén deacetylation, whereas **2** was obtained after treatment of the bromo derivative with sodium azide before deprotection.<sup>[39]</sup> We next prepared four different glyoclusters with valencies of four and six (Scheme 1). Azide-containing cyclopeptide scaffolds  $R_4$  (**3**)<sup>[40]</sup> and  $R_6$  (**4**), as well as lysine-based dendron  $D_4$  (**5**) were first prepared adapting previously reported procedures.<sup>[41]</sup> Cyclotriphosphazene-based  $P_6$  scaffold **6** was synthesized in one step by nucleophilic substitution between

the hexachloro derivative and 4-(prop-2-yn-1-yloxy)phenol.<sup>[36]</sup> Coupling of these four platforms with mannosides **1** and **2** using standard CuAAC conditions<sup>[37]</sup> yielded glyoclusters **7–10**, respectively (Scheme 1).

In previous molecular modeling studies it was shown that RAFT cyclodecapeptides such as **3** adopt an antiparallel  $\beta$ -sheet structure owing to the presence of two L-proline–glycine  $\beta$ -turns which stabilize the peptide in a constrained conformation by intramolecular hydrogen bonding.<sup>[30]</sup> This was confirmed by circular dichroism (CD) experiments, which revealed characteristic  $\beta$ -sheet profiles.<sup>[42,43]</sup> In order to confirm that cyclotetradecapeptide-based compounds adopt the same conformation, we recorded and compared the CD spectra of compounds **7** and **8**. Both glyoclusters exhibited a similar CD profile with maximum and minimum molar ellipticity values around 200 and 225 nm, respectively (Figure 2). This observation confirms that the addition of four amino acids in the cyclopeptide does not affect the conformational stability of the scaffold.

In order to synthesize glycodendrimers with extended valency, we chose a convergent strategy using compounds **7** and **9** as glycosylated dendrons (Scheme 2). Compounds **13** and **14** were first obtained after the coupling of aminooxyacetic acid activated as the *N*-hydroxysuccinimidyl ester **11**.<sup>[44]</sup> Boc group removal afforded aminooxylated dendrons **15** and **16**. Similarly, compounds **17** and **18** were prepared using the activated ester **12** bearing an azido group.<sup>[45]</sup>

Next, cyclopeptide **20** bearing six aldehydes was obtained by oxidative cleavage of the serine residues of **19** as described previously.<sup>[33]</sup> Oxime ligation with aminooxylated dendrons **15** and **16** occurred within a short time and under mild acidic conditions to provide tetracosavalent glycodendrimers **21** ( $RR_{24}$ ) and **22** ( $RD_{24}$ ) in excellent yields (Scheme 3). Finally, the CuAAC reaction between hexapropargylated cyclotriphosphazene **10** and azido-functionalized dendrons **17** and **18** led to glycodendrimers **23** ( $PR_{24}$ ) and **24** ( $PD_{24}$ ).



**Scheme 1.** Synthesis of tetravalent ( $R_4$  and  $D_4$ ) and hexavalent ( $R_6$  and  $P_6$ ) glyoclusters **7–10**. [a] Reagents and conditions: a)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , tris(3-hydroxypropyl)triethylammonium (THPTA), **2**, PBS buffer (pH 7.5), RT, 1 h; b)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , THPTA, **3**, DMF/PBS buffer (1:1, pH 7.5), RT, 1 h. All amino acids have the L-configuration.

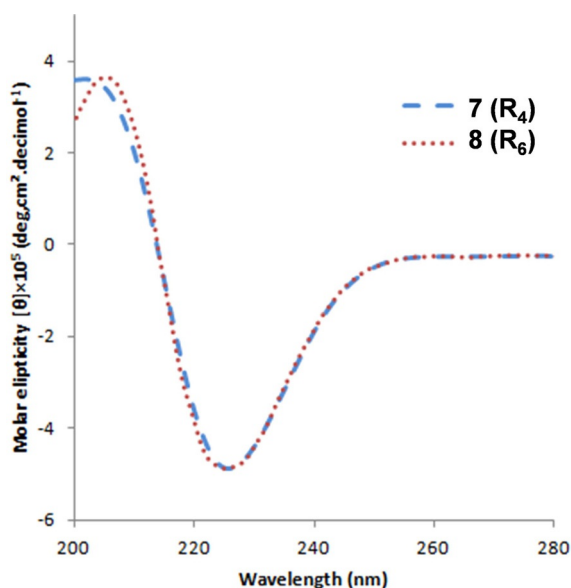


Figure 2. CD spectra of compounds 7 and 8.

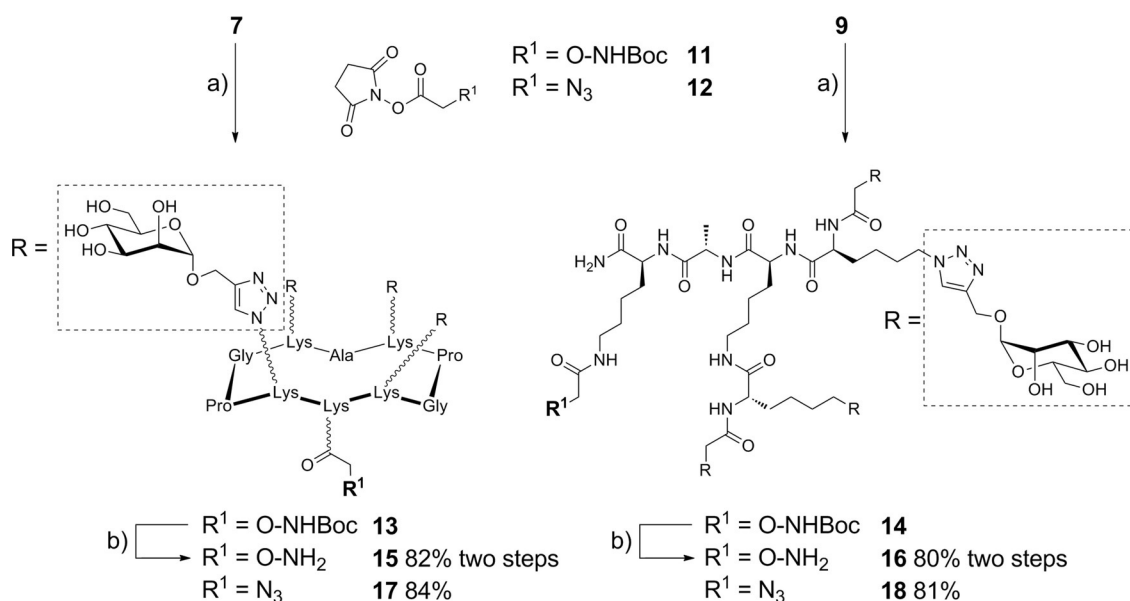
All the final compounds were purified by preparative HPLC and characterized by mass spectrometry (see the Supporting Information). Whereas clusters assembled by CuAAC only (i.e., 7–10, 23 and 24) showed clear mass spectra by ESI and/or MALDI-TOF MS, tetracosavalent glycodendrimers 21 and 22 showed many other signals as well as the expected peak. We have previously shown that these signals are the result of oxime fragmentation rather than partially substituted structures.<sup>[46]</sup> This observation was confirmed by <sup>1</sup>H NMR spectroscopy, which showed the complete disappearance of the two singlets corresponding to the hydrated aldehydes around 5.30 ppm and the presence of a singlet around 7.70 ppm characteristic of the oxime protons with the expected integral value.

### Isothermal Titration Calorimetry

Mannosylated glycoclusters 7–10 and glycodendrimers 21–24 were used in titration microcalorimetry experiments to determine the stoichiometry and thermodynamic parameters of their binding to BC2L-A in solution. Monovalent methyl  $\alpha$ -D-mannoside was used as a reference to calculate the relative potency of each multivalent compound. Titrations were performed in the direct injection mode (i.e., ligand in syringe and protein in cell). When aggregation was observed, it happened after the titration stage, therefore not lowering the quality of the results (Figure 3 and Table 1).

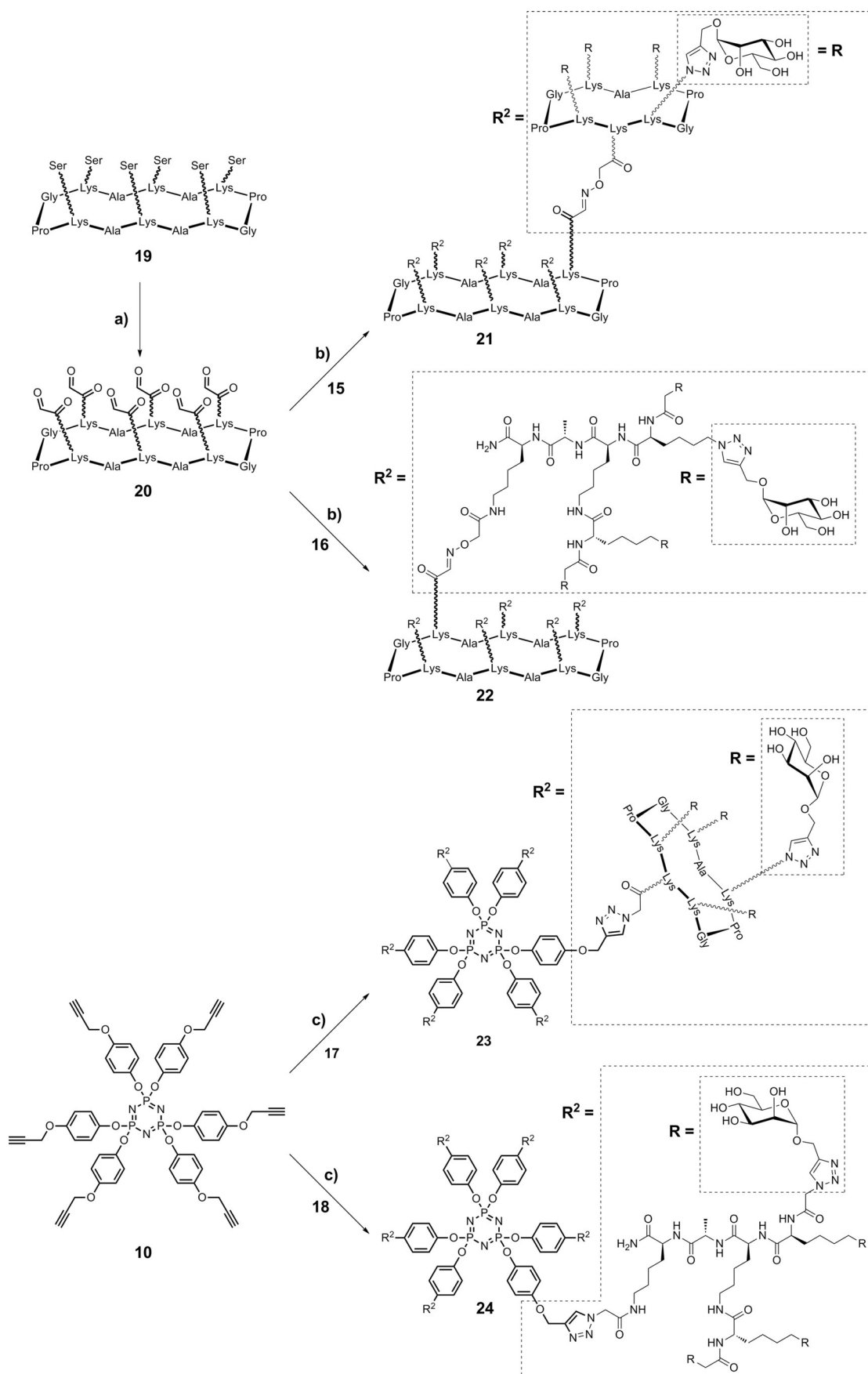
The analysis of the thermodynamic contributions showed that for all the compounds the binding is driven by enthalpy but is counterbalanced by a strong entropy barrier resulting from the loss of flexibility induced upon lectin binding. Interestingly, tetravalent compounds 7 and 9 that are built on different scaffolds exhibited a significant difference in binding entropy contribution ( $16.2 \text{ kJ mol}^{-1}$ ) in favor of the cyclopeptide-based cluster. This difference is also noticeable between hexavalent compounds 8 and 10 ( $12.9 \text{ kJ mol}^{-1}$ ), also in favor of the cyclopeptide-based cluster. The well-known rigidity of cyclopeptide scaffolds seems to provide a more favorable geometry for the binding with BC2L-A. Of these four glycoclusters, compound 8 has the highest affinity ( $K_d = 199 \text{ nM}$ ), albeit with a modest relative potency factor (17.9). These data suggest that the interaction is due to an aggregative effect, implying one binding site per lectin rather than a real chelate complex in which both binding sites are occupied by the same glyco-cluster molecule.

More interestingly, thermodynamic data obtained with the tetracosavalent glycodendrimers 21–24 revealed that compound 23 ( $\text{PR}_{24}$ ) has a significantly less favorable binding enthalpy ( $-\Delta H = -273.5 \text{ kJ mol}^{-1}$ ) than the three other compounds ( $-\Delta H = -335 \text{ kJ mol}^{-1}$  for each). This observation suggests that despite a lower entropy barrier ( $-T\Delta S =$

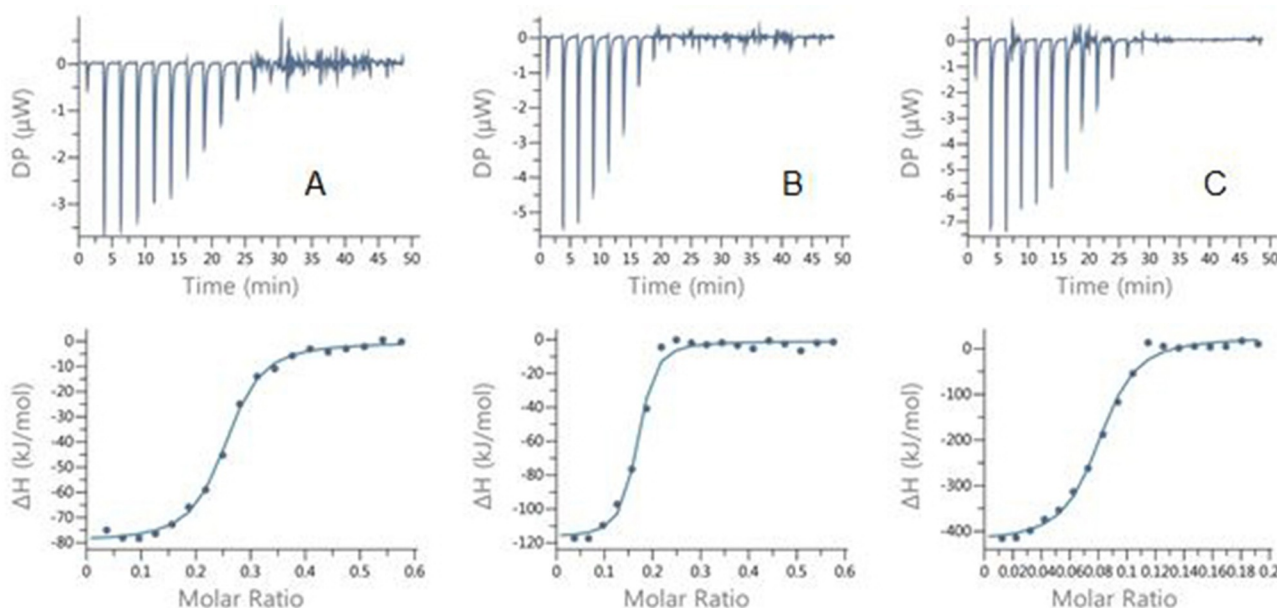


Scheme 2. Synthesis of aminoxy- and azido-functionalized dendrons. Reagents and conditions: a) DIPEA, DMF, RT, 2 h; b) TFA/ $\text{CH}_2\text{Cl}_2$  (6:4), RT, 15 min.





**Scheme 3.** Synthesis of the tetracosavalent glycodendrimers. [a] Reagents and conditions: a)  $\text{NaIO}_4$ ,  $\text{H}_2\text{O}$ , RT, 30 min, 80%; b) 0.1% TFA in  $\text{H}_2\text{O}$ ,  $37^\circ\text{C}$ , 2 h, 77–84%; c)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , THPTA, DMF/PBS buffer (1:1, pH 7.4), RT, 1 h, 68–72%.



**Figure 3.** Isothermal titration calorimetry data: thermograms (top) and corresponding integrated titration curves (bottom) obtained by injections of A) 300  $\mu\text{M}$  **7**, B) 300  $\mu\text{M}$  **8** and C) 100  $\mu\text{M}$  **24** into a solution of BC2L-A (100  $\mu\text{M}$ ). Molar ratio is defined as the number of glycocluster molecules per lectin monomer.

Table 1. Isothermal titration microcalorimetry data for glycoconjugates binding to BC2L-A. <sup>[a]</sup>								
Compound	Valency	$K_d$ [nM]	$n$	$-\Delta H$ [kJ mol <sup>-1</sup> ]	$-\Delta G$ [kJ mol <sup>-1</sup> ]	$-T\Delta S$ [kJ mol <sup>-1</sup> ]	$\alpha$	$\beta$
$\alpha$ -Me-Man	1	3570	0.90	24.1	31.1	-7.1	1	1
<b>7</b> (R)	4	481 $\pm$ 9	0.27	81.1 $\pm$ 0.4	36.1	45.0	7.4	1.9
<b>9</b> (D)	4	449 $\pm$ 32	0.26	99.0 $\pm$ 2	36.3	62.8	7.9	2.0
<b>8</b> (R)	6	199 $\pm$ 3	0.16	119.0 $\pm$ 2	38.2	80.6	17.9	3.0
<b>10</b> (P)	6	368 $\pm$ 6	0.20	130.5 $\pm$ 2.5	36.75	93.7	9.7	1.6
<b>21</b> (RR)	24	256 $\pm$ 16	0.09	335.0 $\pm$ 1	37.7	297	13.9	0.6
<b>22</b> (RD)	24	195 $\pm$ 2	0.061	335.0 $\pm$ 2	38.4	296.5	18.3	0.8
<b>23</b> (PR)	24	215 $\pm$ 6	0.10	273.5 $\pm$ 10.5	38.3	235.5	16.6	0.7
<b>24</b> (PD)	24	51 $\pm$ 3	0.077	335.0 $\pm$ 1	41.7	293	70	2.9

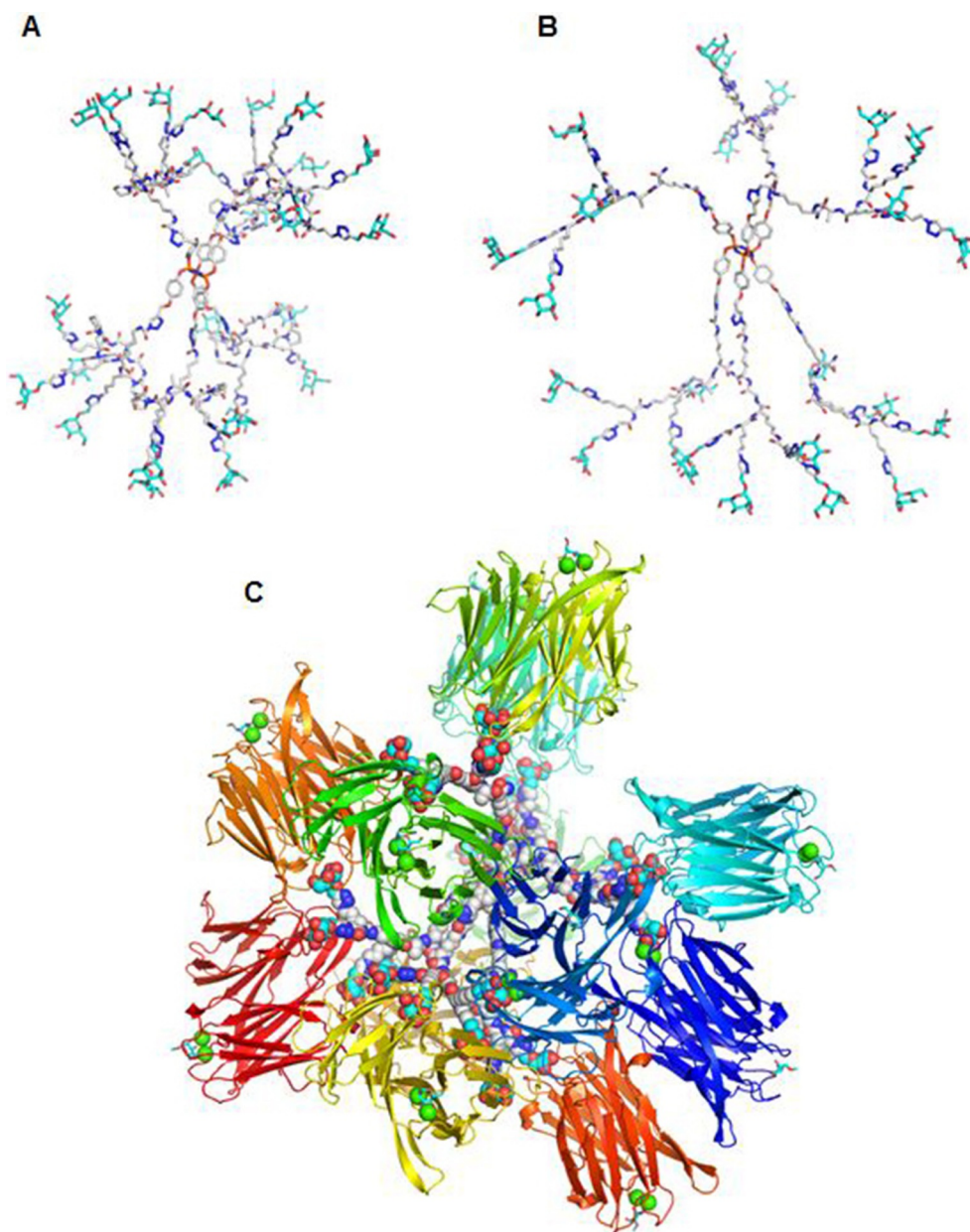
[a] Thermodynamic data refer to moles of glycoclusters and stoichiometry is expressed as the number of glycocluster molecules per lectin monomer. Standard deviations are indicated on experimentally derived values (at least two experiments). Coefficient  $\alpha$  is the relative potency compared to the monovalent compound. Coefficient  $\beta$  is the relative potency per mannose residue.

235.5 kJ mol<sup>-1</sup> versus  $\approx$ 295 kJ mol<sup>-1</sup>) the sugar presentation does not favor binding with BC2L-A, as confirmed by its dissociation constant ( $K_d$ =215 nM). In comparing the  $K_d$  values of dendrimers constructed around the same core structure but with different peripheral scaffolds (**21** versus **22** and **23** versus **24**), we observed that the sugar presentation through the lysine-based dendron leads to stronger binding in each series. This effect can be explained by the flexibility of the dendron, which might facilitate the positioning of the sugars in the lectin binding site. Among the glycodendrimers, the highest affinity ligand is compound **24** ( $K_d$ =51 nM), which contains a cyclotriphosphazene core functionalized with six lysine-based dendrons. The binding of this molecule is between three and five times stronger than the others, each sugar being almost

three times more potent than monovalent methyl  $\alpha$ -D-mannoside.

The stoichiometry data for tetravalent compounds **7** and **9** indicated that all of the four mannose residues can bind to a monomer of lectin. The same accessibility is observed with hexavalent clusters **8** and **10** in which each lectin monomer binds one of the mannose residue, that is, one cluster binds five or six lectins. In the case of the tetracosavalent dendrimers, the stoichiometry was different between compounds **21** and **23**, which can bind approximately 10 lectin monomers, and compounds **22** and **24**, which interact with approximately 15 lectin monomers. This indicates a higher accessibility for the peptide-presented mannose residues, than for the RAFT-presented ones.

In order to rationalize this analysis, models of compounds **23** and **24** were built using Sybyl molecular graphics editor (Certara, Munich, Germany). Starting from the crystal structure of the  $N_3P_3$  scaffold,<sup>[36]</sup> the linkers were built and terminal mannose residues added either onto a model of branched peptide or onto the structure of a cyclic peptide taken from an NMR model.<sup>[41]</sup> The models were constructed in order to alleviate any steric contact and to orientate all torsional angles in reasonable conformations, but no energy calculations were performed (Figure 4). The differences in global size is not significant, with compound **23** extending to approximately 65 Å, whereas compound **24** spans at a maximum distance of 80 Å. Nevertheless, variations occur at the local level, with mannose residues being clearly closer to each other in model **23** than in model **24**. Fitting of mannose-bound BC2L-A to the accessible mannose residues in model **24** allowed for positioning of 11 BC2L-A dimers with no significant



**Figure 4.** Possible conformations of A) **23** and B) **24** represented as sticks with mannose residues colored in blue. C) Same conformation of **24** with 11 BC2L-A dimers positioned on accessible mannose residues by superposition with mannose in binding sites. Compound **24** and calcium ions are represented as spheres and lectins are represented as ribbons. Hydrogen atoms have been omitted in all panels.

steric clashes, illustrating that this compound presents optimal accessibility to the glycans.

## Conclusion

In summary, cyclodecapeptide- and tetradecapeptide-based as well as cyclotriphosphazene- and lysine-based glycoclusters were prepared, affording a new series of tetra- and hexavalent mannosylated ligands. Both hexavalent scaffolds were then combined with tetravalent dendrons to afford a set of four tetracosavalent glycodendrimers. These eight compounds, displaying various flexibilities and architectures were then tested for their binding affinity with BC2L-A lectin using isothermal ti-

tration microcalorimetry. We first demonstrated that both hexa- and tetravalent architectures allowed a good presentation of the sugar residues to the binding site of the lectin, forming complexes in which all mannosides are involved in the binding, albeit with modest enhancements of affinity compared to the monovalent interaction. Within this set of glycoclusters, cyclopeptide-based hexavalent compound **8** exhibited the best binding properties ( $K_d=199$  nM) where each sugar residue is three times more potent than the monovalent methyl  $\alpha$ -D-mannoside. The evaluation of the tetracosavalent glycodendrimers showed that the core architecture does not have a significant effect on the binding affinity. On the contrary, the scaffold used to present the sugars at the periphery



seems to have a significant influence. The binding is stronger if mannose residues are exposed through the more flexible lysine-based dendron. The combination of this architecture with the cyclotriphosphazene core afforded compound **24** with the lowest binding constant in this study ( $K_d = 51$  nM), which also represents the highest affinity known to date for a fully synthetic multivalent glycodendrimer binding to BC2L-A (compared to  $K_d = 204$  nM<sup>[28]</sup>). Analysis of thermodynamic contributions to the binding tend to show that the affinity arises from an aggregative binding mode rather than a chelate complex, which typically leads to stronger affinities. This is not surprising considering that BC2L-A is a dimer in which the two sugar-binding pockets are located on opposite sides of the protein, therefore preventing the ligand to reach both sites at the same time. Nevertheless, high affinities can be achieved if the sugar residues are presented with enough flexibility to accommodate several protein monomers, as it is the case for compound **24**. This compound represents an excellent candidate for structural optimization to achieve higher affinity and to expect anti-adhesive properties against *B. cenocepacia*.

## Experimental Section

### General Methods

All chemical reagents were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France) and were used without further purification. All protected amino acids and Fmoc-Gly-Sasrin resin was obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France).

For peptides and glycopeptides, analytical RP-HPLC was performed on a Waters alliance 2695 separation module, equipped with a Waters 2489 UV/Vis detector. Analyses were carried out at a flow rate of 1.23 mL min<sup>-1</sup> (Interchim Uptisphere X-Serie column, C<sub>18</sub>, 5 µm, 125 × 3.0 mm) with UV absorbance monitoring at 214 and 250 nm using a linear A–B gradient (solvent A: 0.09% CF<sub>3</sub>CO<sub>2</sub>H in water; solvent B: 0.09% CF<sub>3</sub>CO<sub>2</sub>H in 90% acetonitrile). Preparative HPLC was performed on a Gilson GX-281 liquid handler equipped with a fraction collector or on Waters equipment consisting of a Waters 600 controller and a Waters 2487 dual absorbance detector. Purifications were carried out at a flow rate of 22.0 mL min<sup>-1</sup> (VP 250 × 21 mm Nucleosil 100-7 C<sub>18</sub> column) with UV absorbance monitoring at 214 and 250 nm using a linear A–B gradient. For carbohydrate, progress of reactions was monitored by thin layer chromatography using silica gel 60 F254 pre-coated plates (Merck). Spots were visualised by charring with 10% H<sub>2</sub>SO<sub>4</sub> in EtOH. Silica gel 60 (0.063–0.2 mm or 70–230 mesh, Merck) was used for column chromatography.

<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker Avance 400 MHz or Bruker Avance III 500 MHz spectrometer and chemical shifts  $\delta$  are reported in parts per million (ppm). Spectra are referenced to the residual proton solvent peaks relative to the signal of CDCl<sub>3</sub> ( $\delta = 7.27$  and 77.23 ppm for <sup>1</sup>H and <sup>13</sup>C, respectively) and D<sub>2</sub>O (4.79 ppm for <sup>1</sup>H), assignments were facilitated by GCOSY and GHMQC experiments. Standard abbreviations s, d, t, dd, brs, and m refer to singlet, doublet, triplet, doublet of doublets, broad singlet, and multiplet, respectively. ESI mass spectra of peptides and glycopeptides were measured on a Bruker Esquire 3000 spectrometer or on a Waters Acquity UPLC/MS system equipped with an SQ Detec-

tor 2. MALDI-TOF MS was performed on a Bruker AutoFlex I mass spectrometer after sample pre-treatment on an OligoR3 microcolumn (Applied Biosystems) using a 2,5-dihydroxybenzoic acid matrix. HRMS analyses were performed on a Waters Xevo G2-S QToF mass spectrometer at the Mass Spectrometry facility, PCN-ICMG, Grenoble.

Procedures for solid-phase peptide synthesis and peptide cyclization are described in the Supporting Information along with the characterization data.

### Procedure for CuAAC ligation

A solution of CuSO<sub>4</sub> (1.0 equiv. per alkyne) and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 5.0 equiv. per alkyne) in phosphate-buffered saline (PBS, pH 7.5, 10 mM) was added to a mixture of azido-containing cluster (1–5 mM) and propargyl mannoside **1**<sup>[31,32]</sup> (1.5 equiv. per azide) in DMF at room temperature. A solution of sodium ascorbate (7 equiv. per alkyne) in PBS was added to the reaction mixture. All solutions had been degassed under argon. The reaction was stirred at room temperature under argon, and after 2 h, analytical RP-HPLC indicated complete coupling. Chelex resin was added to remove the excess of Cu<sup>2+</sup> ions and the reaction mixture was purified by semipreparative RP-HPLC to afford pure compounds as white foams after lyophilization.

### Synthesis of compound 7

The synthesis was performed using **3** (50.0 mg, 44.5 µmol, see the Supporting Information) and **1**<sup>[31,32]</sup> (58.5 mg, 268 µmol) by following the procedure for CuAAC ligation. Compound **7** (66.7 mg, 33.4 µmol, 75% yield) was obtained as a white powder after purification (semipreparative RP-HPLC, 5–40% solvent B in 30 min) and lyophilization. HRMS (ESI<sup>+</sup>-TOF):  $m/z$ : calcd for C<sub>55</sub>H<sub>90</sub>N<sub>19</sub>O<sub>14</sub>: 1996.9464 [M+H]<sup>+</sup>; found: 1996.9457; RP-HPLC:  $R_t = 6.79$  min (C<sub>18</sub>,  $\lambda = 214$  nm, 0–30% solvent B in 15 min). <sup>1</sup>H NMR spectra showed consistent integrals between anomeric and triazole protons (Figure S10).

### Isothermal titration microcalorimetry

BC2L-A was expressed in *Escherichia coli* and purified on affinity column as previously described.<sup>[24]</sup> ITC experiments were performed with a PEAQ-ITC isothermal titration microcalorimeter (Malvern Instruments, Malvern, UK). The experiments were performed at 25 °C. Lyophilized glycoconjugates and BC2L-A were dissolved in the same buffer composed of Tris-HCl (20 mM) containing NaCl (100 mM) and CaCl<sub>2</sub> (100 µM) at pH 7.5. BC2L-A (100–300 µM) was placed in the 200 µL sample cell operating at 25 °C. Titrations were performed with 19 injections of sugar derivatives (65 µM to 3 mM, 2 µL) spaced by 150 s. The experimental data were fit to a theoretical titration curve using the supplied MicorCal PEAQ-ITC analysis software, with  $\Delta H$  (enthalpy change),  $K_d$  (dissociation constant) and  $n$  (number of binding sites per monomer) as adjustable parameters. Free energy change ( $\Delta G$ ) and entropy contributions ( $T\Delta S$ ) were derived from the equation  $\Delta G = \Delta H - T\Delta S = -RT \ln K_d$  (where  $T$  is the absolute temperature and  $R = 8.314$  J mol<sup>-1</sup> K<sup>-1</sup> and  $K_d = 1/K_a$ ). Two or three independent titrations were performed for each tested ligand.

## Circular dichroism

CD spectra were acquired with signal averaging on a Jasco J-810 spectropolarimeter equipped with a Jasco Peltier PTC-423S temperature controller, and a baseline was recorded separately and subtracted. Far-UV spectra were recorded from 340 to 200 nm at 25 °C, in a quartz cell with 1 mm pathlength. Spectra are reported as the averages of four scans and ellipticities are reported as molar ellipticity. To reduce noise, the data were smoothed with a Savitzky–Golay smoothing algorithm using a convolution window of seven data points. Spectra were acquired using solutions of glycoclusters in Milli-Q H<sub>2</sub>O at a concentration of 200 µM.

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