Multivalent Glycomimetics with Affinity and Selectivity toward Fucose-Binding Receptors from Emerging Pathogens

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S *Supporting Information*

ABSTRACT: Bacterial and fungal pathogens involved in lung infection in cystic fibrosis patients utilize a particular family of glycan-binding proteins, characterized by the presentation of six fucose-binding sites on a ring-shaped scaffold. These lectins are attractive targets for anti-infectious compounds that could interfere in the recognition of host tissues by pathogens. The design of a cyclopeptide-based hexavalent structure allowed for the presentation of six fucose residues. The synthetic hexavalent compound displays liable geometry resulting in high-avidity binding by lectins from *Aspergillus fumigatus* and *Burkholderia ambifaria*. Replacing the fucose residue with a conformationally constrained fucomimetic does not alter the affinity and provides fine specificity with no binding to other fucose-specific lectins.
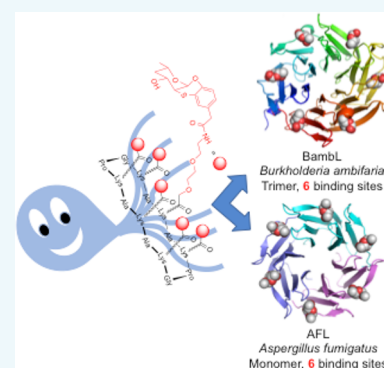
INTRODUCTION

Many microorganisms use lectins as adhesins to interact with host glycoconjugates and to trigger the first step of infection. Among carbohydrate epitopes, fucose has a prominent role because it is an ubiquitous ligand that can be the target of microorganisms receptors on human tissue[2] but also in plant cell walls.[3] Most opportunistic pathogens are indeed able to spread from soil to plants and humans. In mammal tissues, fucose is a marker of inflammation,[4] and fucosylated glycoconjugates are over-expressed in the airways of cystic fibrosis patients.[5]

Pathogens responsible for lung infection in those patients produce soluble lectins that are involved in adhesion of pathogens to fucosylated glycoconjugates such as human histo-blood group epitopes.[6] Soluble fucose-binding lectins have been characterized in *Pseudomonas aeruginosa*,[7] *Burkholderia ambifaria*,[8] and airborne fungus *Aspergillus fumigatus*.[9] Among them, one structural family is of high interest: *B. ambifaria* BambL and *A. fumigatus* AFL (FleA) are made by the repeat of similar small β-sheets of approximately 40 amino acids (Figure 1A,B). Both lectins are able to bind six fucose residues with high affinity.[8,9] The AFL sequence is longer, consisting in six repeats in tandem,[9] while BambL is formed by trimerization of a two-repeat peptides.[10] In this family, the affinity for fucose is in the micromolar range, an unusually strong binding for protein−carbohydrate interaction.

To hamper pathogen adhesion to host cells, fucose mimetics become attractive tools with which to design synthetic ligands to intercept fucose-binding bacteria. Monomeric fucoside derivatives have been tested against AFL demonstrating that (2E)-hexenyl α-L-fucopyranoside is a potent functional inhibitor of FleA.[10] With respect to native glycans, glycomimetics are designed to be more resistant to glycosidases to present a higher bioavailability and to be tailored to decorate scaffolds of different chemical constitutions.[11,12]

In this context, we recently reported the synthesis of a fucose-based glycomimetic **1** (Figure 2) presenting a non-natural aryl α-O-fucosyl linkage.[13] The fucosyl derivative **1** binds to BambL lectin with an affinity comparable to that of the natural ligand because its fucoside moiety establishes the same contact with BambL active site as the natural ligand (Figure 1C). Moreover, it presents a never-before-described specificity for the BambL binding site and does not bind to other fucose-specific lectins such as *P. aeruginosa* LecB or human DC-SIGN.

Because lectins are multimeric, higher affinity can be obtained by synthesizing multivalent glycocompounds.[14,15] For example, calixarene-based fucosylated clusters display strong affinity for LecB (45 nM) and provide an almost complete protection against *P. aeruginosa* in a mice infection model.[16] Fewer efforts have been devoted to competing with β-propeller lectins. Fucosylated glycocluster exhibiting 4, 6, or 8 residues were synthesized based on mannose-centered and
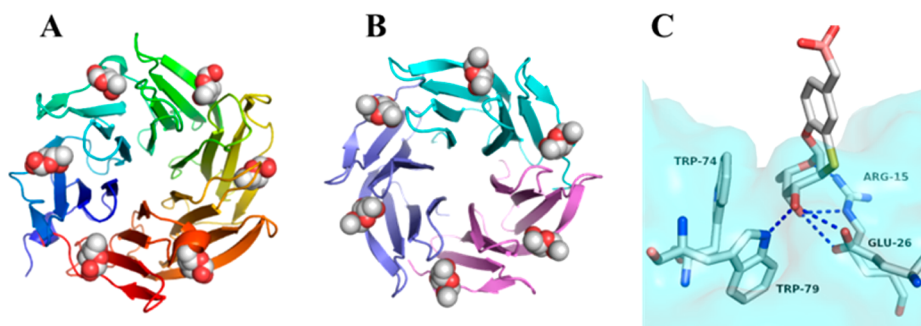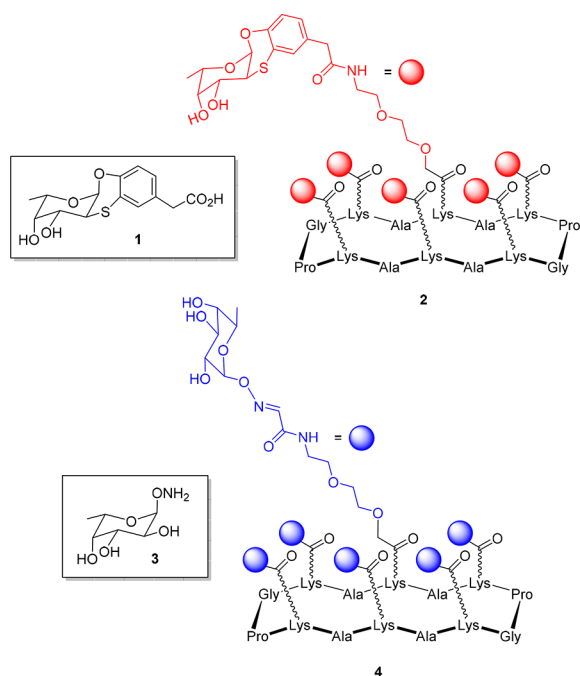
Figure 1. Crystal structures of (A) BambL and (B) AFL, complexed with fucose or fucoside (PDB codes 4AHA and 3ZW2). (C) Model of compound **1** in intramonomeric site of BambL with amino acids of interest. Hydrogen bonds are represented as blue dotted lines.

Figure 2. Structure of the aryl fucose mimetic **1**, the hexavalent conjugate **2**, the aminooxylated fucoside **3**, and the hexavalent conjugate **4**.

branched-phosphodiester scaffolds with best affinities of approximately 43 nM.[17] Additionally, hexavalent cyclotrivera-trylene[18] and penta- and decavalent pillarenes[19,20] have also shown very strong affinity for BambL (ca. 20 nM). Multivalent
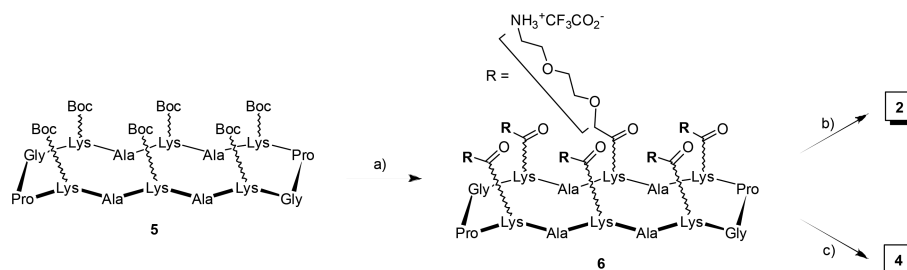
compounds obtained by dynamic assembly in the presence of BambL also blocked the entry in epithelial cells with high efficiency.[21]

The selectivity and the simple synthesis of ligand **1** motivated our interest in synthesizing a multivalent system containing the fucose mimetic **1**. This compound was diastereoselectively obtained by relying on an efficient inverse electron-demand Diels−Alder reaction. It is stable in the 4−8 pH range and presents a carboxylic hook to introduce a spacer, being linked to a scaffold, or both. To achieve this purpose, we have selected cyclopeptide-based scaffolds that have been proven useful for the construction of tetra-,[22] hexa-,[23] and hexadecavalent[24,25] glycoconjugates with nanomolar affinity for diverse vegetal and bacterial lectins. Herein, we report the synthesis of a cyclopeptide-based hexavalent structure displaying fucose mimetic **1** (conjugate **2**) and the binding studies with BambL, AFL, and LecB by isothermal titration calorimetry (ITC). For comparison, the analogue **4**, conjugated with aminooxylated fucose **3** has also been prepared, as previously described.[26,27]

■ RESULTS AND DISCUSSION

Because the target lectins contain six binding sites, we have synthesized two cyclopeptide scaffolds containing 14 amino acid residues (Scheme 1) instead of the usual cyclodecapeptide as previously described.[28,29] In a recent study, we have indeed demonstrated that the expansion of the cycle does not disturb the conformation stability of the scaffold, which still shows a characteristic β-sheet profile as proved by circular dichroism.[23] The cyclopeptide **5** has been synthesized on a solid phase following a previously described procedure.[23] After Boc

Scheme 1. Reagents and Conditions[a]

[a](a) i: TFA/CH$_2$Cl$_2$/H$_2$O (47.5/47.5/5), rt, 2 h. ii: Boc−NH−PEG−CO$_2$H, PyBOP, N,N-diisopropylethylamine (DIPEA), dimethylformamide (DMF), rt, 30 min; iii, TFA/CH$_2$Cl$_2$/H$_2$O (47.5/47.5/5), rt, 2 h, **6** (69% over three steps). (b) 1, PyBOP, DIPEA, DMF, rt, 30 min, **2** (50%). (c) i: BocSer(tBu)OH, PyBOP, DIPEA, DMF, rt, 2 h. ii: TFA/TIS/H$_2$O (95/2.5/2.5), rt, 2 h. iii: NaIO$_4$, H$_2$O, rt, 30 min. iv: **3**, H$_2$O, 0.1% TFA, **4** (> 90%).

deprotection of **5** upon treatment with trifluoroacetic acid (TFA), the Boc−NH−poly(ethylene glycol) (PEG)−CO_2H linker has been conjugated by standard amide coupling with PyBOP to the lysine side chains and the Boc groups have been successively removed by treatment with TFA to provide scaffold **6**. Hexavalent compound **2** was next prepared from **6** by PyBOP-promoted coupling with fucose mimetic **1**. The reaction occurred quantitatively at room temperature in 30 min and provided **2** in 50% yield after semipreparative high-performance liquid chromatography (HPLC) purification. Finally, conjugate **4** was synthesized following an oxime strategy. Boc-protected serine residues were first conjugated to **6** then successively deprotected and treated with sodium periodate to generate oxo-aldehyde functions. The resulting compound was finally ligated with aminooxy fucose **3**[23,24] in water with 0.1% TFA (pH 2.2) to provide the oxime-linked compound **4** in quantitative yield after purification by HPLC.

Monovalent compound **1** was previously demonstrated to be efficiently bound by BambL with affinity in the same order than the one measured for α-methyl-L-fucoside (αMeFuc) with a K_d close to 1 μM (Table 1).[13] In the present work, we tested the efficiency of compound **1** to bind AFL and compared it to αMeFuc.

Table 1. Microcalorimetry Data for Monovalent Compounds Interacting with BambL and AFL

compound	lectin	K_d (μM)	ΔG (kJ/mol)	ΔH (kJ/mol)	$-T\Delta S$ (kJ/mol)
1	BambL[a]	1.54	−33.2	−28.9	−4.3
	AFL	10.3	−28.5	−28.7	0.2
αMeFuc	BambL[a]	0.99	−34.3	−50.1	15.8
	AFL	43.3	−24.9	−39.2	14.3

[a]from[8]

Compound **1** was previously demonstrated to not bind to bacterial lectin LecB and human lectin DC-SIGN.[13] The precise specificity of **1** for BambL and AFL makes it a specific inhibitor for the fucose-binding site present in this family of β-propeller lectin. When the thermodynamics contribution is analyzed, the binding mode of **1** does not present the entropy barrier, classically observed in protein−carbohydrate interactions. AFL and BambL interaction with αMeFuc is enthalpy-driven with an unfavorable entropy term, while binding to compound **1** does not involve any significant entropy. Multivalent compounds **2** and **4** were evaluated in titration microcalorimetry experiments to determine the stoichiometry and thermodynamic parameters of their binding with LecB, BambL, and AFL (Figure 3 and Table 2). Compounds **1** and αMeFuc were used as monovalent references to calculate the relative potency of **2** and **4**, respectively. Titrations were performed in the direct injection mode (i.e., ligand in syringe and protein in cell).

The analysis of the thermodynamic contributions showed that for both compounds, binding is driven by enthalpy but is counterbalanced by a strong entropy barrier resulting from the loss of flexibility induced upon lectin binding. Interestingly, the selectivity of monovalent compound **1** toward BambL and AFL was still observed with hexavalent compound **2**, indicating that the fucose mimetic cannot bind to LecB, even at a high local concentration. On the contrary, hexafucosylated compound **4** exhibits a K_d of 165 nM for LecB, which confirms that
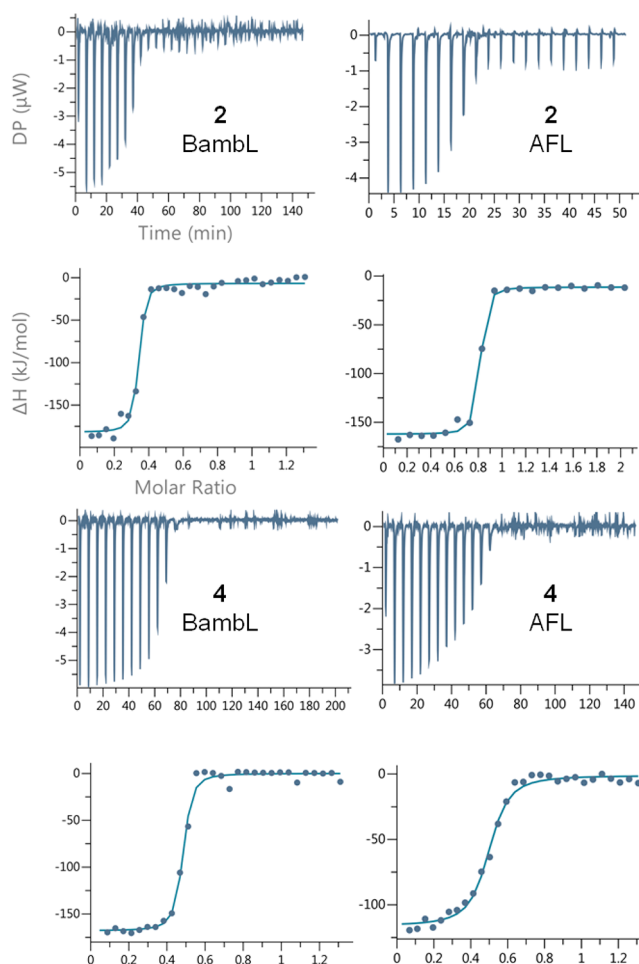
Figure 3. Isothermal titration calorimetry data. Thermograms and corresponding titration curves obtained by injections of compounds **2** and **4** into solutions of BambL (left) and AFL (right). Molar ratio is defined as the number of glycocluster molecules per lectin monomer.

the lack of binding of **2** does not come from an unfavorable geometry of the scaffold.

ITC measurements also showed that hexavalent compounds **2** and **4** bind very strongly to both BambL and AFL. Surprisingly, they both exhibit similar K_d values despite their different epitopes. Compound **4** shows a K_d of 18.5 nM on AFL, making it 1886 times more potent than αMeFuc and meaning that each sugar is 314 times more efficient than the monovalent reference. Studies with BambL show that compound **2** binds with a K_d of 13.8 nM, resulting in an improvement factor of 112 when compared to **1** and a relative potency of 19 per sugar residue. Stoichiometries for all measurements show an approximate ratio of 1:1 and an enthalpy of binding that is approximately four time stronger that the one measured for α-methyl-fucoside (−162 kJ/mol versus −47; see ref 8). This would indicate that at least four of the six fucose residues are involved in lectin binding. This strong enthalpy is counterbalanced by an important entropy factor necessary to accommodate the clusters' flexible arms in the appropriate direction. Altogether, these data indicate that compounds **2** and **4** are the best-known ligands for these lectins BambL and AFL to date.

In view of the large increase of affinity observed for hexavalent ligands, molecular modeling was performed to evaluate if a single hexameric compound could reach the six

Table 2. Microcalorimetry Data for Multivalent Compounds Interacting with LecB, BambL, and AFL

compound	lectin	K_d (nM)	N^a	ΔG (kJ/mol)	ΔH (kJ/mol)	$T \times \Delta S$ (kJ/mol)	α
2	LecB	no binding					
	BambL	13.8 ± 3.8	1.01 ± 0.06	−45	−162.5 ± 12.5	−117.5	112
	AFL	44 ± 10	0.68 ± 0.01	−42.1	−115.5 ± 0.5	−73.7	234
4	LecB	165 ± 5	0.60 ± 0.01	−38.7	−133.5 ± 5.5	−94.6	2.6
	BambL	16.8 ± 2.2	1.53 ± 0.1	−44.5	−165 ± 3	−120.5	59.1
	AFL	18.5 ± 8.5	0.77 ± 0.01	−44.4	−154.5 ± 3.5	−109.5	1886[1]

^{a}The stoichiometry has been calculated as ratio of multivalent compound for the whole lectin (four binding sites for LecB and six fucose binding sites for trimer of BambL and monomer of AFL). Coefficient α is the relative potency of the glycocluster compared to its monomeric epitope.

binding site of one BambL trimer. As displayed in Figure 4, glycocluster **4** in extended conformation brings the fucose-analog extremities at distances that are compatible with the fucose binding site on BambL. However, the orientation of the fucose mimetics does not bring their extremities in a favorable location compared to the geometry of the glycoclusters. It is therefore unlikely that the six fucose analogs would bind at the same time to the same BambL and cross-linking to different proteins is probably occurring.

We describe for the first time glycomimetics with nM affinity for lectins from pathogen organisms, and with precise specificity toward a class of lectins. At the present, the six-bladed fucose-specific propeller that is targeted by these compounds has been described in two human pathogenic microorganisms, i.e., *B. ambifaria* and *A. fumigatus*. The lectin has also been characterized from organisms that interact with plants cell wall, such as RSL from bacterium *Ralstonia solanacearum*, AOL from fungus *Aspergillus oryzae* (both plant pathogens), and AAL mushroom *Aleuria aurantia*. We previously demonstrated that RSL binds to αFuc1,2Gal disaccharide, an epitope present in both human blood group oligosaccharides, and plant cell wall xyloglucan. Such soluble lectins could be therefore involved in adhesion to different substrates.

B. ambifaria and *A. fumigatus* are both lung pathogens that cause serious infections in immunosuppressed patients, and those with cystic fibrosis. To identify other possible target for glycomimetic compounds mimicking fucose, a search based on BambL sequence has been performed in NIH Microbial database. Sequences with similar length and strong identity

scores could be identified in several bacteria, some of them being considered as emergent pathogens (Figure 5).

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B.amb_1    ---MQTAAISWGTT--PSIRVYTAN-GNKITERCYDGS-NWYTGAFNQ
B.stab_1   ---MQTAAISWGTT--PSIRVYTAN-GNKITERCYDGA-NWYTGAFNQ
B.pyr_1    ---MQTAAISWGTT--PSIRVYTAN-GNKITERCYDGS-NWYTGAFAQ
B.cep_1    ---MQTAAISWGST--PSIRVYTAN-GNKITERCYDGQ-NWYTGAFNQ
R.pic_1    MSNVQTAATSWGTV--PSIRVYTAN-GGKITERCYDGK-GWYTGAFNE
R.man_1    MSNVQTAATSWGTV--PSIRVYTAN-GGKITERCYDGK-GWYTGAFNE
A.fum_1    LFRTGIAAVNSTN----HLRVYFQDVYGSIRPSLYEG--SWANGTEKNVIGNA

B.amb_2    AGDNVSATCWLSGSAVHIRVYAT-SGGSTTEWCWDGD-GWTRGAYTGL
B.stab_2   AGDNVSATCWLVGSAVHIRVYAT-SGGTTTEYCWDGD-GWTRGGYTGS
B.pyr_2    AGDNVSATCWLVGSAVHIRVYAT-SGGTTTEYCWDGD-GWTRGGYTGS
B.cep_2    AGDNVSATCWLVGSAIHIRVYAT-SGGTTTEYCWDGD-GWTRGGYTGS
R.pic_2    PGDNVSVTSWLVGSAIHIRVYAT-SGGNTTEWCWDGN-SWTKGGYTATN
R.man_2    PGDSVSVTSWLVGSAIHIRVYAT-SGGSTTTEWCWDGN-SWTKGGYTATN
A.fum_2    KLGSPVAATSKEL---KHIRVYTLTEGNTLQEFAYDSGTGWYNGGLGGAK
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Figure 5. Multiple-sequence alignment of BambL related sequences identified in the genomes of *Burkholderia* and *Raltsonia* pathogenic species. The two blocks correspond to blade repeats, and the four amino acids directly involved in fucose binding are highlighted. Accession numbers are BAX62607.1 for *B. stabilis*, WP_034182107.1 for *B. pyrrocinia*, WP_059237490.1 for *B. cepacia*, WP_024976975.1 for *R. picketti*, and WP_063392503.1 for *R. mannitolilytica*. The sequences of the two first blades of AFL ("A.fum", amino acids 10 to 103) are included for comparison.

B. stabilis, *B. pyrrocinia*, and *B. cepacia* are members of BCC, the *Burkholderia cepacia* complex, a group of closely related species that are found in various environments, including hospitals, responsible for grave infection in cystic fibrosis patients, and often multi-resistant to antibiotics.[31] *R. picketti* and *R. mannitolilytica* are also emergent pathogens, now
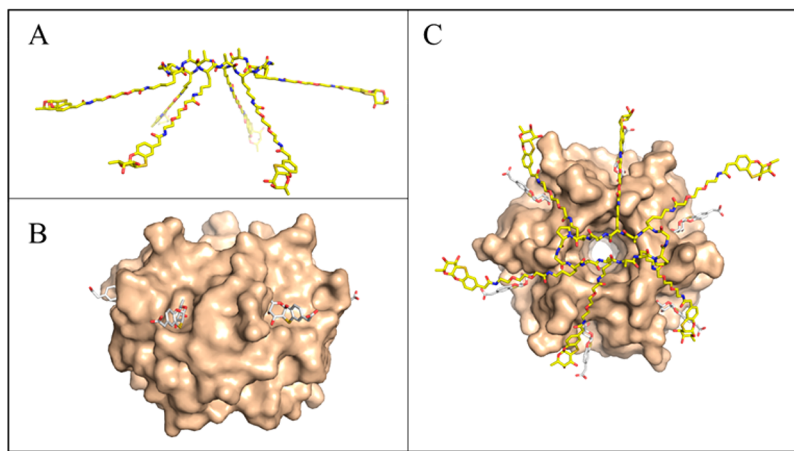

Figure 4. (A) Model of compound **4** built with extended conformation of linkers. The cyclopeptide was built by extended a related model (see ref 30) obtained from NMR studies and molecular modeling. (B) Model of BambL with compound **1** in the six fucose binding sites (adapted from ref 13). (C) Superimposition of the two models with the same scale for size evaluation.

causing more-frequent and more-severe respiratory infections in cystic fibrosis patients, with most *Ralstonia* identified strains being multi-drug resistant.[32]

As displayed in Figure 5, the amino acids involved in fucose binding in this family of lectins are conserved in all described pathogens. It can be therefore expected that the glycomimetics described here and the ones that could be developed from them in the future will be active on several emergent lung pathogens. Strategies for the use of such anti-infectious compounds are getting more urgent because many strains present increasing multi-resistance to antibiotics.

CONCLUSIONS

In summary, we report here a convergent approach that brings together the concepts of glycomimetic and of multivalency for reaching both high affinity and high specificity in targeting the interaction between pathogens and human tissues. Such approach was used previously with well-characterized glyco-mimetics for blocking the interaction of human DC-SIGN with HIV glycoprotein[33] and the interaction of cholera toxin with glycolipids.[34] Nevertheless, this is the first report for specific targeting of the fucose-specific β-propeller lectin present in both fungal and bacterial pathogens involved in lung infection in immunocompromised patients and cystic fibrosis ones. The affinities for BambL and AFL of the glycomimetic hexavalent clusters are in the same range as the fucoclusters, as evaluated via isothermal titration calorimetry studies with dissociation constants in the nanomolar range. The increase in affinity in comparison to the monovalent epitope is very high, with a gain of almost 2000 for binding to AFL, indicating that the cyclopeptide raft scaffold provides efficient multivalency for binding to this lectin family. The non-natural aryl α-O-fucosyl linkage of the glycomimetic adopts a constrained flat conformation that could fit only in the crevasse-shaped binding site of this lectin family, resulting in very precise specificity. Because genes coding for highly similar lectin have been identified in the genome of several emergent pathogens, the present study can be the basis of anti-infectious therapy.

EXPERIMENTAL PROCEDURES

General Method. Reaction progress was monitored by reverse-phase HPLC—mass spectrometry (MS) using C_{18} columns, at 1.3 mL min^{-1} (Nucleosil 120 Å, 3 μm C_{18} particles, 30 × 4.6 mm^2) with UV monitoring at 214 and 250 nm and with electrospray ionization—MS in positive mode using a linear A—B gradient (5 to 40% B in 15 min, buffer A: 0.09% CF_3COOH in water; buffer B: 0.09% CF_3COOH in acetonitrile). Analytical HPLC analyses were performed by using C_{18} columns and a linear gradient (5 to 40% B in 25 min), with UV monitoring at 214 and 250 nm.

Synthesis of Compound 2. To a solution of **6** (30 mg, 0.01 mmol) in dry DMF (3 mL), **1**[9] (22 mg, 0.072 mmol), PyBOP (37 mg, 0.072 mmol) and DIPEA (31 μL, 0.18 mmol) were added. The mixture was stirred at rt and after 30 min, and analytical HPLC found the quantitative conversion of **6** in **2**. The solvent was evaporated, and the peptide was precipitated in Et_2O, centrifuged, and washed three times with Et_2O. The crude was purified by semipreparative HPLC (gradient: 5% to 70% B in 30 min) to obtain **2** (20 mg, 0.005 mmol, 50%). Analytical HPLC: R_t = 14.5 min; matrix-assisted laser desorption/ionization—time-of-flight—mass spectrometry

(MALDI-TOF-MS) (m/z): $[M+2H]^{2+}$ calcd for $C_{182}H_{264}N_{26}O_{62}S_6{}^{2+}$, 1999.33; found, 2000.00.

Synthesis of Compound 4. To a solution of **6** (10 mg, 0.003 mmol) in H_2O (2 mL), $NaIO_4$ (38 mg, 0.18 mmol) was added and the mixture was stirred at rt. After 30 min, the mixture was purified by semipreparative HPLC (gradient: 5% to 40% B in 30 min) to give a crude, which was used in the next reaction without further purification. A solution of crude and **3** (5 mg, 0.027 mmol) was stirred at rt in a mixture of H_2O + 0.1% TFA (2 mL). After 30 min, analytical HPLC found the quantitative conversion of the intermediate in **4**. The mixture was purified by semipreparative HPLC (gradient: 5% to 100% B in 30 min) to give pure **4** (11 mg, 0.003 mmol, >90%). MALDI-TOF-MS (m/z): $[M+2H]^{2+}$ calcd for $C_{146}H_{246}N_{32}O_{68}{}^{2+}$, 1768.34; found, 1768.40.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconj-chem.7b00616.

> Additional details on isothermal titration calorimetry, synthesis, RP-HPLC, and ESI-MS results of compounds **2**, **4**, **5′**, **6**, **8**. Figures showing thermograms. (PDF)

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Notes
The authors declare no competing financial interest.

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