

Organic & Biomolecular Chemistry

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

Fucofullerenes as tight ligands of RSL and LecB, two bacterial lectins

Kevin Buffet,^[1] Emilie Gillon,^[2] Michel Holler,^[3] Jean-François Nierengarten*,^[3] Anne Imberty*,^[2] Stéphane P. Vincent*^[1]

[1] University of Namur (UNamur), Académie Louvain, Département de Chimie, Laboratoire de Chimie Bio-Organique, rue de Bruxelles 61, B-5000 Namur, Belgium; Fax: +32 81 72 45 17, E-mail: stephane.vincent@unamur.be

[2] CERMAV - CNRS (affiliated with Université Grenoble Alpes and ICMG); BP 53, 38041, Grenoble (France); Fax: (+33) 476-547-203; E-mail: anne.imberty@cermav.cnrs.fr

[3] Laboratoire de Chimie des Matériaux Moléculaires, Université de Strasbourg et CNRS (UMR 7509), Ecole Européenne de Chimie, Polymères et Matériaux (ECPM), 25 rue Becquerel, 67087 Strasbourg Cedex 2, France; E-mail: nierengarten@unistra.fr

Abstract: a series of water-soluble glycofullerenes containing up to 24 fucose residues have been prepared. These compounds were tested against the two bacterial fucose-binding lectins LecB and RSL and $C_{60}(E)_{12}$ bearing 24 fucose residues appeared to be the best known inhibitor of both lectins to date. We have shown that increasing both the valency and the length of the spacer between the central core and the peripheral sugars can be beneficial for the affinity.

Keywords – lectins, fullerenes, pathogens, multivalency.

Introduction –

Functionalized nanoscaffolds, and their controlled access, have recently witnessed a growing interest.^[1] The advent of click chemistry^[2] technologies has significantly contributed to the successful synthesis of structurally defined nano-objects with applications spanning from biomedical to material sciences.^[3] In particular, poly-glycosylated nanoscaffolds, sometimes coined glycoclusters, have been designed to take advantage of the so-called “multivalent effect” that often occurs between carbohydrate binding proteins (lectins) and their ligands, if the latter is displayed in a multimeric fashion.^[4] Indeed, as observed in many biological events such as cell-cell interactions, the multivalent presentation of a ligand gives rise to a strong, sometimes dramatic, enhancement of its binding affinity to its receptor.^[5] Since many lectins play critical roles in life sciences, glycoclusters have then emerged as privileged structures^[6] for various applications such as imaging,^[7] drug-delivery,^[8] diagnostics^[9] and antiviral/antibio-therapy.^[10]

To explore the effectiveness of novel nanoscaffolds, we have recently prepared C₆₀ derivatives bearing 12 peripheral reactive groups^[11] and demonstrated that a click coupling reaction with unprotected ligands directly gave dodecafunctionalized fullerenes in high yields.^[12] Shortly after, the proof that glycofullerenes could exhibit good affinities was brought with three different lectins: FimH,^[13] ConA^[14] and LecA.^[15] The high local concentration around the fullerene core was likely at the origin of this tight binding. Importantly, further studies demonstrated that these glycofullerenes could be also efficient in cell-based assays against pathogenic bacteria^[13, 15] or viruses.^[16] Interestingly, glycofullerenes bearing iminocyclitols or heptosides were also found to be strong glycosidase^[17] and glycosyltransferase inhibitors,^[18] respectively, although the enhancement of binding affinities of enzyme inhibitors through multivalent effect are extremely rare.^[19] However, all these studies clearly showed that strong binding affinities, at the protein and cell levels, highly depend on the way the ligands are distributed as well as on the carbohydrate density around the fullerene core structure.

Therefore, we addressed the key question of ligand distribution by the synthesis of a novel family of fucose functionalized C₆₀ (fucosfullerenes), and the study of their binding affinities towards lectins. The target molecules (Figure 1) present 12 or 24 fucoside subunits in order to determine the effect of the valency on the affinity towards the selected bacterial lectins. Moreover, spacers of different lengths have been introduced between the peripheral sugar moieties and the core to evaluate the effect of this structural parameter on the biological properties.

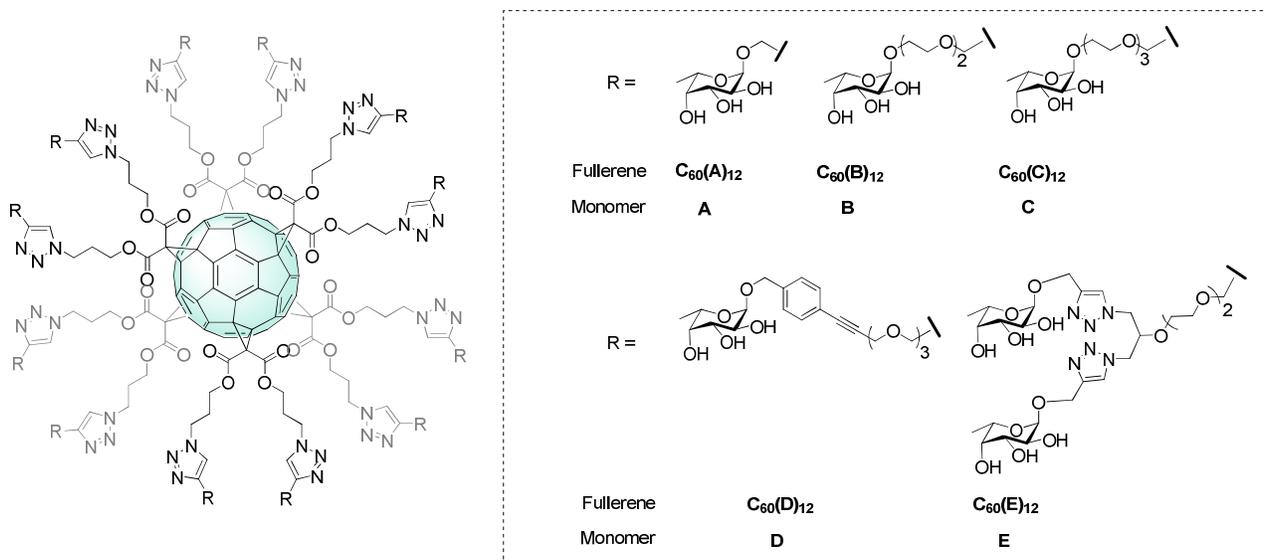


Figure 1 – Targeted fucofullerenes and their corresponding monomeric controls.

The protein receptors selected for the binding assays are two fucose-specific lectins from opportunistic gram negative bacteria responsible of nosocomial infection in patients with immunosuppression and in recurrent pneumonia in cystic fibrosis patients.^[20] LecB (also called PA-III) from *Pseudomonas aeruginosa*, is a tetramer consisting of four subunits, each one presenting a binding site specific for L-fucose. The LecB crystal structure revealed the occurrence of two bridging calcium ions in the carbohydrate binding site.^[21] This unique mode of binding is responsible for unexpected high affinity, with an observed dissociation constant (K_d) of 0.43 μM for α -methyl-fucoside (αMeFuc).^[22] RSL from *Ralstonia solanacearum* is a trimeric lectin, each monomer consisting of two similar β -sheets, and therefore presenting two fucose binding sites.^[23] RSL has similar affinity for fucoside (K_d of 0.73 μM for αMeFuc) than LecB, but presents a different binding site with no calcium requirement. The topologies of the two lectins are completely different with six binding sites on the same face of a functional β -propeller for RSL, while the LecB tetramer has a ball shape with the four binding sites away one from each other. RSL, as the very similar lectin BamBL from *Burkholderia ambifaria*^[24], presents strong similarity in topology and binding sites as AFL1 from *Aspergillus fumigatus*,^[25] which is a target of therapeutical interest. Many multivalent glycomolecules were targeted towards LecB (see review^[10a]) while the BamBL/RSL topology has been the focus of more recent works^[26] but this is the first report of a comparative study for assessing the effect of these two different topologies for binding to the same multivalent ligand.

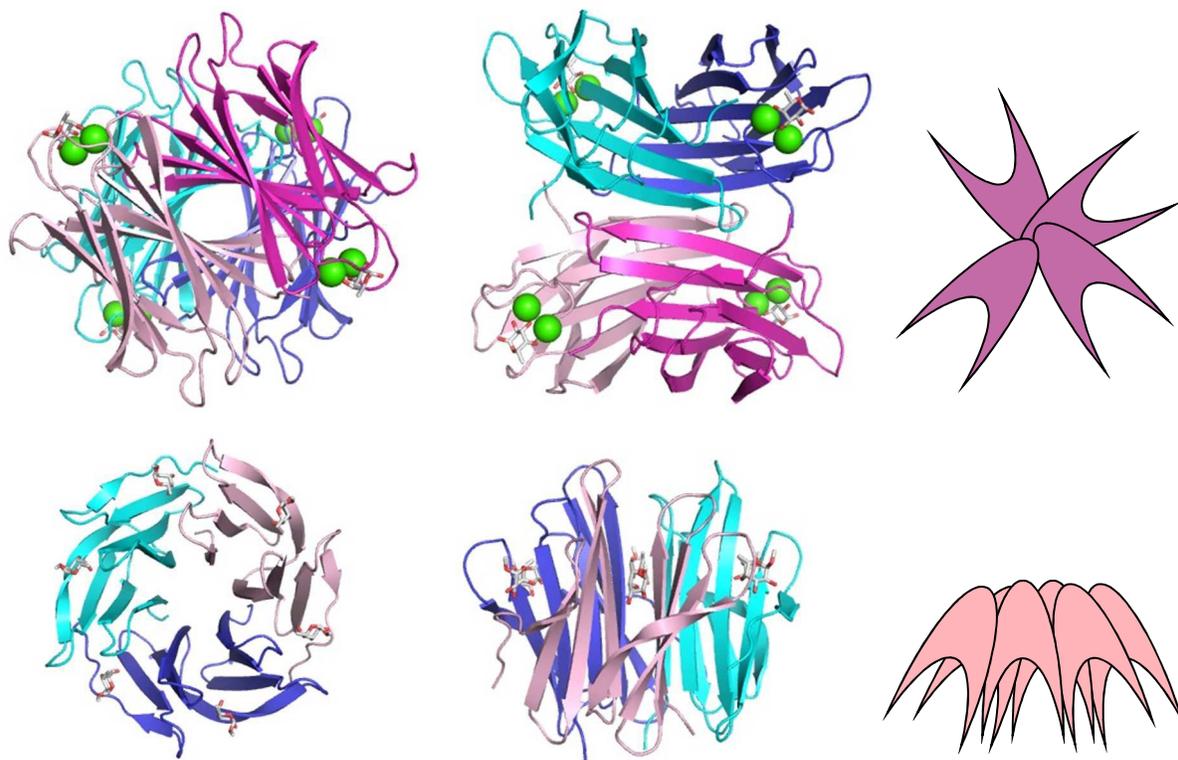
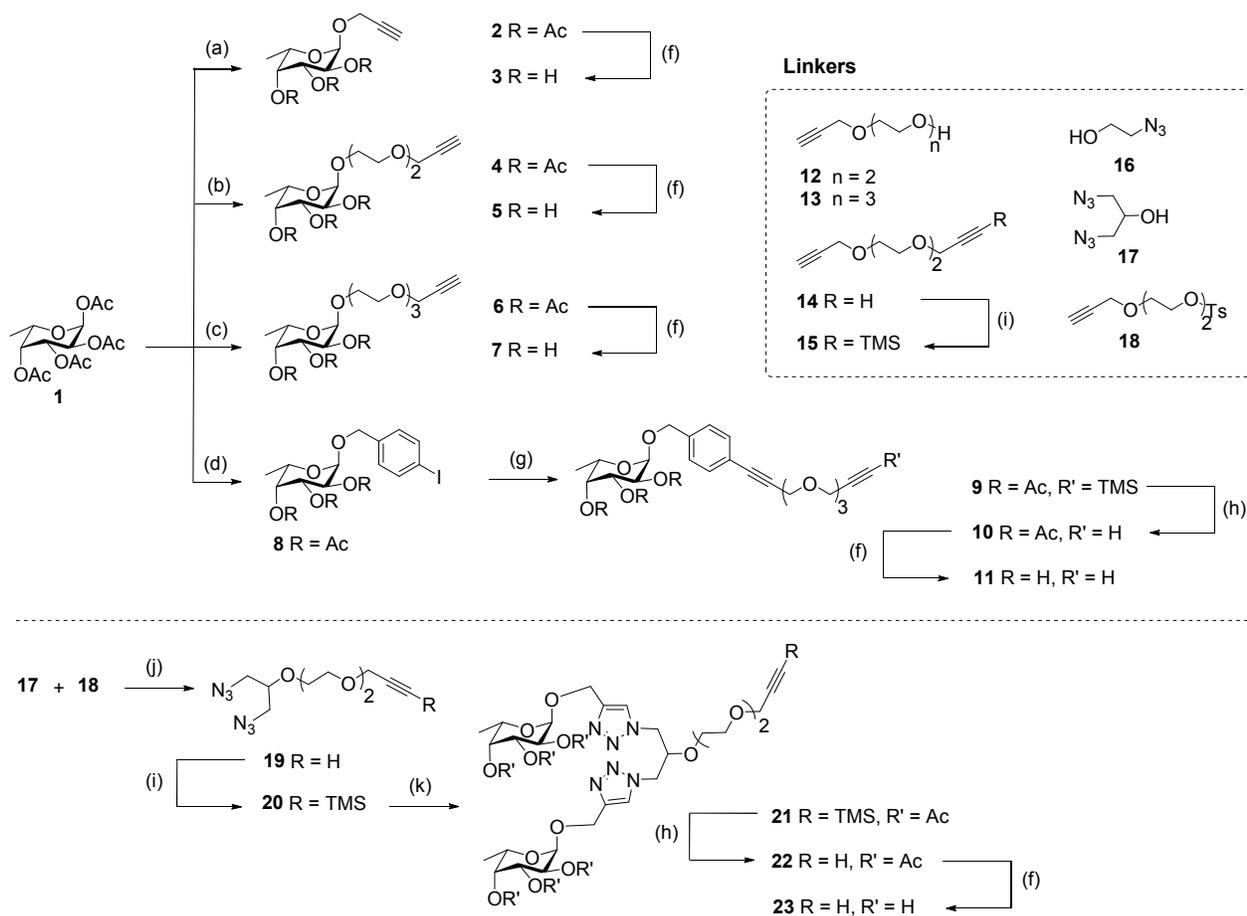


Figure 2 – Graphical representation of the crystal structures of the bacterial lectins complexed with fucose or methyl-fucoside together with schematic representation of topology. Two orthogonal views of LecB/fucose (top: PDB code 1GZT) and RSL/ α MeFuc (bottom, pdb code 2BT9). The peptide chains are represented as ribbons with different colors according to oligomerization, the found fucose as stick and the calcium ions as green spheres.

Synthesis: As previously reported for the inhibition of the mannolectin FimH^[13] and for the inhibition of the heptosyltransferase WaaC,^[18] we took advantage of the copper-catalyzed azide-alkyne cycloaddition for the grafting of twelve unprotected sugar residues on the fullerene core **24** bearing twelve azides that we previously reported.^[12]

A family of fucosides bearing a terminal alkyne (**3**, **5**, **7**, **11** and **23**) were thus designed to allow their grafting to azidofullerene **24**. The spacers **12-18** were designed to explore the effect of distance between the fullerene core and the carbohydrate unit but also to define whether an aromatic group would be beneficial for the interaction with the lectins.

Moreover, we also prepared a divalent fucoside **23** to obtain a final 24-mer glycofullerene in order to assess whether increasing the density of fucose residues around the fullerene would enhance the binding affinity towards the targeted fucolectins.

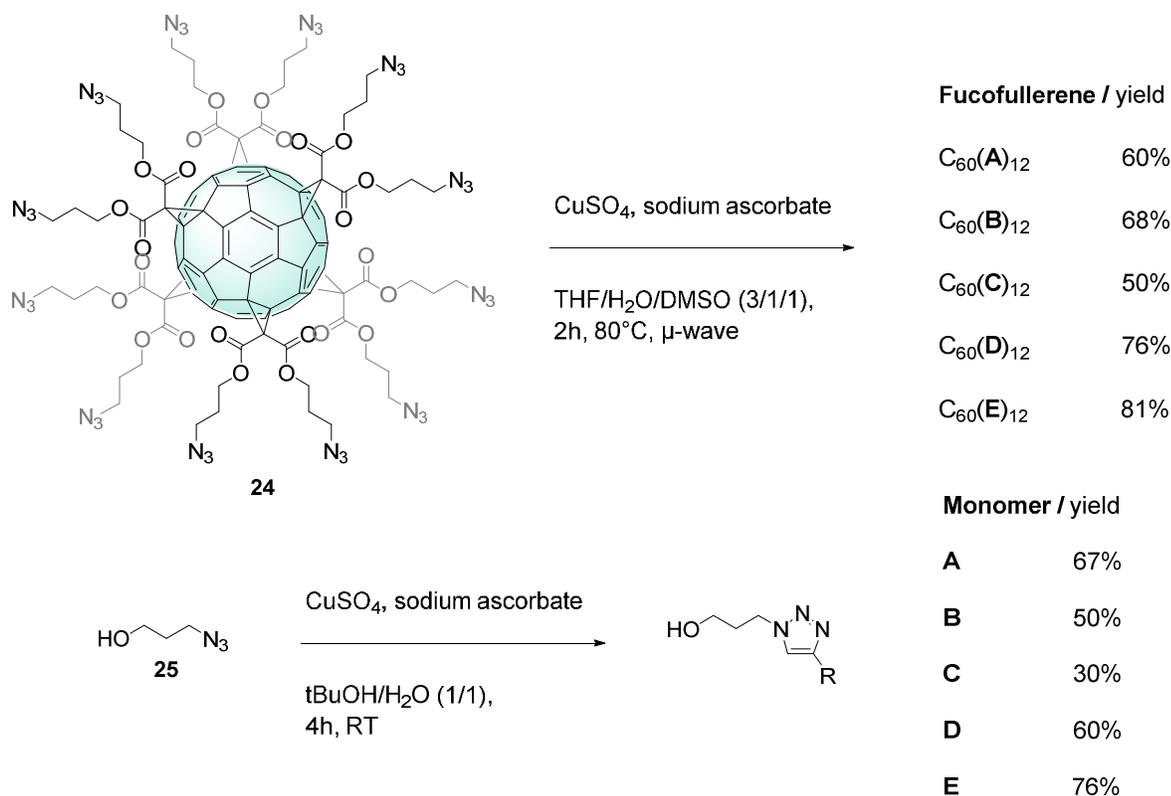


Scheme 1: Synthesis of the monovalent carbohydrates. *Reagents and conditions:* (a) propargyl alcohol, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , 15h, r.t. (35%). (b) **12**, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , 7d, r.t. (23%). (c) **13**, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , 72h, r.t. (24%). (d) *p*-iodobenzyl alcohol, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , 15h, r.t. (26%). (f) MeONa, MeOH, 2h, 0°C to r.t. (**3**: from **2**, 99%; **5**: from **4**, 99%; **7**: from **6**, 97%; **11**: from **10**, 98%, **23**: from **22**, 99%). (g) **15**, $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$, CuI, NEt_3 , 7min, 80°C, $\mu\text{-w}$ (83%). (h) TBAF $\cdot 3\text{H}_2\text{O}$, THF, 5min, r.t. (**10**: from **9**, 73%; **22**: from **21**, 86%). (i) *n*-BuLi, TMSCl, THF, -78°C (**15**: from **14**, 30%; **20**: from **19**, 39%). (j) NaH, DMF, 0°C to r.t., 2h (92%). (k) **2**, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, *t*-BuOH/ H_2O 3:1, 60°C, 15h (72%).

The key step was the glycosylation reaction of peracetylated L-fucose **1** with the different spacers (Scheme 1) that yielded molecules **2**, **4** and **6** which were then deprotected under Zemplén conditions to afford **3**, **5** and **7**, respectively. The synthesis of fucoside **11** was achieved by a Sonogashira coupling between aryl iodide **8** and monoprotected bis-alkyne **15**. The trimethylsilyl and acetyl groups of the resulting fucoside **9** were then removed to give **11**. Building block **20** was obtained by double azidation of 1,3-dibromopropan-2-ol afford **17**¹ followed by reaction with

¹ The synthesis and transformation of bis-azides such as **17** should be realized with special care, due to the hazardous manipulation of azidoalcohols in general.

tosylate **18** and protection of the terminal alkyne function with a trimethylsilyl group. The protected divalent fucoside **21** was prepared from fucoside **2** and diazide **20** under CuAAC conditions. Compound **23** was then prepared through a desilylation-deacetylation sequence. The deprotected fucosides **3**, **5**, **7**, **11** and **23** were then grafted onto known hexa-adduct **24**^[11a] to give the desired final compounds **C₆₀(A-E)₁₂** (Scheme 2). Interestingly, we were able to obtain each of the dodecafucosylated fullerene under the same reaction conditions using a ternary mixture of solvent (THF/DMSO/H₂O 3:1:1) with a catalytic amount of copper sulfate (0.1 equiv.) and sodium ascorbate (0.3 equiv.). The starting terminal alkyne building blocks were used in excess (13 equiv.) to ensure the complete functionalization of dodeca-azide **24**. After complete conversion, the product was precipitated with acetone and extensively washed with acetone and methanol. Afterwards the product was purified by size-exclusion chromatography on a Sephadex™ column to remove any potentially remaining copper salt after washing. This purification step allowed for the separation of two fractions: the first one corresponding to the high molecular mass fraction, i.e. the desired product, and a second one corresponding to low molecular mass compounds which was pale blue indicating the presence of copper salts. Finally, model compounds **A-E** were prepared from azide **25** and the corresponding terminal alkynes (**3**, **5**, **7**, **11** and **23**) under CuAAC conditions.



Scheme 2. Synthesis of the target fucofullerenes and their corresponding monomers (See Figure 1 for structures of A-E).

The final molecules were fully characterized by ^1H and ^{13}C NMR, IR and mass spectrometry (see ESI). For all the fucofullerenes, the absence of diagnostic signals for azide groups at ca. 2092 cm^{-1} in the IR spectra revealed that no defected compounds containing unreacted N_3 group(s) are present. The ^1H NMR spectra of $\text{C}_{60}(\text{A-E})_{12}$ show the appearance of a broad singlet with a chemical shift between 7.8 and 8.0 ppm characteristic of the 1,4-triazole formed during the CuAAC reactions. Owing to the diagnostic signals of the hexasubstituted C_{60} core and of the six equivalent malonates, the ^{13}C -NMR spectra were particularly helpful to show the octahedral T -symmetry of $\text{C}_{60}(\text{A-E})_{12}$. As a typical example, the ^{13}C NMR spectra of compound $\text{C}_{60}(\text{E})_{12}$ is depicted in Figure 3.

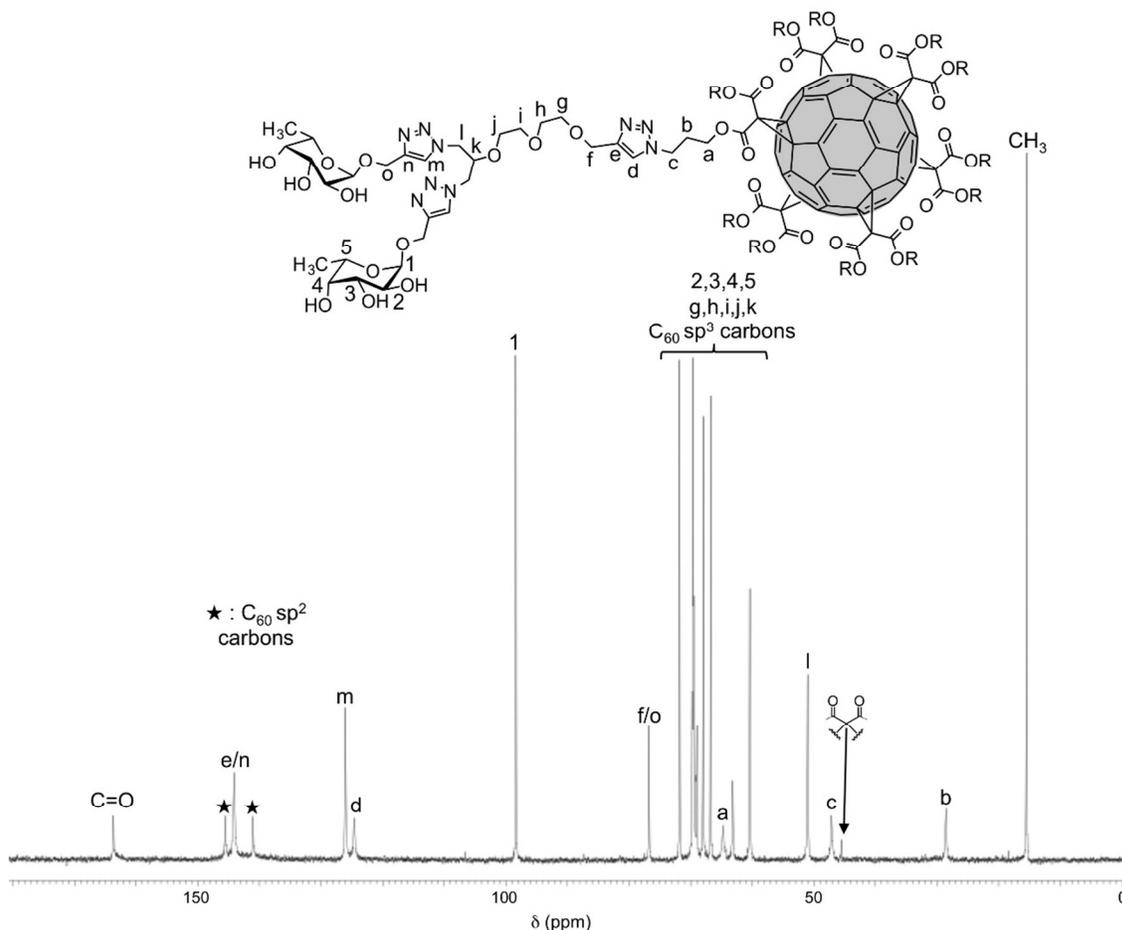


Figure 3. ^{13}C NMR spectra of $\text{C}_{60}(\text{E})_{12}$ recorded in D_2O (100 MHz, 20°C).

Importantly, the structure of glycofullerenes $C_{60}(A-E)_{12}$ was confirmed by MALDI-ToF mass spectrometry. The molecular ion peak was observed for all the compounds together with several peaks resulting from the typical fragmentation of such compounds.^[18] This is illustrated in Figure 4 for compound $C_{60}(E)_{12}$. The expected pseudo-molecular ion peak is observed at m/z 10428.1 ($[M+Na]^+$). A first series of typical fragments corresponds to peaks resulting from retro-Bingel reactions with the loss of one or more malonate addends ($[M+Na-(C_{65}H_{92}N_{18}O_{30})_n]^+$, $n = 1$ to 4). Further fragmentation resulting from the hydrolysis of a malonic ester unit followed by decarboxylation and corresponding to a loss of m/z 796.8 ($C_{32}H_{46}N_9O_{15}$) are systematically observed. Importantly, all these fragments can only arise from compound $C_{60}(E)_{12}$ and cannot be associated to defected products thus showing the monodispersity of the compound.

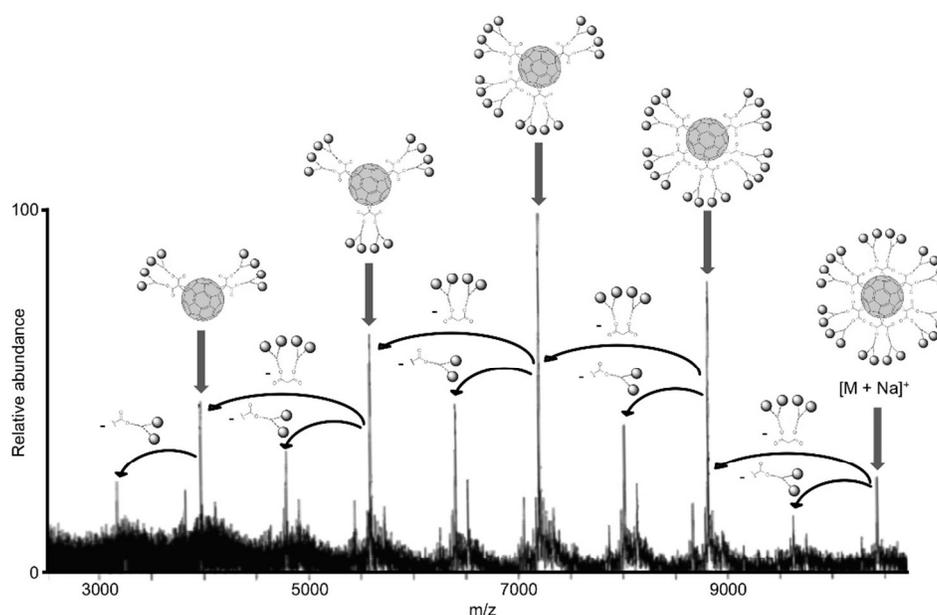


Figure 4. MALDI-ToF mass spectrum of compound $C_{60}(E)_{12}$. The typical fragments of retro-Bingel reaction and hydrolysis of a malonic ester followed by decarboxylation can be attributed.

ITC experiments –

To define whether a multivalent effect occurs between the fucofullerenes and fucolectins, the dissociation constant K_D of the multivalent ligand has to be compared to a control monomeric

ligand. However, in some cases,^[13, 27] the nature of the linker significantly affects the K_D of the control monosaccharide. For this study, we thus selected fucosides **A-E** (Figure 1) as control monomeric structures.

The binding of LecB and RSL to monovalent carbohydrates **A** to **D**, the divalent **E** and all fucosylated fullerenes were investigated by titration microcalorimetry in order to get information about the affinity of the interaction, the thermodynamics but also the stoichiometry involved. Monovalent carbohydrates were assayed on ITC 200 using normal mode (protein in cell and ligand in syringe). In order to limit aggregation, reverse mode was used for multivalent ligand^[28] (protein in syringe). The curves obtained are of good quality, but precipitates were observed at the end of titration indicating that the measured K_D should be considered as apparent dissociation constants.

Titration of LecB by monovalent compounds resulted in titration curves (Figure 5 for selected thermograms and Figure S1 in ESI) with strong exothermic peaks and binding of one compound per protein monomer as expected from previous microcalorimetry results on fucose derivatives^[22] and from crystal structure.^[21]

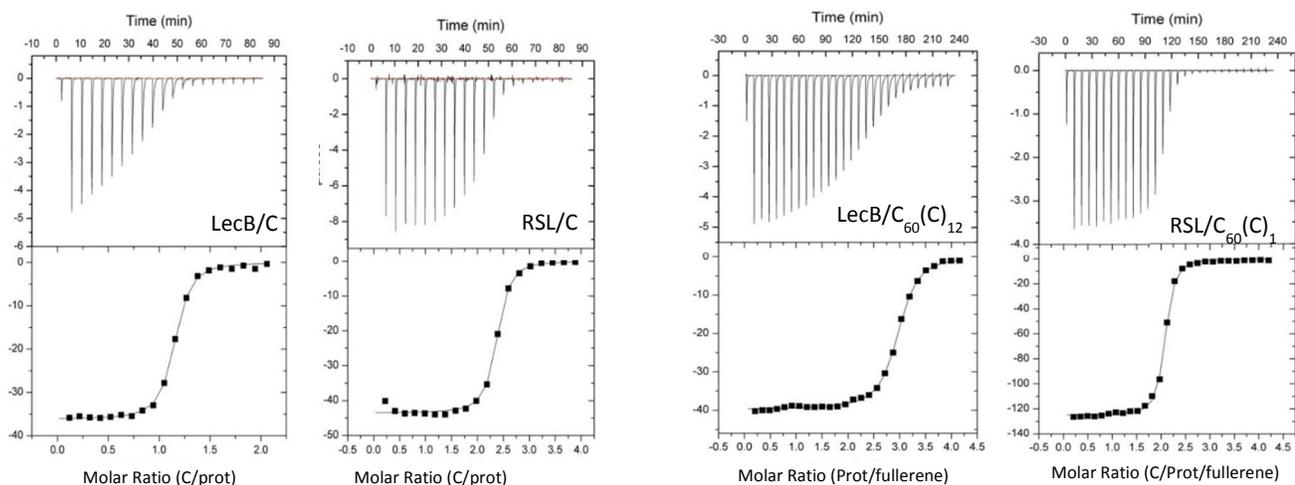


Figure 5: ITC data for the interaction of LecB and RSL with monovalent of fullerene-supported compound **C**. Left: Thermograms obtained by injection of compound **C** (1 mM) in LecB (100 μ M) or RSL (50 μ M). Right: Thermograms obtained by injection of LecB (400 μ M) or RSL (200 μ M) in $C_{60}(C)_{12}$ solution (20 μ M). The corresponding integrated titration curves and calculated fit are displayed below.

The monovalent fucosylated ligand with ethylene glycol chains of different lengths (compounds **A**, **B** and **C**) display rather similar binding behaviour to LecB (Table 1) with dissociation constants in

the submicromolar range, in the same range than the K_D of 430 nM previously obtained for α -methyl-fucoside (α MeFuc).^[22] Stoichiometry values close to 1 confirms the presence of one ligand in the binding site of each monomer. The presence of an aromatic ring in the aglycone **D** slightly increases the affinity, resulting in a K_D of 176 nM, representing a 16 fold increase compared to fucose ($K_D = 2.9 \mu\text{M}$).^[29] Binding of all monovalent ligands is driven by enthalpy term (Table 1) with very little contribution from the entropy term as observed previously for LecB binding to fucose derivatives.^[22]

The divalent compound **E** behaves rather differently and displays a much stronger interaction with LecB with a dissociation constant of 43 nM. Indeed, this 67 fold improvement compared to fucose has never been obtained for a divalent compound and compares to the 100 fold improvement obtained with tetravalent glycopeptide by Reymond and coll.^[30] A stoichiometry of 0.5 (compound **E**/LecB) is obtained indicating that compound **E** is efficiently bridging two LecB tetramers, as confirmed by the aggregation observed at the end of the titration. The very strong enthalpy ($\Delta H = -81 \text{ kJ/mol}$) is larger than twice the amount observed for a monomeric interaction, suggesting some additional protein-protein contacts induced by the clustering effect.

Table 1. Isothermal titration microcalorimetry data for monovalent and divalent compounds binding to LecB. Standard deviations have been estimated from at least two independent experiments (deviations on $T\Delta S$ are similar to those on ΔH).

Ligand	Valency	K_D (nM)	$-\Delta G$ (kJ/mol)	$-\Delta H$ (kJ/mol)	$T\Delta S$ (kJ/mol)
A	1	281 ± 7	37.4	32.4 ± 1.4	5.0
B	1	975 ± 98	34.3	34.6 ± 0.4	0.3
C	1	432 ± 71	36.3	36.8 ± 0.6	-0.5
D	1	176.2 ± 0.5	38.5	38.4 ± 1.5	0.1
E^a	2	43 ± 2	42.0	81.5 ± 0.5	-39.5

^a All experiments were run in direct mode with ITC200 except for compound **E** that has been characterized using inverse mode with VPITC.

All fucosylated fullerenes interact strongly with LecB with the exception of $C_{60}(\mathbf{D})_{12}$ that could not be tested because of poor solubility in buffer. All titration data were collected in reverse mode in order to attenuate aggregation (Figure S2). Stoichiometry of binding varies from 0.8 to 0.32

fullerene/LecB monomer, i.e 3 to 1 LecB monomer per fullerene. Only part of the available fucose residues (between 10 to 20%) are therefore engaged in binding to the protein, probably because of steric hindrance and/or aggregation.

The affinity of fucosylated fullerenes to LecB appears to be dependent on the size of the linker presenting the fucose (Table 2), with K_D values varying from 234 nM for the shorter chain (A) to 68 nM for the longer chain (C). Nevertheless, the observed affinities are in the same range as those obtained with monovalent compounds indicating that multivalent presentation did not result in a large affinity gain for LecB. However, if one takes into account the contribution of each fucose residues of the multimer and compare it to the corresponding monomeric species (this ratio has been defined, in Table 2, as the relative inhibition potency), the affinity of fucoside A is significantly lower when displayed in a multivalent fashion in $C_{60}(A)_{12}$. Interestingly, compound $C_{60}(E)_{12}$ presenting 24 fucose residues on the surface, displays a K_D of 23 nM that is the lowest reported for LecB.

Table 2. Isothermal titration microcalorimetry data for LecB interacting with glycosylated fullerenes. Standard deviations have been estimated from at least two independent experiments (deviations on $T\Delta S$ are similar to those on ΔH). Reverse titration was used (lectin in the cell of VPITC) and stoichiometry N is evaluated as number of bound glycoclusters per protein monomer.

ligand	Valency	K_D (nM)	N	$-\Delta G$ (kJ/mol)	$-\Delta H$ (kJ/mol)	$T\Delta S$ (kJ/mol)	RIP ^a
$C_{60}(A)_1$	12	234 ± 58	0.79 ± 0.05	37.9	84.7 ± 3.8	-46.8	0.10
$C_{60}(B)_{12}$	12	98 ± 17	0.37 ± 0.01	40.0	125 ± 3	-85	0.83
$C_{60}(C)_1$	12	68 ± 9	0.36 ± 0.01	41.0	119 ± 3	-82	0.53
$C_{60}(D)_1$	12	Not soluble					
$C_{60}(E)_{12}$	24	23 ± 5	0.32 ± 0.01	43.7	158 ± 3	-114	0.16

a- RIP = Relative Inhibition Potency. This number represents the relative affinities of each fucose ligand of the fucofullerene $C_{60}(X)_{12}$ compared to the fucose moiety of the monomeric ligand X. $RIP = (K_D(\text{monomer}))/12 * K_D(\text{multimer})$.

All monovalent fucosylated ligand (compounds A to D) bind efficiently to RSL with dissociation constants between 240 and 340 nM (Table 3), which is 2 to 3 times better than αMeFuc ($K_D = 0.73 \mu\text{M}^{[23a]}$). Stoichiometry values close to 2 (Figure S3) are in agreement with the presence of two fucose binding sites per RSL monomer.^[23a] However, if the free energy of binding does not present much variations as a function of the linker, the values of entropy contribution, and therefore

of counterbalancing entropy, are more variable. Compound **B** has the strongest enthalpy contribution ($\Delta H = -60$ kJ/mol) while compound **C** with a longer spacers or compound **D** with a more rigid one have weaker enthalpy contributions ($\Delta H = -40$ kJ/mol). This could be correlated to contact established between the spacer and crevasse shaped binding site, as already proposed for the related lectin BambL.^[31]

The divalent fucosylated compound **E** is a very efficient ligand with K_D of 74 nM (Table 3). Stoichiometry values of 1.5 indicate that a significant portion of the compound is bridging neighbouring RSL β -propellers, as confirmed by observation of some aggregation.

Table 3. Isothermal titration microcalorimetry data for monovalent and divalent compounds binding to RSL. Standard deviations have been estimated from at least two independent experiments (deviations on $T\Delta S$ are similar to those on ΔH).

ligand	Valency	K_D (nM)	$-\Delta G$ (kJ/mol)	$-\Delta H$ (kJ/mol)	$T\Delta S$ (kJ/mol)
A	1	240 ± 4	37.8	50.0 ± 0.2	-12.2
B	1	269 ± 13	37.5	60.6 ± 0.8	-23.1
C	1	337 ± 43	37.0	41.1 ± 2.5	-4.1
D	1	243 ± 13	37.8	40.2 ± 3.6	-2.4
E	2	74 ± 16	40.8	86.7 ± 1.4	-46.1

^a All experiments were run in direct mode with ITC200 except for compound **E** that has been characterized using inverse mode with VPITC.

All fucosylated fullerenes interact strongly with RSL with stoichiometry values varying from 0.9 to 0.34 fullerene/RSL monomer (Table 4 and Figure S4). Again, steric hindrance likely prevents most of the fucose residues to reach a RSL binding site.

Nevertheless, the affinities are very strong with dissociation constants varying from 17 nM for fucosylated fullerene with a short spacer $C_{60}(\mathbf{A})_{12}$ to 10 nM for $C_{60}(\mathbf{C})_{12}$ with a longer spacer. An increased spacer length allows more fucose residues to reach RSL binding sites as indicated by the variations in stoichiometry values, and in the associated ΔH contribution. The fullerene $C_{60}(\mathbf{E})_{12}$ with 24 fucose residues is an excellent ligand for RSL with dissociation constant of about 2 nM. This represents a 300 fold affinity increase compared to fucose, i.e more than 12 fold increase when corrected by multivalency factor.

Table 4. Isothermal titration microcalorimetry data for RSL interacting with glycosylated fullerenes. Standard deviations have been estimated from at least two independent experiments (deviations on $T\Delta S$ are similar to those on ΔH). Reverse titration is used (lectin in the cell of VPITC) and stoichiometry N is evaluated as a number of bound glycofullerenes per protein monomer.

ligand	Valency	K_D (nM)	N	$-\Delta G$ (kJ/mol)	$-\Delta H$ (kJ/mol)	$T\Delta S$ (kJ/mol)	RIP ^a
$C_{60}(A)_1$	12	17.5 ± 1.8	0.89 ± 0.03	44.3	129 ± 8	-85	1.14
$C_{60}(B)_{12}$	12	15.6 ± 0.7	0.53 ± 0.01	44.6	237 ± 9	-192	1.44
$C_{60}(C)_1$	12	10.2 ± 0.2	0.47 ± 0.02	45.6	255 ± 3	-210	2.75
$C_{60}(D)_1$	12	Not soluble					
$C_{60}(E)_{12}$	24	2.4 ± 0.3	0.34 ± 0.05	49.2	326 ± 5	-277	2.57

a- RIP = Relative Inhibition Potency. This number represents the relative affinities of each fucose ligand of the fucosylated fullerene $C_{60}(X)_{12}$ compared to the fucose moiety of the monomeric ligand X . $RIP = (K_D(\text{monomer}))/12 * K_D(\text{multimer})$.

Discussion and conclusions –

The comparison of the two lectins showed that they globally display similar behaviour regarding the various ligands assayed in this study. The K_D values are in the same range for the monomeric fucosides, although the entropic variation is higher in the case of RSL. However, in the case of the fucosylated fullerenes, the affinities are usually stronger for RSL thanks to a much higher enthalpic contribution.

The data shown here clearly indicate that the use of multivalent glycofullerenes allow to enhance the binding affinity of the ligand for the lectin RSL, as already reported for LecA with galactofullerenes.^[15] Although the efficiency per ligand epitope was better in the latter, the difference can be easily explained by the fact that α -methyl-L-fucose is already a very good ligand of LecB, with a K_D of 430 nM, thanks to two calcium cations well-positioned in the binding pocket of LecB. On the contrary, LecA possesses only one calcium ion in the binding site therefore β -methyl-D-galactose has a higher K_D (70 μ M).^[32] Thus, the opportunity to observe large multivalent effects is stronger in the case of LecA. We already showed similar results when we used mannofullerenes as ligands of the lectin FimH^[13]: the monomeric analogues were almost as good as the corresponding glycofullerenes, with K_D in the low nanomolar range. However, while no multivalent effect is observed on LecB, significant enhancement of affinity per carbohydrate epitope is occurring with the glycofullerenes $C_{60}(C)_{12}$ and $C_{60}(E)_{12}$ on RSL, the K_D value being 33-

fold lower compared to the corresponding monomers. It means that almost a 3-fold affinity enhancement is observed for each fucose of the multimers. Moreover, a small enhancement per sugar residue is observed in the case of RSL with the divalent compound **E** compared to the corresponding monomer **A** (1.6-fold enhancement) and this increase of affinity is still stronger in the case of LecB (3.2).

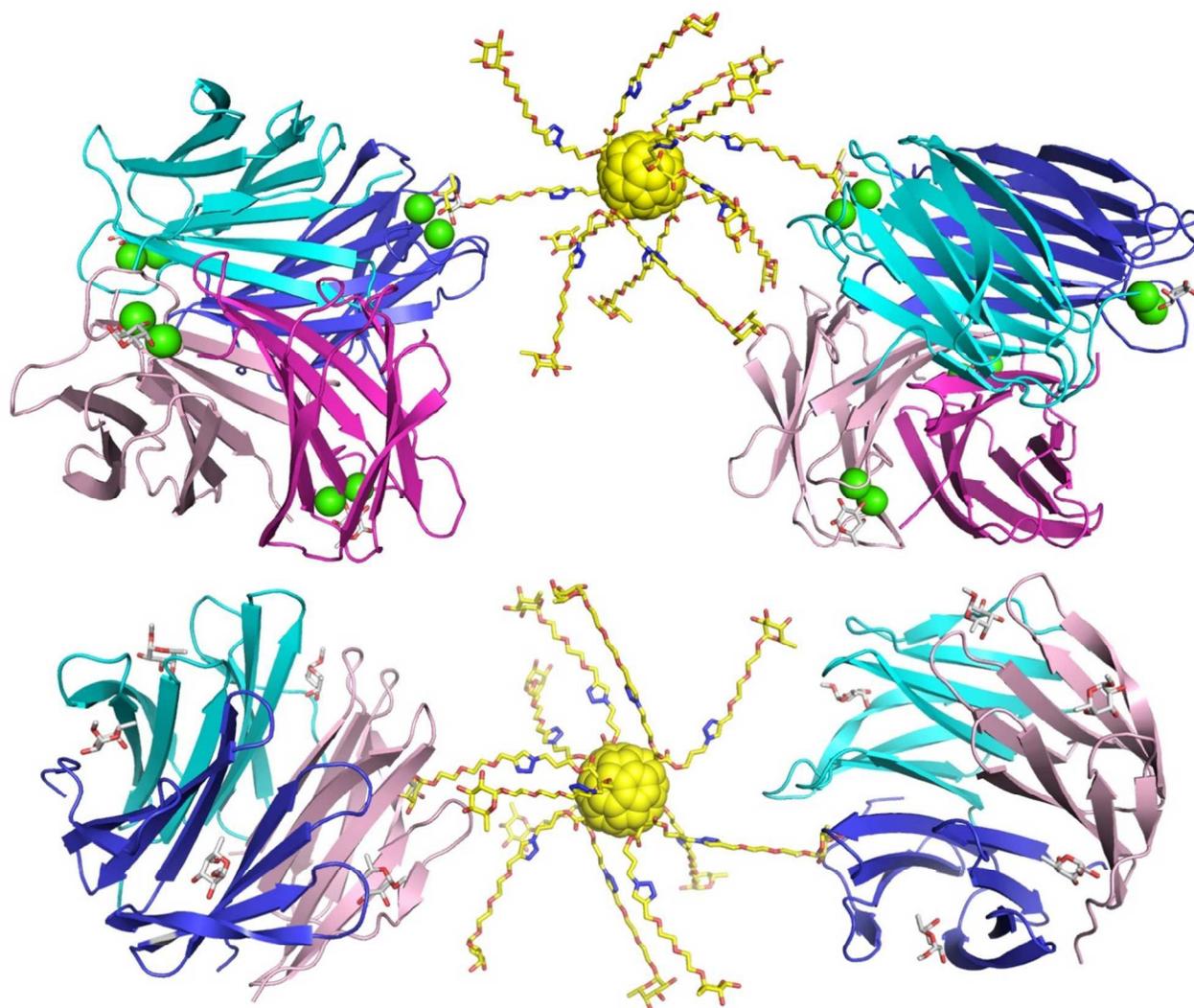


Figure 7: Simplified binding mode of fullerene $C_{60}(B)_{12}$ bridging either two tetramers of LecB (top) or two trimers of RSL (bottom). Peptide chains are represented as ribbon, carbohydrates and spacer as sticks, fullerene and calcium ions as spheres.

As mentioned above, some glycofullerenes have also been assayed against the plant lectin ConA^[14] and DC-SIGN.^[16] It was found that the valency number is not the only factor influencing

the affinity for the lectins. Indeed, these studies showed a decrease of affinity with the increase of valency (from 12 to 36 ligands). Increasing the length between the central core and the sugar allowed to recover the activity, keeping the same valency (36).^[14, 16] The authors explained these results by the variable accessibility of the ligands depending on the chain length.

Interestingly, our study shows that the glycofullerenes with longer chains are able to bridge up to three lectins simultaneously. A simplified binding mode of fullerene $C_{60}(B)_{12}$ bridging either two tetramers of LecB or two trimers of RSL is illustrated in Figure 7. However, only a small amount of the fucose epitopes are participating in the same time, probably due to steric hindrance around the fullerene after binding of three lectins.

In conclusion, we prepared a series of water-soluble glycofullerenes containing up to 24 fucose residues. These compounds were tested against the two fucose-binding lectins LecB and RSL and $C_{60}(E)_{12}$ appeared to be the best known inhibitor of both lectins to date. We showed that increasing the length between the central core and the sugar and increasing the valency of the glycofullerene are both beneficial for the affinity. Therefore, depending on the lectin, increasing the valency can be favourable or not, as showed by our results and previous works.^[14, 16]

Acknowledgements - A.I. acknowledges support from GDR Pseudomonas, Labex ARCANE (ANR-11-LABX-003). A.I. and S.V. acknowledge COST Action CM-1102 MultiGlycoNano. S.V. acknowledges support from FNRS (FRIA grant to K.B.) and from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° [289033].

Bibliography –

- [1] a) J.-L. Reymond and T. Darbre, *Org. Biomol. Chem.* **2012**, *10*, 1483–1492; b) A. Joshi, D. Vance, P. Rai, A. Thiagarajan and R. S. Kane, *Chem. Eur. J.* **2008**, *14*, 7738 – 7747.
- [2] H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021.
- [3] a) J. D. Badji, A. Nelson, S. J. Cantrill, W. B. Turnbull and J. F. Stoddart, *Acc. Chem. Res.* **2005**, *38* 723–732; b) A. Dondoni and A. Marra, *Chem. Rev.* **2010**, *110*, 4949–4977; c) C. Papin, G. Doisneau and J.-M. Beau, *Chem. Eur. J.* **2009**, *15*, 53 – 57.
- [4] a) C. Fasting, C. A. Schalley, M. Weber, O. Seitz, S. Hecht, B. Kocsch, J. Dornedde, C. Graf, E.-W. Knapp and R. Haag, *Angew. Chem. Int. Ed.* **2012**, *51*, 10472 – 10498; b) A. Barnard and D. K. Smith, *Angew. Chem. Int. Ed.* **2012**, *51*, 6572 – 6581; c) P. Braun, B. Nägele, V. Wittmann and

- M. Drescher, *Angew. Chem. Int. Ed.* **2011**, *50*, 8428–8431; d) S. Cecioni, A. Imberty and S. Vidal, *Chem. Rev.* **2015**, *115*, 525-561.
- [5] a) J. J. Lundquist and E. J. Toone, *Chem. Rev.* **2002**, *102*, 555-578; b) M. Mammen, S. K. Choi and G. M. Whitesides, *Angew. Chem. Int. Ed.* **1998**, *37*, 2755-2794; c) R. J. Pieters, *Org. Biomol. Chem.* **2009**, *7*, 2013-2025; d) D. Schwefel, C. Maierhofer, J. G. Beck, S. Seeberger, K. Diederichs, H. M. Möller, W. Welte and V. Wittmann, *J. Am. Chem. Soc.* **2010**, *132*, 8704–8719; e) P. I. Kitov and D. R. Bundle, *J. Am. Chem. Soc.* **2003**, *125*, 16271-16284; f) A. K. Adak, H.-J. Lin and C.-C. Lin, *Org. Biomol. Chem.* **2014**, *12*, 5563.
- [6] a) J. E. Gestwicki, C. W. Cairo, L. E. Strong, K. A. Oetjen and L. L. Kiessling, *J. Am. Chem. Soc.* **2002**, *124*, 14922-14933; b) M. Hartmann and T. K. Lindhorst, *Eur. J. Org. Chem.* **2011**, 3583–3609; c) A. Imberty, Y. M. Chabre and R. Roy, *Chem. Eur. J.* **2008**, *14*, 7490-7499; d) L. L. Kiessling, J. E. Gestwicki and L. E. Strong, *Angew. Chem. Int. Ed.* **2006**, *45*, 2348-2368; e) M. Lahmann, *Top. Curr. Chem.* **2009**, *288*, 17-65; f) D. Deniaud, K. Julienne and S. G. Guin, *Org. Biomol. Chem.* **2011**, 966.
- [7] a) S. I. van Kasterena, S. J. Campbellb, S. Serresc, D. C. Anthonyb, N. R. Sibsonc and B. G. Davis, *Proc. Nat. Acad. Sci. U.S.A.* **2009**, *106*, 18–23; b) D. C. Kennedy, D. Grünstein, C.-H. Lai and P. H. Seeberger, *Chem. Eur. J.* **2013**, *19*, 3794 – 3800.
- [8] a) H. Surangi, N. Jayawardena, K. W., Jayawardana, X. Chen and M. Yan, *Chem. Commun.* **2013**, *49*, 3034-3036; b) W. Yu, N. Zhang, C. Li *Curr. Phar. Design* **2009**, *15*, 3826-3836 b) L. Gallego-Yerga, M. Lomazzi, F. Sansone, C. Ortiz Mellet, A. Casnati, J. M. García Fernández *Chem. Commun.* **2014**, *50*, 7440-7443 d) C. Ortiz Mellet, J.M. Benito, and J. M. García Fernández *Chem. Eur. J.* **2010**, *16*, 6728 – 6742.
- [9] a) E. Mahon, T. Aastrup and M. Barboiu, *Chem. Commun.* **2010**, *46*, 2441-2443; b) E. Mahon, Z. Mouline, M. Sillion, A. Gilles, M. Pinteala and M. Barboiu, *Chem. Commun.* **2013**, *49*, 3004-3006; c) N. Parera Pera and R. J. Pieters, *Med. Chem. Commun.* **2014**, *5*, 1027.
- [10] a) A. Bernardi and A. C. J. Jiménez-Barbero, C. De Castro, T. Darbre, F. Fieschi, J. Finne, H. Funken, K.-E. Jaeger, M. Lahmann, T. K. Lindhorst, M. Marradi, P. Messner, A. Molinaro, P. Murphy, C. Nativi, S. Oscarson, S. Penadés, F. Peri, R. J. Pieters, O. Renaudet, J.-L. Reymond, B. Richichi, J. Rojo, F. Sansone, C. Schäffer, W. B. Turnbull, T. Velasco-Torrijos, S. Vidal, S. Vincent, T. Wennekes, H. Zuilhof, A. Imberty, *Chem. Soc. Rev.* **2013**, *42*, 4709-4727; b) S. Cecioni, R. Lalor, B. Blanchard, J. P. Praly, A. Imberty, S. E. Matthews and S. Vidal, *Chem. Eur. J.* **2009**, *15*, 13232-13240; c) S. G. Guin, A. Wellens, J. Bouckaert and J. Kovensky, *ChemMedChem* **2009**, *4*, 749-755; d) M. Hartmann, P. Betz, Y. Sun, S. N. Gorb, T. K. Lindhorst and K. A., *Chem. Eur. J.* **2012**, *18*, 6485-6492; e) C. D. Heidecke and T. K. Lindhorst, *Chem. Eur. J.* **2007**, *13*, 9056-9067; f) R. Kikkeri, X. Y. Liu, A. Adibekian, Y. H. Tsai and P. H. Seeberger, *Chem. Commun.* **2010**, *46*, 2197-2199; g) I. Nierengarten, K. Buffet, M. Holler, S. P. Vincent and J.-F. Nierengarten, *Tetrahedron Lett.* **2013**, 2398-2402; h) C.-C. Lin, Y.-C. Yeh, C.-Y. Yang, C.-L. Chen, G.-F. Chen, C.-C. Chen and Y.-C. Wu, *J. Am. Chem. Soc.* **2002**, *124*, 3508-3509; i) O. Martinez-Avila, K. Hijazi, M. Marradi, C. Clavel, C. Champion, C. Kelly and S. Penadés, *Chem. Eur. J.* **2009**, *15*, 9874-9888; j) M. Reynolds, M. Marradi, A. Imberty, S. Penadés and S. Pérez, *Chem. Eur. J.* **2012**, *18*, 4264 – 4273; k) R. U. Kadam, M. Bergmann, M. Hurley, D. Garg, M. Cacciarini, M. A. Swiderska, C. Nativi, M. Sattler, A. R. Smyth, P. Williams, M. Camara, A. Stocker, T. Darbre and J.-L. Reymond, *Angew. Chem. Int. Ed.* **2011**, *50*, 10631 –10635.
- [11] a) J. Iehl, R. P. de Freitas, B. Delavaux-Nicot and J. F. Nierengarten, *Chem. Commun.* **2008**, 2450-2452; b) J. Iehl and J. F. Nierengarten, *Chem. Eur. J.* **2009**, *15*, 7306-7309.

- [12] J. F. Nierengarten, J. Iehl, V. Oerthel, M. Holler, B. M. Illescas, A. Munoz, N. Martin, J. Rojo, M. Sanchez-Navarro, S. Cecioni, S. Vidal, K. Buffet, M. Durka and S. P. Vincent, *Chem. Commun.* **2010**, *46*, 3860-3862.
- [13] M. Durka, K. Buffet, J. Iehl, M. Holler, J.-F. Nierengarten, J. Taganna, J. Bouckaert and S. P. Vincent, *Chem. Commun.* **2011**, *47*, 1321 - 1323.
- [14] M. Sanchez-Navarro, A. Munoz, B. M. Illescas, J. Rojo and N. Martin, *Chem. Eur. J.* **2011**, *17*, 766-769.
- [15] S. Cecioni, V. Oerthel, J. Iehl, M. Holler, D. Goyard, J. P. Praly, A. Imberty, J. F. Nierengarten and S. Vidal, *Chem. Eur. J.* **2011**, *17*, 3252-3261.
- [16] J. Luczkowiak, A. Muñoz, M. Sánchez-Navarro, R. Ribeiro-Viana, A. Ginieis, B. M. Illescas, N. Martín, R. Delgado and J. Rojo, *Biomacromolecules* **2013**, *14*, 431-437.
- [17] a) P. Compain, C. Decroocq, J. Iehl, M. Holler, D. Hazelard, T. M. Barragan, C. Ortiz Mellet and J. F. Nierengarten, *Angew. Chem. Int. Ed.* **2010**, *49*, 5753-5756; b) R. Riquez-Cuadro, J. M. García Fernández, J.-F. Nierengarten and C. Ortiz Mellet, *Chem. Eur. J.* **2013**, *19*, 16791-16803.
- [18] M. Durka, K. Buffet, J. Iehl, M. Holler, J.-F. Nierengarten and S. P. Vincent, *Chem. Eur. J.* **2012**, *18*, 641 - 651.
- [19] J. Diot, M. I. Garcia-Moreno, S. G. Gouin, C. Ortiz Mellet, K. Haupt and J. Kovensky, *Org. Biomol. Chem.* **2009**, *7*, 357-363 b) P. Compain and A. Bodlenner, *ChemBioChem* **2014**, *15*, 1239 - 1251; c) S. G. Gouin, *Chem. Eur. J.* **2014**, *20*, 11616 - 11628.
- [20] J. R. Govan, A. R. Brown and A. M. Jones, *Future Microbiol.* **2007**, *2*, 153-164.
- [21] E. Mitchell, C. Houles, D. Sudakevitz, M. Wimmerova, C. Gautier, S. Pérez, A. M. Wu, N. Gilboa-Garber and A. Imberty, *Nature Struct. Biol.* **2002**, *9*, 918-921.
- [22] C. Sabin, E. P. Mitchell, M. Pokorná, C. Gautier, J.-P. Utille, M. Wimmerová and A. Imberty, *FEBS Lett.* **2006**, *580*, 982-987.
- [23] a) N. Kostlanova, E. P. Mitchell, H. Lortat-Jacob, S. Oscarson, M. Lahmann, N. Gilboa-Garber, G. Chambat, M. Wimmerova and A. Imberty, *J. Biol. Chem.* **2005**, *280*, 27839-27849 b) J. Arnaud, K. Tröndle, J. Claudinon, A. Audfray, A. Varrot, W. Rçmer and A. Imberty, *Angew. Chem. Int. Ed.* **2014**, *53*, 9267-9270; c) J. Arnaud, J. Claudinon, K. Tröndle, M. Trovaslet, G. r. Larson, A. Thomas, A. Varrot, W. Römer, A. Imberty and A. Audfray, *ACS Chem. Biol.* **2013**, *8*, 1918-1924.
- [24] A. Audfray, J. Claudinon, S. Abounit, N. Ruvoën-Clouet, G. Larson, D. F. Smith, M. Wimmerová, J. Le Pendu, W. Römer, A. Varrot and A. Imberty, *J. Biol. Chem.* **2012**, *287*, 4335-4347.
- [25] a) J. Houser, J. Komarek, N. Kostlanova, G. Cioci, A. Varrot, S.C. Kerr, M. Lahmann, V. Balloy, J.V. Fahy, M. Chignard, A. Imberty and M. Wimmerova, *PLoS ONE* **2013**, *8*, e83077; b) A. Varrot, S. M. Basheer and A. Imberty, *Curr. Opin. Struct. Biol.* **2013**, *23*, 678-685.
- [26] a) B. Richichi, A. Imberty, E. Gillon, R. Bosco, I. Sutkeviciute, F. Fieschi and C. Nativi, *Org. Biomol. Chem.* **2013**, *11*, 4086-4094; b) C. Ligeour, A. Audfray, E. Gillon, A. Meyer, N. Galanos, S. Vidal, J. J. Vasseur, A. Imberty and F. Morvan, *RSC Advances* **2013**, *3*, 19515-19524.
- [27] a) M. Touaibia, A. Wellens, T. C. Shiao, Q. Wang, S. Sirois, J. Bouckaert and R. Roy, *ChemMedChem* **2007**, *2*, 1190-1201; b) M. Almant, V. Moreau, J. Kovensky, J. Bouckaert and S. G. Gouin, *Chem. Eur. J.* **2011**, *17*, 10029-10038.
- [28] T. K. Dam and C. F. Brewer, *Methods enzymol.* **2004**, *379*, 107-128.
- [29] S. Perret, C. Sabin, C. Dumon, M. Pokorná, C. Gautier, O. Galanina, S. Iliia, N. Bovin, M. Nicaise, M. Desmadril, N. Gilboa-Garber, M. Wimmerová, E. P. Mitchell and A. Imberty, *Biochem. J.* **2005**, *389*, 325-332.

- [30] E. M. V. Johansson, S. A. Crusz, E. Kolomiets, L. Buts, R. U. Kadam, M. Cacciarini, K.-M. Bartels, S. P. Diggle, M. Camara, P. Williams, R. Loris, C. Nativi, F. Rosenau, K.-E. Jaeger, T. Darbre and J.-L. Reymond, *Chemistry & Biology*, **2008**, *15*, 1249–1257.
- [31] B. Richichi, A. Imberty, E. Gillon, R. Bosco, I. Sutkeviciute, F. Fieschi and C. Nativi, *Org. Biomol. Chem.* **2013**, *11*, 4086-4094.
- [32] S. Cecioni, J.-P. Praly, S. E. Matthews, M. Wimmerova, A. Imberty and S. Vidal, *Chem. Eur. J.* **2012**, *18*, 6250 – 6263.