Organic & Biomolecular Chemistry

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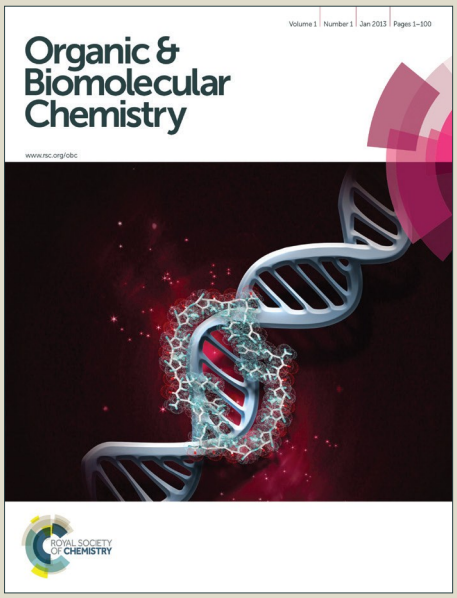

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Pentavalent pillar[5]arene-based glycoclusters and their multivalent binding to pathogenic bacterial lectins

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Anti-adhesive glycoclusters offer potential as therapeutic alternatives to classical antibiotics in treating infections. Pillar[5]arenes functionalised with either five galactose or five fucose residues were readily prepared using CuAAC reactions and evaluated for their binding to three therapeutically relevant bacterial lectins: LecA and Lec B from *Pseudomonas aeuruginosa* and BambL from *Burkholderia ambifaria*. Steric interactions were demonstrated to be a key factor in achieving good binding to LecA with more flexible galactose glycoclusters showing enhanced activity. In contrast binding to the fucose-selective lectins confirmed the importance of topology of the glycoclusters for activity with the pillar[5]arene ligand proving a selective ligand for BambL.

Introduction

Pillararenes,[1-5] the newest members of the calixarene family, have, since their introduction in 2008,[6] been rapidly developed for a wide range of applications including sensors, rotaxane wheels and supramolecular polymers.[1-5] In contrast to calix[4]arene and resorcin[4]arene, pillar[5]arene offers a highly rigid and symmetrical structure with two identical faces for functionalisation, and thus higher valency on a small platform, and is inherently chiral. Synthetic procedures have been developed for the preparation of both fully and partially functionalised pillar[5]arenes. In particular, use of non-symmetrically functionalised alkoxybenzene derivatives in the condensation reaction provides platforms featuring five regularly distributed points of functionalisation[7] whereas further selective functionalisation can be achieved using statistical mixtures[8, 9] in the condensation and through post cyclisation approaches.

The biological property of multivalency has attracted significant attention for the development of cluster molecules capable of high-affinity binding, based on a wide range of topologically different scaffolds.[10-12] One particular area of interest that has recently come to the fore is the development of anti-adhesive molecules against pathogen infections. Glycoclusters capable of disrupting bacterial-to-human cell interactions and formation of bacteria biofilms through interaction with bacterial lectins have been designed.[13-22] We have previously developed a topologically diverse series of such glycoclusters based on calix[4]-[23, 24] and calix[6]arenes,[25] resorcin[4]arenes,[26] fullerenes,[27, 28] and porphyrins.[25, 29-32] The calix[4]arene-based glycoclusters have been demonstrated to alter the lectin-mediated infectivity of *Pseudomonas aeruginosa* in lungs.[23]

P. aeruginosa is an opportunistic pathogen, with emerging drug resistance, responsible for severe lung infections in immune-compromised patients.[33] Two major soluble lectins involved in infectivity have been identified; the galactose specific LecA[34] and LecB,[35, 36] which binds to fucose and fucose-containing oligosaccharides, particularly Lewis a trisaccharide. Whilst LecA has been implicated in damage to the alveolae and uptake of the bacteria into cells,[37, 38] LecB has been demonstrated to be involved in cell adhesion[15] making both lectins ideal targets for therapeutic intervention.

In contrast to the tetrameric LecB, the fucose selective lectin BambL[39] from *Burkholderia ambifaria* self assembles into a trimeric β-propeller fold presenting six binding sites. *B. ambifaria*, a gram negative organism, is a component of the *Burkholderia cepacia* complex (BCC) which is responsible for both acute and chronic respiratory disease and, in some cases, cepacia syndrome particularly in patients with cystic fibrosis.[40] Although, the role of BambL in infection has not been fully characterised, it has been shown to bind to blood group associated oligosacacharides[39] and structurally similar lectins[41] have been implicated in inflammation in epithelial cells making it an interesting target for multivalent glycoclusters. Additionally, comparison of binding to tetrameric LecB versus hexameric BambL provides fundamental insights into the role of topology of glycoclusters in achieving selective lectin binding.

The potential of pillar[5]arene scaffolds for the development of antimicrobial glycoclusters was first shown using a deca-mannose functionalised pillar[5]arene which was able to inhibit adhesion of *Escherichia coli* to red blood cells through interaction with the FimH adhesin.[42] Similarly, pillar[5]arene-based glycoclusters, featuring five galactose moieties, facilitated agglutination of *E. coli*, although the specific lectin interactions were not identified.[43] The potential to use pillar[5]arene based rotaxanes to simultaneously provide inhibition of both LecA and LecB has also been described. [2]Rotaxanes featuring *bis*-fucosylated stoppers combined with deca-galactosylated wheels[44] provided the ideal arrangement for good binding with LecB,

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through an aggregative interaction and LecA, through clustering.

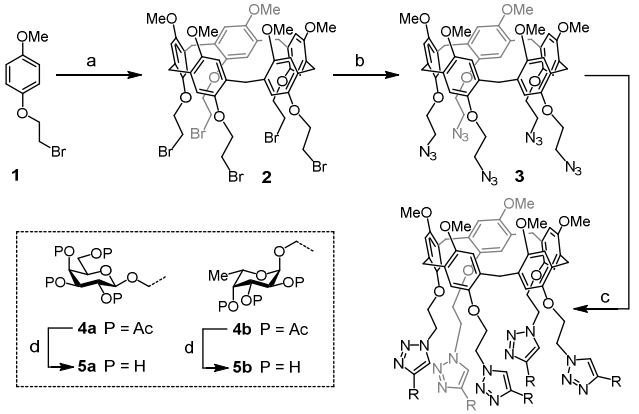
More recently, we have reported the development of decavalent pillar[5]arenes, featuring either galactose or fucose epitopes, and evaluated binding against LecA, LecB and BambL.[45] In this series, increased valency resulted in higher affinity as did increasing flexibility of the sugar linkers. The present study describes the synthesis and characterisation of pentavalent pillar[5]arenes functionalised with either galactose or fucose and investigates their binding to LecA, LecB and BambL. The use of a combination of biophysical techniques, including hemagglutination inhibition assay (HIA), enzyme-linked lectin assay (ELLA), isothermal titration microcalorimetry (ITC) and surface plasmon resonance (SPR) to determine the binding interactions is also reported.

Results and Discussion

Synthesis of glycoclusters

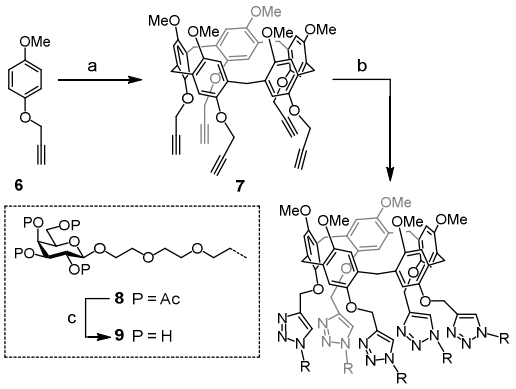
Building on our previous experience[24-28, 46, 47] in the development of glycoclusters using Cu(I)-assisted azide-alkyne cycloaddition (CuAAC),[48] the synthetic route focused on the preparation of pillar[5]arenes bearing either five azido or five alkynyl substituents for subsequent reaction with the corresponding alkynyl or azido carbohydrate derivatives.

In both cases functionalisation was introduced prior to cyclisation by using non-symmetrical alkoxybenzenes. The penta-azido pillar[5]arene **3** was accessed through the preparation of the penta-bromo derivative **2.** BF_3•OEt_2 mediated macrocyclization of the bromo-precursor **1** with para-formaldehyde gave pillar[5]arene **2** (Scheme 1). Subsequent azidation readily provided **3** suitable for CuAAC conjugation with the previously reported[45] propargyl galactoside and fucoside affording the desired acetylated glycoclusters **4a-b.** Solvolysis of the acetates provided the water soluble glycoclusters **5a-b** for binding assays.



Scheme 1. Synthesis of pentavalent pillar[5]arene-based glycoclusters using a penta-azido core scaffold. *Reagents and conditions*: (a) paraformaldehyde, BF_3•OEt_2, $Cl(CH_2)_2Cl$, r.t., 84%; (b) NaN_3, DMF, 100°C, 12 h, 91%; (c) propargyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside or propargyl 2,3,4-tri-*O*-acetyl-α-D-fucopyranoside, CuI, *i*Pr_2NEt, DMF, 110°C, 15 min, μwaves, 87% (**4a**), 93% (**4b**); (d) MeOH, Et_3N, H_2O, r.t., 16 h, >95% (**5a** and **5b**).

More conveniently, the penta-alkynyl pillar[5]arene scaffold **7** was obtained directly from its precursor **6** and paraformaldehyde (Scheme 2).[49] Conjugation with the azido-functionalized triethyleneglycol galactoside afforded the desired acetylated glycocluster **8**. Unmasking of the protecting groups provided the desired galactose glycocluster **9**.



Scheme 2. Synthesis of a galactosylated pentavalent pillar[5]arene-based glycocluster using a penta-propargylated core scaffold. *Reagents and conditions*: (a) paraformaldehyde, BF_3•OEt_2, $Cl(CH_2)_2Cl$, r.t., 50%; (b) 1-azido-3,6-dioxa-octyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside, $CuSO_4$, sodium ascorbate, DMF, 110°C, 15 min, μwaves, 76%; (c) MeOH, Et_3N, H_2O, r.t., 16 h, >95%.

It is important to note that the chirality induced by the pillar[5]arene ring system[50] results in the formation of diastereoisomers upon conjugation with carbohydrates.[42] It has previously been reported[51] that separation of non-symmetrical pillar[5]arenes can be achieved, but only when there are substantial differences between the two substituents. Separation of **7** has not proved possible[52] although the diastereoisomers of a specific click conjugate have been isolated. Not surprisingly, the separation of the individual diastereoisomers of **5** and **9,** bearing a methyl group**,** was not successful even after careful column chromatography. Although these isomeric mixtures could affect the interpretation of the binding studies towards lectins, the spatial arrangements of the carbohydrate epitopes for pentavalent scaffolds will not alter greatly the general presentation of the binding partners to the lectins.

In addition to the two enantiomers generated during the macrocyclization process, several conformers can also arise from the rotation of each arene ring (Figure 1).[7] The formation of the pillar[5]arene macrocycle provides a mixture of 4 possible constitutional isomers $\mathbf{A_{1\text{-}4}}$ (Figure 1, top). These four isomers can rotate each of their arene moieties to provide a series of eight rotational isomers (e.g. $\mathbf{A_1}$ to $\mathbf{F_1}$, Figure 1, bottom). The conformer $\mathbf{A_1}$ can have one aromatic ring rotated by 180° to generate the conformer $\mathbf{B_1}$. Further rotations of aromatic moieties toward the right hand side provide a series of rotational conformers $\mathbf{C_1}$, $\mathbf{D_1}$, $\mathbf{E_1}$ and finally $\mathbf{F_1}$. When considering conformers $\mathbf{A_1}$ and $\mathbf{F_1}$, they are mirror images of each other and therefore enantiomers, similarly for $\mathbf{B_1}$ and $\mathbf{E_1}$ and also $\mathbf{C_1}$ and $\mathbf{D_1}$. These rotations are possible due to the small size of the methoxy substituents in an in-cavity rotation. Several other conformations can also be adopted but are not depicted. These occur from rotation at non-contiguous aromatic moieties and the conformers presented in Figure 1 illustrate the most simple and step-by-step process. Hence, variable temperature NMR experiments can provide an insight on such isomeric mixtures.

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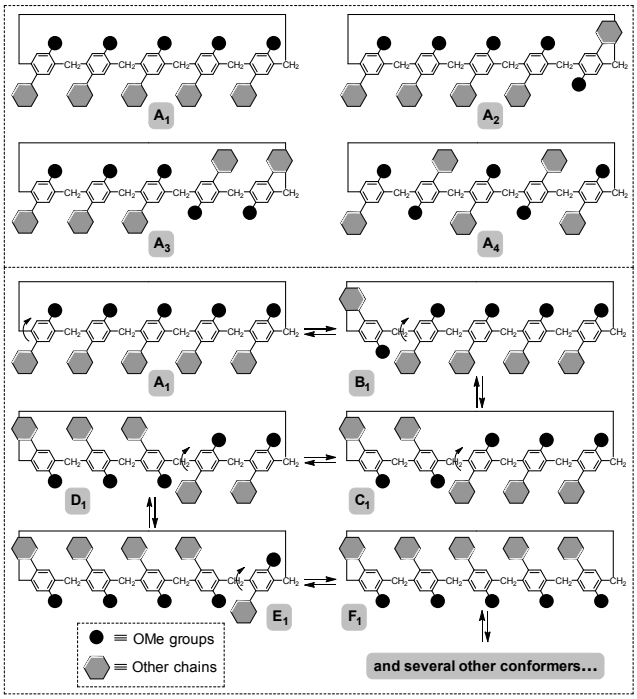

Figure 1. Schematic diagram of the potential isomers that can be formed during the macrocyclization.

This conformational isomerism could be easily observed in both ^{1}H and ^{13}C NMR (Figures 2, S1 and S2). Several peaks appeared as collections of singlets showing the conformer population in solution indicating that interconversion was slower than the NMR timescale. A slight dependence on temperature was observed since heating from 20 to 100°C accelerated the equilibrium and the NMR spectra displayed sharper peaks both in the ^{1}H and ^{13}C NMR data (Figures 2, S1 and S2).

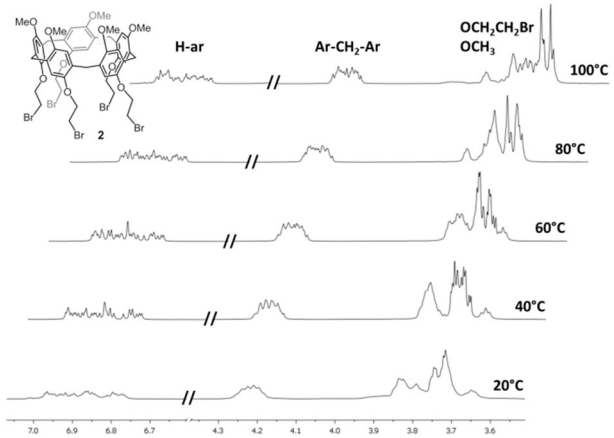

Figure 2. Partial ^{1}H NMR data (400 MHz, DMSO-d_6) measured for the brominated pillar[5]arene **2**.

Binding studies

The binding properties of glycoclusters **5a** and **9** with LecA were evaluated using four complementary techniques; hemagglutination inhibition assay (HIA), enzyme-linked lectin assay (ELLA), isothermal titration microcalorimetry (ITC) and surface plasmon resonance (SPR). In contrast ITC was chosen as the single technique for evaluation of the dependence of binding of glycocluster **5b** to LecB and BambL, giving access to the stoichiometry of the binding partners for each multimeric complex formed. In addition, two decavalent pillar[5]arene-based glycoclusters **PillarGal$_{10}$** and **PillarFuc$_{10}$** were also included in the study for comparison (Figure 3).

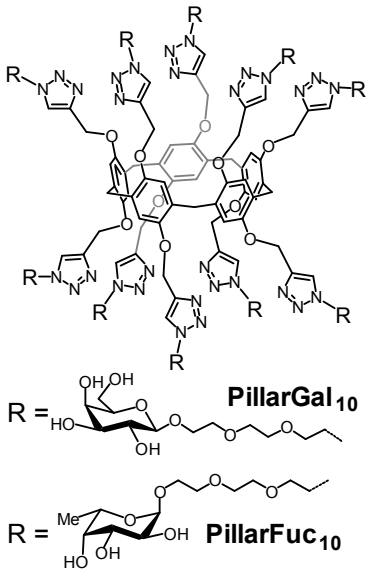

Figure 3. Structures of the galactosylated (**PillarGal$_{10}$**) and fucosylated (**PillarFuc$_{10}$**) decavalent pillar[5]arene-based glycoclusters.

LecA Binding studies

Two galactosylated glycoclusters (**5a** and **9**) were evaluated for their ability to bind to LecA (Table 1) and compared with the previously described PillarGal$_{10}$. The initial HIA study highlighted efficient binding of these multivalent ligands to LecA which was confirmed in ELLA studies. Both new ligands showed moderate improvement (**5a** β = 23, **9** β = 73) in binding based on their multivalency. The significantly enhanced activity of **9** could be ascribed to the longer triethyleneglycol linker arms in this conjugate providing greater flexibility for binding of the galactose moieties to the lectin.

ITC was used to gain an insight into the thermodynamic parameters governing the binding to LecA and also the stoichiometry of binding. The same enhancement in binding was observed for the more flexible glycocluster **9** over **5a** although both ligands gave K_d values in the sub-micromolar range.

The stoichiometry (N) for the complex generated between the lectin and the glycocluster also varied depending on the linker. Thus compound **5a** interacted with two or three LecA monomers (N = 0.40), while, in contrast, **9** bound to five LecA monomers (N = 0.17) indicating that all the carbohydrate epitopes are engaged. This variation is probably a consequence of the longer triethyleneglycol linker arm allowing reduced steric hindrance in the binding of multiple LecA monomers.

SPR experiments using LecA attached on the chip were also undertaken and affinity was evaluated using a thermodynamic approach. Typical curves were observed as the glycoclusters **5a** and **9** were circulated. The SPR and ITC affinities were in the same range demonstrating that the immobilization of LecA on the

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chip surface did not modify the binding to multivalent ligands. As with the other techniques, enhancement of binding was again seen for the more flexible **9** over **5a** in SPR studies reinforcing the importance of flexibility to LecA binding.

It is interesting to compare the results seen here with those of our previously reported decavalent pillar[5]arenes.[45] Whilst it might be expected that increasing the valency would enhance binding further, in the case of glycocluster **9** in comparison to its analogous deca-functionalized pillar[5]arene[45] (**PillarGal$_{10}$**) this is not the case in both HIA and ELLA studies and only a moderate improvement is seen in ITC. . The stoichiometry observed in ITC may provide a rationale for this as despite ten groups being available for binding, only five LecA tetramers interact with the glycoclusters and thus no advantage of the further five available carbohydrate epitopes is seen.

Table 1. HIA, ELLA ITC and SPR measurements for binding of pillar[5]arene-based glycoclusters to **LecA**

Compound	HIA	ELLA		ITC					SPR
	MIC[a] (μM)	IC_{50} (μM)	β[b]	N[c]	$-\Delta H$ (kJ.mol^{-1})	$-T\Delta S$ (kJ.mol^{-1})	K_d (nM)	β[b]	K_d th (nM)
β-GalOMe[d]	6250	658	1	0.8	39	15	70 000	1	n.m.[e,f]
5a	10	29	23	0.40±0.02	82±3	47.4	931±30	75	508
9	2	9	73	0.17±0.01	174±9	138	586±38	119	337
PillarGal$_{10}$[g]	5	218	3	0.20±0.01	115±2	78	366±129	191	47

[a] Minimum inhibitory concentration (MIC) required to inhibit the agglutination of erythrocytes. [b] Calculated using methyl β-D-galactopyranoside (β-GalOMe) as monovalent reference. [c] Stoichiometry of binding defined as the number of glycoclusters per monomer of LecA. [d] Data from previous report.[47] [e] n.m. = Not measured. [f] RU shift was too weak due to the low molecular weight of β-GalOMe. [g] Data from previous report.[45]

LecB and BambL Binding studies

The fucosylated glycocluster **5b** was evaluated for its potential as a LecB and BambL ligand by ITC (Table 2) and showed considerable selectivity between the two topologically different fucose binding lectins, with high affinity only towards BambL. The millimolar K_d value for LecB was disappointing compared to the monosaccharide ligand and to our previously reported calix[4]arene derivatives with no enhancement achieved through presentation on a multivalent scaffold.[11] The stoichiometry (N) of the lectin-glycocluster complex indicated three LecB monomers bound to the pentavalent ligand **5b** (N = 0.29) but clearly the orientation of the carbohydrate epitopes is not favorable and resulted in a loss of affinity at each site. The importance of ligand orientation is further demonstrated through comparison with **PillaFuc$_{10}$** which is able to interact with additional binding sites and shows enhanced binding and a positive multivalent effect.

In contrast, **5b** proved to be an excellent ligand for BambL (Table 2) surpassing previously reported glycoclusters based on mannose and phosphodiester cores[53] and the higher valency pillar[5]arene derivatives including **PillarFuc$_{10}$**.[45] Interestingly the β value of 45 for the pentavalent ligand **5b** with a stoichiometry (N = 0.71) indicated that only a fraction of the five carbohydrate epitopes were used in the binding and that one or two lectin monomers are bound.

Table 2. ITC measurements for binding of pillar[5]arene-based glycoclusters to **LecB** and **BambL**

Compound	Lectin	N[b]	$-\Delta H$ (kJ.mol^{-1})	$-T\Delta S$ (kJ.mol^{-1})	K_d (nM)	β[a]
α-FucOMe[c]	LecB	0.77±0.03[c]	41±1	5[c]	430±10	1
5b	LecB	0.29±0.01	106±19	73	1402±45	0.3
PillarFuc$_{10}$[d]	LecB	0.13±0.01	250±2	213	280±33	1.5
α-FucOMe[e]	BambL	2.02	48±2	14	960±30	1
5b	BambL	0.71±0.01	79±3	35	21±7	45
PillarFuc$_{10}$[d]	BambL	0.35±0.02	201±5	160	57±16	17

[a] Calculated using monovalent methyl α-L-fucopyranoside (α-FucOMe) as reference. [b] Stoichiometry of binding defined as the number of glycoclusters per monomer of LecB. [c] Data from previous report.[54] [d] Data from previous report.[45] [e] Data from previous report.[39]

Conclusions

The potential of multivalent anti-adhesive drugs as therapeutics for drug-resistant bacterial infections in immune-compromised patients is an area of considerable interest. Here we have shown that the pillar[5]arene scaffold, when non-symmetrically functionalised to present five glycosides, acts as an excellent platform for interaction with bacterial lectins. Pillar[5]arenes were prepared bearing either five alkynyl or azido functional groups and glycoclusters featuring either galactose or fucose moieties were readily produced using CuAAC. Binding of galactose functionalised pillar[5]arenes to LecA from *P. aeruginosa* was shown to be heavily dependent on linker length between the core and carbohydrate and that this also plays a role

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in stoichiometry, with the best ligand demonstrating the ability to interact simultaneously with five separate LecA tetramers. Considerable variation in binding of the fucosylated glycoclusters to the two fucose specific lectins LecB from *P. aeruginosa* and BambL from *B. ambifaria* was observed. This glycocluster proved to be an excellent ligand for BambL with low nanomolar affinity whereas no improvement in binding compared to a monovalent ligand was observed for LecB. This result demonstrates the importance of lectin topology in analysing binding selectivity.

An interesting aspect of these glycoclusters is their stereochemistry as each was obtained as a 1:1 diastereoisomer mixture due to the cyclisation of non-symmetric arenes. Some selectivity in binding may be observed between the diastereoisomers and thus the data presented here can be considered to be an average result. Our further studies will focus on the preparation of pillar[5]arene derivatives which can be chromatographically separated in order to determine whether the chirality of the glycocluster affects binding strength and selectivity.

Acknowledgments

The authors thank the Université Claude Bernard Lyon 1 and the CNRS for financial support. This project was also supported by COST Action CM-1102 MultiGlycoNano. A.I. acknowledges support from GDR Pseudomonas and Labex ARCANE (ANR-11-LABX-003). Dr F. Albrieux, C. Duchamp and N. Henriques are gratefully acknowledged for mass spectrometry analyses. N.G. thanks the Région Rhône-Alpes (Cluster de Recherche Chimie) for additional funding and the Royal Society for funding work undertaken in S.E.M.'s laboratory (JP090449). Prof J.-F. Nierengarten (Université de Strasbourg and CNRS) is gratefully acknowledged for helpful and critical discussions of the chirality of pillar[5]arenes.

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† Electronic Supplementary Information (ESI) available: Synthesis and characterization of compounds, SPR and ITC data. See DOI: 10.1039/b000000x/

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DOI: 10.1039/C6OB00220J

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