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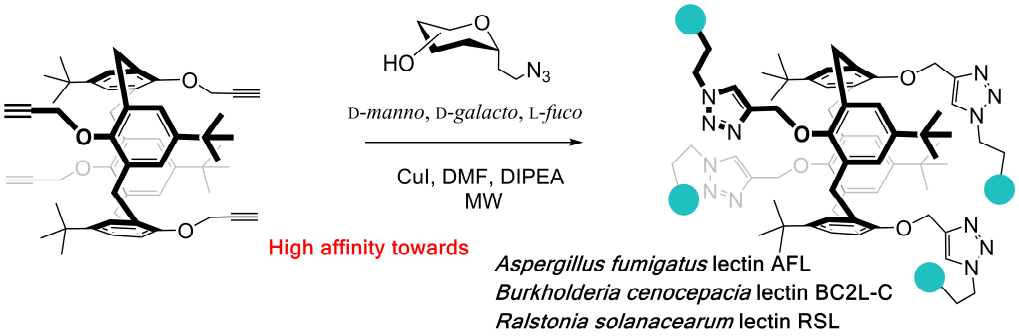

Selectivity of Original *C*-Hexopyranosyl Calix[4]arene Conjugates towards Lectins of Different Origin

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Abstract

As a part of ongoing activities towards the design of ligands against pathogenic lectins, a synthesis of original α-*C*-galacto/α-*C*-manno/α-*C*-fucopyranosyl glycomimetics based on a calix[4]arene scaffold and their binding evaluation is described. The interactions of the glycomimetics with seven lectins of various origins were carried out using agglutination inhibition assays. The 1,3-*alternate* tetra-*C*-fucosylated ligand and its derivative having a *tert*Bu group at the upper rim of the calix[4]arene scaffold were the most potent towards the AAL lectin family (RSL, AFL, AAL, AOL) and BC2L-C. As AFL and RSL originate from important human (*Aspergillus fumigatus*) and plant (*Ralstonia solanacearum*) pathogens, the inhibition potency of both leading structures was assessed by surface plasmon resonance. With AFL, both structures exhibited an approximately three orders of magnitude increase in affinity compared to the reference L-fucose. The role of *tert*Bu groups as "aglycon-assisted" events was illustrated by NMR. Furthermore, both compounds showed significant increased ability to inhibit BC2L-C (from human pathogen *Burkholderia cenocepacia*) cell agglutination and were able to cross-link whole *B. cenocepacia* cells. Although the ligands failed to significantly inhibit the agglutination activity of LecA and LecB from *Pseudomonas aeruginosa*, tetra-*C*-galactosylated calix[4]arene with *tert*Bu groups at the upper rim of the 1,3-*alternate* conformation inhibited *P. aeruginosa* biofilm formation efficiently. This systematic and comprehensive study highlights the fact that hydrolytically stable polyvalent *C*-glycomimetics should be regarded as potent and selective ligands capable of acting as antiadhesive agents.

Keywords: glycomimetics; lectin; *C*-glycosides; polyvalency; calix[4]arene.

1. Introduction

To date, considerable evidence has been accumulated showing that the recognition at the cell level is accomplished by the interaction of carbohydrate-binding proteins, e.g. lectins, on the surface of one type of cell with complementary sugars incorporated into glycoconjugates present on the surface of another cell. Lectins from the pathogens could be important virulence factors and therefore they are also a suitable therapeutic target [1]. Lectins are multivalent proteins frequently displaying an avidity effect, resulting in significantly increased affinity towards their ligands. Consequently, the synthesis of multivalent glycosylated constructs and their affinity to lectins have been extensively studied over the past decade [2].

In the synthesis of complex glycomolecules with controlled topology and valency, one of the major considerations is the choice of appropriate scaffolds. Recently, there has been a noticeable growth in interest in using calix[n]arene scaffolds [3]. It appears clear that the easy availability in particular of calix[4]arene, the possibility of controlling their conformational properties as well as topological presentation of the epitope, and the possibility of modifications at the lower and/or upper rim make them attractive candidates for polyvalent neoglycoconjugate synthesis. Moreover, calix[4]arenes are non-toxic and exhibit good stability. The only limitation is the low solubility of some glycosylated calix[n]arenes in water that can be dependent on their conformation [3e]. The NMR study of tetragalactosyl calix[4]arene in a *cone* conformation indicated a tendency for the aggregation of these amphiphilic substances in water [4]. Another important consideration in the synthesis of polyvalent structures is an appropriate selection of a linker between the sugar moiety and calixarene scaffold, as the simultaneous interactions of a multivalent ligand to several binding sites of a multivalent receptor has been proven to have a strong impact. Even when the

topology and the distance between the binding sites are known, the design of a matching multivalent ligand does not always meet expectations [5]. The flexibility of a polyvalent structure can be also influenced by the synthetic approach used for the ligation of the sugar units. In this respect, a Huisgen cycloaddition reaction, later introduced as Cu(I) catalyzed azide−alkyne cycloaddition (CuAAC) by Sharpless and Meldal [6], seems to occupy a central position [7].

The affinity of neoglycoconjugates based on a calix[4]arene scaffold can be illustrated by several examples from the literature. Isomeric galactosyl calix[4]arenes were designed as potential antiadhesive drugs of the bacterium *Pseudomonas aeruginosa* [8]. The 1,3-*alternate* tetravalent glycocluster was identified as the best inhibitor of the LecA (PA-IL) lectin with a 800-fold increase in affinity compared to a monovalent galactoside as assessed by isothermal titration calorimetry (ITC). Subsequently, an aggregative chelate binding mode was evidenced using atomic force microscopy [9]. The use of these neoglycoconjugates for pharmaceutical compositions was patented [10]. Calix[4]arene-based glycoclusters with four terminal β-D-galactopyranosyl units were further examined as ligands for LecA using surface plasmon resonance (SPR). The best affinity in terms of IC_{50} was found in the range 0.5 - 1.2 μM for the 1,3-*alternate* conformation depending on a linker [11].

The overwhelming majority of polyvalent ligands described so far are based on the connection of sugar units with a linker through a *O*-glycosidic bond [2,3]. Generally, the *O*-glycosidic bonds are unstable to mild acid and to glycosidase enzymes *in vivo*, thus this fact can seriously limit their biomedical applications. *C*-glycosides are attractive mimetics due to their potential stability towards acids and hydrolytic enzymes. Although the first C-glycosyl clustering on a calix[4]arene scaffold was reported in 2002 [12] and a 1,2,3-triazole linker was introduced in 2006 [13], the synthesis and/or biological assessment of any other polyvalent *C*-glycopyranosyl constructs has rarely been published so far. A series of

multivalent β-*C*-galactopyranosyl mimetics was synthesized and tested towards LecA [14]. The most potent nonavalent construct exhibited dissociation constants below 500 nM, corresponding to a 400-fold increase in affinity compared to the methyl β-D-galactopyranoside using ITC.

Based on the results from the literature [14] and our own findings [15], we introduce here original polyvalent calix[4]arene-based *C*-glycomimetics with different three-dimensional shapes. The readily prepared alkyne functionalized calix[4]arenes, being tuneable platforms, were coupled with 2-(α-hexopyranosyl)ethylazide with D-*manno*, D-*galacto*, and L-*fuco* configurations via CuAAC cycloaddition. To explore whether these constructs are adequate candidates for the interaction with lectins, we decided to perform a broad initial screening using an agglutination inhibition test. The collection of lectins consisted of BC2L-C (*Burkholderia cenocepacia*), LecA (PA-IL) and LecB (PA-IIL) (*P. aeruginosa*), RSL (*Ralstonia solanacearum*), AFL (*Aspergillus fumigatus*), AOL (*Aspergillus oryzae*), and AAL (*Aleuria aurantia*). The most promising constructs were subjected to surface plasmon resonance evaluation, *in vivo* cross-linking tests with whole cells, and their ability to inhibit biofilm formation was assessed. This approach allowed us to obtain a deep insight into the selectivity between lectins and to simultaneously identify promising structures for follow-up optimization.

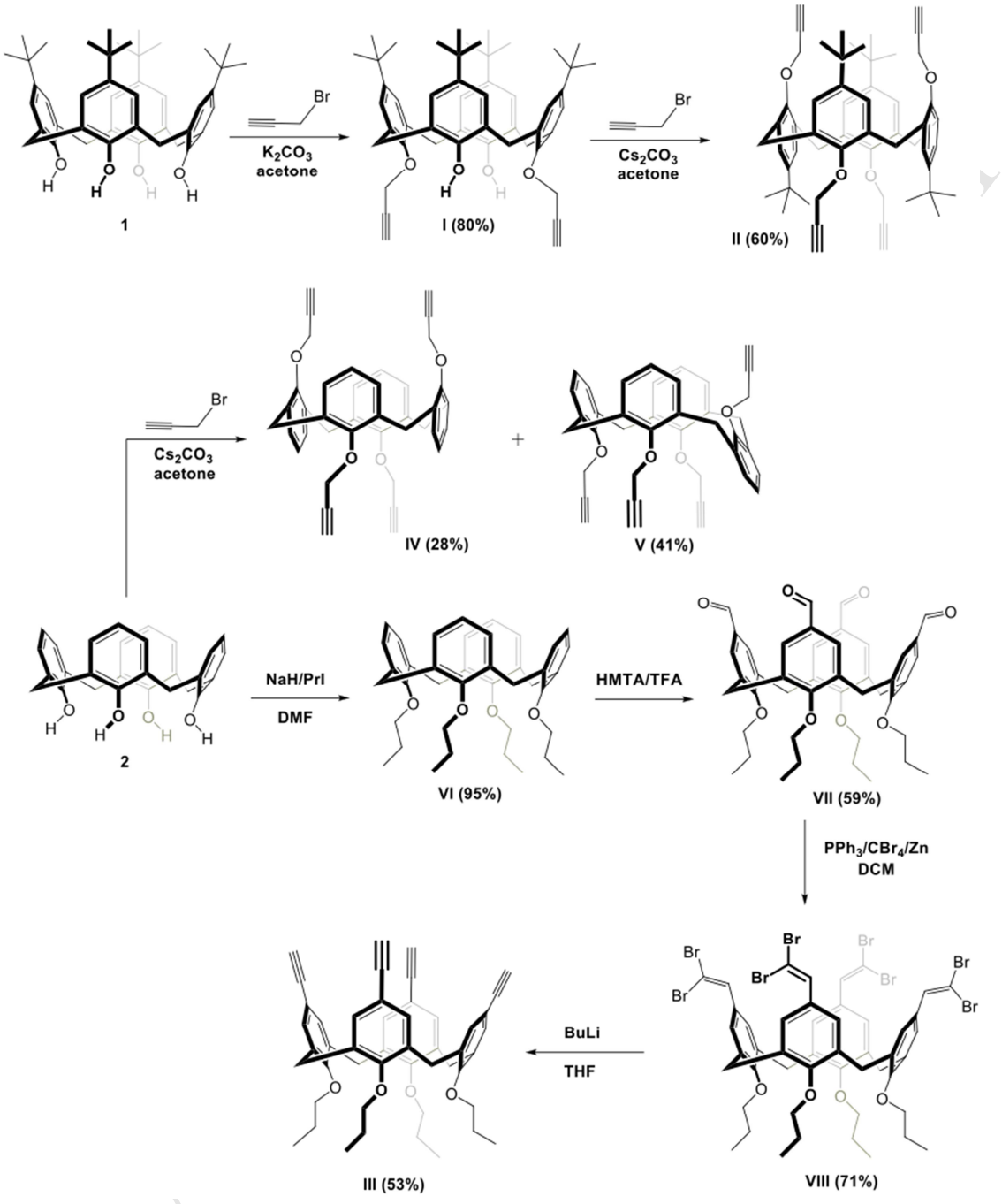
2. Results and Discussion

2.1. Synthesis of calix[4]arene polyvalent glycomimetics

The title glycomimetics were synthesized by a highly efficient copper-catalyzed azide-alkyne cycloaddition (CuAAC) protocol developed previously [15]. Our strategy required the preparation of alkynyl calix[4]arene **I**, **II**, **III**, and **IV** (**Scheme 1**) and 2-(α-D-galactopyranosyl)ethylazide (**A**), 2-(α-D-mannopyranosyl)ethylazide (**B**), and 2-(α-L-

fucopyranosyl)ethylazide (**C**). The synthesis of calix[4]arenes **I**, **II**, and **IV** was carried out using a modified version of reported procedures. Thus, the slow addition of propargyl bromide (3.5 eq.) to a stirred solution of starting *tert*-butylcalix[4]arene **1** (1 eq.) and K_2CO_3 (2.4 eq.) in acetone at room temperature, and subsequent reflux of the reaction mixture overnight gave disubstituted compound **I** in an 80% yield [16]. Tetrapropargyl derivative **II** was obtained by the alkylation of **I** with propargyl bromide (3.5 eq.) in acetone under reflux, this time using Cs_2CO_3 (3 eq.) as a base. Under these conditions, the corresponding 1,3-*alternate* conformer **II** was obtained in a 60% yield [17]. Analogous conditions (excess of propargyl bromide (16 eq.) and Cs_2CO_3 (20 eq.) in refluxing acetone) were used for the preparation of derivative **IV** starting from calix[4]arene **2**. The mixture of the *partial cone* and *1,3-alternate* conformations was separated using column chromatography on silica gel to give compound **IV** in a 28% yield, and the corresponding *partial cone* analogue **V** in a 41% yield [18]. The synthesis of the *cone* conformer **III** bearing four acetylene units on the upper rim started with the alkylation of basic calix[4]arene **2** with an excess of n-propyl iodide in DMF in the presence of sodium hydride (**Scheme 1**) [19]. Due to the template effect of the sodium cation, the *cone* product **VI** was obtained in an excellent yield (95%) after precipitation with methanol. Refluxing **VI** with hexamethylenetetramine in trifluoroacetic acid (Duff reaction) gave tetraformyl derivative **VII** in a good yield of 59% after precipitation from methanol [20]. This compound could be transformed into derivative **VIII** using the so-called Corey-Fuchs procedure. The reaction with 3 equiv. of dibromomethylene triphenylphosphorane, generated *in situ* from Zn, CBr_4 and PPh_3, led to compound **VIII** in a 71% yield. The reaction could be scaled up to 2.0 g of starting compound, as no chromatographic separation was necessary for the isolation of product **VIII**, which was simply obtained by recrystallization from a DCM/MeOH mixture. The bromoalkene **VIII** was further treated with n-BuLi (8 equiv.) to

give the ethynyl-substituted calix[4]arene **III** [21] in the *cone* conformation in a 53% yield after precipitation from methanol (**Scheme 1**).



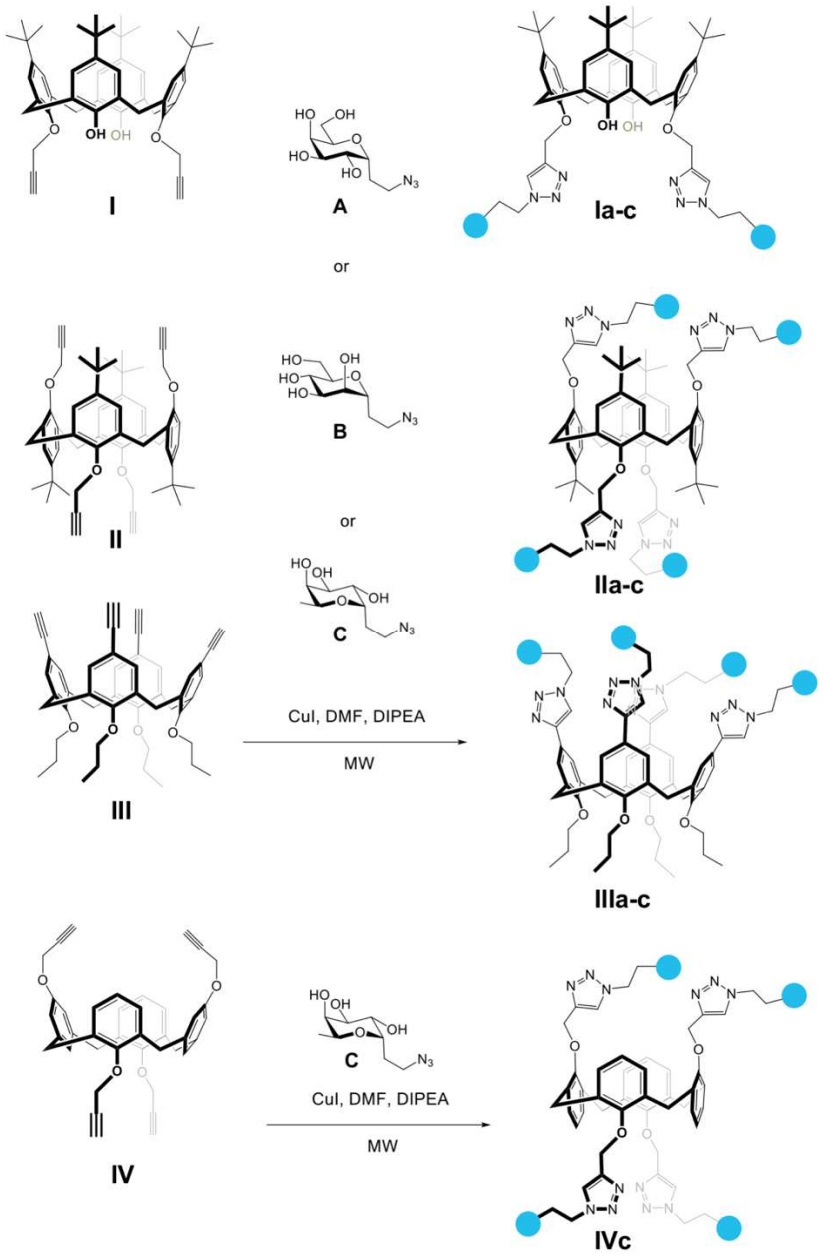
Scheme 1. Synthesis of propargyl calix[4]arene scaffolds

The conformation of calix[4]arenes was established using the ^{1}H NMR spectra of compounds. Starting compounds II and IV immobilised in the *1,3-alternate* conformation possessed a typical singlet of methylene bridges at around 3.50 - 3.70 ppm. The

corresponding *cone* conformer III exhibited typical doublets of the Ar-CH_2-Ar moiety at 4.37 ppm (*axial*) and 3.10 ppm (*equatorial*) with a geminal interaction constant 13.5 Hz.

The preparation of pyranosyl azides **A**, **B** and **C** has already been described. Briefly, galactosyl azide **A** [22] and mannosyl azide **B** [15] were prepared from the respective α-*C*-allyl glycoside [23] by one-pot ozonolysis and reduction [24] followed by azidation with phosphoryl azide [22] in an overall yield of 45%. Fucopyranosyl azide **C** was synthesized from L-fucose by a sequence of acetylation, *C*-allylation, ozonolysis and reduction in a one-pot arrangement, azidation and deacetylation in an overall yield of 23% [15].

The synthesis of all title glycomimetics followed an optimized procedure using CuI as the catalyst, *N*,*N*-diisopropylethylamine (DIPEA) as the base and *N*,*N*-dimethylformamide (DMF) as the solvent. Microwave irradiation was applied to accelerate the reaction rate (**Scheme 2**).



Scheme 2. Synthesis of glycomimetics **Ia-c, IIa-c**, **IIIa-c** and **IVc**

All final compounds were purified by preparative HPLC and characterized by mass spectrometry, optical rotation, ^{1}H and ^{13}C NMR spectrometry. The isolated yields varied from 46 to 77%. Interestingly, both ^{1}H and ^{13}C NMR spectra revealed a doubling of certain resonances as the result of the diastereotopicity, due to the presence of chiral sugar moieties at the linker. The fact that the diastereotopicity has been observed not only on methylene protons

but also on other protons and carbons of the molecule (e.g. cavity aromatic protons H3 and carbons C2 and C3 and bridge carbons) is unique.

Besides the glycomimetics depicted (**Scheme 2**), the synthesis of tetravalent glycomimetics with tetrapropargyloxy calix[4]arene in the 1,2-*alternate* and *cone* conformation derivatized at the lower rim was tried, but all attempts failed. Only inseparable mixtures of partially *C*-glycosylated calix[4]arene were obtained. Such failure is known from the literature and is attributed to the high density of alkyne which promotes the side reaction, the homocoupling of scaffolded alkynes [25]. Because the preliminary agglutination test indicated that L-fucosyl glycomimetic **IIc** could be a promising ligand, a pool of structures was completed with the additional ligand **IVc**.

2.2. Lectins

We chose a pool of economically and/or medically important lectins from bacteria and fungi that are selective for L-fucose or D-mannose or D-galactose.

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen causing lethal airway infections in cystic fibrosis and immunosuppressed patients [26]. *P. aeruginosa* produces two soluble lectins, LecA (PA-IL) and LecB (PA-IIL), specific for D-galactose and L-fucose, respectively [27]. The crystallographic structures of both lectins revealed a tetrameric arrangement with a requirement for calcium in the carbohydrate binding site [28]. It has been found that LecB exhibits a clear preference for the α-anomer; methyl α-L-fucopyranoside was 770 times more potent a ligand than the β-anomer [29]. Furthermore, LecB is considered to be L-fucose-specific but it can also bind D-mannose with high affinity [28c] .

Burkholderia cenocepacia is a Gram-negative bacterium and belongs to the *B. cepacia* complex containing at least 18 species [30]. *B. cenocepacia* causes lung infections in immunosuppressed patients, especially those suffering from cystic fibrosis [31]. Both of the above-mentioned pathogens often infect patients simultaneously [32]. Looking at *B. cenocepacia*, three soluble lectins were identified and designed as BC2L-A, -B, and -C. Each of them contains at least one domain with a strong sequence similarity to LecB from *P. aeruginosa* [27b]. Lectin BC2L-C has two distinct domains with unique selectivity [33]. The *N*-terminal domain is a novel TNF-α-like L-fucose binding domain, while the *C*-terminal part exhibits selectivity for D-mannose and L-*glycero*-D-*manno*-heptose in a calcium-dependent manner.

Another opportunistic human pathogen associated with cystic fibrosis is *Aspergillus fumigatus*, which is also responsible for invasive pulmonary aspergillosis in immunodeficient patients [34]. It produces a soluble L-fucose-specific lectin AFL, which crystallizes as a homodimer and contains six non-equivalent binding sites per monomer [35].

Aspergillus oryzae is a fungus widely used in the fermentation industry and its L-fucose-specific lectin AOL might be involved in *A. oryzae*-induced allergic responses [36]. The crystal structure of AOL was recently solved in a complex with seleno α- and β-fucosides [37]. There are six L-fucose-binding sites in the monomer of AOL, and each of them has a different carbohydrate binding affinity.

Ralstonia solanacearum is one of the most destructive phytopathogenic bacteria, causing great agricultural and economic losses in potato and tomato production [38]. Its L-fucose binding lectin RSL is believed to be responsible for the infection, although its function was not yet elucidated. However, it could be involved in the adhesion, possibly via binding to the terminal fucosides of xyloglucans [39]. RSL is a trimer with two almost identical sugar-

binding sites on each monomer [39]. RSL was found to interact with both the α- and the β-anomer of L-fucose as well as D-arabinose and L-galactose in the NMR study [40].

The orange peel fungus *Aleuria aurantia* possesses an L-fucose-specific lectin AAL that is widely used as a specific probe for L-fucose [41]. AAL has been described as a dimer in solution [42] and also in its crystal structure [43]. The crystal structure of the complex between AAL and L-fucose revealed five L-fucose residues bound per monomer [43a], while a crystal with a different space group contained only three L-fucose molecules per monomer [43b].

2.3. Agglutination tests

The *cone* calix[4]arene ligands **I** displaying only two sugar units at the lower rim were not soluble enough in water. Even their solution in 50% dimethyl sulfoxide (DMSO) in water had a tendency to precipitate. Glycomimetics **III** were soluble in 50% DMSO, while the 1,3-*alternate*-based glycomimetics **II** exhibited a higher water solubility and no aggregation activity. Glycomimetic **IVc** was fully soluble in water. The low solubility of calix[4]arene-based glycoclusters, in particular those in a cone conformation, was already cited in the literature [8,11,44].

Our initial screening was based purely on a simple and robust agglutination inhibition test, using red blood cells or yeast if the solubility of ligands was insufficient and DMSO had to be added. Microscopy was utilised as the detection method as described previously [45]. This method is quick and the consumption of lectins and inhibitors is significantly lower compared to classical hemagglutination in a microtiter plate. The lowest concentration of inhibitor able to completely inhibit hemagglutination/yeast agglutination was determined, and the potency of the inhibitors was calculated via comparison with a standard monosaccharide.

The inhibition potency of tetragalactosyl-*C*-calix[4]arene **IIa** towards lectin LecA was 16-times higher than that of D-galactose (Table 1), only a two times increase in inhibition potency of tetrafucosyl-*C*-calix[4]arene **IIc** towards LecB was observed. However, even the monovalent compound **C** showed twice higher MIC compared to L-fucose (Table 1) suggesting additional interactions with the lectin in comparison to free fucose. For D-mannose and LecB, inhibitory effect of **B** also increased two times. Unfortunately, the *tetra*mannosyl ligand **IIb** was not completely soluble in the buffer at the concentration necessary for the inhibition of LecB and precipitated. Compounds **IIIb** and **IIIc** were not directly soluble in the working buffer. To test the inhibitory potential of our ligands against the LecB lectin, they were dissolved in DMSO and MIC was determined via yeast agglutination in the buffer and in a 50% DMSO environment (Table 2). The corresponding control values were routinely determined under identical conditions so that the test system was able to answer the question of whether the sugar moieties in the calix[n]arenes are capable of acting as lectin inhibitors. As the concentration of inhibitors necessary for the yeast agglutination was lower than in the hemagglutination experiment, the affinity of ligand **IIb** was also evaluated in the buffer. The results revealed a low inhibition potency of all the tested compounds for LecB.

Table 1. Minimal inhibitory concentration (MIC) for inhibition of hemagglutination caused by LecA and LecB lectins (*P. aeruginosa*), and BC2L-C (*B. cenocepacia*) and potency of tested ligands (MIC of standard/MIC of ligand)

Lectin	Inhibitor	MIC (mM)	Potency	Lectin	Inhibitor	MIC (mM)	Potency
LecA	D-galactose[a]	6.25	1	LecB	D-mannose[a]	50	1
	A	3.125	2		**B**	25	2
	IIa	0.391	16		L-fucose[a]	0.781	1
BC2L-C	L-fucose[a]	50	1		**C**	0.391	2
	C	25	2		**IIc**	0.391	2
	IIc	0.195	256		**IVc**	0.391	2
	IVc	1.563	32				

[a]Standard

Table 2. MIC (minimal inhibitory concentration) values for inhibition of yeast agglutination caused by LecB and potency of tested ligands (MIC of standard/MIC of ligand)[a]

	Buffer		50% DMSO	
Inhibitor	MIC (mM)	Potency	MIC (mM)	Potency
D-mannose[b]	6.25	1	0.391 (0.195)[c]	1
B	3.125	2	0.391	1
IIb	1.5625	4	0.391	1
IIIb	n.d.[d]	n.d.	6.25 (1.5625)[e]	0.06 (0.25)[e]
L-fucose[b]	0.195	1	0.09766	1
C	0.09766	2	0.09766	1
IIc	0.195	1	0.195	0.5
IIIc	n.d.	n.d.	0.391	0.25

[a]Experiments were performed in the buffer (20 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5) and in the mixture buffer : DMSO (1 : 1). [b]Standard. [c]MIC was not clearly distinguishable. [d]MIC not detected due to solubility problems. [e]Observed agglutination was weak until concentration of **IIIb** dropped to 0.781 mM. The inhibitory effect at the higher concentration was therefore significant, but MIC was not clearly determined.

Based on the results, we focused exclusively on the affinity of fucopyranosyl calix[4]arene constructs **IIc** and **IVc**. Four homologous representatives of the fucose-specific AAL lectin family (AFL, RSL, AAL and AOL) were subjected to hemagglutination tests (Table 3). The construct **IIc** exhibited a sizeable affinity to all these fucose-binding lectins and was the best ligand for RSL compared to the standard. Moreover, the relative potency of **IIc** decreased in the order RSL>AFL~AAL>AOL. The affinity of **IVc** was very similar to that of **IIc**, giving a relative potency in the order RSL~AAL>AOL>AFL.

Table 3. MIC (minimal inhibitory concentration) values for inhibition of hemagglutination caused by AFL, RSL, AAL, and AOL lectins and potency (MIC of standard/MIC of ligand) of tested ligands **IIc** and **IVc**

Inhibitor	MIC (mM)	Potency	MIC (mM)	Potency
	Lectin AFL		Lectin RSL	
L-fucose[a]	12.5	1	1.5625	1
C	6.25	2	1.5625	1
L-fucose[a,b]	25	1	3.125	1
IIc	0.781	32	0.04883	64
L-fucose[a,b]	12.5	1	0.781	1
IVc	0.781	16	0.0122	64
	Lectin AAL		Lectin AOL	
L-fucose[a]	25	1	12.5	1
C	6.25	4	6.25	2
IIc	0.781	32	0.781	16
IVc	0.391	64	0.391	32

[a]Standard. [b]New batch of lectins was used.

Finally, the affinity of **IIc** and **IVc** towards the lectin BC2L-C (*B. cenocepacia*) was also established in the hemagglutination test. An MIC value of 50 mM was determined for L-fucose, whilst 0.195 mM was the value found for **IIc,** resulting in a 256-fold affinity increase in inhibitory potency. An MIC value of 1.563 mM was found for **IVc,** which corresponds to a 32-fold increase in inhibitory potency.

2.4. Surface plasmon resonance

As AFL and RSL originate from important human (*A. fumigatus*) and plant (*R. solanacearum*) pathogens, their interactions with calix[4]arene tetravalent glycomimetics **IIc** and **IVc** were further examined in detail by SPR inhibition assay using immobilized α-L-fucopyranoside (Figure 1, Table 4). The SPR inhibition assay can be considered to be a better model for lectin binding than agglutination tests, due to the fact that the binding to a complex glycan surface in the flow-through arrangement mimics interactions with host tissues. The binding potency of **IIc** and **IVc** for AFL was approximately three orders of magnitude higher than that of L-fucose. With RSL, the increase in the potency was much lower. This observation could reflect possible differences in the affinity of native ligands and particular functions of lectins in host-pathogen interactions. These data correspond with the affinities of both lectins, when AFL has a lower affinity to monosaccharides than RSL, and also suggest that AFL exhibits a higher avidity (or multivalency) effect than RSL [39,46]. Therefore, compounds **IIc** and **IVc** are suitable candidate inhibitors of AFL, which may be able to compete with natural complex ligands on host cells.

Table 4. IC_{50} and potency (IC_{50} standard/IC_{50} inhibitor) for fucosyl azide **C** and construct **IIc** and **IVc** for inhibition of binding of AFL and RSL to immobilized α-L-fucopyranoside

Inhibitor	Lectin AFL		Lectin RSL	
	IC_{50} (μM)	Potency	IC_{50} (μM)	Potency
L-fucose[a]	3,110	1	12.3	1
C	716	4	16.1	0.8
IIc	3.0	1,037	3.6	3.4
IVc	3.4	915	2.5	4.9

[a]Standard.

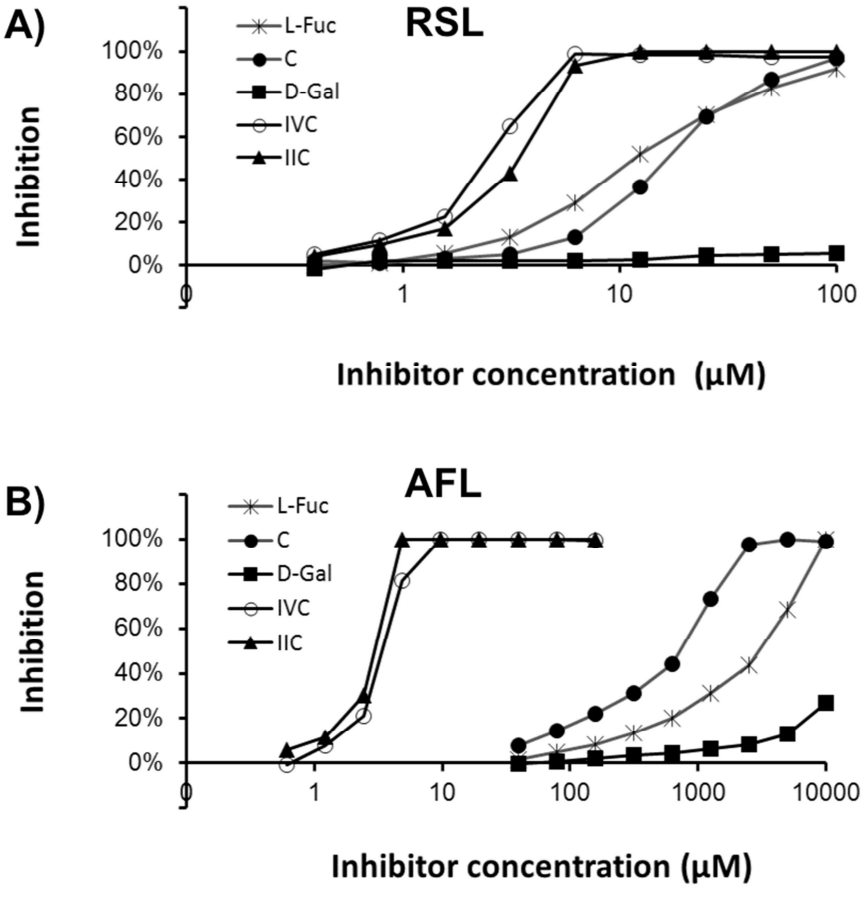


Figure 1. Inhibitory effects of monosaccharides and compounds **IIc** and **IVc** on RSL (A) and AFL (B) binding to immobilized α-L-fucopyranoside assessed by SPR. The monosaccharide D-galactose, which is not specifically recognized by lectins, was used as a negative control.

2.5. STD NMR

To obtain a deeper insight into the interactions of both **IIc** and **IVc** with lectins AFL and RSL, an STD NMR experiment was performed. STD experiments revealed the interaction of the nonpolar fucosyl C5-methyl group of both calix[4]arene glycomimetics **IIc** and **IVc** with both types of lectins AFL and RSL, which corresponds with published crystal structures [39,46]. In addition, the *tert*-Bu group in **IIc** behaves as an "aglycon-assisted" moiety, and this interaction with both lectins is even stronger than the interaction of the fucopyranosyl methyl group. This was documented by the binding experiment of **IIc** with RSL, where the ratio of integral intensities of the C5-methyl group and *tert*-Bu groups in the STD experiment was 3:18, while in the ^{1}H NMR spectrum it was 3:9 (**Figure 2**). A slightly weaker STD effect (3:12) was observed for **IIc** interacting with AFL. Furthermore, it was found that the methyl group of L-fucopyranose of both **IIc** and **IVc** interact similarly with both lectins. On the other hand, no interaction with the aromatic protons of the calix[4]arene scaffold was detected.

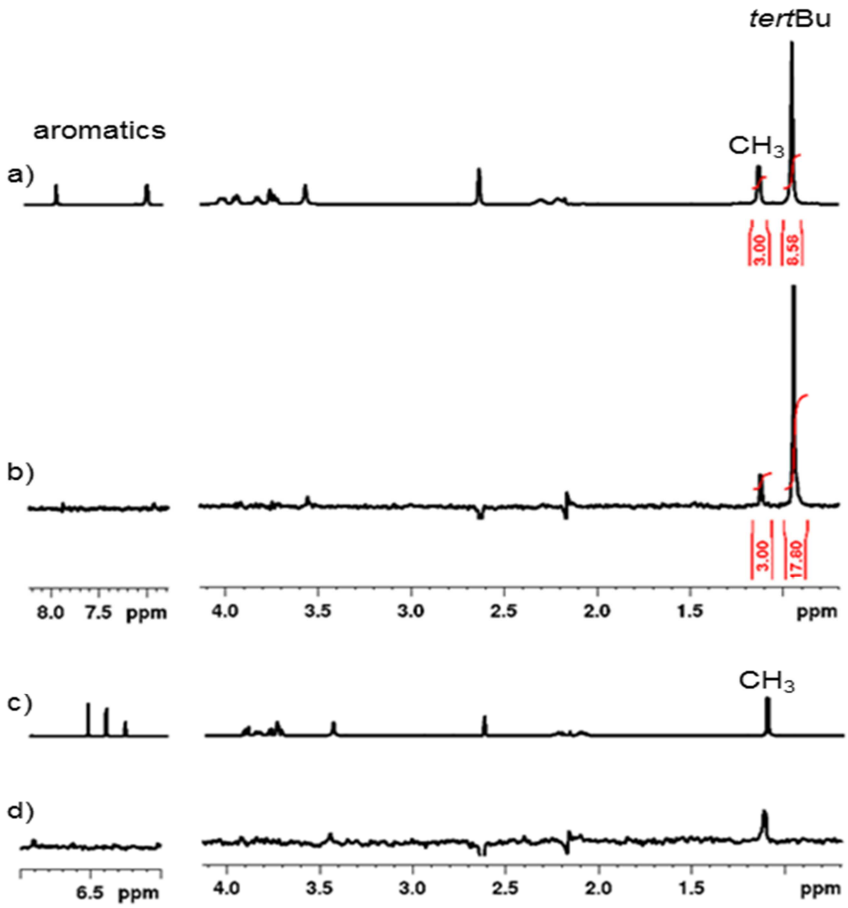


Figure 2. STD NMR screening of ligands **IIc** and **IVc** (500 µM) and 5µM RSL; a) Reference ^{1}H NMR spectrum of **IIc**; b) STD spectrum of **IIc** with RSL selectively saturated at 0 Hz; c) Reference ^{1}H NMR spectrum of **IVc**; d) STD spectrum of **IVc** with RSL selectively saturated at 0 Hz.

2.6. Cross-linking tests

As the lectin BC2L-C was confirmed to be immunogenic and located on the bacterial surface [33], inhibitors **IIc** and **IVc**, which showed increased inhibitory potency, were further investigated directly via cross-linking of whole *B. cenocepacia* cells to determine whether they could interact with BC2L-C at the cell level. Compound **IIc** at a concentration of 10 mM was able to aggregate 5% (w/w) *B. cenocepacia* cells grown in LB medium. Significantly

better results were obtained with cells cultivated in minimal M9 medium. Both compounds **IIc** and **IVc** were able to cross-link *B. cenocepacia* cells in a concentration-dependent manner with **IIc** (**Figure 3**), resulting in an apparently higher aggregation rate than **IVc**. Cross-linking and aggregation was not observed using simple monovalent L-fucose even at a concentration of 1M. These results are in agreement with the hemagglutination inhibitory assay, where compound **IIc** is a 256-fold better inhibitor than L-fucose, whereas **IVc** is only 32-fold better.

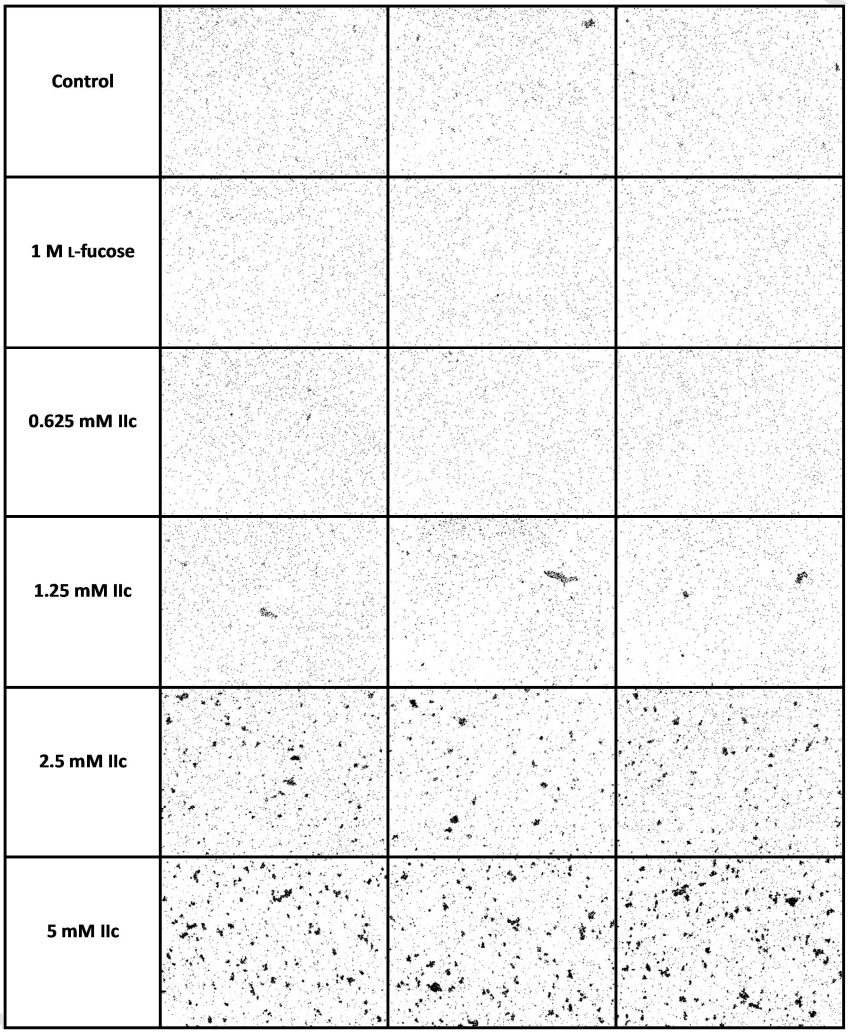


Figure 3. Cross-linking of *Burkholderia cenocepacia* cells with compound **IIc**. *B. cenocepacia* cells alone and in presence of L-fucose were used as a control. Cells were cultivated in minimal M9 medium. Magnification 100x, bright field, background subtraction in GIMP, triplicates.

2.7. Biofilm inhibition

The ability of the ligands **IIa**, **IIc**, and **IVc** to inhibit the biofilm formation of the reference strain *P. aeruginosa* ATCC 10145 was investigated by Microtiter Dish Biofilm Formation Assay [47].

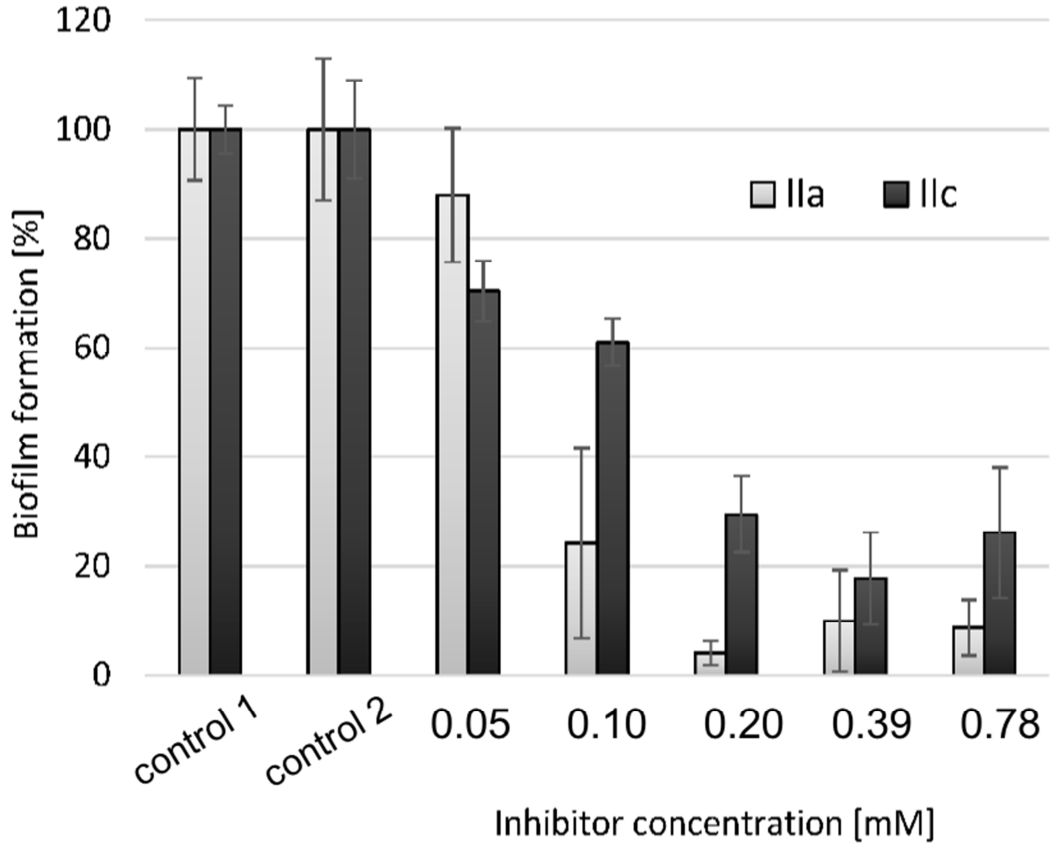


Figure 4. *P. aeruginosa* biofilm formation in the presence of compounds **IIa** and **IIc** (control 1, cells in medium without inhibitor; control 2, cells in medium with 5% DMSO without inhibitor)

The data showed that both substances **IIa** and **IIc** (**Figure 4**) were able to inhibit the biofilm formation of *P. aeruginosa* in a dose-dependent manner. Over the concentration range 0.05–0.78 mM, the percentage of biofilm inhibition increased from 9% to 88% for the galactopyranosyl ligand **IIa**. Fucopyranosyl ligand **IIc** also inhibited the biofilm formation, but to a lesser degree – the inhibition was from 26 % to 70 % over the same concentration range. In contrast, fucopyranosyl ligand **IVc** was only a weak inhibitor of biofilm formation. 50% inhibition was achieved with a concentration of 6.24 mM. Therefore, although only a

weak inhibitory activity of calix[4]arene glycomimetics was observed during agglutination inhibition assay for LecA and LecB, these compounds could inhibit biofilm formation. The higher efficiency of galactosylated inhibitors compared to fucosylated suggests that LecA is a more suitable target for inhibition of biofilm formation than LecB.

3. Conclusions

This article addresses one limitation that can be found in the literature published so far: the selectivity of ligand-lectin interactions. To address this issue, we synthesized ten new polyvalent glycomimetics built on calix[4]arene scaffolds using a CuAAC procedure. D-Galactose, D-mannose and L-fucose, being frequently occurring sugar epitopes, were attached through potentially stable *C*-glycosidic bonds. Their affinity towards a pool of seven different lectins was assessed by agglutination inhibition tests, and the most promising candidates were subjected to SPR. The biological evaluation was completed with tests at the cell level. We discovered three promising tetravalent glycomimetics based on calix[4]arene (1,3-*alternate*): **IIa** against LecA (*P. aeruginosa*), **IIc** against BC2L-C (*B. cenocepacia*) and both **IIc** and **IVc** against AFL (*A. fumigatus*) as well as RSL (*R. solanacearum*) lectins. Work is currently in progress on modulating the structural parameters of ligands **IIa**, **IIc** and **IVc**, which could lead to even more potent candidates for further research, especially at the cell level and *in vivo*.

4. Experimental

4.1. General Methods

Optical rotations were measured with a Autopol VI (Rudolph Research Analytical, USA) digital polarimeter in appropriate solvents, at temperature 25 ºC and 589 nm sodium line, in 1 dm cuvette and are given at 10^{-1}.deg.cm².g^{-1}. Concentrations (*c*) are given in g/100 ml. ^{1}H,

^{13}C, COSY, HMQC and HMBC spectra were measured on a Bruker DPX-300, DRX-400, DRX-500, or Bruker Advance III 600 (Bruker Corporation, Germany) spectrometer. All spectra were acquired at 298 K. Chemical shifts are given in δ-units (ppm) and are referenced to TMS. Coupling constants (J) are reported in Hz. The ESI high resolution mass spectra were measured with a LTQ Velos Orbitrap XL (Thermo Fisher Scientific, UK) instrument equipped with LockSpray in ES^+ and ES^- modes with methanol as a mobile phase at flow rate 150 $\mu L.min^{-1}$. Nominal and exact *m*/*z* values are reported in Daltons and were locked at palmitic acid (255.2329) and diisooctyl phthalate mass (413.2662) for ES^- and ES^+ modes, respectively. For preparative HPLC, a chromatograph Waters 600 equipped with UV/VIS detector was used. The instrument was connected to a column (250 x 25 mm) packed with LiChrosorb RP-18 (5 µm; Merck, Germany).

4.2. General procedure for the CuAAC reaction

In the optimized procedure, the free azide derivative (1.1 eq. per alkyne), alkynyl calix[4]arene (1 eq.), CuI (0.5 eq.) were dissolved in DMF. Subsequently *N*,*N*-diisopropylethylamine (DIPEA) (5 eq.) was added. Reaction mixture was heated at 110 °C for 30 min under microwave irradiation. Then the mixture was concentrated and a residue purified on column chromatography (SiO_2, $CHCl_3$: CH_3OH = 4:1) followed by a preparative HPLC (C18, CH_3OH : H_2O = 7:3) unless otherwise stated. Numbering of nuclei for NMR assignment of pseudoglycoconjugates is depicted in Figure 5.

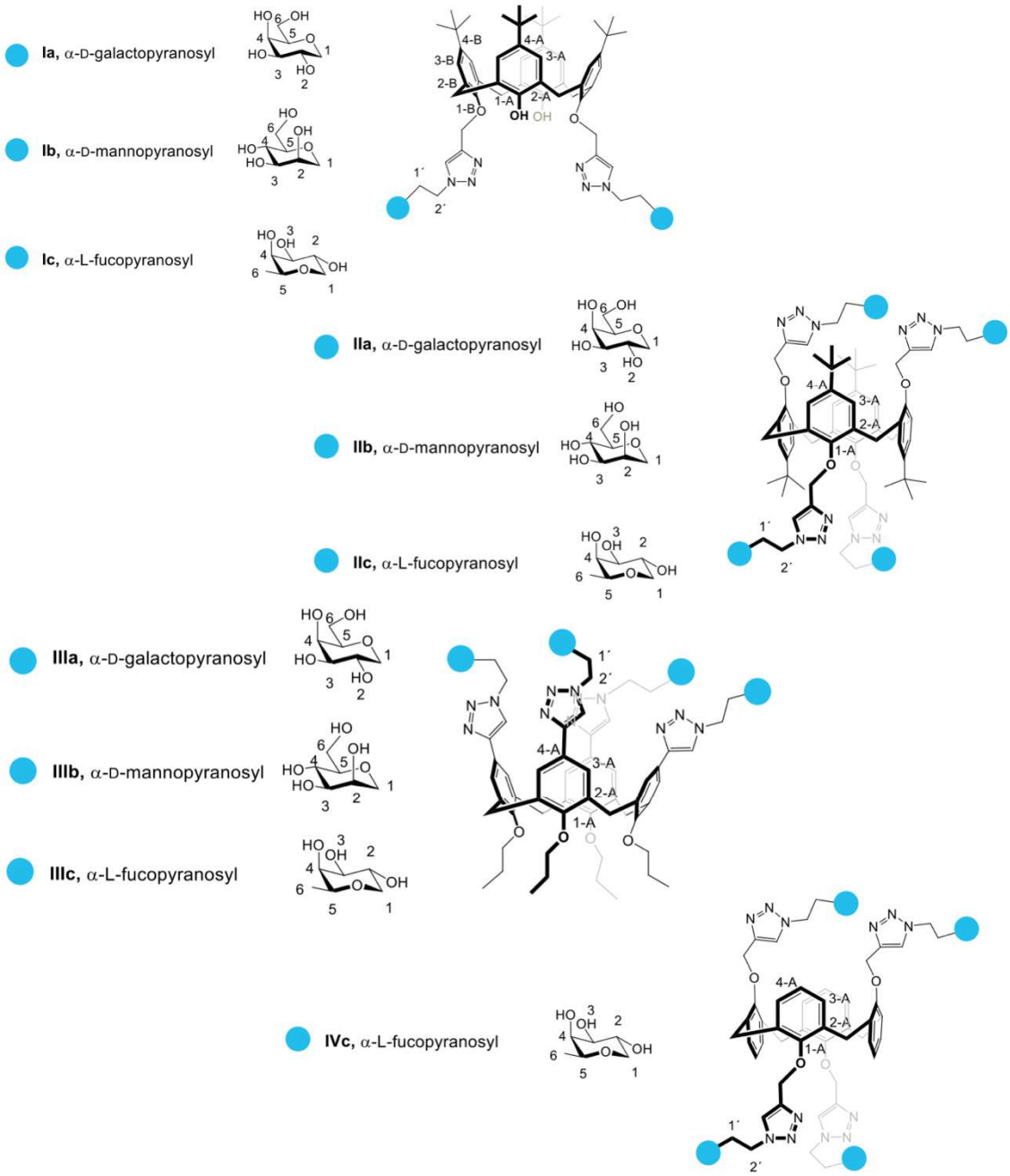


Figure 5. Numbering of nuclei for NMR shift assignment in synthesised pseudoglycoconjugates.

4.2.1. 25,27-Bis-{1*N*-[2-(α-D-galactopyranosyl)ethyl]-triazol-4-yl}-methoxy-26,28-dihydroxy-5,11,17,23-tetra-*tert*butyl-calix[4]arene (*cone*) (Ia)

Starting from 2-azido-(α-D-galactopyranosyl)ethane (**A**) (110 mg, 0.47 mmol) pseudoglycoconjugate **Ia** (131 mg, 53 %) was isolated as a glassy product: $[\alpha]_D^{25}$ 21.7 (*c* 1.9,

MeOH); ^{1}H NMR (CD_3OD): δ 8.30 (s, 2H, triazolyl-H), 7.15 (s, 4H; H_A-3), 7.00 (s, 4H; H_B-3), 5.17 (s, 4H; O-CH_2), 4.70-4.60 (m, 4H; H-2´), 4.28 (2 x d, J 13.0 Hz, 4H; H bridge ax), 4.10 (ddd, $J_{1,2}$ 5.2 Hz, $J_{1,1´}$ 10.9 Hz, $J_{1,1´}$ 4.3 Hz, 2H; H-1), 3.96 (dd, 4H, $J_{3,4}$ = $J_{4,5}$ 2.8 Hz, 2H; H-4), 3.92 (dd, $J_{1,2}$ 5.2 Hz, $J_{4,3}$ 8.5 Hz, 2H; H-2), 3.88 (dd, $J_{6,6´}$ 11.5 Hz, $J_{5,6}$ 7.6 Hz, 2H; H-6), 3.83-3.81 (m, 2H; H-5), 3.73-3.69 (m, 4H; H-3, H-6), 3.36-3.31 (m, 4H; H bridge eq), 2.40-2.33 (m, 2H; H-1´), 2.31-2.25 (m, 2H; H-1´), 1.32 (s, 18H; *tert*Bu-A), 1.07 (s, 18H; *tert*Bu-B);^{13}C NMR (CD_3OD), δ (ppm): 149.68 (C1-A) 149.44 (C1-B), 147.70 (C4-B), 143.10 ($C_{triazol}$) and 142.04 (C4-A), 133.19 (C2-B), 128.15 (C2-A), 125.56 (C3-B), 125.23 ($CH_{triazol}$), 124.82 (C3-A), 73.47 (C-5), 70.98 (C-1), 70.62 (C-3), 68.80 (C-2), 68.72 (CH_2-O) 68.66 (C-4), 60.82 (C-6), 47.19 (C-2´), 33.61, 33.34 (*C*(CH_3)$_3$), 31.14, 31.12 (C_{bridge}), 30.72, 30.22 (C(*C*H_3)$_3$), 26.51 (C-1´); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{66}H_{91}N_6O_{14}$ ($M+H^+$): 1191.6588; Found: 1191.6604; Calcd. for $C_{66}H_{90}N_6NaO_{14}$ ($M+Na^+$): 1213.6407; Found: 1213.6418; Calcd. for $C_{66}H_{90}KN_6O_{14}$ ($M+K^+$): 1229.6147; Found: 1229.6082.

4.2.2. 25,27-Bis-{1*N*-[2-(α-D-mannopyranosyl)ethyl]-triazol-4-yl}-methoxy-26,28-dihydroxy-5,11,17,23-tetra-*tert*butyl-calix[4]arene (*cone*) (Ib)

Starting from 2-azido-(α-D-mannopyranosyl)ethane (**B**) (76 mg, 0.32 mmol) pseudoglycoconjugate **Ib** (57 mg, 56 %) was isolated as a glassy product: $[\alpha]_D^{25}$ 13.4 (*c* 1.2, MeOH); ^{1}H NMR (CD_3OD): δ 8.35 (s, 2H, triazolyl-H), 7.13 (s, 4H, H_A-3), 7.05 (s, 4H, H_B-3), 5.21 (s, 4H, O-CH_2), 4.71 (dd, 4H, J 6.0 Hz, J 7.8 Hz, H-2´), 4.27 and 4.25 (2 x d, J 12.9 Hz, 4H, H bridge ax), 3.88-3.86 (m, 2H, H-1), 3.85 (dd, J $_{6,6}$ 11.9 Hz, $J_{5,6}$ 6.8 Hz, 2H, H-6), 3.79 (dd, $J_{6,6}$ 11.9 Hz, $J_{5,6}$ 2.8 Hz, 2H, H-6) 3.77-3.74 (m, 4H, H-2, H-3), 3.68 (dd, $J_{3,4}$ 7.3 Hz, 2H, H-4), 3.66-3.60 (m, 2H, H-5), 3.35-3.30 (m, 4H, H bridge eq), 2.42-2.36 (m, 2H, H-1´), 2.28-2.22 (m, 2H, H-1´), 1.25 (s, 18H, *tert*Bu-A), 1.08 (s, 18H, *tert*Bu-B); ^{13}C NMR (CD_3OD): δ 149.63 (C1-A) 149.41 (C1-B), 147.79 (C4-B), 143.15 ($C_{triazol}$), 142.09 (C4-A), 133.25 (C2-B), 128.15 and 128.13 (C2-A), 125.61 and 125.58 (C3-B), 125.28 ($CH_{triazol}$),

124.79 (C3-A), 75.85 (C-5), 72.53 (C-1), 71.20 (C-2), 70.77 (C-3), 68.67 (CH_2-O), 68.34 (C-4), 61.18 (C-6), 46.87 (C-2´), 33.63 ($C(CH_3)_3$-B), 33.33 ($C(CH_3)_3$-A), 31.15 and 31.14 (C_{bridge}), 30.66 ($C(CH_3)_3$-A), 30.20 ($C(CH_3)_3$-B), 29.68 (C-1´); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{66}H_{90}N_6NaO_{14}$ ($M+Na^+$): 1213.6407; Found: 1213.6409.

4.2.3. 25,27-Bis-{1*N*-[2-(α-L-fucopyranosyl)ethyl]-triazol-4-yl}-methoxy-26,28-dihydroxy-5,11,17,23-tetra-*tert*butyl-calix[4]arene (*cone*) (Ic)

Starting from 2-azido-(α-L-fucopyranosyl)ethane (**C**) (66 mg, 0.30 mmol) pseudoglycoconjugate **Ic** (107 mg, 67 %) was isolated as a glassy product: $[\alpha]_D^{25}$ -21.6 (*c* 1.2, MeOH); ^{1}H NMR (CD_3OD): δ 8.24 (s, 2H, triazolyl-H), 7.13 (d, J 2.3 Hz, 2H, H_A-3), 7.11 (d, J 2.3 Hz, 2H, H_A-3), 7.03 (d, J 2.2 Hz, 2H, H_B-3), 7.02 (d, J 2.2 Hz, 2H, H_B-3), 5.19 (d, $J_{Ha,Hb}$ 11.8 Hz, 2H, O-C*Ha*Hb), 5.15 (d, $J_{Ha,Hb}$ 11.8 Hz, 2H, O-CHa*Hb*), 4.52 – 4.65 (m, 4H, H-2´), 4.29 and 4.27 (2 x d, $J_{Hax,Heq}$ 12.9 Hz, 4H, H bridge ax), 3.99-3.92 (m, 2H, H-1), 3.95-3.90 (m, 2H, H-2), 3.88-3.83 (m, 2H, H-5) 3.71-3.60 (m, 4H, H-3, H-4), 3.35-3.30 (2x overlapped by MeOH, 4H, H bridge eq), 2.38-2.35 (m, 2H, H-1´), 2.31-2.29 (m, 2H, H-1´), 1.27 (s, 18H, *tert*Bu-A), 1.16 (d, $J_{6,5}$ 6.3 Hz, 6H, H-6), 1.06 (s, 18H, *tert*Bu-B); ^{13}C NMR (CD_3OD): δ 149.70 (C1-A), 149.44 (C1-B), 147.70 (C4-B), 143.05 ($C_{triazol}$), 142.01 (C4-A), 133.18 (C2-B), 128.10 and 128.09 (C2-A), 125.59 and 125.53 (C3-B), 124.99 ($CH_{triazol}$), 124.83 and 124.81 (C3-A), 72.23 (C-1), 71.14 (C-3), 70.84 (C-4), 68.79 (CH_2-O), 68.26 (C-2), 67.81 (C-5), 47.18 (C-2´) 33.61 and 33.33 ($C(CH_3)_3$), 31.13 and 31.10 (C_{bridge}), 30.71 ($C(CH_3)_3$-A), 30.20 ($C(CH_3)_3$-B), 25.96 (C-1´), 15.32 (C-6); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{66}H_{91}N_6O_{12}$ ($M+H^+$): 1159.6690; Found: 1159.6710 Calcd. for $C_{66}H_{90}N_6NaO_{12}$ ($M+Na^+$): 1181.6509; Found: 1181.6525.

4.2.4. 25,26,27,28-Tetrakis-{1*N*-[2-(α-D-galactopyranosyl)ethyl]-triazol-4-yl}-methoxy-5,11,17,23-tetra-*tert*butyl-calix[4]arene (*1,3-alternate*) (IIa)

Starting from 2-azido-(α-D-galactopyranosyl)ethane (**A**) (133 mg, 0.57 mmol) pseudoglycoconjugate **IIa** (115 mg, 47 %) was isolated as a glassy product. A gradient elution was used for a column chromatography (SiO_2, $CHCl_3$: CH_3OH 4:1→2:1): $[\alpha]_D^{25}$ 64.4 (*c* 0.8, MeOH); ^{1}H NMR (CD_3OD): δ 7.36 (s, 4H, triazolyl-H), 6.96 (d, J 2.6 Hz, 4H, H_A-3), 6.94 (d, J 2.6 Hz, 4H, H_A-3), 4.67 (s, 8H, O-CH_2), 4.58-4.50 (m, 8H, H-2´), 3.99-3.97 (m, 8H, H-4, H-1), 3.92- 3.87(m, 8H, H-6, H-2) 3.83-3.68 (m, 4H, H-5), 3.72-3.67 (m, 8H, H-3, H-6), 3.61 and 3.51 (2 x s, 8H, H bridge), 2.37-2.28 (m, 4H, H-1´), 2.18-2.11 (m, 4H, H-1´), 1.08 (s, 36H, *tert*Bu); ^{13}C NMR (CD_3OD), δ (ppm): 153.28 (C-1A), 144.61 (C-4A), 144.36 ($C_{triazol}$), 133.46 and 133.18 (C-2A), 126.41 and 126.31 (C-3A), 124.47 ($CH_{triazol}$), 73.86 (C-5), 70.71 (C-3), 70.11 (C-1), 69.06 (C-2), 68.27 (C-4), 63.59 (OCH_2), 60.38 (C-6), 46.99 (C-2´), 37.66 and 37.60 (C_{bridge}), 33.35 ($C(CH_3)_3$), 30.73 ($C(CH_3)_3$), 27.13 (C-1´); HRMS/ESI (negative mode): *m/z* Calcd. for $C_{88}H_{122}N_{12}O_{24}$ ($M\text{-}2H^+$): 865.4353; Found: 865.4345; Calcd. for $C_{88}H_{123}N_{12}O_{24}$ ($M\text{-}H^+$): 1731.8779; Found: 1731.8774.

4.2.5. 25,26,27,28-Tetrakis-{1*N*-[2-(α-D-mannopyranosyl)ethyl]-triazol-4-yl}-methoxy-5,11,17,23-tetra-*tert*butyl-calix[4]arene (*1,3-alternate)* (IIb)

Starting from 2-azido-(α-D-mannopyranosyl)ethane (**B**) (117 mg, 0.50 mmol) pseudoglycoconjugate **IIb** (166 mg, 77 %) was isolated as a glassy product. A gradient elution was used for a column chromatography (SiO_2, $CHCl_3$: CH_3OH 4:1→2:1): $[\alpha]_D^{25}$ 21.2 (*c* 1.7, MeOH); ^{1}H NMR (CD_3OD), δ (ppm): 7.39 (s, 4H, triazolyl-H), 6.95 (d, J 2.4 Hz, 4H, H_A-3), 6.94 (d, J 2.4 Hz, 4H, H_A-3), 4.66 (d, $J_{Ha,Hb}$ 12.2 Hz, 4H, O-C*Ha*Hb), 4.62 (d, $J_{Ha,Hb}$ 12.2 Hz, 4H, O-CHa*Hb*), 4.60-4.50 (m, 8H, H-2´), 3.87-3.82 (m, 8H, H-1, H-6_a), 3.78 (dd, 4H, $J_{5,6a}$ 2.8 Hz, $J_{6a,6b}$ 11.9 Hz, H-6_b), 3.75-3.72 (m, 8H, H-2, H-3), 3.67 (dd, $J_{3,4}$ 7.3 Hz, $J_{4,5}$ 7.3 Hz, 4H, H-4), 3.61 (s, 4H, H bridge), 3.62-3.55 (m, 4H, H-5), 3.54 (s, 4H, H bridge), 2.40-2.30 (m, 4H, H-1´), 2.18-2.11 (m, 4H, H-1´), 1.12-1.03 (s, 36H, *tert*Bu); ^{13}C NMR (CD_3OD): δ 153.42 (C-1A), 144.55 and 144.45 (C-4A; $C_{triazol}$), 133.39 and 133.23 (C-2A), 126.37(C-

3A), 124.39 ($CH_{triazol}$), 75.75 (C-5), 72.59 (C-1), 71.25 (C-2), 70.84 (C-3), 68.26 (C-4), 63.72 (OCH_2), 61.18 (C-6), 46.62 (C-2´), 37.70 (C_{bridge}), 33.35 ($C(CH_3)_3$), 30.70 ($C(CH_3)_3$), 29.79 (C-1´); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{88}H_{124}N_{12}Na_2O_{24}$ ($M+2Na^+$): 889.4318; Found: 889.9331; *m/z* Calcd. for $C_{88}H_{124}N_{12}NaO_{24}$ ($M+Na^+$): 1755.8744; Found: 1755.8731.

4.2.6. 25,26,27,28-Tetrakis-{1*N*-[2-(α-L-fucopyranosyl)ethyl]-triazol-4-yl}-methoxy-5,11,17,23-tetra-*tert*butyl-calix[4]arene (*1,3-alternate*) (IIc)

Starting from 2-azido-(α-l-fucopyranosyl)ethane (**C**) (110 mg; 0.50 mmol) pseudoglycoconjugate **IIc** (136 mg, 68 %) was isolated as a glassy product. A gradient elution was used for a column chromatography (SiO_2, $CHCl_3$: CH_3OH 4:1→2:1): $[\alpha]_D^{25}$ -50.2 (*c* 1.0, MeOH); ^1H NMR (CD_3OD): δ 7.26 (s, 4H, triazolyl-H), 6.95 and 6.97-6.91 (m, 8H, H_A-3), 4.67 (s, 8H, OCH_2), 4.52-4.43 (m, 8H, H-2´), 4.02-3.98 (m, 4H, H-1), 3.92 (dd, $J_{1,2}$ 7.5 Hz, $J_{2,3}$ 8.9 Hz, 4H, H-2), 3.88 (dq, $J_{4,5}$ 1.9 Hz, $J_{5,6}$ 6.5 Hz, 4H, H-5), 3.73 (br dd, 4H, H-4), 3.70 (dd, $J_{2,3}$ 8.9 Hz, $J_{3,4}$ 3.4 Hz, 4H, H-3), 3.60 (s, 4H, H bridge), 3.54 (s, 4H, H bridge), 2.38-2.31 (m, 4H, H-1´), 2.28-2.21 (m, 4H, H-1´), 1.25 (d, $J_{5,6}$ 6.5 Hz, 12H, H-6), 1.08 (s, 36H, *tert*Bu); ^{13}C NMR (CD_3OD): δ 153.35 (C-1A), 144.65 and 144.30 (C-4A, $C_{triazol}$), 133.42 and 133.27 (C-2A), 126.26 and 126.24 (C-3A), 124.19 ($CH_{triazol}$), 71.83 (C-1), 70.93 (C-3, C-4), 68.31 (C-2), 67.93 (C-5), 63.61 (OCH_2), 47.18 (C-2´), 37.76 and 37.72 (C_{bridge}), 33.35 ($C(CH_3)_3$) 30.71 ($C(CH_3)_3$), 26.26 (C-1´), 15.27 (C-6); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{88}H_{124}N_{12}Na_2O_{20}$ ($M+2Na^+$): 857.9420; Found: 857.9431; Calcd. for $C_{88}H_{125}N_{12}NaO_{20}$ ($M+H^++Na^+$): 846.9549; Found: 846.9526; Calcd. for $C_{88}H_{124}N_{12}NaO_{20}$ ($M+Na^+$): 1691.8947; Found: 1691.8960.

4.2.7. 5,11,17,23-Tetrakis-{1*N*-[2-(α,D-galactopyranosyl)ethyl]}-triazol-4-yl-25,26,27,28-tetrapropoxy-calix[4]arene (*cone*) (IIIa)

Starting from 2-azido-(α-D-galactopyranosyl)ethane (**A**) (114 mg, 0.49 mmol) pseudoglycoconjugate **IIIa** (132 mg, 67 %) was isolated as a glassy product. A gradient

elution for a column chromatography (SiO_2, $CHCl_3$: CH_3OH 4:1→2:1→ CH_3OH) and mobil phase CH_3OH : H_2O 1:1 for preparative C18 HPLC separation were used: $[\alpha]_D^{25}$ 44.0 (*c* 1.0, pyridine/MeOH 2:1); ^1H NMR (CD_3OD): δ 7.92 (s, 4H, triazolyl-H), 7.19 (s, 8H, H_A-3), 4.60 (d, J 13.1 Hz, 4H, H bridge ax), 4.52-4.45 (8H, H-2´), 4.01-3.95 (m, 12H, O-C*H*$_2$CH$_2$CH$_3$, H-1), 3.94-3.83 (m, 12H, H-6, H-4, H-3), 3.81-3.77 (m, 4H, H-5), 3.72-3.68 (m, 8H, H-6, H-2), 3.35-3.30 (overlapped d, 4H, H bridge eq), 2.31-2.23 (m, 4H, H-1´), 2.22-2.10 (m, 4H, H-1´), 2.06-1.98 (m, 8H, O-CH$_2$C*H*$_2$CH$_3$), 1.09 (t, J 7.4 Hz, 12H; O-CH$_2$CH$_2$C*H*$_3$); ^{13}C NMR (CD_3OD): δ 156.82 (C-1A), 147.38 ($C_{triazol}$), 135.31 (C-2A), 125.40 (C-3A); 120.48 (C-4A), 116.03 ($CH_{triazol}$), 76.74 (O-*C*H$_2$CH$_2$CH$_3$), 73.55 (C-5), 70.57 (C-2, C-3), 68.79 (C-4), 68.57 (C-1), 60.63 (C-6), 46.53 (C-2´), 30.73 (C_{bridge}), 26.28 (C-1´), 23.07 (O-CH$_2$*C*H$_2$CH$_3$), 7.81 (O-CH$_2$CH$_2$*C*H$_3$); HRMS/ESI (negative mode): *m/z* Calcd. for $C_{80}H_{106}N_{12}O_{24}$ (M-2H$^+$): 809.3727; Found: 809.3730; Calcd. for $C_{80}H_{107}N_{12}O_{24}$ (M-H$^+$): 1619.7527; Found: 1619.7500.

4.2.8. 5,11,17,23-Tetrakis-{1*N*-[2-(α,D-mannopyranosyl)ethyl]}-triazol-4-yl-25,26,27,28-tetrapropoxy-calix[4]arene (*cone*) (IIIb)

Starting from 2-azido-(α-D-mannopyranosyl)ethane (**B**) (130 mg, 0.55 mmol) pseudoglycoconjugate **IIIb** (144 mg, 64 %) was isolated as a glassy product. A gradient elution for a column chromatography (SiO_2, $CHCl_3$: CH_3OH 4:1→2:1→ CH_3OH) and mobil phase CH_3OH : H_2O 1:1 for preparative C18 HPLC separation were used: $[\alpha]_D^{25}$ 13.1 (*c* 1.0, pyridine/MeOH 2:1); ^1H NMR (CD_3OD): δ 7.95 (s, 4H, triazolyl-H), 7.19 (s, 8H, H_A-3), 4.59 (d, J 13.3 Hz, 4H, H bridge ax), 4.49 (t, J 6.2 Hz, 8H, H-2´), 3.98 (t, J 7.4 Hz, 8H, O-C*H*$_2$CH$_2$CH$_3$), 3.83 (dd, $J_{5,6}$ 6.8 Hz, $J_{6,6'}$ 11.5 Hz, H-6), 3.80-3.70 (16 H, H-6', H-3, H-2, H-1), 3.69 (dd, $J_{3,4}$ 7.3 Hz, $J_{4,5}$ 7.3 Hz, 4H, H-4), 3.60-3.55 (m, 4H, H-5), 3.35-3.30 (overlapped d, 4H, H bridge eq), 2.36-2.26 (m, 4H, H-1´), 2.17-2.10 (m,4H, H-1´), 2.05-1.98 (m, 8H, O-CH$_2$C*H*$_2$CH$_3$), 1.08 (t, J = 7.4 Hz, 12H, 4 x O-CH$_2$CH$_2$C*H*$_3$), ^{13}C NMR (CD_3OD), δ (ppm): 156.84 (C-1A), 147.39 ($C_{triazol}$), 135.31 (C-2A), 125.43 and 125.38 (C-3A), 124.15 (C-4A),

120.66 ($CH_{triazol}$), 76.74 (O-*C*$H_2CH_2CH_3$), 75.81 (C-5), 72.54 (C-1), 71.13 (C-2), 70.75 (C-3), 68.30 (C-4), 61.04 (C-6), 46.66 (C-2´), 30.73 (C_{bridge}), 29.39 (C-1´), 23.07 (O-CH_2*C*H_2CH_3), 9.45 (O-CH_2CH_2*C*H_3); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{80}H_{108}N_{12}Na_2O_{24}$ ($M+2Na^+$): 833.3692; Found: 833.3693; Calcd. for $C_{80}H_{108}N_{12}NaO_{24}$ ($M+Na^+$): 1644.7525; Found: 1644.7539.

4.2.9. 5,11,17,23-Tetrakis-{1*N*-[2-(α,L-fucopyranosyl)ethyl]}-triazol-4-yl-25,26,27,28-tetrapropoxy-calix[4]arene (*cone*) (IIIc)

Starting from 2-azido-(α-L-fucopyranosyl)ethane (**C**) (126 mg, 0.58 mmol) pseudoglycoconjugate **IIIc** (144 mg, 64 %) was isolated as a glassy product. A gradient elution for a column chromatography (SiO_2, $CHCl_3$: CH_3OH 4:1→2:1) was used: $[\alpha]_D^{25}$ -48.3 (*c* 1.0, MeOH); ^{1}H NMR (CD_3OD): δ 7.84 (s, 4H, triazolyl-H), 7.16 (s, 8H, H_A-3), 4.59 (d, J 13.2 Hz, 4H, Hbridge ax), 4.47-4.43 (m, 8H, H-2´), 3.98 (t, J 7.4 Hz, 8H, O-C*H*$_2CH_2CH_3$), 3.94-3.87 (m, 8H, H-2, H-1), 3.83 (dq, J 1.9 Hz, J 6.9 Hz, 4H, H-5), 3.73-3.69 (m, 4H, H-4), 3.68 (dd, J 3.5 Hz, J 8.7 Hz, 4H, H-3), 3.35-3.30 (overlapped d, 4H, H bridge eq), 2.38-2.33 (m, 4H, H-1´), 2.23-2.17 (m, 4H, H-1´), 2.06-1.96 (m, 8H, O-CH_2C*H*$_2CH_3$) 1.18 (d, J = 6.4 Hz, 12H, H-6), 1.08 (t, J = 7.5 Hz, 12H, O-CH_2CH_3C*H*$_3$); ^{13}C NMR (CD_3OD): δ 156.80 (C-1A), 147.32 ($C_{triazol}$), 135.31 (C-2A), 125.41 (C-3A) 124.21 (C-4A), 120.19 ($CH_{triazol}$), 76.74 (O-*C*$H_2CH_2CH_3$), 72.27 (C-1), 71.15 (C-4), 70.77 (C-3), 68.16 (C-2), 67.73 (C-5), 47.18 (C-2´), 30.73 (C_{bridge}), 25.53 (C-1´), 23.07 (O-CH_2*C*H_2CH_3), 15.29 (C-6), 9.46 (O-CH_2CH_2*C*H_3); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{80}H_{109}N_{12}NaO_{20}$ ($M+H^++Na^+$): 790.3884; Found: 790.3876; Calcd. for $C_{80}H_{108}N_{12}Na_2O_{20}$ ($M+2Na^+$): 801.3794; Found: 801.3792; Calcd. for $C_{80}H_{108}N_{12}NaO_{20}$ ($M+Na^+$): 1580.7729; Found: 1580.7743.

4.2.10. 25,26,27,28-Tetrakis-{1*N*-[2-(α-L-fucopyranosyl)ethyl]-triazol-4-yl}-methoxy-calix[4]arene (*1,3-alternate*) (IVc)

Starting form 2-azido-(α-L-fucopyranosyl)ethane (**C**) (150 mg, 0.69 mmol) pseudoglycoconjugate **IVc** (115 mg, 46 %) was isolated as a glassy product. A gradient elution for a column chromatography (SiO_2, $CHCl_3$: CH_3OH 4:1→2:1→ CH_3OH) and mobil phase CH_3OH : H_2O 1:1 for preparative C18 HPLC separation were used: $[\alpha]_D^{25}$ -52.5 (*c* 0.8, MeOH); ^{1}H NMR (CD_3OD): δ 6.88 (d, J 7.6 Hz, 4H, H_A-3), 6.86 (d, J 7.6 Hz, 4H, H_A-3), 6.75 (s, 4H, triazolyl-H), 6.47 (dd, $J_{3,4}$ 7.6 Hz, $J_{3',4}$ 7.6 Hz, 4H, H_A-4), 4.86 (overlapped d, 4H, $J_{Ha,Hb}$ 12.5 Hz, O-C*Ha*Hb), 4.82 (d, $J_{Ha,Hb}$ 12.5 Hz, 4H, O-CHa*Hb*), 4.51-4.40 (m, 8H, H-2´), 4.00-3.91 (m, 8H, H-1, H-2), 3.89-3.85 (dq, $J_{4,5}$ 1.9 Hz, $J_{5,6}$ 6.6 Hz, 4H, H-5), 3.77-3.70 (m, 8 H, H-4, H-3), 3.58 (s, 4H, H bridge), 3.56 (s, 4H, H bridge), 2.35-2.16 (m, 8H, H-1´), 1.26 (d, $J_{5,6}$ 6.5 Hz, 12H, H-6); ^{13}C NMR (CD_3OD) δ (ppm): 154.82 (C-1A), 144.107 ($C_{triazol}$), 134.33 (C-2A), 129.06 and 129.00 (C-3A) 124.41 ($CH_{triazol}$), 122.79 (C-4A), 71.45 (C-1), 70.98 and 70.90 (C-4, C-3), 68.37 (C-2), 67.97 (C-5), 47.11 (C-2´), 37.06 and 37.04 (C_{bridge}), 26.67 (C-1´), 15.20 (C-6); HRMS/ESI (positive mode): *m/z* Calcd. for $C_{72}H_{94}N_{12}O_{20}$ ($M+2H^+$): 723.3348; Found: 723.3350; Calcd. for $C_{72}H_{93}N_{12}NaO_{20}$ ($M+H^++Na^+$): 734.3255; Found: 734.3258; Calcd. for $C_{72}H_{93}KN_{12}O_{20}$ ($M+H^++K^+$): 742.3128; Found: 742.3084; Calcd. for $C_{72}H_{93}N_{12}O_{20}$ ($M+H^+$): 1445.6624; Found: 1445.6638; *m/z* Calcd. for $C_{72}H_{92}N_{12}NaO_{20}$ ($M+Na^+$): 1467.6443; Found: 1467.6454; Calcd. for $C_{72}H_{92}KN_{12}O_{20}$ ($M+K^+$):1483.6182; Found: 1483.6191.

4.3. Procedure for STD NMR

STD spectra were acquired on a Bruker Avance III 600 MHz spectrometer at 298 K using 0.57 mL samples of 5 μM AFL or RSL lectin in the presence of a 100-fold excess (500 μM) of small molecule ligand **IIc** or **IVc** in PBS buffer, with 2.5 s irradiation alternating between 0.0 ppm (on-resonance) and 20 ppm (off-resonance) using 50 ms Gaussian pulses.

4.4. Lectins production and purification

The LecB, BC2L-C, RSL, AFL and LecA lectins in recombinant forms were produced and purified as previously described [29c,33,39,46,48]. Briefly, transformed *Escherichia coli* cells bearing plasmid for the particular lectin were cultured in LB broth medium containing an appropriate antibiotic at 37 °C. When the culture reached an OD_{600} of ≈ 0.5, cells were induced by isopropyl 1-thio-β-D-galactopyranoside (IPTG) added to a final concentration of 0.5 mM. Cells were incubated at 30 °C for 3 hours, harvested by centrifugation and re-suspended in the suitable buffer. Cells were then disintegrated by sonication and the cytosolic fraction containing soluble lectin was separated by centrifugation. Lectins were then purified by affinity chromatography, dialyzed and further processed according to the previously published procedures. Freeze-dried lectins were stored at -20 °C. Lectins AAL (Vector Laboratories) and AOL (TCI) were purchased in freeze-dried form.

4.5. Yeast agglutination inhibition assay

Freeze-dried lectin LecB was dissolved in the buffer (20 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5). Carbohydrate inhibitors serially diluted in the buffer were mixed with the lectin (2.5 $mg.mL^{-1}$) in a 5 µL : 5 µL ratio. 10 µL of 5% commerciallyavailable baker's yeast (UNIFERM) was then added; the mixture was thoroughly mixed and incubated for 10 min at room temperature. After incubation, the mixture was again mixed, transferred to a microscope slide and examined. The examination was conducted using the Levenhuk D2L NG Digital Microscope (Levenhuk). Images were obtained with a Levenhuk D2L digital camera (Levenhuk) using the software ToupView for Windows (Levenhuk). The positive (experiment without inhibitor) and negative control (experiment without lectin) were prepared and processed in the same way using the appropriate volume of dissolving buffer instead of the omitted components. The lowest concentration of inhibitor able to inhibit agglutination was determined, compared with the standard (L-fucose) and potency of the inhibitor was calculated. Freeze-dried lectin LecB dissolved in the buffer was then mixed in 1:1 ratio with

100% DMSO. Yeast cells and inhibitors were also dissolved in the buffer/DMSO mixture and whole experiment was repeated in this 50% DMSO environment, including positive and negative controls.

4.6. Hemagglutination inhibition assay

Hemagglutination inhibition assays was performed with lectins using human erythrocytes treated with 0.1% papain according to a method described previously [46]. Red blood cells (RBC) were diluted to 50% with PBS, stabilized with 0.01% sodium azide and stored at 4 °C. The desired concentration of RBC suspension was obtained by further dilution with the appropriate buffer. Freeze-dried lectins AFL, AOL, AAL and RSL were dissolved in the PBS buffer to the concentration 0.1 $mg.mL^{-1}$. Lectins were mixed with carbohydrate inhibitors serially diluted in PBS buffer in a 5 µL : 5 µL ratio. The final (working) concentration of the lectins was therefore 0.05 $mg.mL^{-1}$. 10 µL of 20% papain-treated, azid-stabilized red blood cells 0^+ in PBS buffer was then added, the mixture was thoroughly mixed and incubated for 5 min at room temperature. After incubation, the mixture was again mixed, transferred to a microscope slide and examined. The examination was conducted using the Levenhuk D2L NG Digital Microscope (Levenhuk). Images were obtained with a Levenhuk D2L digital camera (Levenhuk) using the software ToupView for Windows (Levenhuk). The positive (experiment without inhibitor) and negative control (experiment without lectin) were prepared and processed in the same way using the appropriate volume of dissolving buffer instead of the omitted components. The lowest concentration of inhibitor able to inhibit hemagglutination was determined, compared with the standard (L-fucose) and potency of the inhibitor was calculated.

Lectins BC2L-C and LecA were dissolved in the PBS buffer to the concentrations 0.2 $mg.mL^{-1}$ and 0.5 $mg.mL^{-1}$ and processed in the same way resulting in the working concentrations 0.1 $mg.mL^{-1}$ and 0.25 $mg.mL^{-1}$ respectively. As LecA is a galactose-specific lectin, D-galactose

was used as standard for the calculation of potencies of inhibitors. Considering lectin LecB, this protein was dissolved in the buffer with calcium ions suitable for the activity of the lectin (20 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5) to the concentration 2.5 $mg.mL^{-1}$. Lectin was mixed with carbohydrate inhibitors serially diluted in the Tris buffer in a 5 µL :5 µL ratio. The final (working) concentration of the lectins was therefore 1.25 $mg.mL^{-1}$. 10 µL of 20% papain-treated, azide-stabilized red blood cells 0^+ in the Tris buffer was then added, the mixture was thoroughly mixed and incubated for 5 minutes at room temperature. The examination was conducted and evaluated as mentioned above.

4.7. Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed on a BIAcore 3000 instrument (GE Healthcare) at 25 °C, using 10 mM HEPES, 150 mM NaCl, 0.005% TWEEN 20, pH 7.4 as a running buffer. The flow rate was 5 $\mu L.min^{-1}$. L-Fucose was immobilized onto a HC200M sensor chip (XanTec) covered with a linear polycarboxylate hydrogel matrix. The channels 1 and 2 were first coated with streptavidin using a standard amine coupling method. The polycarboxylate surface was activated with an *N*-hydroxysuccinimide/*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride solution (NHS/EDC, GE Healthcare, 100 µL) and streptavidin diluted in 10 mM sodium acetate, pH 5.0 to a concentration of 50 $\mu g.mL^{-1}$ was injected onto the chip (100 µL). Unreacted groups were blocked with 1 M ethanolamine-HCl, pH 8.5 (50 µL). Biotinylated α-L-fucose (Lectinity) was then used for the immobilization on channel 2. The compound was diluted in the running buffer to the concentration of 0.25 mM, and 50 µL of this mixture was injected onto the channel 2. Channel 1 coated only with streptavidin was used as a blank in subsequent inhibition experiments. The amount of immobilized streptavidin was similar on both channels (≈9000 RU).

Lectins RSL and AFL were dissolved in the PBS buffer with 0.005% TWEEN to the concentrations 0.1 $mg.mL^{-1}$ and 0.2 $mg.mL^{-1}$, respectively. Lectins were mixed with 10x concentrated inhibitors in a 9:1 ratio. 30 µL of this mixture was injected onto the sensor chip with immobilized α-L-fucoside at a flow rate of 5 $\mu L.min^{-1}$. PBS with 0.005% TWEEN was used as running buffer. The sensor chip was washed with 200 mM L-fucose or 10 mM methyl α-L-fucopyranoside after each injection. The response on the blank channel was subtracted before evaluation. The IC_{50} (concentration of inhibitor resulting for a 50% inhibition of the binding) was determined. The potency of a certain inhibitor is the ratio of IC_{50} of a chosen standard inhibitor (in this case α-L-fucose) and the inhibitor in question. The monosaccharide D-galactose was chosen as a negative control.

4.8. Cross-linking of *Burkholderia cenocepacia* cells

Burkholderia cenocepacia CCM 4899 (LMG 16656, J. Govan J2315, Czech Collection of Microorganisms) was cultivated overnight in Luria-Bertani medium at 37 °C with shaking. Cells were centrifuged, washed three times with the buffer (20 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5) and resuspended in the buffer. 10 µL of 10% (w/w) cells was mixed with 10 µL of compound **IIc**, incubated for 2 minutes at 25 °C and thoroughly mixed again. 10 µL of the mixture was transferred on the microscope slide and examined by motorized inverted microscope IX81 (Olympus). *Burkholderia cenocepacia* cells mixed with the buffer were used as a negative control. Alternatively, *B. cenocepacia* cells were cultivated at 37 °C with shaking for four days in M9 minimal medium with micronutrients (see Table 5). Cells were concentrated in semipermeable dialysis tubing (SnakeSkin, cut-off 3.5K MWCO, ThermoFisher) via solution removal by hygroscopic solid PEG 10000 to the OD_{600} 0.7. Cells were mixed in 9:1 ratio with 10x concentrated compound **IIc**, incubated for 5 min at 25 °C and thoroughly mixed again. 10 µl of the mixture was transferred on the microscope slide and examined by the microscope. *B. cenocepacia* cells without **IIc** were used as a negative

control. Influence of carbohydrates on the cells was tested via addition of simple L-fucose to the cells to the concentration 1M. Images were obtained by microscope digital camera DP72 (Olympus) using bright field and phase contrast observation methods. The images obtained by bright field method were edited in GIMP. The stock solution of compound **IIc** was prepared by dissolving in DMSO. Therefore, DMSO was added to the negative controls to the same residual concentrations as resulted from the dilution of compound **IIc**.

Table 5. The composition of M9 minimal medium with micronutrients

Nutrient	Final concentration	Micronutrient	Final concentration
$Na_2HPO_4.2H_2O$	4.2 x 10^{-3} M	$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	3x10^{-9} M
KH_2PO_4	7.3 x 10^{-3} M	H_3BO_3	4x10^{-7} M
NaCl	8,5 x 10^{-3} M	$CoCl_2.6H_2O$	3x10^{-8} M
NH_4Cl	1.8 x 10^{-2} M	$CuSO_4.5H_2O$	1x10^{-8} M
D-glucose	0,2% (w/v)	$MnCl_2.4H_2O$	8x10^{-8} M
$MgSO_4$	10^{-3}M	$ZnSO_4.7H_2O$	1x10^{-8} M
$CaCl_2$	10^{-4} M	$FeSO_4.7H_2O$	1x10^{-6} M

4.9. Microtiter dish biofilm formation assay

The biofilm formation of *Pseudomonas aeruginosa* ATCC 10145 (American Type Culture Collection, USA) was observed by microtiter dish biofilm assay [47]. Biofilm inhibition was determined as percentage in comparison to control (cells in LB medium without compounds). The microbial strain was pre-cultured for 24 h to achieve exponential phase of growth in LB medium (Tryptone 10 g.L^{-1}, yeast extract 5 g.L^{-1}, NaCl 10 g.L^{-1}; 100 mL of medium in Erlenmeyer flask, 100 rpm, 37 °C). The culture was centrifuged and diluted in LB medium to OD_{600nm} = 0.6. The inoculum (210 μL) was pipetted into 96-well microtiter plates along with the predetermined concentration of the compound (80 μL). The compounds were diluted in 20 % DMSO in LB medium. The final concentration of DMSO (5%) in the experiments was verified not to influence the cell growth and biofilm formation. The plates were covered with

a plastic lid and incubated at 37 °C for 24 h. After the incubation, the plates were washed with saline three times to remove planktonic cells. Crystal violet solution (0.1%, 200 μL) was added into each well for the biofilm biomass determination. The plate was incubated for 20 minutes at room temperature. Afterwards, each well was washed three times with saline to remove unbound crystal violet. Crystal violet bound to the biofilm biomass was released by adding 200 μL of 96% ethanol and incubating for 10 minutes at room temperature. The quantification of biofilm formation was accomplished by transferring the contents of each well into new microtiter plate. The absorbance of each sample was measured at 580 nm and a percentage of biofilm inhibition was calculated. Each biofilm inhibition assay was performed in 5 replicates.

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Original *C*-α-(D-manno/D-galacto/L-fuco)pyranosyl calix[4]arene-based constructs were synthesized.

The affinity towards 7 lectins was evaluated using *in vitro* and *in vivo* assays.

Synthesised fucopyranosyl calix[4]arenes are able to cross-link *B. cenocepacia* cells in a concentration-dependent manner.