

1 **Exploring calixarene-based clusters for efficient functional presentation of *Streptococcus pneumoniae***
2 **saccharides**

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15 **ABSTRACT**

16 Calixarenes are promising scaffolds for an efficient clustered exposition of multiple saccharide antigenic
17 units. Herein we report the synthesis and biological evaluation of a calix[6]arene functionalized with six
18 copies of the trisaccharide repeating unit of *Streptococcus pneumoniae* (SP) serotype 19F. This system has
19 demonstrated its ability to efficiently inhibit the binding between the native 19F capsular polysaccharide
20 and anti-19F antibodies, despite a low number of exposed saccharide antigens, well mimicking the epitope
21 presentations in the polysaccharide. The calix[6]arene mobile scaffold has been selected for
22 functionalization with SP 19F repeating unit after a preliminary screening of four model glycolixarenes,
23 functionalized with N-acetyl mannosamine, and differing in the valency and/or conformational properties.
24 This work is a step forward towards the development of new fully synthetic calixarenes comprising small
25 carbohydrate antigens as potential carbohydrate-based vaccine scaffold.

26

27 **KEYWORDS:** saccharide antigens, serotype 19F, *Streptococcus pneumoniae*, calixarenes, multivalent ligands

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29 **1. INTRODUCTION:**

30 Encapsulated bacterial infections are still one of the most prevalent causes of serious disease in humans,
31 especially in young children. Immunization is the most appropriate way to prevent bacterial infections [1].
32 In the early stages of microbiology, capsular polysaccharides (CPSs) were recognized as relevant virulence
33 factors, and found to be able to stimulate protective immunity against infections, laying the bases for the
34 development of current antibacterial vaccines [2]. Nevertheless, the study of the chemical determinants of
35 immunogenicity to CPSs still requires detailed molecular insights. CPSs are cell-surface polymers consisting
36 of oligosaccharide repeating units, characterized by a huge number of possible structural modifications and
37 linkage combinations and associated to a relative high degree of flexibility. Typically, anti-carbohydrate
38 antibodies show affinities in their recognition that are several factors lower than those observed for
39 antibodies specific for proteins or peptide antigens. This peculiar weakness is partially compensated by the
40 multivalent nature of such antibodies, that face in a clustered form a multiple number of densely displayed
41 antigen molecules to increase the effect of the response [3]. This can explain the existing relationship
42 between molecular weight and antigenicity, established for polysaccharide antigens. In fact, high molecular
43 mass repetitive polysaccharides are able to simultaneously display a greater number of epitopes capable to
44 effectively interact with specific antibodies. This kind of model where the binding of a glycan-binding

1 protein to a clustered saccharide patch enhances the overall affinity of the interaction has been widely
2 described [4], for instance between tumor associated saccharide antigens and lectins. Lectin-glycan
3 interactions are stabilized by weak hydrogen bonding and van der Waals intermolecular forces, and the
4 multivalent presentation is the basis for a biologically relevant binding. There are several examples
5 demonstrating that lectins bind their targets only when glycans are clustered at high density in a
6 multivalent glycoconjugate backbone [5]. This concept has been exploited for instance in the field of cancer
7 vaccine development. For example, the Tn tumor associated antigen is overexpressed on the surface of
8 tumor cells. It is exposed on the Mucine surface in clusters of 2 to 5 units. Data show that at least two
9 contiguous antigen molecules are crucial for the binding of the anti-Tn monoclonal antibodies, with an even
10 greater affinity in the presence of three residues [6]. Different studies have been oriented to the selection
11 of the proper scaffold for the presentation of multiple copies of the Tn antigenic unit to generate an
12 anticancer vaccine [7, 8, 9]. The choice of the scaffold for multivalent presentation of glycans to target
13 proteins is crucial in determining the biological activity [10, 11, 12, 13]. In this context, the use of
14 calixarenes represents a viable approach explored in the literature for the display of multiple glycan units
15 [14]. This type of macrocycle in fact, due to peculiar structural properties and selective synthetic
16 procedures [15], allows the achievement of small libraries of potential multivalent ligands bearing the same
17 epitopes but with a controlled modulation of their number (valency) and orientation in the space. The
18 conformational behavior of the different possible types of calixarenes strongly impacts on the presentation
19 mode of the conjugated saccharide moieties [14, 16], which can be exploited to increase the avidity of the
20 biological recognition. This versatility can make possible the comparison of subtly modified parameters and
21 their effects on selectivity and efficiency in biological activities like interaction with carbohydrate
22 recognition proteins [14]. A calix[4]arene in the so called cone geometry conjugated to LacNAc fragments,
23 for example, was found extremely selective in the inhibition of galectin-3 while totally unable to bind to
24 galectin-1 [17]. Calixarenes functionalized with lactose units showed inhibition activity towards human
25 galectins characterized, in some cases, by an impressive selectivity strictly dependent on their
26 conformational properties and related arrangement of the epitopes [18]. Analogously, different sized and
27 conformationally featured calixarenes exposing galactose units resulted in different efficiency in the
28 binding to *Pseudomonas aeruginosa* lectin A (PA-IL) [19]. Ten years ago, a calix[4]arene decorated with four
29 Tn units has indeed been described as a promising synthetic multivalent vaccine candidate [20]. In this
30 framework, we have decided to explore the potential of calixarenes as scaffolds for the multipresentation
31 of bacterial CPS fragments. Our hypothesis was to verify if such macrocyclic scaffold, presenting multiple,
32 but however limited copies of short CPS fragments, is able to gain affinity and potency towards the natural
33 antibodies approaching those observed for the natural polysaccharide. In principle, a positive assessment
34 would open the possibility to work with shorter saccharide fragments, which can be obtained by chemical
35 synthesis, instead of the longer oligosaccharides, used in vaccine formulations, obtained by size-reduced
36 purified natural polysaccharides [21]. Moreover, the use of calixarenes and short saccharide fragments
37 would have the advantage of giving structurally well-defined and easily reproducible systems.

38 We focused on the gram positive bacterium *Streptococcus pneumoniae* (SP) which is among the major
39 responsible of severe forms of bacterial infectious diseases [1, 22, 23, 24]. In particular, serotype 19F
40 (SP19F) is one of the most common causes of invasive pneumococcal disease in children, and included in
41 the current commercial pneumococcal conjugate vaccines. Its CPS is a linear polymer made up of β -D-
42 ManpNAc-(1 \rightarrow 4)- α -D-Glcp-(1 \rightarrow 2)- α -L-Rha trisaccharide repeating units, linked through phosphodiester
43 bridges (Figure 1). Herein, we report on the preparation and biological evaluation of a family of calixarenes
44 functionalized with saccharide fragments related to the trisaccharide repeating unit of SP19F with the aim
45 to explore the effect of the multipresentation of the ligand mediated by calixarenes on the binding to the
46 antibodies. We found that a calix[6]arene, functionalized with six copies of the trisaccharide repeating unit
47 of SP19F, is capable to effectively inhibit the binding between the native 19F CPS and the anti-19F
48 antibodies.

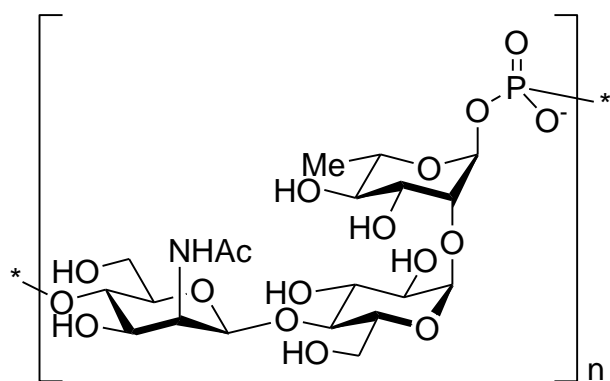


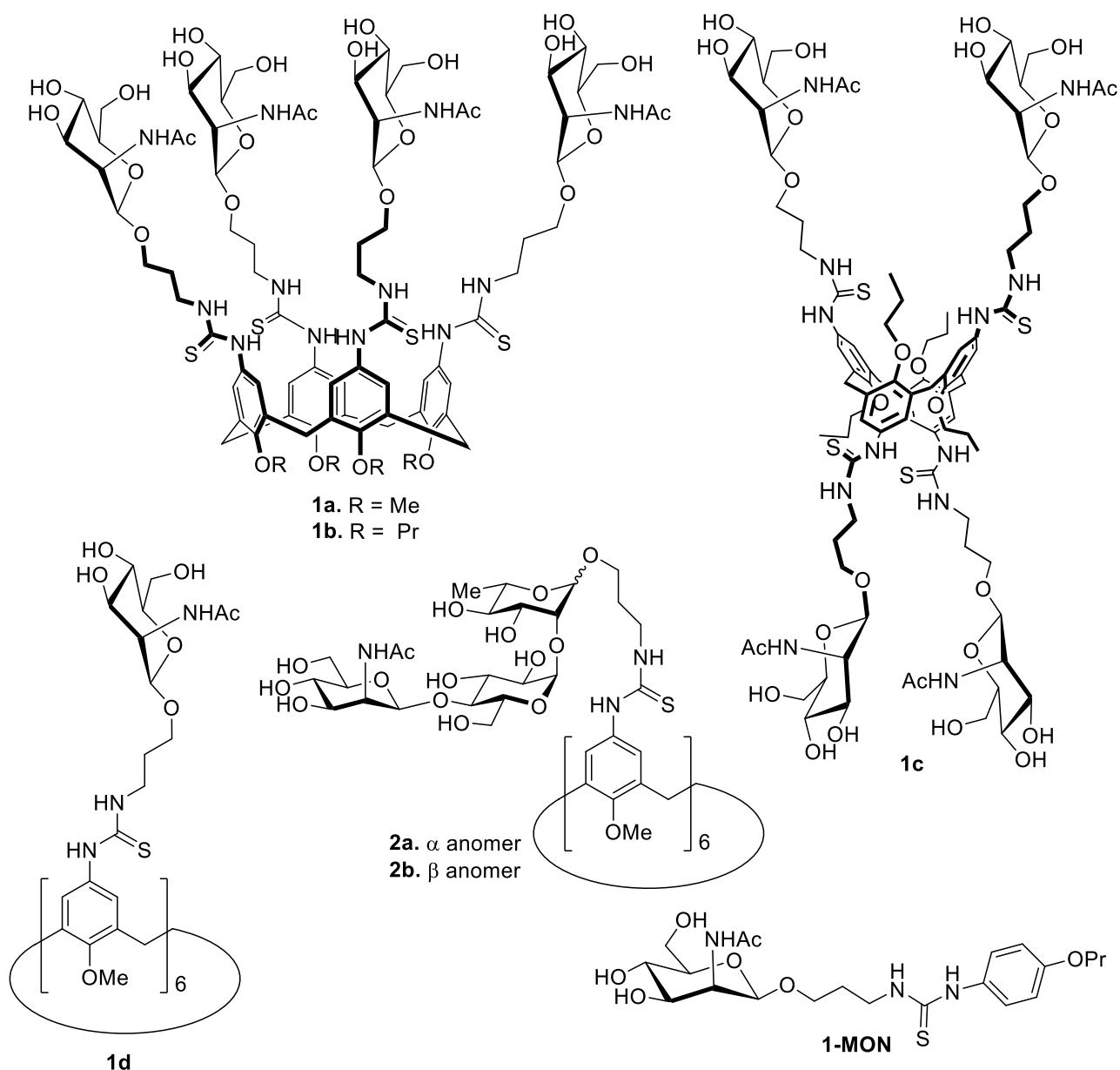
Figure 1: *Streptococcus pneumoniae* 19F capsular polysaccharide repeating unit

2. RESULTS AND DISCUSSION

2.1. Chemistry

We report the synthesis of a small library of glycolixarenes (compounds **1a-d** and **2a-b**, Figure 2) functionalized at the upper rim with saccharide units present in the CPS structure of SP19F. To this aim, both calix[4]- and calix[6]arenes were selected as scaffolds in order to have a modulation of the valency. Moreover, the calix[4]arene based derivatives were designed with three different conformational features, one conformationally mobile, one blocked in the cone geometry, one blocked in the 1,3-alternate one, in order to compare a different spatial arrangement of the epitope units. Also the calix[6]arene based glyoclusters are conformationally mobile. In addition, a monovalent acyclic analogue was planned to have a reference for verifying the role of the multivalency in the interaction with the antibodies.

In a first step of the project, all the selected scaffolds were functionalized with the N-acetyl- β -D-mannosamine residue (compounds **1a-d** and **1-MON**, Figure 2), while subsequently, based on a preliminary set of inhibition studies, only the calixarene scaffold of the best inhibitor of the binding between the 19 F polysaccharide and the corresponding anti-19F antibodies was functionalized with the SP19F trisaccharide repeating unit. Thus, two additional glycolixarenes (**2a** and **2b**, Figure 2), were obtained, with increased similarity with respect to the natural structure. This two-steps approach was pursued because of the demanding synthesis of the trisaccharide and the consequent difficulty in producing a sufficient amount for the functionalization of all the available structures.

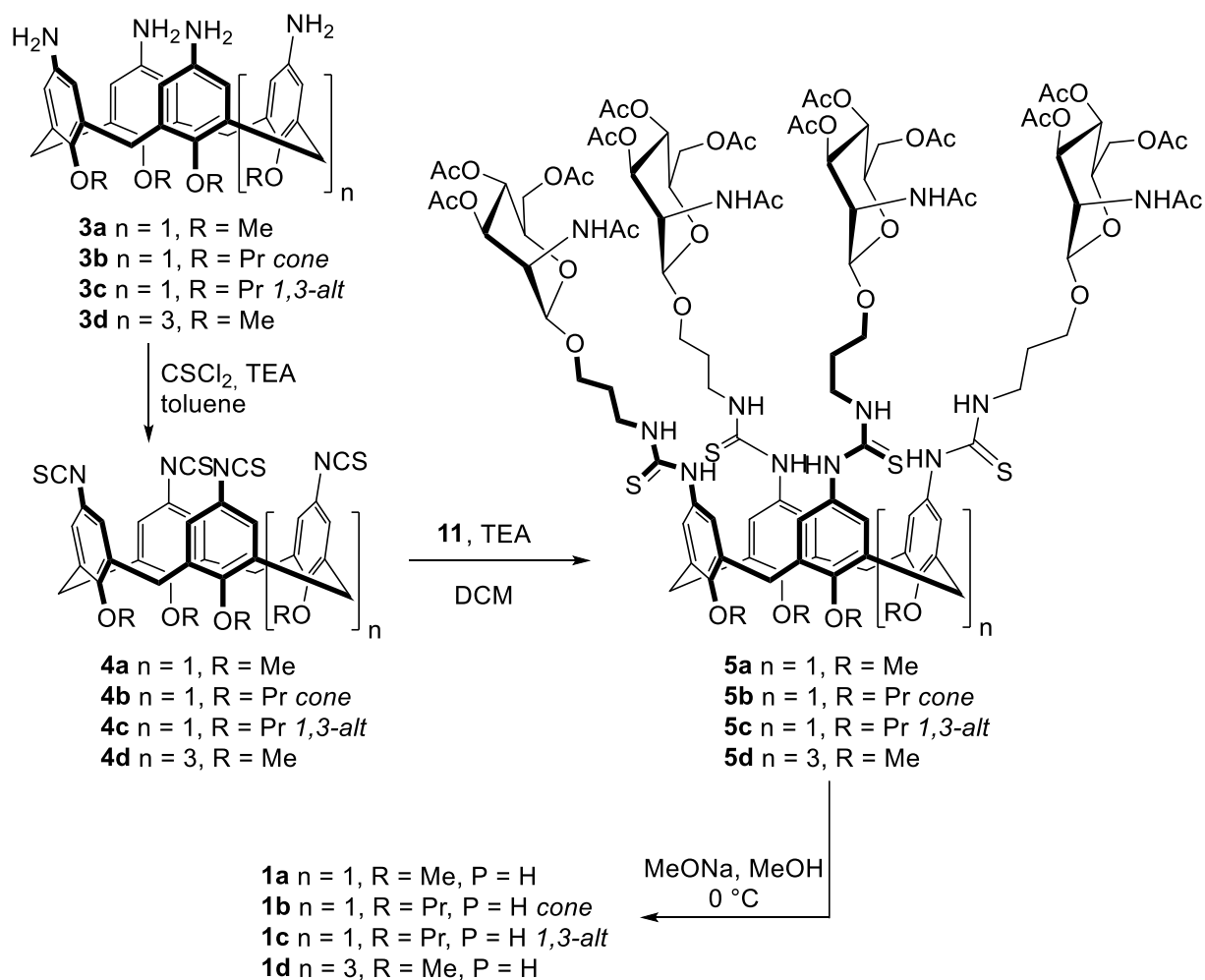


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Figure 2. Library of multivalent glycolixarenes decorated with saccharide units of SP 19F CPS

3 In order to have an efficient and clean reaction for the coupling between the calixarene scaffolds and the
 4 sugar units, we decided to exploit the amine-isothiocyanate condensation. To this aim, the calixarene
 5 isothiocyanates **4a-d** and the sugar unit **6**, functionalized at the reducing end with an aminopropyl linker,
 6 were synthesized (Scheme 1). Compounds **4a-d** were prepared by treatment of the corresponding, already
 7 known aminocalixarenes **3a-d** [25, 26, 27] with thiophosgene (*caution*) in the presence of a base, the cone
 8 isomer following a previously reported procedure [28] and the others through equivalent strategies. It may
 9 be interesting to underline that for the conformationally mobile tetra-isothiocyanate calixarene **4a** the ^1H
 10 NMR spectrum in CDCl_3 highlighted the presence of two conformers, identified as the *cone* and the *partial*
 11 *cone*, in equilibrium and in slow exchange on the NMR timescale, in a 1:4 ratio.



Scheme 1. Synthesis of glycolalix[n]arenes **1a-d**.

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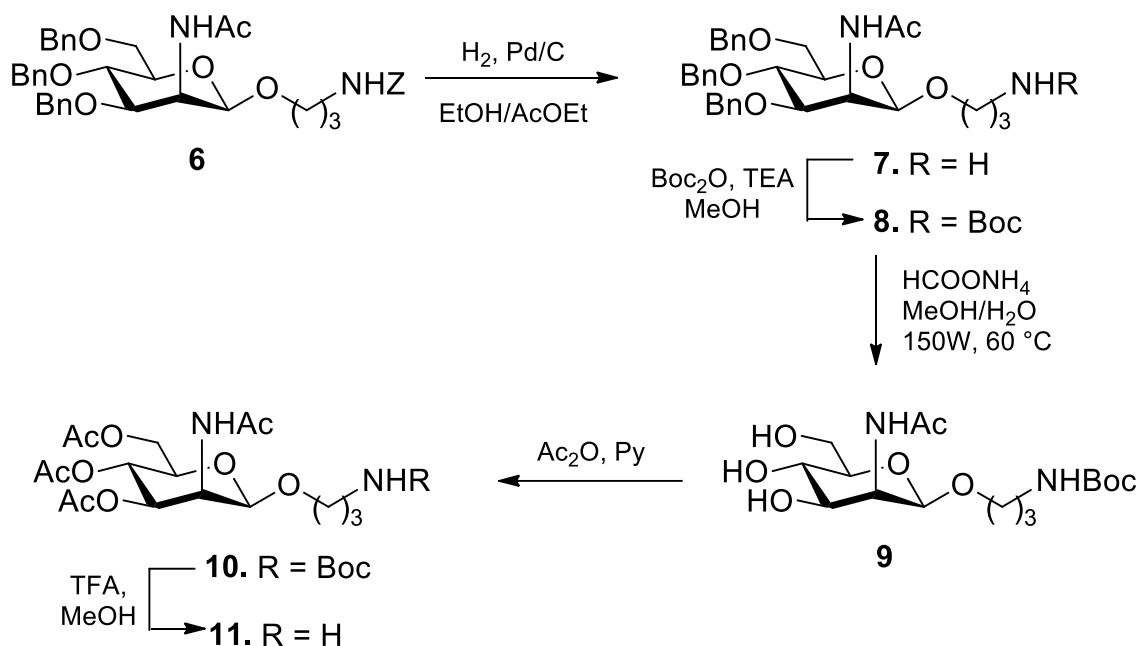
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4 On the basis of procedures reported in the literature [29], we then prepared compound **6**, a protected
 5 derivative of 3-aminopropyl N-acetyl- β -D-mannosamine, benzylated at the sugar hydroxyls.

6 Monosaccharide **6** was deprotected from the Cbz group by hydrogenolysis producing the amino derivative
 7 **7** that was subsequently reacted with cone and 1,3-alternate tetra-isothiocyanate calix[4]arenes **4b** and **4c**,
 8 obtaining in good yields the corresponding tetraglycosylated macrocycles (see SI). Nevertheless,
 9 unexpectedly, their deprotection from benzyl groups to give the two planned glycoclusters failed, despite
 10 the use of different methods, discouraging the use of this pathway for the preparation of the target
 11 calixarenes **1a-d**.

12 Therefore, we went back to monosaccharide **6** to replace benzyl protecting groups with acetyls being
 13 proved that the latter one can efficiently be removed from saccharide units attached to a calixarene
 14 scaffold [13, 14, 30, 31]. Therefore, a sequence of protection and deprotection reactions starting from **6**
 15 was carried out to finally obtain compound **11** with an overall yield of 50% (Scheme 2).

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Scheme 2. Sequence of protection and deprotection steps to obtain compound **11**.

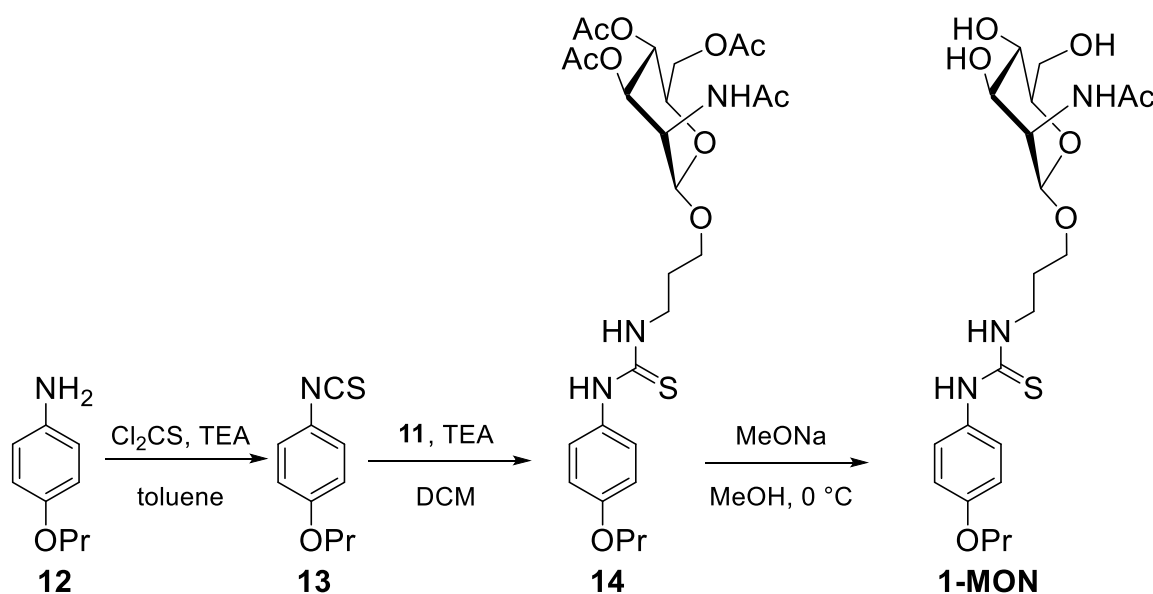
More in detail, after the selective removal of N-Cbz from **6** by hydrogenolysis, compound **7** was protected with a Boc group to give **8**. The subsequent catalytic hydrogen transfer under MW irradiation yielded **9**, fully deprotected on the OH groups, without the need of purification. The acetylation step, performed with acetic anhydride and pyridine, afforded in high yield sugar **10**, which was treated with trifluoroacetic anhydride to remove the Boc group providing **11** in quantitative yield.

The mannosamine derivative **11** was then reacted with the isothiocyanate calixarenes **4a-d**. The click chemistry reaction between isothiocyanate and amine, used for the conjugation reaction, is one of the most commonly employed methodology for the design and synthesis of multivalent glycoconjugates [32, 33]. This methodology does not involve the anomeric position of the glycoside and the newly formed thioureido unit presents an enhanced potential for hydrogen bonding that can increase both the solubility in water of the ligand and the number of interactions with the receptor. Glycocalixarenes **5a** [34], **5b**, **5c** [34] and **5d** were obtained in moderate yields (from 25% to 71%) after column chromatography performed to remove the sugar excess and traces of partially functionalized derivatives. Finally, compounds **5a-d** were deacetylated under Zemplén conditions at 0 °C to give the target calixarenes **1a-d**. For the two conformationally mobile derivatives **1a** and **1d**, we proceeded in the investigation by ¹H NMR spectroscopy of their behavior in solution. The ¹H NMR spectrum in MeOD of the former one did not give any clear information about the conformation adopted by the calixarene in solution. A spectrum in D₂O was then recorded, knowing that conformationally mobile calix[4]arene amphiphiles usually tend to adopt in water a *1,3-alternate* geometry which minimizes the lipophilic surfaces exposed to the solvent [35]. At room temperature the spectrum still presented broad signals, difficult to assign, while by raising the temperature up to 80 °C a rather well-defined spectrum was obtained (see SI at page SI19). It was possible to identify the signals of the H₁ and H₂ protons of the sugar moiety, at 4.69 and 4.43 ppm respectively, and, at the same time, the single signal at around 7 ppm for all the aromatic protons together with the absence of the typical doublets for the methylene bridge of cone and partial cone conformers demonstrated the adoption of a *1,3-alternate* geometry. This should plausibly be the geometry adopted by glycocalixarene **1a** also in the aqueous environment of the biological tests.

As observed for **1a**, the ¹H NMR spectrum in D₂O at room temperature of calix[6]arene **1d** was characterized by broad signals that became sharp by increasing the temperature at 80 °C (see SI at page SI25). This behaviour could be explained with the low mobility of the aromatic units through the macrocyclic annulus, slowed down by the steric hindrance of the bulky substituents at the upper rim

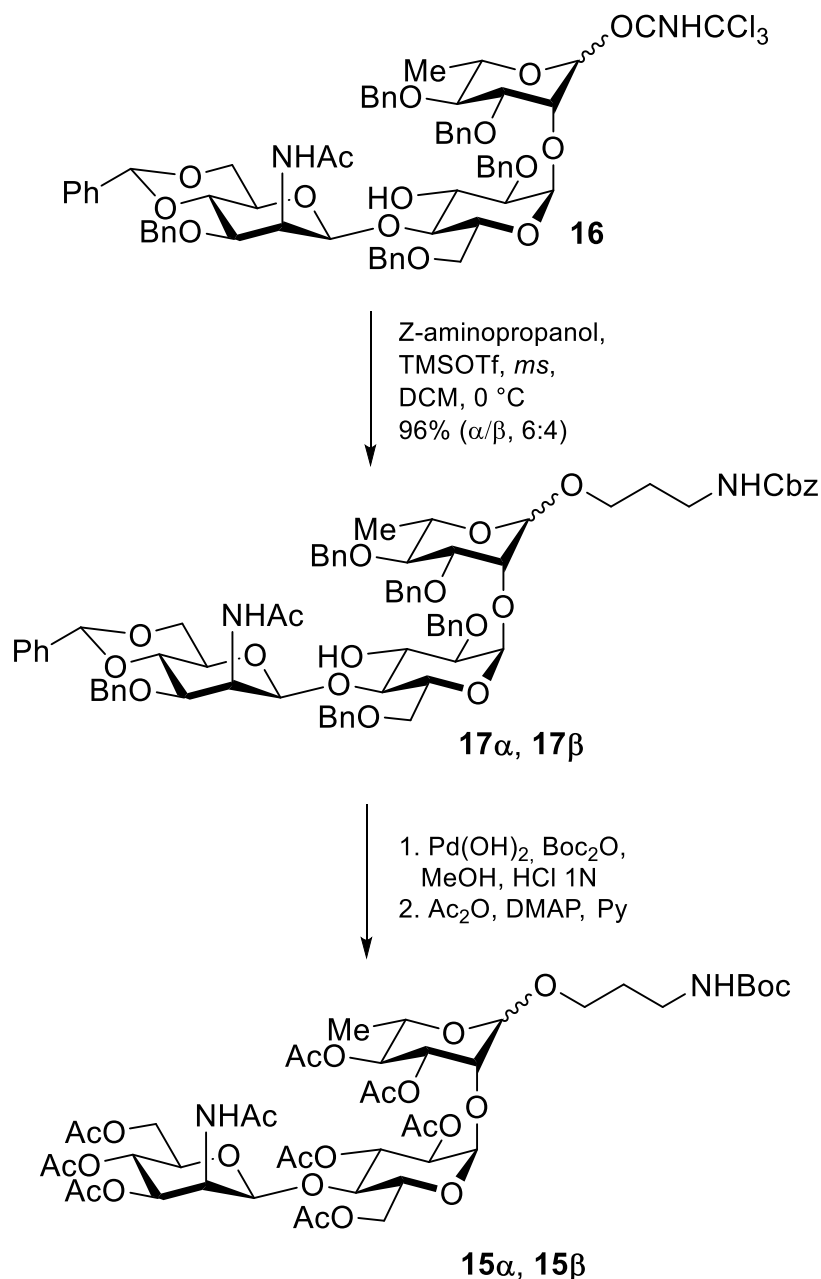
1 despite the bigger size of the macrocycle. On the other hand, interestingly, in the past it was observed,
2 rather unexpectedly, that methoxyglucocalix[6]- and [8]arenes tend to self-assemble in aqueous solution
3 [36], despite the absence of a well-defined amphiphilicity, as indeed for **1a** and **1d**. For glyco-calixarenes **1a**
4 and **1d** self-aggregation cannot then be excluded, determining or at least contributing to the broadening of
5 the signals in the ¹H NMR spectrum. In both cases, the sharpening of the signals by increasing the
6 temperature can be read as a consequence of the assembly disaggregation and/or of the increased mobility
7 of these derivatives in solution.

8 Following a similar synthetic strategy, monomeric derivative **1-MON** was prepared (Scheme 3) starting from
9 4-propoxyaniline **12** that was transformed into the corresponding isothiocyanate **13**. Compound **13** was
10 then reacted with sugar unit **11** to give compound **14** which was subsequently deprotected to the final **1-**
11 **MON** in 78% yield.



Scheme 3. Synthesis of monomer **1-MON**

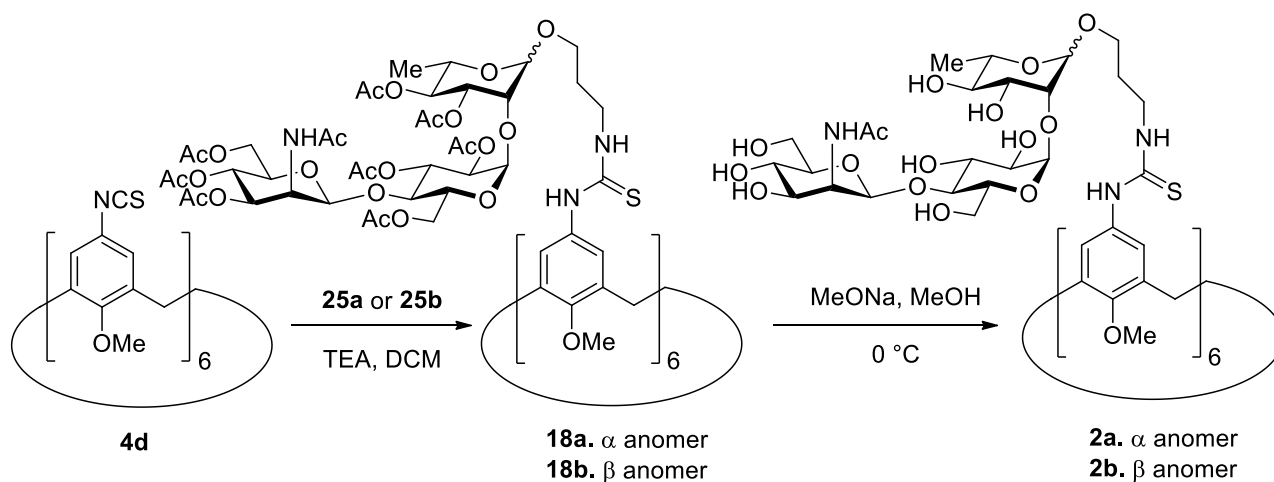
14 On the basis of the results obtained from biological tests on compounds **1a-d** (see below), the
15 conformationally mobile calix[6]arene was selected as the best scaffold for obtaining the glyco-calixarenes
16 displaying the trisaccharide repeating unit of SP19F. To this aim, exploiting our expertise in the synthesis of
17 synthetic fragments of *Streptococcus pneumoniae* [37, 38, 39], we planned the preparation of compound
18 **15**, the peracetylated derivative of the SP19F trisaccharide repeating unit, functionalized at the
19 downstream residue with an amino propyl linker. Compound **15**, was synthesized starting from the
20 trisaccharide trichloroacetimidate donor **16** [37], which was glycosylated with Z-amino propanol (Scheme 4)
21 [38]. The reaction was promoted with trimethylsilyl triflate and trisaccharide **17** was obtained in high yields
22 (96%) as an alpha/beta mixture of the two anomers **17 α** and **17 β** , which were separated by flash
23 chromatography. Each anomer was separately subjected to initial exchange of the CBZ amino protecting
24 group of the linker for the BOC one, followed by hydrogenolysis of the benzyl ethers, and final acetylation
25 of the hydroxyl groups with acetic anhydride. This protecting group manipulation sequence was very
26 efficient and allowed to recover in both cases the final trisaccharides **15 α** and **15 β** in 88% yield.



Scheme 4

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4 Hexaisothiocyanate **4d** was separately reacted in good yields with both the α and the β anomer of
 5 compound **15**, to evaluate if a different stereochemistry of the anomeric position on the rhamnose residue
 6 may have an influence on the biological activity of the glycoligand. If not, in perspective, the calixarene
 7 could be reacted with the mixture of the two anomers that is produced in the final step of the synthesis of
 8 the trisaccharide when the aminopropyl chain is introduced at the anomeric position of rhamnose. The two
 9 glycoligandenes **18a** and **18b** obtained by this condensation were deprotected under Zemplén conditions
 10 at 0 °C to give the final glycoligandenes **2a** and **2b** in 81% and 82% yield respectively (Scheme 5).



Scheme 5. Synthesis of **2a** and **2b**.

2.2. Binding affinity measurements

The ability of increasing concentrations (from 10^{-7} mg/mL to 1 mg/mL) of each new compound to inhibit the binding between the native 19F CPS, coated onto plates, and the mouse anti-19F polyclonal antibodies was evaluated in a classical competitive ELISA. Figure 3 shows the inhibition curves obtained with the compounds under evaluation.

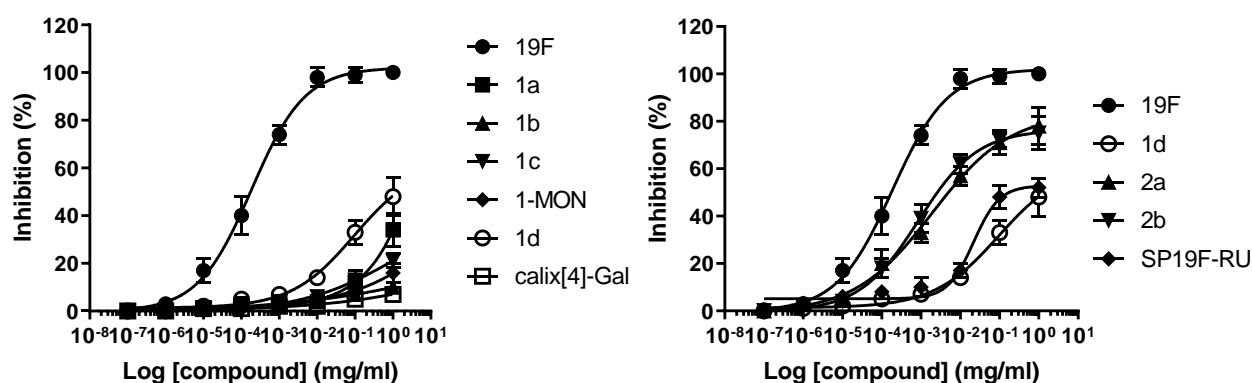


Figure 3. Results of the Elisa experiments. Concentration/response curves of tested compounds on the inhibition of the binding between SP19F native polysaccharide, coated onto the plates, and the anti-19F antibodies, evaluated by a competitive ELISA method. Values are means of at least four experiments run in triplicate

The relative efficacy of each compound was assessed by determining its maximum effect of inhibition at 1 mg/mL, while the concentration that produces the 50% of the possible maximum effect (IC_{50}) was calculated when the curve reaches a plateau and taken as indirect index of the relative potency (Table 1). The activities of the new compounds were compared with those of the natural 19F polysaccharide and the 19F trisaccharide repeating unit (**SP19F-RU**, see structure in SI) [38, 40] as the reference compound. The maximum inhibition observed for 19F CPS was fixed as the 100%.

| Compound | IC ₅₀ (mg/mL) | Max inhibition (%) ^a |
|---------------------|--------------------------|---------------------------------|
| 19F | 1.8 x 10 ⁻⁴ | 100 ± 2 |
| 1a | | 34 ± 7 |
| 1b | | 10 ± 2 |
| 1c | 1.2 x 10 ⁻¹ | 21 ± 3 |
| 1d | 9.5 x 10 ⁻² | 48 ± 8 |
| 1-MON | | 16 ± 7 |
| calix[4]-Gal | | 7 ± 2 |
| SP19F-RU | 2.1 x 10 ⁻² | 52 ± 4 |
| 2a | 1.9 x 10 ⁻³ | 78 ± 8 |
| 2b | 8.6 x 10 ⁻⁴ | 75 ± 7 |

^a The maximum inhibition elicited by each compound at 1 mg/mL

Table 1: Results of the competitive Elisa assay

Our results demonstrate that the spatial preorganization of the mannosamide units on the calixarene effectively increases their efficacy in a way that depends on the valency and on the tridimensional architecture of the macrocycle scaffold. In fact, comparing the maximum % of inhibition (Table 1) found for the monomeric model **1-MON** (16%) with that of calixarene **1a** (34%), also considering that the concentration of the ManAc epitope is substantially the same for the two compounds (2.1 and 2.2 mM for **1-MON** and **1a**, respectively), the single mannosamide unit evidences an efficacy almost 2 fold higher when it is displaced on the calix[4]arene scaffold, which raises to 3 fold with the glycolix[6]arene **1d**. The efficacy of the tetravalent calixarenes **1a-c** appears to be related to their conformational properties and the consequent geometry of presentation of the mannosamine units. In fact, the percentage of inhibition increases from the cone isomer to the 1,3-alternate and to the conformationally mobile (**1b** 10 ± 2 %, **1c** 21 ± 3%, **1a** 34 ± 7). This suggests that, despite the same number of saccharide units in these three ligands, the upper rim of ligand **1b** is too crowded for an effective interaction with the antibodies resulting even worse than the monovalent model **1-MON**. The better behavior of **1c** supports this hypothesis since it exposes only two sugar units per part of the space with a reduced steric hindrance and perhaps an overall more convenient arrangement with respect to **1b**. However, also this blocked display of **1c** seems not to be the optimal one, as reflected in its lower level of efficacy with respect to the mobile isomer **1a** that evidently can adapt itself for a better interaction with the antibodies as allows the epitope units to better adapt to the binding sites. Flexibility appears then to play an important role in determining the efficacy. The inability of compound **calix[4]-Gal**, a tetra thioureidoglycolixarene based on the same calix[4]arene scaffold as **1a** but bearing a non-related sugar (galactose), in inhibiting the binding between 19F CPS and anti-19F antibodies confirms the specificity of the sugar recognition and excludes an unspecific cross-reaction with the calixarene scaffold. On the whole, the conformational properties, the related geometry of exposition of the mannosamine units, the flexibility together with the higher valency could explain the highest efficacy (maximal inhibition 48 ± 8%) and potency (IC₅₀= 9.5x10⁻² mg/mL) showed by glycolix[6]arene **1d**. Calix[6]arene was then selected as the more promising scaffold for the functionalization with the SP19F trisaccharide repeating unit analogs **2a** and **2b**.

As shown in Figure 3 and reported in Table 1, glycolix[6]arene **2a** and **2b**, decorated with six trisaccharide SP19F repeating units, significantly improve efficacy (75-78% of inhibition) and affinity (IC₅₀= 1.9x10⁻³ and 8.6x10⁻⁴ mg/mL, respectively) towards the antibodies with respect to the simpler cluster **1d** (48% of inhibition, IC₅₀= 9.5x10⁻² mg/mL). This result can be explained considering the nature of the saccharide ligands on the two clusters: the trisaccharide repeating unit is a more specific epitope for the anti-19F antibodies, whereas the lower, but still non-negligible, activity of compound **1d** could be ascribed to a

1 predominant role of the mannosamine unit into the trisaccharide. Moreover, and more importantly, both
2 glycolixarenes **2a** and **2b** are more active than the single trisaccharide unit (52% of inhibition and $IC_{50} =$
3 2.1×10^{-2} mg/mL) showing once more and more significantly that the presentation of the epitope units on
4 this type of scaffold increases the strength and the efficacy of antibody binding. In this framework, at the
5 maximum of inhibition, the trisaccharide attached to the calix[6]arene platform is at a very similar
6 concentration (1.31 mM) as when used alone (1.47 mM), but induces an efficacy 1.5 fold higher than
7 **SP19F-RU**, determining an inhibition of the binding over 70% significantly higher than the 52% of the
8 **SP19F-RU**. These data strongly suggest that the glycolix[6]arene thanks to its peculiar conformational
9 mobility enables a proper presentation of the trisaccharide repeating units that improves the strength of
10 the ligand-receptor interactions well mimicking the conformational organization of the epitopes generated
11 by the natural 19F polysaccharide and recognized by the specific antibodies. This result is even more
12 significant if we consider that the chemical structure of calixarenes **2a** and **2b** is much simpler than the one
13 of the natural polysaccharide and yet able to provide a significant inhibitory effect. As a consequence, the
14 maximum of inhibition compared with that of 19F CPS is relevant, 75% of inhibition versus the 100%.
15 Furthermore, the structural difference due to the α and β anomeric connection of the trisaccharide to the
16 spacer has no significant influence on the efficacy of the two ligands **2a** and **2b**, although resulted in slightly
17 different potencies. This means that, in perspective, the separation of the two anomers **15 α** and **15 β** could
18 even be avoided, proceeding in the coupling as a mixture of both with the calixarene isothiocyanate. The
19 statistic products containing randomly both anomers should substantially show the same biological activity
20 as **2a** and **2b** with a not negligible save in the procedure of preparation.

21 22 23 **3. CONCLUSIONS**

24 Fully synthetic carbohydrate-based vaccines offer the advantage to allow site-selective conjugation of
25 saccharide epitopes [37, 41, 42] and to incorporate into the nanosystems active mediators to increase
26 vaccine efficacy. The possibility to accurately control the number and type of vaccine active species ensures
27 homogeneous composition, which is important to induce highly reproducible biological properties with a
28 better safety profile. In this work, the calix[6]arene scaffold represents a valuable platform for the
29 simultaneous presentation of multiple copies of minimized portions of the 19F CPS. In fact, this system,
30 bearing six trisaccharide repeating units linked through a thiourea group to the macrocycle via an
31 aminopropyl spacer, has been able to efficiently bind to anti-19F antibodies with a significant improvement
32 of the inhibition activity compared to the one of the single repeating unit. The overall efficiency is very
33 high, especially considering the low number of exposed saccharide antigens compared to the large number
34 of repeating units that are present in the natural polymer. Evidently, the structural properties of the
35 calixarene can properly present the exposed saccharide units in a tridimensional arrangement that is
36 significantly similar to that of the natural epitopes in CPS. Our data clearly suggest that, in perspective,
37 glycolixarenes **2a** and **2b**, or even closely related systems with slightly longer saccharide fragments, could
38 be functionalized with immunogenic peptides in order to elicit an antibacterial specific immune response. A
39 structurally well-defined and easily reproducible multivalent glycolixarene should avoid the use of
40 complex oligosaccharide species, which are generally required for immunogenicity. Calixarenes seem then
41 to have great potential as carriers for the development of fully synthetic carbohydrate-based vaccines, and
42 this work contributes towards this direction.

43 44 **4. EXPERIMENTAL SECTION**

45 *4.1. Chemical procedures*

46 **General information.** All moisture sensitive reactions were carried out under a nitrogen or argon
47 atmosphere, using previously oven-dried glassware. All dry solvents were prepared according to standard
48 procedures, distilled before use and stored over 3 or 4 Å molecular sieves. All other reagents were
49 commercial samples and used without further purification. Analytical TLC were performed using prepared
50 plates of silica gel (Merck 60 F-254 on aluminum) and then, according to the functional groups present on
51 the molecules, revealed with UV light or using staining reagents: $FeCl_3$ (1% in $H_2O/MeOH$ 1:1), H_2SO_4 (5% in

1 EtOH), ninhydrin (5% in EtOH), basic solution of KMnO_4 (0.75% in H_2O), molybdic acid solution
2 (molybdatophosphorus acid and Ce(IV)sulphate in 4% sulphuric acid). Reverse phase TLC were performed
3 using silica gel 60 RP-18 F-254 on aluminium sheets. Merck silica gel 60 was used for flash chromatography
4 (40-63 μm) and for preparative TLC plates (10-12 μm). Sigma Aldrich C18 reverse phase silica gel was used
5 for flash chromatography. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AV300 and Bruker AV400
6 spectrometers (observation of ^1H nucleus at 300 MHz and 400 MHz, respectively, and of ^{13}C nucleus at 75
7 MHz and 100 MHz, respectively) and partially deuterated solvents were used as internal standards to
8 calculate the chemical shifts (δ values in ppm). All ^{13}C NMR spectra were performed with proton
9 decoupling. For ^1H NMR spectra recorded in D_2O at temperatures higher than 25°C the correction of
10 chemical shifts was performed using the expression $\delta = 5.060 - 0.0122 \times T(^{\circ}\text{C}) + (2.11 \times 10^{-5}) \times T^2(^{\circ}\text{C})$ [43] to
11 determine the resonance frequency of water protons. Electrospray ionization (ESI) mass analyses were
12 performed with a Waters single-quadrupole spectrometer in positive or negative mode using MeOH or
13 CH_3CN as solvents or with a LTQ Orbitrap XL spectrometer in positive ionization mode. Melting points were
14 determined on an Electrothermal apparatus in closed capillaries. Microwave reactions were performed
15 using CEM Discovery System reactor.

16 5,11,17,23-Tetraamino-25,26,27,28-tetramethoxy-calix[4]arene [25], 5,11,17,23-tetraamino-25,26,27,28-
17 tetrapropoxy-calix[4]arene *cone* [27], 5,11,17,23-tetraamino-25,26,27,28-tetrapropoxy-calix[4]arene *1,3-*
18 *alternate* [25], 5,11,17,23,29,35-hexaamino-37,38,39,40,41,42-hexamethoxycalix[6]arene [26], 1-amino-4-
19 propoxy benzene [44], 5,11,17,23-tetraisothiocyanate-25,26,27,28-tetrapropoxy-calix[4]arene *cone* [28], N-
20 (benzyloxycarbonyl)aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy- β -D-mannopyranoside [29] were
21 prepared according to literature procedures.

22 **4.1.1. 5,11,17,23-Tetraisothiocyanate-25,26,27,28-tetramethoxy-calix[4]arene (4a):**

23 In a two-neck round-bottom flask 5,11,17,23-tetraamino-25,26,27,28-tetramethoxy-calix[4]arene **3a** (0.82
24 g, 1.22 mmol) was dissolved in 65 mL of dry toluene under N_2 atmosphere. Then thiophosgene (1.11 mL,
25 14.64 mmol, *caution*) and Et_3N (4.07 mL, 29.28 mmol) were added and the mixture was allowed to react at
26 room temperature for 48 h. The solvent was removed under reduced pressure and the crude was
27 redissolved in dichloromethane. The organic phase was washed with water (2x70 mL), 1 N HCl (70 mL) and
28 then with a saturated solution of NaCl (2x80 mL). The solvent was removed under reduced pressure and
29 the residue purified by column chromatography (hexane/DCM 7/3, v/v) to afford a yellowish solid (0.19 g,
30 0.28 mmol, 23% yield). Mp: dec > 180°C . ^1H NMR (400 MHz, CDCl_3) δ (ppm): 7.14 (s, 1.6H, ArH, partial
31 cone), 7.02 (s, 1.6H, ArH, partial cone), 6.81 (s, 1.6H, ArH, partial cone), 6.65 (s, 1.6H, ArH, cone), 6.29 (s,
32 1.6H, ArH, partial cone), 4.26 (d, $J = 13.5$ Hz, 0.8H, $\text{ArCHH}_{\text{ax}}\text{Ar}$, cone), 3.97 (d, $J = 14.0$ Hz, 1.6H, $\text{ArCHH}_{\text{ax}}\text{Ar}$,
33 partial cone), 3.78 (s, 2.4H, OCH_3 , cone), 3.74 (s, 2.4H, OCH_3 , partial cone), 3.68 (s, 4.8H, OCH_3 , partial cone),
34 3.56 (s, 3.2H, $\text{ArCHH}_{\text{eq}}\text{Ar}$, partial cone), 3.13 (d, $J = 13.5$ Hz, 0.8H, $\text{ArCHH}_{\text{eq}}\text{Ar}$, cone), 3.06 (d, $J = 14.0$ Hz,
35 1.6H, $\text{ArCHH}_{\text{eq}}\text{Ar}$, partial cone), 3.04 (s, 2.4H, OCH_3 , partial cone). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 156.9,
36 156.4 (C_{Ar} ipso), 135.6, 134.3, 132.5, 127.7, 126.5, 126.2, 125.7, 125.6 (C_{Ar} , NCS), 61.9, 61.4, 60.2, 59.8
37 (OCH_3), 35.0 (ArCH_2Ar , partial cone), 30.3, 30.1 (ArCH_2Ar , cone and partial cone). HRMS (ESI-TOF) m/z : calcd
38 for $\text{C}_{36}\text{H}_{28}\text{N}_4\text{O}_4\text{S}_4\text{Na}$ [$(4\mathbf{a}+\text{Na})^+$] 731.0886, found 731.0861.

39 **4.1.2. 5,11,17,23-Tetraisothiocyanate-25,26,27,28-tetrapropoxy-calix[4]arene 1,3-alternate (4c):**

40 In a two-neck round-bottom flask 5,11,17,23-tetraamino-25,26,27,28-tetrapropoxy-calix[4]arene *1,3-*
41 *alternate* **3c** (1.23 g, 1.96 mmol) was dissolved in 25 mL of dry toluene under N_2 atmosphere. Then
42 thiophosgene (1.8 mL, 23.47 mmol, *caution*) and Et_3N (6.53 mL, 46.94 mmol) were added and the mixture
43 was allowed to react at room temperature for 48 h. The solvent was removed under reduced pressure and
44 the crude was redissolved in dichloromethane. The organic phase was washed twice with distilled H_2O (30
45 mL), once with 1 N HCl (30 mL) and then twice with a saturated solution of NaCl (40 mL). The solvent was
46 removed under reduced pressure and the desired compound was obtained in 33% yield as an off-white
47 solid after purification by column chromatography (cyclohexane/DCM 4/1 v/v) (0.53 g, 0.65 mmol). Mp:
48 $220\text{--}221^\circ\text{C}$. ^1H NMR (400 MHz, CDCl_3) δ (ppm): 6.90 (s, 8H, ArH), 3.65 (t, $J = 7.2$ Hz, 8H, OCH_2), 3.47 (s, 8H,
49 ArCH_2Ar), 1.87-1.78 (m, 8H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.09 (t, $J = 7.2$ Hz, 12H, CH_2CH_3). ^{13}C NMR (100 MHz, CDCl_3) δ
50 (ppm): 155.3 (C_{Ar} ipso), 134.0 (NCS), 127.1 (C_{Ar} ortho), 125.0 (C_{Ar} para), 74.7 (OCH_2), 35.1 (ArCH_2Ar), 23.9

1 (OCH₂CH₂CH₃), 10.7 (CH₂CH₃). HRMS (ESI-TOF) m/z: calcd for C₄₄H₄₄N₄O₄S₄Na [(4c+Na)⁺] 843.2138, found
2 843.2156.

3 **4.1.3. 5,11,17,23,29,35-Hexaisothiocyanate-37,38,39,40,41,42-hexamethoxy-calix[6]arene (4d):**

4 In a round-bottom flask **3d** (0.15 g, 0.185 mmol) was dissolved in DCM (5 mL) under N₂ atmosphere. Then
5 thiophosgene (254 μL, 3.33 mmol, *caution*), BaCO₃ (0.657 g, 3.33 mmol) and H₂O (3 mL) were added with
6 the remaining amount of DCM (5 mL). The mixture was allowed to react at rt for 48 h and then diluted
7 with DCM/H₂O. The organic phase was separated from the aqueous layer and evaporated under reduced
8 pressure. The crude thus obtained was purified by flash chromatography (hexane/EtOAc 8/2, v/v) and
9 crystallized with CH₃CN to yield the pure product as a white solid (39 mg, 0.037 mmol, 20%). Mp: dec >
10 200°C. ¹H NMR (300 MHz, CDCl₃) δ (ppm): 6.74 (s, 12H, ArH), 3.89 (s, 12H, ArCH₂Ar), 3.54 (s, 18H, OCH₃). ¹³C
11 NMR (75 MHz, CDCl₃) δ (ppm): 155.3 (C_{Ar} ipso), 135.2 (C_{Ar} ortho), 133.9 (NCS), 126.6 (C_{Ar} para), 126.2 (C_{Ar}
12 meta), 61.0 (OCH₃), 30.2 (ArCH₂Ar). HRMS (ESI-TOF) m/z: calcd for C₅₄H₄₂N₆O₆S₆Na [(4d+Na)⁺] 1085.1388,
13 found 1085.1414 (80%); calcd for C₅₄H₄₂N₆O₆S₆K [(4d+K)⁺] 1101.1127, found 1101.1151 (100%).

14 **4.1.4. Aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside (7):**

15 To a solution of N-(Benzyloxycarbonyl)aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-
16 mannopyranoside (**6**) (0.54 g, 0.79 mmol) in a 4:1 mixture of AcOEt/EtOH (10 mL), Pd/C (10%) was added
17 and the suspension was shaken in a Parr hydrogenator for 90 minutes under 1.5 bar of H₂ at room
18 temperature, after which the catalyst was filtered off and the filtrate evaporated under vacuum, to give the
19 desired product as an orange oil (0.373 g, 0.68 mmol, 86%). ¹H NMR (300 MHz, CDCl₃) δ (ppm): 7.38-7.13
20 (m, 15H, ArH), 6.20 (d, *J* = 9.9 Hz, 1H, NHAc), 4.90-4.79 (m, 3H, H₂, 2 × CHHPh), 4.57 (d, *J* = 11.9 Hz, 1H,
21 CHHPh), 4.52-4.42 (m, 4H, H₁, 3 × CHHPh), 3.93-3.82 (m, 1H, OCHHCH₂), 3.73 (br, 2H, H_{6a,b}), 3.68-3.62 (m,
22 2H, H₄, H₅), 3.61-3.51 (m, 1H, OCHHCH₂), 3.46-3.38 (m, 1H, H₃), 2.83-2.74 (m, 2H, CH₂CH₂NH₂), 2.03 (s, 3H,
23 CH₃CO), 1.77-1.69 (m, 2H, CH₂CH₂CH₂). ¹³C NMR (75 MHz, CDCl₃) δ (ppm): 171.9 (COCH₃), 138.2, 137.9,
24 137.7, 128.4, 128.33, 128.31, 127.9, 127.85, 127.8, 127.7 (C_{Ar}), 99.5 (C₁), 79.8 (C₅), 75.0 (C₃), 74.8 (CH₂Ph),
25 74.1 (C₄), 73.3 (CH₂Ph), 71.1 (CH₂Ph), 68.9 (C₆), 67.6 (OCH₂), 49.2 (C₂), 38.5 (CH₂NH₂), 27.9 (CH₂CH₂CH₂), 23.6
26 (COCH₃). ESI-MS m/z: calcd for C₃₂H₄₁N₂O₆ [(7+H)⁺] 549.3, found 549.0.

27 **4.1.5. N-(Boc)aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside (8):**

28 Aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside (**7**) (0.34 g, 0.63 mmol) was
29 dissolved in MeOH (8 mL), then Et₃N (348 μL, 2.5 mmol) and Boc₂O (0.68 g, 3.13 mmol) were subsequently
30 added. The reaction was allowed to react for 2 h, then the solvent was removed under vacuum. The pure
31 product was obtained as a pale oil in quantitative yield (0.40 g, 0.61 mmol). ¹H NMR (300 MHz, CDCl₃) δ
32 (ppm): 7.26-7.03 (m, 15H, ArH), 6.18 (d, *J* = 9.4 Hz, 1H, NHAc), 5.26 (br, 1H, NHBoc), 4.78-4.68 (m, 3H, H₂,
33 2 × CHHPh), 4.44 (d, *J* = 12.0 Hz, 1H, CHHPh), 4.40-4.31 (m, 4H, 3 × CHHPh, H₁), 3.75-3.66 (m, 1H, OCHHCH₂),
34 3.64-3.58 (m, 2H, H_{6a,b}), 3.56-3.51 (m, 2H, H₄, H₅), 3.50-3.39 (m, 1H, OCHHCH₂), 3.37-3.29 (m, 1H, H₃), 3.11-
35 3.05 (m, 2H, CH₂CH₂NHBoc), 1.89 (s, 3H, COCH₃), 1.66-1.54 (m, 2H, CH₂CH₂CH₂), 1.31 (s, 9H, C(CH₃)₃). ¹³C
36 NMR (100 MHz, CDCl₃) δ (ppm): 170.8 (COCH₃), 156.1 (CO(CH₃)₃), 146.7, 138.2, 137.9, 137.8, 128.4, 128.3,
37 128.2, 127.8, 127.78, 127.73, 127.6 (C_{Ar}), 99.5 (C₁), 85.0 (C(CH₃)₃), 80.3 (C₅), 74.9 (C₃), 74.7 (CH₂Ph), 74.0 (C₄),
38 73.3 (CH₂Ph), 71.0 (CH₂Ph), 68.8 (C₆), 67.1 (OCH₂), 49.2 (C₂), 37.6 (CH₂NH₂), 29.7 (CH₂CH₂CH₂), 27.2 (C(CH₃)₃),
39 23.3 (COCH₃). ESI-MS m/z: calcd for C₃₇H₄₈N₂O₈Na [(8+Na)⁺] 671.3, found 671.1.

40 **4.1.6. N-(Boc)aminopropyl 2-acetamido-2-deoxy-β-D-mannopyranoside (9):**

41 In a MW sealed vessel N-(Boc)aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside
42 (**8**) (0.60 g, 0.93 mmol) was dissolved in a MeOH/H₂O mixture (5 mL, 1/1 v/v) and Pd/C (10%) in catalytic
43 amount and then NH₄COOH (0.23 g, 3.7 mmol) were added. The mixture was heated at 60 °C under
44 microwave irradiation (150 W) for 1.5 h. The catalyst was filtered off and the solvent removed under
45 reduced pressure, to give the desired product as white foam (0.30 g, 0.79 mmol, 85%) ¹H NMR (400 MHz,
46 MeOD) δ (ppm): 4.65 (s, 1H, H₁), 4.47 (d, *J* = 3.2 Hz, 1H, H₂), 3.87 (br, 3H, H_{6a,b}, OCHHCH₂), 3.68 (dd, *J*_{3,2} =
47 4.0, *J*_{3,4} = 9.5 Hz, 1H, H₃), 3.63-3.57 (m, 1H, OCHHCH₂), 3.53 (t, *J* = 9.5 Hz, 1H, H₄), 3.31-3.25 (m, 1H, H₅), 3.13
48 (t, *J* = 6.4 Hz, 2H, CH₂CH₂NHBoc), 2.05 (s, 3H, COCH₃), 1.73 (t, *J* = 6.0 Hz, 2H, CH₂CH₂CH₂), 1.45 (s, 9H,
49 C(CH₃)₃). ¹³C NMR (100 MHz, MeOD) δ (ppm): 173.4 (COCH₃), 157.1 (CO(CH₃)₃), 99.4 (C₁), 78.5 (C(CH₃)₃), 76.9

1 (C₅), 73.0 (C₃), 66.9 (C₄), 66.3 (OCH₂), 60.5 (C₆), 53.5 (C₂), 36.9 (CH₂NH₂), 29.5 (CH₂CH₂CH₂), 27.4 (C(CH₃)₃),
2 21.4 (COCH₃). ESI-MS m/z: calcd for C₁₆H₃₂N₂O₈Na [(9+Na)⁺] 401.2, found 401.3.

3 **4.1.7. N-(Boc)aminopropyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopyranoside (10):**

4 N-(Boc)aminopropyl 2-acetamido-2-deoxy-β-D-mannopyranoside (9) (0.18 g, 0.47 mmol) was dissolved in
5 pyridine (6 mL) and then acetic anhydride was added (740 μL, 7.91 mmol). The reaction mixture was stirred
6 at room temperature for 1 h, then the solvent removed under reduced pressure. The pure compound was
7 obtained after purification via flash column chromatography (EtOAc/hexane 4/1, v/v) as a white foam in
8 quantitative yield (0.23 g, 0.46 mmol). ¹H NMR (300 MHz, CDCl₃) δ (ppm): 5.94 (br, 1H, NHAc), 5.06 (t, J =
9 9.2 Hz, 1H, H₄), 4.97 (dd, J_{3,2} = 4.0, J_{3,4} = 9.2 Hz, 1H, H₃), 4.67-4.60 (m, 3H, H₁, H₂, NHBoc), 4.27 (dd, J_{6a,5} = 6.0,
10 J_{6a,6b} = 15.0 Hz, 1H, H_{6a}), 4.12 (dd, J_{6b,5} = 6.0, J_{6b,6a} = 15.0 Hz, 1H, H_{6b}), 3.90-3.83 (m, 1H, OCHHCH₂), 3.68-3.58
11 (m, 1H, H₅), 3.55 (br, 1H, OCHHCH₂), 3.22-3.15 (m, 2H, CH₂CH₂NHBoc), 2.09 (s, 3H, CH₃CO), 2.06 (s, 3H,
12 CH₃CO), 2.04 (s, 3H, CH₃CO), 2.00 (s, 3H, NHCOCH₃), 1.77-1.68 (m, 2H, CH₂CH₂CH₂), 1.42 (s, 9H, C(CH₃)₃). ¹³C
13 NMR (75 MHz, CDCl₃) δ (ppm): 171.4, 170.5, 170.2, 169.7 (COCH₃), 156.1 (CO(CH₃)₃), 98.7 (C₁), 78.9 (C(CH₃)₃),
14 72.2 (C₅), 71.4 (C₃), 67.0 (OCH₂), 66.2 (C₄), 62.6 (C₆), 49.9 (C₂), 37.3 (CH₂NH₂), 29.5 (CH₂CH₂CH₂), 28.3
15 (C(CH₃)₃), 22.9, 20.6, 20.5 (COCH₃). ESI-MS m/z: calcd for C₂₂H₃₆N₂O₁₁Na [(22+Na)⁺] 527.2, found 527.4.

16 **4.1.8. Aminopropyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopyranoside (11):**

17 N-(Boc)aminopropyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopyranoside (10) (0.24 g, 0.47 mmol)
18 was dissolved in dry DCM (15 mL), then trifluoroacetic acid (1.27 mL, 16.45 mmol) was added dropwise.
19 The reaction was allowed to stir at room temperature for 1 h, then it was quenched by the addition of Et₃N.
20 The solvent was evaporated under reduced pressure and the desired compound was obtained as a yellow-
21 orange oil in quantitative yield (0.19 g, 0.46 mmol). ¹H NMR (300 MHz, CDCl₃) δ (ppm): 6.85 (d, J = 9.0 Hz,
22 1H, NHAc), 5.08 (t, J = 9.6 Hz, 1H, H₄), 4.99 (dd, J_{3,2} = 4.6, J_{3,4} = 9.6 Hz, 1H, H₃), 4.78-4.70 (m, 2H, H₁, H₂), 4.24
23 (dd, J_{6a,5} = 6.0, J_{6a,6b} = 12.4 Hz, 1H, H_{6a}), 4.13 (dd, J_{6b,5} = 2.4, J_{6b,6a} = 12.0 Hz, 1H, H_{6b}), 4.01-3.92 (m, 1H,
24 OCHHCH₂), 3.81-3.74 (m, 2H, H₅, OCHHCH₂), 3.71-3.64 (m, 2H, CH₂CH₂NH₂), 2.07 (s, 3H, CH₃CO), 2.02 (s, 6H,
25 CH₃CO), 1.99 (s, 3H, NHCOCH₃), 2.00-1.96 (m, 2H, CH₂CH₂CH₂). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 161.5,
26 161.2, 160.4 (COCH₃), 98.2 (C₁), 72.3 (C₅), 71.3 (C₃), 66.6 (OCH₂), 66.0 (C₄), 62.4 (C₆), 49.5 (C₂), 37.4 (CH₂NH₂),
27 26.3 (CH₂CH₂CH₂), 22.4, 20.4 (COCH₃). HRMS (ESI-TOF) m/z: calcd for C₁₇H₂₈N₂O₉Na [(11+Na)⁺] 427.1687,
28 found 427.1658.

29 **4.1.9. N-(tert-butoxycarbonyl)-3-amminopropyl (2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-**
30 **mannopyranosyl)-(1→4)-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1→2)-3,4-di-O-acetyl-L-**
31 **rhamnopyranoside (15):** Compound 16 [37] (0.19 g, 0.147 mmol) and N-(benzyloxycarbonyl)-3-
32 amminopropyl (0.12 g, 0.586 mmol), as previously described [38], were dissolved in dry CH₂Cl₂ (3 ml) and
33 activated powder molecular sieves 4 Å (0.10 g) were added. The suspension was stirred under Ar
34 atmosphere at room temperature for 15 minutes, then it was cooled to 0 °C and TMSOTf 0.1 M in dry
35 CH₂Cl₂ (0.29 ml, 0.029 mmol) was added. After 15 minutes, the reaction was quenched by the addition of
36 TEA, filtered over a Celite pad and the solvent evaporated under reduced pressure. Purification of the crude
37 through flash chromatography (Hexane/Ethyl Acetate 6:4) afforded 0.48 g of the less polar α-anomer, 0.80
38 g of a mixture of the two anomers, and 0.63 g of the β-anomer (17 overall yield: 96%). 17α and 17β were
39 reacted separately in the next step. To a solution under Argon of compound SP3 in MeOH (0.01M), Boc₂O
40 (3.5 eq.) and then Pd(OH)₂/C (1:1, w/w_{substrate}) were added. The mixture was stirred under hydrogen
41 atmosphere for 3 h, and checked by TLC (E/A, 1:1) to confirm that the Z-amino protecting group has been
42 exchanged with BOC. Then, one drop of HCl 1N was added, and the reaction was stirred again under
43 hydrogen atmosphere overnight. TLC (DCM/MeOH, 75:25) control showed that the reaction was
44 completed, then few drops of dry Py were added, and the reaction was filtered over filter paper. After
45 evaporation of the solvent, the crude was dissolved in dry Py (0.03M), and acetic anhydride was added
46 (Ac₂O/Py, 1:2) together with a catalytic amount of DMAP. The reaction was stirred at room temperature for
47 19 h, diluted with MeOH, and then the solvent evaporated. Purification of the crude by flash
48 chromatography (hexane/EtOAc, 1:9) gave compound 15 as an amorphous white solid.

1 **15 α** : 0.32 g of **15 α** were obtained starting from 0.48 g of **17 α** (88% yield). $[\alpha]_D^{20} = +22.3$ ($c = 1$ in
2 chloroform). $^1\text{H-NMR}$ (CDCl_3): $\delta = 5.90$ (d, 1H, $J_{2',\text{NH}} = 7.2$ Hz, NH), 5.39 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.4$ Hz, H-3'),
3 5.29-5.20 (m, 2H, H-1', 3), 5.15-5.03 (m, 2H, H-4, 4'), 4.92 (dd, 1H, $J_{2'',3''} = 3.8$ Hz, $J_{3'',4''} = 10.0$ Hz, H-3''),
4 4.76-4.56 (m, 5H, H-1, 1'', 2', 2'' and NH), 4.36 (dd, 1H, $J_{5'',6a''} = 5.3$ Hz, $J_{6a'',6b''} = 12.5$ Hz, H-6a''), 4.30-
5 4.22 (m, 2H, 2 H-6'), 4.15-4.03 (m, 2H, H-5', 6a''), 4.02-3.98 (m, 1H, H-2), 3.84-3.69 (m, 3H, H-a, 4', 5),
6 3.67-3.59 (m, 1H, H-5''), 3.49-3.40 (m, 1H, H-a'), 3.28-3.17 (m, 2H, 2 H-c), 2.21-1.98 (9 s, 27H, 9 CH_3),
7 1.85-1.75 (m, 2H, 2 H-b), 1.46 (s, 9H, $(\text{CH}_3)_3\text{CO}$), 1.20 (d, 3H, $J_{5,6} = 6.0$ Hz, 3 H-6). $^{13}\text{C-NMR}$ (CDCl_3): $\delta =$
8 171.9-169.6 (9C, C=O), 155.9 (C=O), 98.2 (C-1''), 96.4 (C-1), 93.3 (C-1'), 75.5 (C-4'), 73.7 (C-2), 72.7 (C-
9 5''), 71.9 (C-3''). 71.3 (C-4), 71.0 (C-2'), 70.7 (C-3'), 69.9 (C-3), 68.2 (C-5'), 66.6 (C-5), 65.9 (C-a), 65.7
10 (C-4''), 62.2 (C-6''), 62.0 (C-6'), 50.8 (C-2''), 37.83 (C-c), 31.6 (Me_3C), 29.8 (C-b), 28.4 (Me_3C), 21.2-20.6
11 (9C, CH_3CO), 17.7 (C-6). MS (ESI) m/z (%): 1045.3 (100) $[\text{M}+\text{Na}]^+$.

12 **15 β** : 0.42 g of **15 β** were obtained starting from 0.63 g of **17 β** (85% yield). $[\alpha]_D^{20} = +55.4$ ($c = 1$ in
13 chloroform). $^1\text{H-NMR}$ (CDCl_3): $\delta = 5.94$ (d, 1H, $J_{2',\text{NH}} = 7.2$ Hz, NH), 5.62 (br d, 1H, $J_{1',2'} = 3.9$ Hz, H-1'),
14 5.41 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3'), 5.13-5.04 (m, 2H, H-4, 4''), 4.98 (dd, 1H, $J_{2,3} = 3.0$ Hz, $J_{3,4} = 10.0$
15 Hz, H-3), 4.92 (dd, 1H, $J_{2'',3''} = 4.0$ Hz, $J_{3'',4''} = 10.0$ Hz, H-3''), 4.75 (dd, 1H, $J_{1',2'} = 3.9$ Hz, $J_{2',3'} = 10.2$ Hz,
16 H-2'), 4.69 (br s, 1H, H-1''), 4.63-4.58 (m, 1H, H-2''), 4.47 (s, 1H, H-1), 4.36 (dd, 1H, $J_{5'',6a''} = 5.4$ Hz,
17 $J_{6a'',6b''} = 12.4$ Hz, H-6a''), 4.25 (dd, 1H, $J_{5',6a'} = 5.0$ Hz, $J_{6a',6b'} = 11.8$ Hz, H-6a'), 4.21-4.12 (m, 3H, H-2, 5',
18 6b'), 4.06 (dd, 1H, $J_{5'',6a''} = 2.2$ Hz, $J_{6a'',6b''} = 12.4$ Hz, H-6a''), 3.87-3.79 (m, 1H, H-a), 3.69 (t, 1H, $J_{3',4'} =$
19 $J_{4',5'} = 9.5$ Hz, H-4'), 3.65-3.61 (m, 1H, H-5''), 3.50-3.41 (m, 2H, H-a, 5), 3.29-3.19 (m, 1H, H-c), 3.11-
20 3.02 (m, 1H, H-c), 2.17-2.00 (9 s, 27H, 9 CH_3), 1.82-1.66 (m, 2H, 2 H-b), 1.45 (s, 9H, $(\text{CH}_3)_3\text{CO}$), 1.26 (d,
21 3H, $J_{5,6} = 6.0$ Hz, 3 H-6). $^{13}\text{C-NMR}$ (CDCl_3): $\delta = 172.1$ -169.5 (9C, C=O), 156.1 (C=O), 100.9 (C-1), 98.2 (C-
22 1''), 94.3 (C-1'), 75.7 (C-4'), 72.7 (C-5''), 72.3 (C-2), 71.9 (2C, C-3, 3''). 71.1 (C-4), 70.7 (C-5), 70.5 (C-
23 3'), 70.3 (C-2'), 67.8 (C-5'), 67.6 (C-a), 65.8 (C-4''), 62.2 (2C, C-6, 6''), 50.8 (C-2''), 37.3 (C-c), 31.6
24 (Me_3C), 29.4 (C-b), 28.4 (Me_3C), 23.1-20.6 (9C, CH_3CO), 17.7 (C-6). MS (ESI) m/z (%): 1045.3 (100)
25 $[\text{M}+\text{Na}]^+$.

26 **4.1.10. 5,11,17,23-Tetrakis-N-[3-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-mannopiranosyloxy)-**
27 **propyl-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene Cone (5b):**

28 In a two-neck round-bottom flask calixarene **4b** (0.05 g, 0.0609 mmol) was dissolved in dry DCM (6 mL)
29 under N_2 atmosphere, then sugar **11** (0.123 g, 0.305 mmol) and Et_3N (255 μL , 1.83 mmol) were added. The
30 mixture was stirred at rt for 24 h, after which half equivalents of sugar and Et_3N were added and the
31 mixture stirred for additional 16 h. The solvent was removed under reduced pressure and the crude was
32 purified via flash column chromatography (DCM/MeOH 20/1, v/v) yielding cone derivative **5b** in 37% yield as
33 a white solid (0.0547 g, 0.0225 mmol). Mp: dec > 130 $^\circ\text{C}$. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ (ppm): 8.36 (br, 4H,
34 NHCS), 6.65 (br, 8H, ArH), 6.38 (br, 8H, CSNHCH_2 , NHAc), 5.15-5.09 (m, 8H, H_3 , H_4), 4.78-4.74 (m, 8H, H_2 , H_1),
35 4.41 (d, $J = 13.2$ Hz, 4H, $\text{ArCHH}_{\text{ax}}\text{Ar}$), 4.27 (dd, $J_{6a,5} = 5.6$, $J_{6a,6b} = 12.0$ Hz, 4H, H_{6a}), 4.13 (dd, $J_{6b,5} = 2.4$, $J_{6b,6a} =$
36 12.0 Hz, 4H, H_{6b}), 3.97-3.96 (m, 4H, $\text{OCHHCH}_2\text{CH}_2$), 3.84 (br, 12H, $\text{OCH}_2\text{CH}_2\text{CH}_3$, CHHNHCS), 3.70-3.69 (m, 4H,
37 H_5), 3.57 (br s, 4H, $\text{OCHHCH}_2\text{CH}_2$), 3.49-3.48 (m, 4H, CHHNHCS), 3.14 (d, $J = 13.2$ Hz, 4H, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 2.07 (s,
38 12H, CH_3CO), 2.05 (s, 12H, CH_3CO), 2.03 (s, 12H, CH_3CO), 2.01 (s, 12H, CH_3CO), 1.96-1.90 (m, 16H,
39 $\text{OCH}_2\text{CH}_2\text{CH}_3$, $\text{CH}_2\text{CH}_2\text{CH}_2$), 0.94 (t, $J = 7.5$ Hz, 12H, CH_2CH_3). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ (ppm): 180.4 (CS),
40 172.1 (NHCOCH_3), 170.7 (COCH_3), 170.5 (COCH_3), 169.8 (COCH_3), 154.5 (C_{Ar} ipso), 136.2 (C_{Ar} ortho), 132.1
41 (C_{Ar} para), 124.8 (C_{Ar} meta), 99.1 (C_1), 77.3 ($\text{OCH}_2\text{CH}_2\text{CH}_3$) 72.4 (C_5), 71.4 (C_4), 68.7 ($\text{OCH}_2\text{CH}_2\text{CH}_2$), 66.0 (C_3),
42 62.5 (C_6), 50.3 (C_2), 43.0 (CH_2NHCS), 30.9 (ArCH_2Ar), 29.9 ($\text{CH}_2\text{CH}_2\text{CH}_2$), 28.9 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 23.4 (COCH_3),
43 23.2 (COCH_3), 20.8 (COCH_3), 20.7 (COCH_3), 10.2 ($\text{CH}_2\text{CH}_2\text{CH}_3$). HRMS (ESI-TOF) m/z : calcd for
44 $\text{C}_{112}\text{H}_{156}\text{N}_{12}\text{O}_{40}\text{S}_4\text{Na}$ [$(\mathbf{5b}+2\text{Na})^{2+}$] 1241.4590, found 1241.4586.

45 **4.1.11. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-mannopiranosyloxy)-**
46 **propyl-thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (5d):**

47 To a solution of compound **4d** (0.0416 g, 0.0392 mmol) in DCM (8 mL) sugar **11** (0.111 g, 0.274 mmol) and
48 NEt_3 (273 μL , 1.96 mmol) were added and the mixture stirred at rt for 72 h. The solvent was removed under
49 reduced pressure and the crude purified via flash column chromatography and preparative TLC
50 (DCM/MeOH 94/6, v/v) to afford **5d** as a yellowish solid (0.0317 mg, 0.0091 mmol, 23%). $^1\text{H NMR}$ (400
51 MHz, CDCl_3) δ (ppm): 8.66 (br, 6H, NHCS), 6.92 (br, 12H, ArH), 6.57 (s, 12H, CSNHCH_2 , NHAc), 5.18 (br, 6H,

1 H₄), 5.03 (br, 6H, H₃), 4.77 (br, 6H, H₂), 4.71 (br, 6H, H₁), 4.29 (br, 6H, H_{6a}), 4.14 (br, 6H, H_{6b}), 4.03-3.17
2 (overlapped, 60H, OCHHCH₂CH₂, CHHNHCS, H₅, ArCH₂Ar, OCH₃), 2.10 (s, 18H, CH₃CO), 2.07 (s, 36H, CH₃CO),
3 2.03 (s, 18H, CH₃CO), 1.81 (br, 12H, CH₂CH₂CH₂). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 180.6 (CS), 172.1
4 (COCH₃), 170.7 (COCH₃), 170.3 (COCH₃), 169.7 (COCH₃), 154.2 (C_{Ar} ipso), 134.8 (br, C_{Ar} ortho, C_{Ar} para), 125.3
5 (C_{Ar} meta), 99.9 (C₁), 72.5 (C₅), 71.3 (C₃), 68.6 (OCH₂CH₂CH₂), 66.1 (C₄), 62.6 (C₆), 61.0 (OCH₃), 50.4 (C₂), 42.9
6 (CH₂NHCS), 29.7 (ArCH₂Ar), 29.0 (CH₂CH₂CH₂), 23.4 (NHCOCH₃), 20.9 (COCH₃), 20.8 (COCH₃), 20.7 (COCH₃).
7 HRMS (ESI-TOF) m/z: calcd for C₁₅₆H₂₁₀N₁₈O₆₀S₆Na₂ [(5d+2Na)²⁺] 1766.6028, found 1766.6047.

8 **4.1.12. N-[3-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopiranosyloxy)-propyl-thioureido]-4-**
9 **propoxy benzene (14):**

10 To a solution of **13** (0.03 g, 0.155 mmol) in dry DCM (4 mL) under N₂ atmosphere, sugar **11** (0.0943 g, 0.233
11 mmol) and NEt₃ (324 μL, 2.33 mmol) were added and the mixture stirred for 12 h at rt. The solvent was
12 removed under reduced pressure and the crude purified by flash column chromatography (DCM/MeOH
13 20/1, v/v) yielding **14** as an off-white solid (0.0490 g, 0.0821 mmol, 53%). Mp: 90-91 °C. ¹H NMR (400 MHz,
14 CDCl₃) δ (ppm): 8.25 (br, 1H, NHCS), 7.18 (d, *J* = 8.0 Hz, 2H, ArH), 6.86 (d, *J* = 8.0 Hz, 2H, ArH), 6.23 (br, 1H,
15 CH₂NHCS), 6.05 (d, *J* = 7.6 Hz, 1H, NHAc), 5.08 (t, *J* = 9.6 Hz, 1H, H₄), 4.99 (dd, *J*_{3,2} = 4.0 Hz, *J*_{3,4} = 10.0 Hz, 1H,
16 H₃), 4.72 (d, *J* = 4.0 Hz, 1H, H₂), 4.63 (s, 1H, H₁), 4.26 (dd, *J*_{6a,5} = 5.2 Hz, *J*_{6a,6b} = 12.4 Hz, 1H, H_{6a}), 4.08 (d, *J* =
17 12.0 Hz, 1H, H_{6b}), 3.89-3.86 (m, 3H, OCHHCH₂CH₂, OCH₂CH₂CH₃), 3.76 (br, 1H, CHHNHCS), 3.67-3.56 (m, 2H,
18 H₅, OCHHCH₂CH₂), 3.53 (br, 1H, CHHNHCS), 2.06 (s, 3H, COCH₃), 2.03 (s, 3H, COCH₃), 2.02 (s, 3H, COCH₃),
19 1.97 (s, 3H, COCH₃), 1.75 (m, 4H, OCH₂CH₂CH₃, CH₂CH₂CH₂), 0.98 (t, *J* = 7.2 Hz, 3H, CH₂CH₃). ¹³C NMR (100
20 MHz, CDCl₃) δ (ppm): 181.7 (CS), 171.7 (NHCOCH₃), 170.6 (COCH₃), 170.1 (COCH₃), 169.7 (COCH₃), 157.9
21 (C_{Ar} ipso), 129.8 (C_{Ar} ortho), 127.3 (C_{Ar} para), 127.3, 125.6 (C_{Ar} meta), 99.1 (C₁), 72.5 (C₅), 71.0 (C₃), 69.8
22 (OCH₂CH₂CH₃), 68.5 (OCH₂CH₂CH₂), 66.0 (C₄), 62.4 (C₆), 50.3 (C₂), 43.1 (CH₂NHCS), 29.7 (CH₂CH₂CH₂), 23.4
23 (COCH₃), 20.8 (OCH₂CH₂CH₃), 20.8, 20.7 (COCH₃), 10.5 (OCH₂CH₂CH₃). HRMS (ESI-TOF) m/z: calcd for
24 C₂₇H₃₉N₃O₁₀SNa [(14+Na)⁺] 620.2254, found 620.2261.

25 **4.1.13. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopyranosyl-**
26 **(1→4)-2,3,6-tri-O-acetyl-α-D-glucopyranosyl-(1→2)-3,4-di-O-acetyl-α-L-rhamnopyranosyloxy)-propyl-**
27 **thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (18a):**

28 **15α** (22 mg, 0.0215 mmol) was dissolved in dry DCM (4 mL) under Ar atmosphere. The temperature was
29 decreased to 0 °C and 0.5 mL of trifluoroacetic were added. The reaction proceeded at room temperature
30 for 1 hour, after which the TLC showed the complete consumption of the reagent, thus the solvent was
31 evaporated at reduced pressure. The obtained deprotected product was dissolved in dry DCM (2 mL) and
32 5,11,17,23,29,35-hexaisothiocyanate-37,38,39,40,41,42-hexamethoxy-calix[6]arene (**4d**) (2.5 mg, 0.00239
33 mmol) and NEt₃ (17 μL, 0.120 mmol) were added under Ar atmosphere. The mixture was stirred for 48
34 hours at room temperature, after which the reaction was quenched by removal of the solvent at reduced
35 pressure. The desired product was obtained after column chromatography (DCM/MeOH 96:4 v/v) as a
36 white solid in 86% yield (13.5 mg, 0.00205 mmol). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.01 (br, 12H, ArH),
37 5.91 (br, 6H, NHAc), 5.38 (t, *J*_{3',4'} = *J*_{3',2'} = 9.5 Hz, 6H, H_{3'}), 5.24 (br, 12H, H₃, H_{1'}), 5.14-5.03 (m, 12H, H₄, H_{4'}),
38 4.93 (br, 6H, H_{3''}), 4.77-4.67 (m, 12H, H_{2'}, H_{1''}), 4.62 (br, 12H, H_{2''}, H₁), 4.37 (dd, *J*_{6a,5} = 5.1, *J*_{6a,6b} = 12.5 Hz, 6H,
39 H_{6a''}), 4.25 (br, 12H, H_{6'}), 4.12-4.03 (m, 12H, H_{5'}, H_{6''}), 4.00 (br, 6H, H₂), 3.84-3.70 (m, 18H, H₅, H_{4'},
40 OCHHCH₂CH₂), 3.67 (br, 12H, H_{5''}, OCHHCH₂CH₂), 3.45 (br, 12H, CH₂NHCS), 2.16 (s, 18H, CH₃CO), 2.13 (s,
41 18H, CH₃CO), 2.12 (s, 18H, CH₃CO), 2.11 (s, 18H, CH₃CO), 2.09 (s, 18H, CH₃CO), 2.06 (s, 36H, CH₃CO), 2.04 (s,
42 18H, CH₃CO), 2.02 (s, 18H, CH₃CO), 1.88 (br, 12H, CH₂CH₂CH₂), 1.19 (d, *J* = 5.8 Hz, 18H, H₆). HRMS (ESI-TOF)
43 m/z: calcd for C₂₈₈H₃₉₀N₁₈O₁₄₄S₆Na₄ [(6a+4Na)⁴⁺] 1672.0388, found 1672.0396.

44 **4.1.14. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopyranosyl-**
45 **(1→4)-2,3,6-tri-O-acetyl-α-D-glucopyranosyl-(1→2)-3,4-di-O-acetyl-β-L-rhamnopyranosyloxy)-propyl-**
46 **thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (6b):**

47 **15β** (29 mg, 0.0283 mmol) was dissolved in dry DCM (4 mL) under Ar atmosphere. The temperature was
48 decreased to 0 °C and 0.5 mL of trifluoroacetic were added dropwise. The reaction proceeded at room
49 temperature for 1 hour, after which the TLC showed the complete consumption of the reagent, thus the
50 solvent was evaporated at reduced pressure. The crude was then dissolved in dry DCM (3 mL) and

1 5,11,17,23,29,35-hexaisothiocyanate-37,38,39,40,41,42-hexamethoxy-calix[6]arene (**4d**) (3.3 mg, 0.00314
2 mmol) and NEt_3 (21 μL , 0.157 mmol) were added under Ar atmosphere. The mixture was stirred for 48
3 hours at room temperature, after which the reaction was quenched by removal of the solvent at reduced
4 pressure. The desired product was obtained after column chromatography (DCM/MeOH 95/5, v/v) as a
5 white solid in 82% yield (17 mg, 0.00258 mmol). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 7.11 (br, 12H, ArH),
6 5.93 (d, $J = 7.3$ Hz, 6H, NHAc), 5.60 (br, 6H, $\text{H}_{1'}$), 5.41 (t, $J = 9.1$ Hz, 6H, H_3) 5.17-5.02 (m, 12H, $\text{H}_{4'}$, H_4), 4.96
7 (dd, $J_{3,2} = 3.0$, $J_{3,4} = 10.1$ Hz, 6H, H_3), 4.93 (dd, $J_{3'',2''} = 5.8$, $J_{3'',4''} = 9.9$ Hz, 6H, $\text{H}_{3''}$) 4.74 (br, 6H, $\text{H}_{1''}$), 4.69 (br,
8 6H, $\text{H}_{2'}$), 4.62 (br, 6H, $\text{H}_{2''}$), 4.50 (br, 6H, H_1), 4.39 (dd, 6H, $\text{H}_{6a''}$) 4.28 (br, 6H, $\text{H}_{6a'}$), 4.13 (br, 18H, H_2 , $\text{H}_{5'}$, $\text{H}_{6b'}$),
9 4.05 (d, $J = 12.0$ Hz, 6H, $\text{H}_{6b''}$), 3.86 (br, 12H, CH_2NHCS), 3.75 (br, 6H, $\text{H}_{4'}$), 3.66 (br, 6H, $\text{H}_{5''}$), 3.45 (br, 18H, H_5 ,
10 OCH_2) 2.16 (s, 18H, CH_3CO), 2.12 (s, 36H, CH_3CO), 2.11 (s, 18H, CH_3CO), 2.061 (s, 18H, CH_3CO), 2.056 (s, 18H,
11 CH_3CO), 2.05 (s, 18H, CH_3CO), 2.03 (s, 18H, CH_3CO), 2.01 (s, 18H, CH_3CO), 1.75 (br, 12H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 1.23 (d,
12 $J = 5.8$ Hz, 18H, H_6). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 172.1, 170.6, 170.5, 170.4, 170.1, 169.7, 169.6,
13 135.2, 125.0, 100.8, 98.3, 94.0, 75.8, 72.6, 72.3, 71.9, 71.8, 71.0, 70.7, 70.4, 67.8, 65.6, 62.3, 62.1, 60.8,
14 50.8, 41.8, 32.2, 29.7, 26.4, 23.4, 23.2, 21.3, 20.9, 20.8, 20.76, 20.68, 17.7, 14.1. HRMS (ESI-TOF) m/z : calcd
15 for $\text{C}_{288}\text{H}_{390}\text{N}_{18}\text{O}_{144}\text{S}_6\text{Na}_4$ [(**6b**+4Na) $^{4+}$] 1672.0388, found 1672.0399.

16 **4.1.15. General procedure for the deacetylation reaction of glycolix[n]arenes 5a-d and monomer 14:**

17 To a solution of the peracetylated compound in MeOH at 0 °C, freshly prepared MeONa was added till pH
18 9. The solution was stirred for 3 hours, after which Amberlite IR-120 (H^+), and 1 mL of H_2O in the case of the
19 hexacalixarenes, were added and the mixture stirred at rt till neutral pH. The resin was filtered off and the
20 solvent removed under reduced pressure.

21 **4.1.15.1. 5,11,17,23-Tetrakis-N-[3-(2-acetamido-2-deoxy- β -D-mannopiranosyloxy)-propyl-thioureido]-** 22 **25,26,27,28-tetramethoxycalix[4]arene (1a):**

23 The crude was first purified by C18 reverse phase column chromatography (MeOH/ H_2O 5.5/4.5, v/v), and
24 then by size exclusion chromatography on Sephadex G-25 as stationary phase (MeOH/ H_2O 1.5/8.5, v/v).
25 The final purification step was performed via standard flash chromatography (*i*-PrOH/ H_2O / Et_3N 8/2/0.5,
26 v/v/v) to afford the mobile calix[4]arene **1a** in 40% yield (0.037 g, 0.0191 mmol). Mp: dec > 140 °C. ^1H NMR
27 (400 MHz, D_2O , 80 °C) δ (ppm): 6.98 (br, 8H, ArH), 4.69 (br, 4H, H_1), 4.43 (br, 4H, H_2), 3.88-3.22 (m, 48H, H_3 ,
28 H_4 , H_5 , H_{6a} , H_{6b} , OCH_2CH_2 , CH_2NHCS , OCH_3), 1.97 (s, 12H, NHCOCH_3), 1.80-1.74 (m, 8H, $\text{CH}_2\text{CH}_2\text{CH}_2$). ^{13}C NMR
29 (100 MHz, MeOD, 55 °C) δ (ppm): 180.6 (CS), 173.6 (NHCOCH_3), 155.7 (C_{Ar} ipso), 135.32 (C_{Ar} ortho), 132.4
30 (C_{Ar} para), 124.4 (C_{Ar}), 99.5 (C_1), 77.0 (C_5), 73.0 (C_3), 67.2 (C_4 , $\text{OCH}_2\text{CH}_2\text{CH}_2$), 61.0 (C_6 , OCH_3), 53.6 (C_2), 42.1
31 (CH_2NHCS , ArCH_2Ar), 29.2 ($\text{CH}_2\text{CH}_2\text{CH}_2$), 22.2 (NHCOCH_3). HRMS (ESI-TOF) m/z : calcd for $\text{C}_{80}\text{H}_{116}\text{N}_{12}\text{O}_{28}\text{S}_4\text{Na}$
32 [(**1a**+Na) $^+$] 1843.6803, found 1843.6812.

33 **4.1.15.2. 5,11,17,23-Tetrakis-N-[3-(2-acetamido-2-deoxy- β -D-mannopiranosyloxy)-propyl-thioureido]-** 34 **25,26,27,28-tetrapropoxycalix[4]arene Cone (1b):**

35 The pure product **1b** was obtained after purification by C18 reverse phase column chromatography
36 (MeOH/ H_2O 4/1, v/v) in 78% yield as a white solid (0.034 g, 0.0176 mmol). Mp: dec > 178 °C. ^1H NMR (400
37 MHz, MeOD) δ (ppm): 6.71 (br, 8H ArH), 4.71 (s, 4H, H_1), 4.53 (s, 4H, H_2), 4.47 (d, $J = 13.2$ Hz, 4H,
38 $\text{ArCHH}_{\text{ax}}\text{Ar}$), 3.98-3.86 (m, 24H, $\text{H}_{6a,b}$, $\text{OCHHCH}_2\text{CH}_2$, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 3.71-3.63 (m, 16H, H_3 , $\text{OCHHCH}_2\text{CH}_2$,
39 CHHNHCS), 3.58 (t, $J = 9.6$ Hz, 4H, H_4), 3.29-3.27 (m, 4H, H_5), 3.18 (d, $J = 13.2$ Hz, 4H, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 2.08 (s,
40 12H, NHCOCH_3), 2.02-1.96 (m, 8H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.86 (br, 8H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 1.06 (t, $J = 6.8$ Hz, 12H,
41 $\text{OCH}_2\text{CH}_2\text{CH}_3$). ^{13}C NMR (100 MHz, MeOD) δ (ppm): 179 (CS), 173.6 (NHCOCH_3), 154.0 (C_{Ar} ipso), 135.32 (C_{Ar}
42 ortho), 132.2 (C_{Ar} para), 123.9 (C_{Ar}), 99.5 (C_1), 76.9 (C_5), 76.8 (C_6), 72.8 (C_3), 67.3 ($\text{OCH}_2\text{CH}_2\text{CH}_2$), 66.9 (C_4),
43 60.7 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 53.6 (C_2), 47.7 (CH_2NHCS), 42.2 (C_2), 30.5 (ArCH_2Ar), 28.7 ($\text{CH}_2\text{CH}_2\text{CH}_2$), 23.1
44 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 21.7 (NHCOCH_3), 9.5 ($\text{OCH}_2\text{CH}_2\text{CH}_3$). HRMS (ESI-TOF) m/z : calcd for $\text{C}_{88}\text{H}_{132}\text{N}_{12}\text{O}_{28}\text{S}_4\text{Na}$
45 [(**1b**+Na) $^+$] 1955.8055, found 1955.8069.

46 **4.1.15.3. 5,11,17,23-Tetrakis-N-[3-(2-acetamido-2-deoxy- β -D-mannopiranosyloxy)-propyl-thioureido]-** 47 **25,26,27,28-tetrapropoxycalix[4]arene 1,3-Alternate (1c):**

48 Compound **1c** was obtained after purification via C18 reverse phase column chromatography (MeOH/ H_2O
49 3/2, v/v) as a white solid (0.023 g, 0.0119 mmol, 83%). Mp: dec > 176 °C. ^1H NMR (400 MHz, MeOD) δ
50 (ppm): 7.09 (s, 8H, ArH), 4.71 (s, 4H, H_1), 4.56 (d, $J = 3.6$ Hz, 4H, H_2), 4.06-3.94 (m, 4H, CHHNHCS), 3.87 (d, J

1 = 3.2 Hz, 8H, H_{6ab}), 3.83-3.64 (m, 24H, H₃, OCH₂CH₂CH₂, CHHNHCS, OCH₂CH₂CH₃), 3.63-3.50 (m, 12H,
2 ArCH₂Ar, H₄), 3.31-3.25 (m, 4H, H₅), 2.10 (s, 12H, NHCOCH₃), 2.01-1.81 (m, 8H, CH₂CH₂CH₂), 1.96-1.80 (m,
3 8H, OCH₂CH₂CH₃), 1.04 (t, *J* = 7.6 Hz, 12H, OCH₂CH₂CH₃). ¹³C NMR (100 MHz, MeOD) δ (ppm): 180.7 (CS),
4 173.5 (NHCOCH₃), 153.9 (C_{Ar} ipso), 133.6 (C_{Ar} ortho), 131.6 (C_{Ar} para), 125.4 (C_{Ar}), 99.52 (C₁), 76.90 (C₅), 74.9
5 (OCH₂CH₂CH₃), 72.8 (C₃), 67.2 (CH₂NCS), 66.9 (C₄), 60.7 (C₆), 53.6 (C₂), 42.2 (OCH₂CH₂CH₂), 35.0 (ArCH₂Ar),
6 28.8 (CH₂CH₂CH₂), 23.6 (OCH₂CH₂CH₃), 21.6 (NHCOCH₃), 9.7 (OCH₂CH₂CH₃). HRMS (ESI-TOF) *m/z*: calcd for
7 C₈₈H₁₃₂N₁₂O₂₈S₄Na [(1c+Na)⁺] 1955.8055, found 1955.8070.

8 **4.1.15.4. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-2-deoxy-β-D-mannopiranosyloxy)-propyl-
9 thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (1d):**

10 Purification by C18 reverse phase column chromatography (MeOH/H₂O 64/36, v/v) afforded **1d** in 54% yield
11 as a off-white solid (0.015 g, 0.00549 mmol). Mp: 186-187 °C. ¹H NMR (400 MHz, D₂O, 80 °C) δ (ppm): 7.45
12 (s, 12H, ArH), 5.20 (s, 6H, H₁), 4.97 (d, *J* = 4.4 Hz, 6H, H₂), 4.44 (br, 12H, ArCH₂Ar), 4.40-4.23 (m, 24H, H₃,
13 H_{6a,b}, OCHHCH₂CH₂), 4.18-4.11 (m, 6H, OCHHCH₂CH₂), 4.07-4.00 (m, 18H, CH₂NHCS, H₄), 3.84 (br, 6H, H₅),
14 3.77 (br, 18H, CH₃), 2.52 (s, 18H, NHCOCH₃), 2.31 (br, 12H, CH₂CH₂CH₂). ¹³C NMR (100 MHz, D₂O, 80 °C) δ
15 (ppm): 180.7 (CS), 175.4 (NHCOCH₃), 154.8 (C_{Ar} ipso), 135.6 (C_{Ar} ortho), 134.1 (C_{Ar} para), 126.8 (C_{Ar}), 99.9
16 (C₁), 77.2 (C₅), 75.0 (C₃), 67.8 (C₄), 67.7 (OCH₂CH₂CH₂), 61.2 (OCH₃), 59.9 (C₆), 53.8 (C₂), 42.5 (CH₂NHCS), 30.7
17 (CH₂CH₂CH₂), 29.2 (ArCH₂Ar), 22.8 (NHCOCH₃). HRMS (ESI-TOF) *m/z*: calcd for C₁₂₀H₁₇₄N₁₈O₄₂S₆K₂ [(1d+2K)²⁺]
18 1404.6177, found 1404.6204.

19 **4.1.15.5. N-[3-(2-acetamido-2-deoxy-β-D-mannopiranosyloxy)-propyl-thioureido]-4-propoxy-benzene (1-
20 MON):**

21 The pure product was obtained after purification by C18 reverse phase column chromatography
22 (MeOH/H₂O 1/1, v/v) as a off-white solid (0.0272 g, 0.0577 mmol, 66%). Mp: dec > 76 °C. ¹H NMR (400
23 MHz, MeOD) δ (ppm): 7.24 (d, *J* = 8.9 Hz, 2H, ArH), 6.93 (d, *J* = 8.9 Hz, 2H, ArH), 4.65 (d, *J* = 1.5 Hz, 1H, H₁),
24 4.48 (dd, *J*_{2,1} = 1.5, *J*_{2,3} = 4.5 Hz, 1H, H₂), 3.95 (t, *J* = 6.5 Hz, 2H, OCH₂CH₂CH₃), 3.93-3.89 (m, 1H,
25 OCHHCH₂CH₂), 3.88-3.84 (m, 1H, H_{6a,b}), 3.69 (br, 1H, CHHNHCS), 3.67 (dd, *J*_{3,2} = 4.4, *J*_{3,4} = 9.6 Hz, 1H, H₃),
26 3.61 (m, 2H, OCHHCH₂CH₂, CHHNHCS), 3.53 (t, *J* = 9.6 Hz, 1H, H₄), 3.29-3.23 (m, 1H, H₅), 2.04 (s, 3H,
27 NHCOCH₃), 1.89-1.76 (m, 4H, OCH₂CH₂CH₃, CH₂CH₂CH₂), 1.06 (t, *J* = 7.2 Hz, 3H, OCH₂CH₂CH₃). ¹³C NMR (100
28 MHz, MeOD) δ (ppm): 181.1 (CS), 173.5 (NHCOCH₃), 157.7 (C_{Ar} ipso), 127.3 (C_{Ar} para), 126.7, 114.5 (C_{Ar}
29 meta), 99.4 (C₁), 76.9 (C₅), 76.8 (OCH₂CH₂CH₃), 69.4 (C₄, OCH₂CH₂CH₂), 60.6 (C₆), 53.5 (C₂), 41.9 (CH₂NHCS),
30 28.6 (CH₂CH₂CH₂), 22.3 (OCH₂CH₂CH₃), 21.4 (NHCOCH₃), 9.4 (OCH₂CH₂CH₃). HRMS (ESI-TOF) *m/z*: calcd for
31 C₂₁H₃₃N₃O₇SNa [(7+Na)⁺] 494.1937, found 494.1955.

32 **4.1.15.5. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-α-D-
33 glucopyranosyl-(1→2)-α-L-rhamnopyranosyloxy)-propyl-thioureido]-37,38,39,40,41,42-
34 hexamethoxycalix[6]arene (2a):**

35 The pure product was obtained after trituration in diethyl ether (7.5 mg, 0.00164 mmol, 80%). ¹H NMR (400
36 MHz, MeOD/D₂O 75:25) δ (ppm): 8.49 (s, NH), 7.01 (br, 12H, ArH), 5.04 (br, OH), 4.94 (br, 6H, H₁'), 4.87 (br,
37 6H, H₁''), 4.85 (br, 6H, H₁), 4.54 (br, 6H, H₂''), 4.14-3.22 (overlapped, 144H, H₃'', H₄'', H₅'', H_{6a,b}'', H₂', H₃', H₄', H₅',
38 H_{6a,b}', H₂, H₃, H₄, H₅, OCH₂CH₂CH₂, CH₂NHCS, OCH₃, ArCH₂Ar), 2.08 (s, 18H, NHCOCH₃), 1.86 (br, 12H,
39 CH₂CH₂CH₂), 1.29 (br, 18H, H₆). ¹³C NMR (100 MHz, MeOD/D₂O 1:1) δ (ppm): 174.5, 99.4, 98.0, 97.7, 78.8,
40 76.9, 76.8, 72.5, 72.3, 71.6, 71.5, 70.5, 70.1, 68.9, 66.7, 65.2, 60.4, 59.9, 53.5, 39.9, 28.7, 21.8, 16.8. HRMS
41 (ESI-TOF) *m/z*: calcd for C₁₉₂H₂₉₄N₁₈O₉₆S₆Na₃ [(2a+3Na)³⁺] 1550.2248, found 1550.2276.

42 **4.1.15.6. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-α-D-
43 glucopyranosyl-(1→2)-β-L-rhamnopyranosyloxy)-propyl-thioureido]-37,38,39,40,41,42-
44 hexamethoxycalix[6]arene (2b):**

45 The pure product was obtained after trituration in diethyl ether (9.6 mg, 0.00209 mmol, 81%). ¹H NMR (400
46 MHz, MeOD/D₂O 9:1) δ (ppm): 7.04 (br, 12H, ArH), 5.11 (br, 6H, H₁'), 4.83 (s, 6H, H₁''), 4.67 (br, 6H, H₁), 4.63
47 (br, OH), 4.53 (br, 6H, H₂''), 4.24-3.27 (overlapped, 144H, H₃'', H₄'', H₅'', H_{6a,b}'', H₂', H₃', H₄', H₅', H_{6a,b}', H₂, H₃, H₄,
48 H₅, OCH₂CH₂CH₂, CH₂NHCS, OCH₃, ArCH₂Ar), 2.07 (s, 18H, NHCOCH₃), 1.90 (br, 12H, CH₂CH₂CH₂), 1.34 (br,
49 18H, H₆). ¹³C NMR (100 MHz, MeOD/D₂O 9:1) δ (ppm): 173.4, 100.7, 100.4, 99.5, 78.9, 78.4, 77.0, 72.9, 72.6,

1 72.4, 72.1, 70.4, 66.7, 60.5, 60.0, 53.5, 29.5, 21.6, 16.8. HRMS (ESI-TOF) m/z: calcd for C₁₉₂H₂₉₄N₁₈O₉₆S₆Na₃
2 [(2b+3Na)³⁺] 1550.2248, found 1550.2273.

3 4.2. Competitive ELISA

4 96-well flat-bottomed plates were incubated overnight at 4-8°C with a mixture of *S. pneumoniae* CPS 19F (1
5 mg/mL, Sanofi-Aventis, France) and methylated human serum albumin (1 mg/mL). A solution of foetal calf
6 serum (5%) in phosphate-buffered saline supplemented with Brij-35 (0.1%) and sodium azide (0.05%) was
7 applied to the plates for blocking of nonspecific binding sites. The plates were incubated overnight at 4-8°C
8 with a solution (1:200) of rabbit polyclonal anti-19F, used as reference serum (Statens Serum Institut,
9 Artillerivej, Denmark). When compounds were tested, they were added to each well immediately before
10 the addition of the reference serum. The plates were then incubated with alkaline phosphatase conjugate
11 goat anti-rabbit IgG (Sigma-Aldrich, Milan, Italy), stained with *p*-nitrophenylphosphate, and the absorbance
12 was measured at 405 nm with an Ultramark microplate reader (Bio-Rad Laboratories S.r.l., Milan, Italy).

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