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

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ABSTRACT

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

1. Introduction

Encapsulated bacterial infections are still one of the most prevalent causes of serious disease in humans, especially in young children. Immunization is the most appropriate way to prevent bacterial infections [1]. In the early stages of microbiology, capsular polysaccharides (CPSs) were recognized as relevant virulence factors, and found to be able to stimulate protective immunity against infections, laying the bases for the development of current antibacterial vaccines [2]. Nevertheless, the study of the chemical determinants of immunogenicity to CPSs still requires detailed molecular insights. CPSs are cell-surface polymers consisting of oligosaccharide repeating units, characterized by a huge number of possible structural modifications and linkage combinations and associated to a relative high degree of flexibility. Typically, anti-carbohydrate antibodies show affinities in their recognition that are several factors lower than those observed for antibodies specific for proteins or peptide antigens. This peculiar weakness is partially compensated by the multivalent nature of such antibodies, that face in a clustered form a multiple number of densely displayed antigen molecules to increase the effect of the response [3]. This can explain the existing relationship between molecular weight and antigenicity, established for polysaccharide antigens. In fact, high molecular mass repetitive polysaccharides are able to simultaneously display a greater number of epitopes capable to effectively interact with specific antibodies. This kind of model where the binding of a glycan-binding protein to a clustered saccharide patch enhances the overall affinity of the interaction has been widely described [4], for instance between tumor associated saccharide antigens and lectins. Lectin-glycan interactions are stabilized by weak hydrogen bonding and van der Waals intermolecular forces, and the multivalent presentation is the basis for a biologically relevant binding. There are several examples demonstrating that lectins bind their targets only when glycans are clustered at high density in a multivalent glycoconjugate backbone [5]. This concept has been exploited for instance in the field of cancer vaccine development. For example, the Tn tumor associated antigen is overexpressed on the surface of tumor cells. It is exposed on the Mucine surface in clusters of 2–5 units. Data show that at least two contiguous antigen molecules are crucial for the binding of the anti-Tn monoclonal antibodies, with an even greater affinity in the presence of three residues [6]. Different studies have been oriented to the selection of the proper scaffold for the presentation of multiple copies of the Tn antigenic unit to generate an anticancer vaccine [7–9]. The choice of the scaffold for multivalent presentation of glycans to target proteins is crucial in determining the biological activity [10–13]. In this context,

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the use of calixarenes represents a viable approach explored in the literature for the display of multiple glycan units [14]. This type of macrocycle in fact, due to peculiar structural properties and selective synthetic procedures [15], allows the achievement of small libraries of potential multivalent ligands bearing the same epitopes but with a controlled modulation of their number (valency) and orientation in the space. The conformational behavior of the different possible types of calixarenes strongly impacts on the presentation mode of the conjugated saccharide moieties [14,16], which can be exploited to increase the avidity of the biological recognition. This versatility can make possible the comparison of subtly modified parameters and their effects on selectivity and efficiency in biological activities like interaction with carbohydrate recognition proteins [14]. A calix[4]arene in the so called cone geometry conjugated to LacNAc fragments, for example, was found extremely selective in the inhibition of galectin-3 while totally unable to bind to galectin-1 [17]. Calixarenes functionalized with lactose units showed inhibition activity towards human galectins characterized, in some cases, by an impressive selectivity strictly dependent on their conformational properties and related arrangement of the epitopes [18]. Analogously, different sized and conformationally featured calixarenes exposing galactose units resulted in different efficiency in the binding to *Pseudomonas aeurginosa* lectin A (PA-IL) [19]. Ten years ago, a calix[4]arene decorated with four Tn units has indeed been described as a promising synthetic multivalent vaccine candidate [20]. In this framework, we have decided to explore the potential of calixarenes as scaffolds for the multipresentation of bacterial CPS fragments. Our hypothesis was to verify if such macrocyclic scaffold, presenting multiple, but however limited copies of short CPS fragments, is able to gain affinity and potency towards the natural antibodies approaching those observed for the natural polysaccharide. In principle, a positive assessment would open the possibility to work with shorter saccharide fragments, which can be obtained by chemical synthesis, instead of the longer oligosaccharides, used in vaccine formulations, obtained by size-reduced purified natural polysaccharides [21]. Moreover, the use of calixarenes and short saccharide fragments would have the advantage of giving structurally well-defined and easily reproducible systems.

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

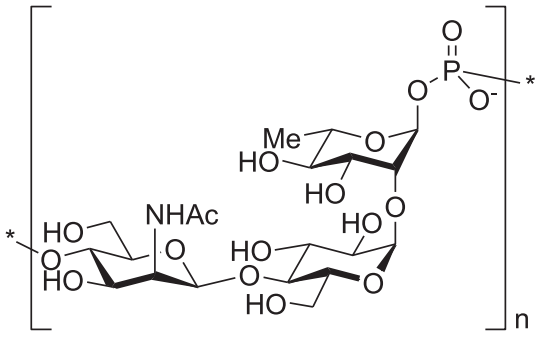


Fig. 1. *Streptococcus pneumoniae* **19F** capsular polysaccharide repeating unit.

2. Results and discussion

2.1. Synthesis

We report the synthesis of a small library of glycocalixarenes (compounds **1a-d** and **2a-b**, Fig. 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]arene 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 glycoclusters 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**, Fig. 2), while subsequently, based on a preliminary set of inhibition studies, only the calixarene scaffold of the best inhibitor of the binding between the 19F polysaccharide and the corresponding anti-19F antibodies was functionalized with the SP19F trisaccharide repeating unit. Thus, two additional glycocalixarenes (**2a** and **2b**, Fig. 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.

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

On the basis of procedures reported in the literature [29], we then prepared compound **6**, a protected derivative of 3-aminopropyl *N*-acetyl-β-D-mannosamine, benzylated at the sugar hydroxyls. Monosaccharide **6** was deprotected from the Cbz group by hydrogenolysis producing the amino derivative **7** that was subsequently reacted with cone and 1,3-alternate tetraisothiocyanate calix[4]arenes **4b** and **4c**, obtaining in good yields the corresponding tetraglycosylated macrocycles (see SI). Nevertheless, unexpectedly, their deprotection from benzyl groups to give the two planned glycoclusters failed, despite the use of different methods, discouraging the use of this pathway for the preparation of the target calixarenes **1a-d**.

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

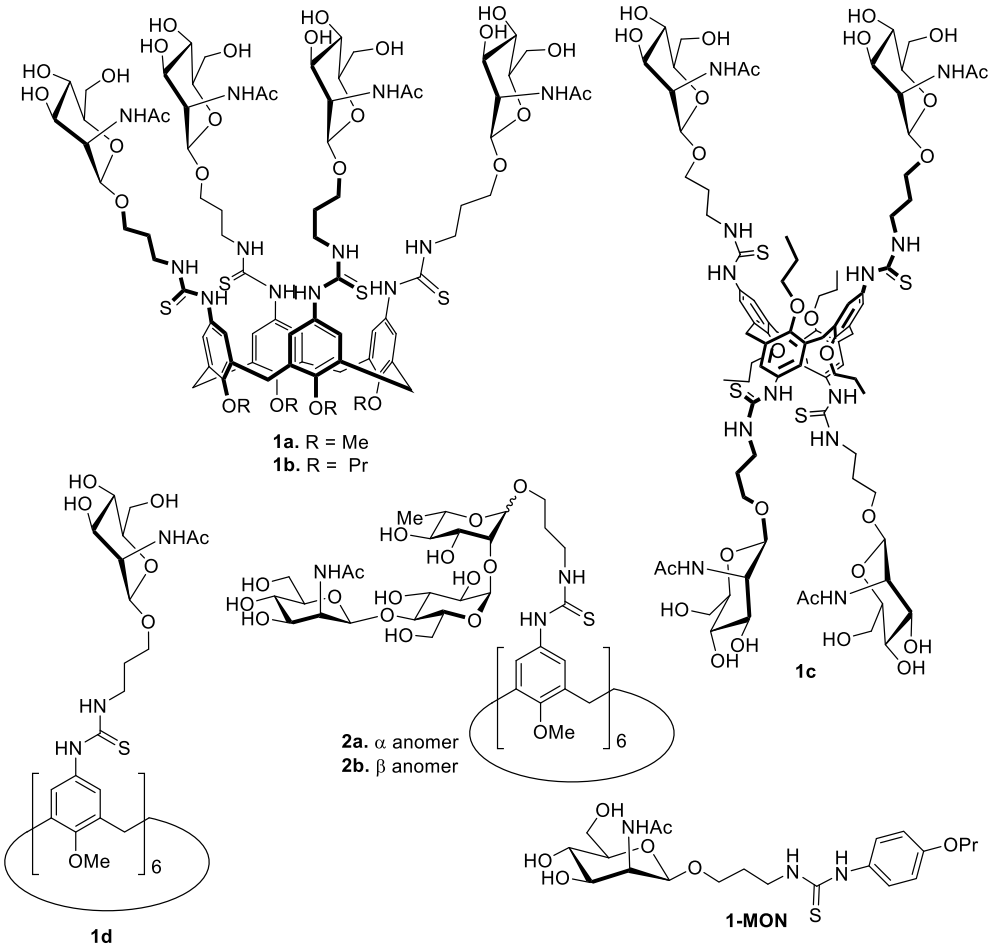


Fig. 2. Library of multivalent glycocalixarenes decorated with saccharide units of SP 19F CPS.

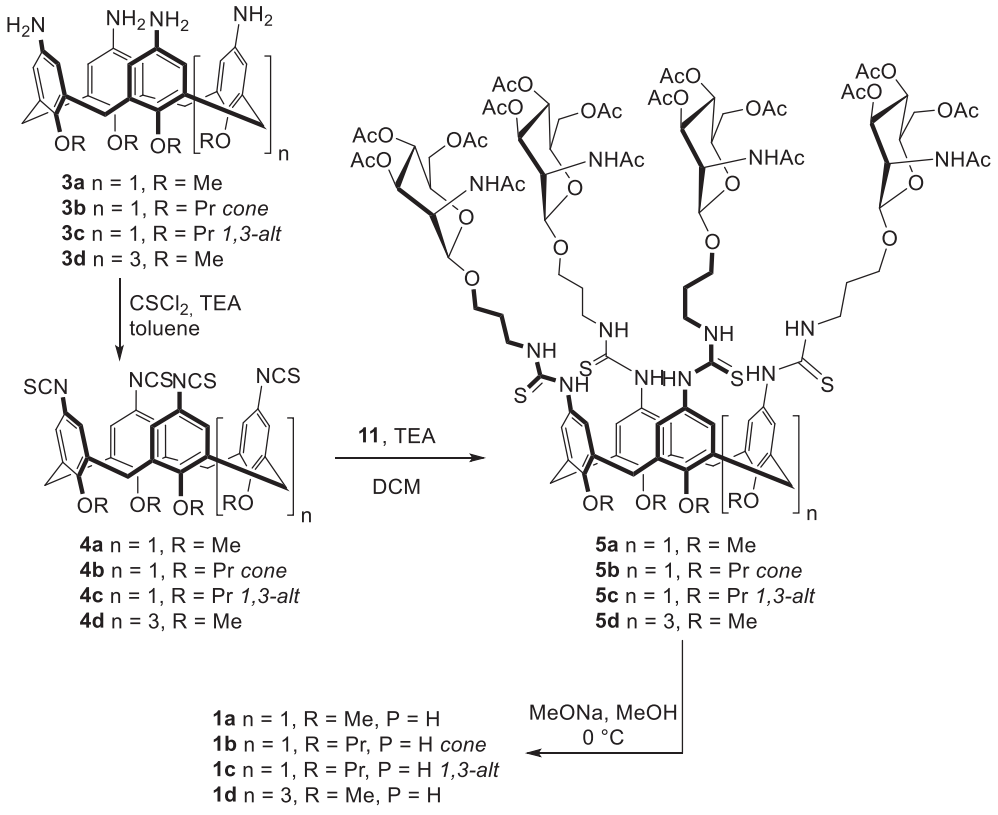
acetylation step, performed with acetic anhydride and pyridine, afforded in high yield sugar **10**, which was treated with trifluoroacetic anhydride to remove the Boc group providing **11** in quantitative yield.

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 Zemplen 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 ^{1}H NMR spectroscopy of their behavior in solution. The ^{1}H 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 D_2O 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 SI22). It was possible to identify the signals of the H_1 and H_2 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 ^{1}H NMR spectrum in D_2O 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 SI24). 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 despite the bigger size of the macrocycle. On the other hand, interestingly, in the past it was observed, rather unexpectedly, that methoxyglucocalix[6]- and [8]-arenes tend to self-assemble in aqueous solution [36], despite the absence of a well-defined amphiphilicity, as indeed for **1a** and **1d**. For glycocalixarenes **1a** and **1d** self-aggregation cannot then be excluded, determining or at least contributing to the broadening of the signals in the ^{1}H NMR spectrum. In both cases, the sharpening of the signals by increasing the temperature can be read as a consequence of the assembly disaggregation and/or of the increased mobility of these derivatives in solution.

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

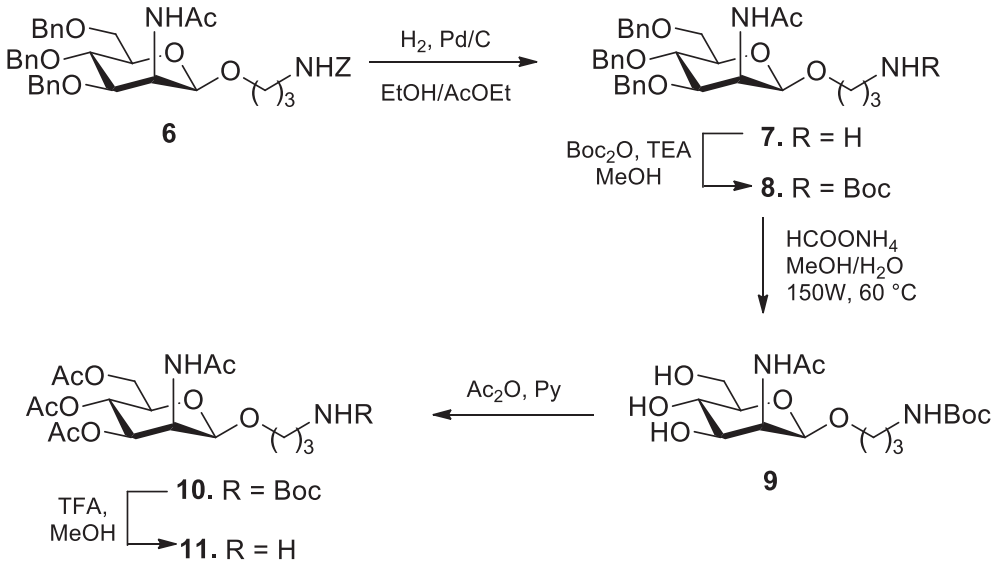
On the basis of the results obtained from biological tests on compounds **1a-d** (see below), the conformationally mobile calix[6]arene was selected as the best scaffold for obtaining the glycocalixarenes



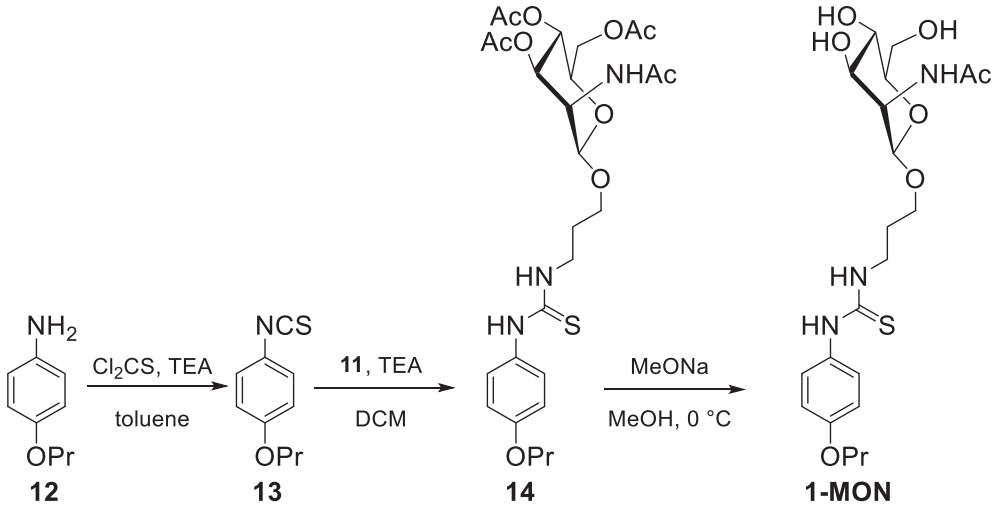
Scheme 1. Synthesis of glycocalix[*n*]arenes **1a–d**.

displaying the trisaccharide repeating unit of SP19F. To this aim, exploiting our expertise in the synthesis of synthetic fragments of *Streptococcus pneumoniae* [37–39], we planned the preparation of compound **15**, the peracetylated derivative of the SP19F trisaccharide repeating unit, functionalized at the downstream residue with an aminopropyl linker. Compound **15** was synthesized starting from the trisaccharide trichloroacetimidate donor **16** [37], which was glycosylated with Z-aminopropanol (Scheme 4) [38]. The reaction was promoted with trimethylsilyl triflate and trisaccharide **17** was obtained in high yields (96%) as an alpha/beta mixture of the two anomers **17α** and **17β**, which were separated by flash chromatography. Each anomer was separately subjected to initial exchange of the CBZ amino protecting group of the linker for the BOC one, followed by hydrogenolysis of the benzyl ethers, and final acetylation of the hydroxyl groups with acetic anhydride. This protecting group manipulation sequence was very efficient and allowed to recover in both cases the final trisaccharides **15α** and **15β** in 88% yield.

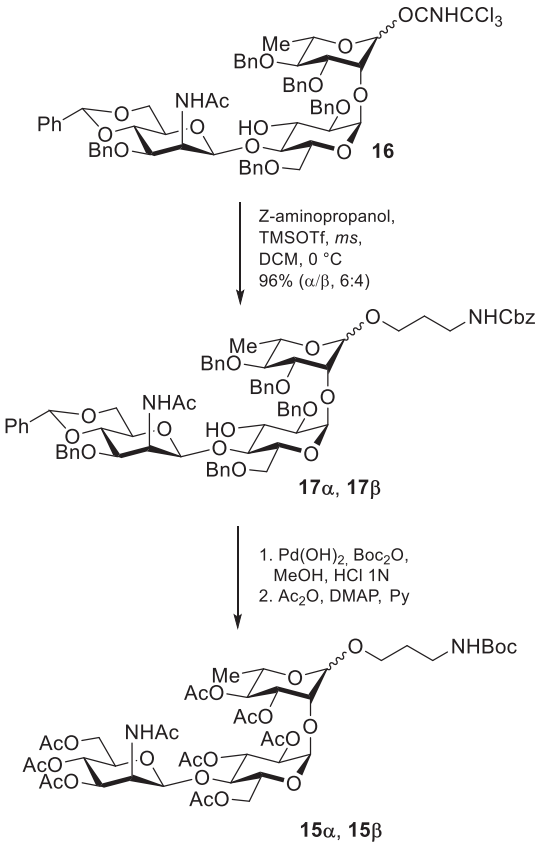
Hexaisothiocyanate **4d** was separately reacted in good yields with both the α and the β anomer of compound **15**, to evaluate if a different stereochemistry of the anomeric position on the rhamnose residue may have an influence on the biological activity of the glycocalixarene. If not, in perspective, the calixarene could be reacted with the mixture of



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



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



Scheme 4. Synthesis of trisaccharides **15α** and **15β**.

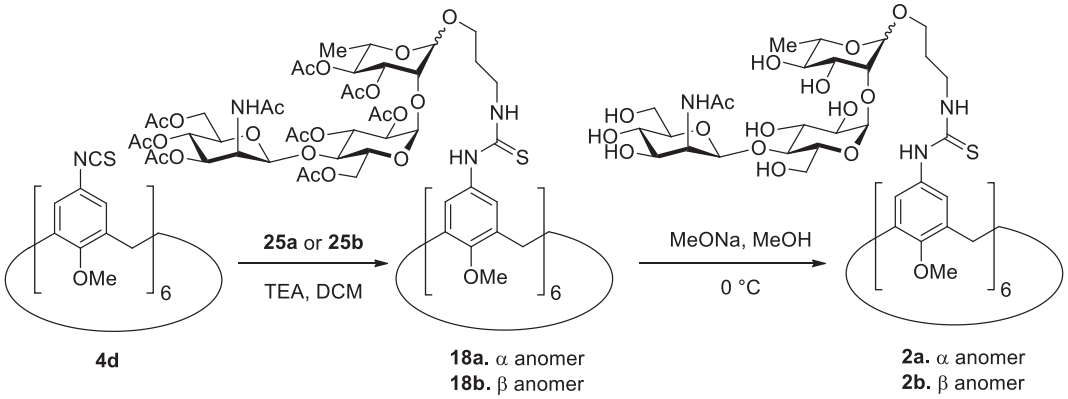
the two anomers that is produced in the final step of the synthesis of the trisaccharide when the aminopropyl chain is introduced at the anomeric position of rhamnose. The two glycocalixarenes **18a** and **18b** obtained by this condensation were deprotected under Zemplen conditions at 0 °C to give the final glycoclusters **2a** and **2b** in 81% and 82% yield respectively (Scheme 5).

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. Fig. 3 shows the inhibition curves obtained with the compounds under evaluation.

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%.

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 macrocyclic 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 glycocalix[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 thioureidoglycocalixarene 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



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

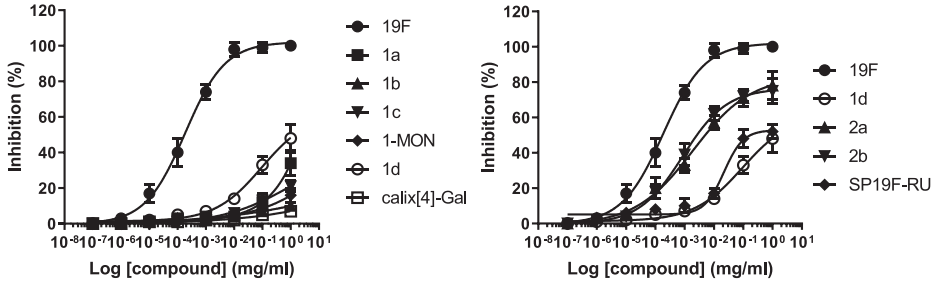


Fig. 3. Results of the Elisa experiments. Concentration/response curves of tested compounds on the inhibition of the binding between SP19F native polysaccharide, coated onto the plates, and the anti-19F antibodies, evaluated by a competitive ELISA method. Values are means of at least four experiments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

Table 1
Results of the competitive Elisa assay.

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

[a] The maximum inhibition elicited by each compound at 1 mg/mL.

valency could explain the highest efficacy (maximal inhibition 48 ± 8%) and potency ($IC_{50} = 9.5 \times 10^{-2}$ mg/mL) showed by glycocalix[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 Fig. 3 and reported in Table 1, glycocalix[6]arenes **2a** and **2b**, decorated with six trisaccharide SP19F repeating units, significantly improve efficacy (75–78% of inhibition) and affinity ($IC_{50} = 1.9 \times 10^{-3}$ and 8.6×10^{-4} mg/mL, respectively) towards the antibodies with respect to the simpler cluster **1d** (48% of inhibition, $IC_{50} = 9.5 \times 10^{-2}$ 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 predominant role of the mannosamine unit into the trisaccharide. Moreover, and more importantly, both glycocalixarenes **2a** and **2b** are more active than the single trisaccharide unit (52% of inhibition and $IC_{50} = 2.1 \times 10^{-2}$ mg/mL) showing once more and more significantly that the presentation of the epitope units on this type of scaffold increases the strength and the efficacy of antibody binding. In this framework, at the maximum of inhibition, the trisaccharide attached to the calix[6]arene platform is at a very similar concentration (1.31 mM) as when used alone (1.47 mM), but induces an efficacy 1.5 fold higher than **SP19F-RU,** determining an inhibition of the binding over 70% significantly higher than the 52% of the **SP19F-RU**. These data strongly suggest that the glycocalix[6]arene thanks to its peculiar conformational mobility enables a proper presentation of the trisaccharide repeating units that improves the strength of the ligand-receptor interactions well mimicking the conformational organization of the epitopes generated by the natural 19F polysaccharide and recognized by the specific antibodies. This result is even more significant if we consider that the chemical structure of calixarenes **2a** and **2b** is much simpler than the one of the natural polysaccharide and yet able to provide a significant inhibitory effect. As a consequence, the maximum of inhibition compared with that of 19F CPS is relevant, 75% of inhibition versus the 100%. Furthermore, the structural difference due to the α and β anomeric connection of the trisaccharide to the spacer has no significant influence on the efficacy of the two ligands **2a** and **2b**, although resulted in slightly different potencies. This means that, in perspective, the separation of the two anomers **15α** and **15β** could even be avoided, proceeding in the coupling as a mixture of both with the calixarene isothiocyanate. The statistic products containing randomly both anomers should substantially show the same biological activity as **2a** and **2b** with a not negligible save in the procedure of preparation.

3. Conclusions

Fully synthetic carbohydrate-based vaccines offer the advantage to allow site-selective conjugation of saccharide epitopes [37,41,42] and to incorporate into the nanosystems active mediators to increase vaccine efficacy. The possibility to accurately control the number and type of vaccine active species ensures homogeneous composition, which is important to induce highly reproducible biological properties with a better safety profile. In this work, the calix[6]arene scaffold represents

a valuable platform for the simultaneous presentation of multiple copies of minimized portions of the 19F CPS. In fact, this system, bearing six trisaccharide repeating units linked through a thiourea group to the macrocycle via an aminopropyl spacer, has been able to efficiently bind to anti-19F antibodies with a significant improvement of the inhibition activity compared to the one of the single repeating unit. The overall efficiency is very high, especially considering the low number of exposed saccharide antigens compared to the large number of repeating units that are present in the natural polymer. Evidently, the structural properties of the calixarene can properly present the exposed saccharide units in a tridimensional arrangement that is significantly similar to that of the natural epitopes in CPS. Our data clearly suggest that, in perspective, glycocalixarenes **2a** and **2b**, or even closely related systems with slightly longer saccharide fragments, could be functionalized with immunogenic peptides in order to elicit an antibacterial specific immune response. A structurally well-defined and easily reproducible multivalent glycocalixarene should avoid the use of complex oligosaccharide species, which are generally required for immunogenicity. Calixarenes seem then to have great potential as carriers for the development of fully synthetic carbohydrate-based vaccines, and this work contributes towards this direction.

4. Experimental section

4.1. Chemical procedures

General information. All moisture sensitive reactions were carried out under a nitrogen or argon atmosphere, using previously oven-dried glassware. All dry solvents were prepared according to standard procedures, distilled before use and stored over 3 or 4 Å molecular sieves. All other reagents were commercial samples and used without further purification. Analytical TLC were performed using prepared plates of silica gel (Merck 60F-254 on aluminum) and then, according to the functional groups present on the molecules, revealed with UV light or using staining reagents: $FeCl_3$ (1% in H_2O/MeOH 1:1), H_2SO_4 (5% in EtOH), ninhydrin (5% in EtOH), basic solution of $KMnO_4$ (0.75% in H_2O), molybdic acid solution (molybdatophosphorus acid and Ce(IV) sulphate in 4% sulphuric acid). Reverse phase TLC were performed using silica gel 60 RP-18F-254 on aluminium sheets. Merck silica gel 60 was used for flash chromatography (40–63 μm) and for preparative TLC plates (10–12 μm). Sigma Aldrich C18 reverse phase silica gel was used for flash chromatography. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AV300 and Bruker AV400 spectrometers (observation of ^{1}H nucleus at 300 MHz and 400 MHz, respectively, and of ^{13}C nucleus at 75 MHz and 100 MHz, respectively) and partially deuterated solvents were used as internal standards to calculate the chemical shifts (δ values in ppm). All ^{13}C NMR spectra were performed with proton decoupling. For ^{1}H NMR spectra recorded in D_2O at temperatures higher that 25 °C the correction of chemical shifts was performed using the expression $\delta = 5.060–0.0122 \times T(°C) + (2.11 \times 10^{-5}) \times T^2(°C)$ [43] to determine the resonance frequency of water protons. Electrospray ionization (ESI) mass analyses were performed with a Waters single-quadrupole spectrometer or with a LTQ Orbitrap XL spectrometer. Melting points were determined on an Electrothermal apparatus in capillaries sealed under N_2 atmosphere. Microwave reactions were performed using a CEM Discovery System reactor.

5,11,17,23-Tetraamino-25,26,27,28-tetramethoxycalix[4]arene [25], 5,11,17,23-tetraamino-25,26,27,28-tetrapropoxycalix[4]arene *cone* [27], 5,11,17,23-tetraamino-25,26,27,28-tetrapropoxycalix[4]arene *1,3-alternate* [25], 5,11,17,23,29,35-hexaamino-37,38,39,40,41,42-hexamethoxycalix[6]arene [26], 4-propoxyaniline [44], 5,11,17,23-tetraisohiocyanate-25,26,27,28-tetrapropoxycalix[4]arene *cone* [28], N-(benzyloxycarbonyl)aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside [29] were prepared according to literature procedures.

4.1.1. 5,11,17,23-Tetraisothiocyanate-25,26,27,28-tetramethoxycalix[4]arene (4a)

In a two-neck round-bottom flask 5,11,17,23-tetraamino-25,26,27,28-tetramethoxycalix[4]arene **3a** (0.82 g, 1.22 mmol) was dissolved in 65 mL of dry toluene under N_2 atmosphere. Then thiophosgene (1.11 mL, 14.64 mmol, *caution*) and Et_3N (4.07 mL, 29.28 mmol) were added and the mixture was allowed to react at room temperature for 48 h. The solvent was removed under reduced pressure and the crude was redissolved in dichloromethane. The organic phase was washed with water (2 × 70 mL), 1 N HCl (70 mL) and then with a saturated solution of NaCl (2 × 80 mL). The solvent was removed under reduced pressure and the residue purified by column chromatography (hexane/DCM 7/3, v/v) to afford a yellowish solid (0.19 g, 0.28 mmol, 23% yield). Mp: dec > 180 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.14 (s, 1.6H, ArH, partial 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, 1.6H, ArH, partial cone), 4.26 (d, J = 13.5 Hz, 0.8H, ArCHH_{ax}Ar, cone), 3.97 (d, J = 14.0 Hz, 1.6H, ArCHH_{ax}Ar, 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), 3.56 (s, 3.2H, ArCHH_{eq}Ar, partial cone), 3.13 (d, J = 13.5 Hz, 0.8H, ArCHH_{eq}Ar, cone), 3.06 (d, J = 14.0 Hz, 1.6H, ArCHH_{eq}Ar, partial cone), 3.04 (s, 2.4H, OCH_3, partial cone). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 156.9, 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 (OCH_3), 35.0 ($ArCH_2Ar$, partial cone), 30.3, 30.1 ($ArCH_2Ar$, cone and partial cone). HRMS (ESI-TOF) *m/z*: calcd for $C_{36}H_{28}N_4O_4S_4Na$ $[M + Na]^+$ 731.0886, found 731.0861.

4.1.2. 5,11,17,23-Tetraisothiocyanate-25,26,27,28-tetrapropoxycalix[4]arene 1,3-alternate (4c)

In a two-neck round-bottom flask 5,11,17,23-tetraamino-25,26,27,28-tetrapropoxycalix[4]arene 1,3-alternate **3c** (1.23 g, 1.96 mmol) was dissolved in 25 mL of dry toluene under N_2 atmosphere. Then thiophosgene (1.8 mL, 23.47 mmol, *caution*) and Et_3N (6.53 mL, 46.94 mmol) were added and the mixture was allowed to react at room temperature for 48 h. The solvent was removed under reduced pressure and the crude was redissolved in dichloromethane. The organic phase was washed twice with distilled H_2O (30 mL), once with 1 N HCl (30 mL) and then twice with a saturated solution of NaCl (40 mL). The solvent was removed under reduced pressure and the desired compound was obtained in 33% yield as an off-white solid after purification by column chromatography (cyclohexane/DCM 4/1 v/v) (0.53 g, 0.65 mmol). Mp: 220–221 °C. ^{1}H 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, $ArCH_2Ar$), 1.87–1.78 (m, 8H, $OCH_2CH_2CH_3$), 1.09 (t, J = 7.2 Hz, 12H, CH_2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (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 ($OCH_2CH_2CH_3$), 10.7 (CH_2CH_3). HRMS (ESI-TOF) *m/z*: calcd for $C_{44}H_{44}N_4O_4S_4Na$ $[M + Na]^+$ 843.2138, found 843.2156.

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

In a round-bottom flask **3d** (0.15 g, 0.185 mmol) was dissolved in DCM (5 mL) under N_2 atmosphere. Then thiophosgene (254 μL, 3.33 mmol, *caution*), $BaCO_3$ (0.657 g, 3.33 mmol) and H_2O (3 mL) were added with the remaining amount of DCM (5 mL). The mixture was was allowes to react at rt for 48 h and then diluted with DCM/H_2O. The organic phase was separated from the aqueous layer and evaporated under reduced pressure. The crude thus obtained was purified by flash chromatography (hexane/EtOAc 8/2, v/v) and crystallized with CH_3CN to yield the pure product as a white solid (39 mg, 0.037 mmol, 20%). Mp: dec > 200 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.74 (s, 12H, ArH), 3.89 (s, 12H, $ArCH_2Ar$), 3.54 (s, 18H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ(ppm): 155.3 (C_{Ar} ipso), 135.2 (C_{Ar} ortho), 133.9 (NCS), 126.6 (C_{Ar} para), 126.2 (C_{Ar} meta), 61.0 (OCH_3), 30.2 ($ArCH_2Ar$). HRMS (ESI-TOF) *m/z*: calcd for $C_{54}H_{42}N_6O_6S_6Na$ [(**4d** + Na)$^+$] 1085.1388, found

1085.1414 (80%); calcd for $C_{54}H_{42}N_6O_6S_6K$ $[M + K]^+$ 1101.1127, found 1101.1151 (100%).

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

To a solution of N-(benzyloxycarbonyl)aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside (**6**) (0.54 g, 0.79 mmol) in a 4:1 mixture of AcOEt/EtOH (10 mL), Pd/C (10%) was added and the suspension was shaken in a Parr hydrogenator for 90 min under 1.5 bar of H_2 at room temperature, after which the catalyst was filtered off and the filtrate evaporated under vacuum, to give the desired product as an orange oil (0.373 g, 0.68 mmol, 86%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.38–7.13 (m, 15H, ArH), 6.20 (d, J = 9.9 Hz, 1H, NHAc), 4.90–4.79 (m, 3H, H_2, 2 × CH*H*Ph), 4.57 (d, J = 11.9 Hz, 1H, C*H*HPh), 4.52–4.42 (m, 4H, H_1, 3 × C*H*HPh), 3.93–3.82 (m, 1H, OC*H*HCH$_2$), 3.73 (br, 2H, $H_{6a,b}$), 3.68–3.62 (m, 2H, H_4, H_5), 3.61–3.51 (m, 1H, OCH*H*CH$_2$), 3.46–3.38 (m, 1H, H_3), 2.83–2.74 (m, 2H, CH$_2$C*H*$_2$NH$_2$), 2.03 (s, 3H, CH_3CO), 1.77–1.69 (m, 2H, CH$_2$C*H*$_2$CH$_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 171.9 (*C*OCH$_3$), 138.2, 137.9, 137.7, 128.4, 128.33, 128.31, 127.9, 127.85, 127.8, 127.7 (C_{Ar}), 99.5 (C_1), 79.8 (C_5), 75.0 (C_3), 74.8 (CH_2Ph), 74.1 (C_4), 73.3 (CH_2Ph), 71.1 (CH_2Ph), 68.9 (C_6), 67.6 (OCH$_2$), 49.2 (C_2), 38.5 (CH_2NH_2), 27.9 (CH$_2$*C*H$_2$CH$_2$), 23.6 (CO*C*H$_3$). ESI-MS *m/z*: calcd for $C_{32}H_{41}N_2O_6$ $[M + H]^+$ 549.3, found 549.0.

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

Aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside (**7**) (0.34 g, 0.63 mmol) was dissolved in MeOH (8 mL), then Et_3N (348 μL, 2.5 mmol) and Boc_2O (0.68 g, 3.13 mmol) were subsequently added. The reaction was allowed to react for 2 h, then the solvent was removed under vacuum. The pure product was obtained as a pale oil in quantitative yield (0.40 g, 0.61 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ (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, H_2, 2 × C*H*HPh), 4.44 (d, J = 12.0 Hz, 1H, C*H*HPh), 4.40–4.31 (m, 4H, 3 × C*H*HPh, H_1), 3.75–3.66 (m, 1H, OC*H*HCH$_2$), 3.64–3.58 (m, 2H, $H_{6a,b}$), 3.56–3.51 (m, 2H, H_4, H_5), 3.50–3.39 (m, 1H, OC*H*HCH$_2$), 3.37–3.29 (m, 1H, H_3), 3.11–3.05 (m, 2H, CH$_2$C*H*$_2$NHBoc), 1.89 (s, 3H, $COCH_3$), 1.66–1.54 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.31 (s, 9H, C(CH$_3$)$_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 170.8 (*C*OCH$_3$), 156.1 (*C*O(CH$_3$)$_3$), 146.7, 138.2, 137.9, 137.8, 128.4, 128.3, 128.2, 127.8, 127.78, 127.73, 127.6 (C_{Ar}), 99.5 (C_1), 85.0 (*C*(CH$_3$)$_3$), 80.3 (C_5), 74.9 (C_3), 74.7 (CH_2Ph), 74.0 (C_4), 73.3 (CH_2Ph), 71.0 (CH_2Ph), 68.8 (C_6), 67.1 (OCH$_2$), 49.2 (C_2), 37.6 (CH_2NH_2), 29.7 (CH$_2$*C*H$_2$CH$_2$), 27.2 (C(*C*H$_3$)$_3$), 23.3 (CO*C*H$_3$). ESI-MS *m/z*: calcd for $C_{37}H_{48}N_2O_8Na$ $[M + Na]^+$ 671.3, found 671.1.

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

In a MW sealed vessel N-(Boc)aminopropyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-mannopyranoside (**8**) (0.60 g, 0.93 mmol) was dissolved in a MeOH/H_2O mixture (5 mL, 1/1 v/v) and Pd/C (10%) in catalytic amount and then NH_4COOH (0.23 g, 3.7 mmol) were added. The mixture was heated at 60 °C under microwave irradiation (150 W) for 1.5 h. The catalyst was filtered off and the solvent removed under reduced pressure, to give the desired product as white foam (0.30 g, 0.79 mmol, 85%) ^{1}H NMR (400 MHz, MeOD) δ (ppm): 4.65 (s, 1H, H_1), 4.47 (d, J = 3.2 Hz, 1H, H_2), 3.87 (br, 3H, $H_{6a,b}$, OC*H*HCH$_2$), 3.68 (dd, $J_{3,2}$ = 4.0, $J_{3,4}$ = 9.5 Hz, 1H, H_3), 3.63–3.57 (m, 1H, OC*H*HCH$_2$), 3.53 (t, J = 9.5 Hz, 1H, H_4), 3.31–3.25 (m, 1H, H_5), 3.13 (t, J = 6.4 Hz, 2H, CH$_2$C*H*$_2$NHBoc), 2.05 (s, 3H, $COCH_3$), 1.73 (t, J = 6.0 Hz, 2H, CH$_2$C*H*$_2$CH$_2$), 1.45 (s, 9H, C(CH$_3$)$_3$). ^{13}C NMR (100 MHz, MeOD) δ (ppm): 173.4 (*C*OCH$_3$), 157.1 (*C*O(CH$_3$)$_3$), 99.4 (C_1), 78.5 (*C*(CH$_3$)$_3$), 76.9 (C_5), 73.0 (C_3), 66.9 (C_4), 66.3 (OCH$_2$), 60.5 (C_6), 53.5 (C_2), 36.9 (CH_2NH_2), 29.5 (CH$_2$*C*H$_2$CH$_2$), 27.4 (C(*C*H$_3$)$_3$), 21.4 (CO*C*H$_3$). ESI-MS *m/z*: calcd for $C_{16}H_{32}N_2O_8Na$ $[M + Na]^+$ 401.2, found 401.3.

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

N-(Boc)aminopropyl 2-acetamido-2-deoxy-β-D-mannopyranoside (**9**) (0.18 g, 0.47 mmol) was dissolved in pyridine (6 mL) and then acetic anhydride was added (740 μL, 7.91 mmol). The reaction mixture was stirred at room temperature for 1 h, then the solvent removed under reduced pressure. The pure compound was obtained after purification via flash column chromatography (EtOAc/hexane 4/1, v/v) as a white foam in quantitative yield (0.23 g, 0.46 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 5.94 (br, 1H, NHAc), 5.06 (t, J = 9.2 Hz, 1H, H_4), 4.97 (dd, $J_{3,2}$ = 4.0, $J_{3,4}$ = 9.2 Hz, 1H, H_3), 4.67–4.60 (m, 3H, H_1, H_2, NHBoc), 4.27 (dd, $J_{6a,5}$ = 6.0, $J_{6a,6b}$ = 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, OC*H*HCH$_2$), 3.68–3.58 (m, 1H, H_5), 3.55 (br, 1H, OC*H*HCH$_2$), 3.22–3.15 (m, 2H, CH$_2$C*H*$_2$NHBoc), 2.09 (s, 3H, CH_3CO), 2.06 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.00 (s, 3H, NHCOC*H*$_3$), 1.77–1.68 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.42 (s, 9H, C(CH$_3$)$_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 171.4, 170.5, 170.2, 169.7 (*C*OCH$_3$), 156.1 (*C*O(CH$_3$)$_3$), 98.7 (C_1), 78.9 (*C*(CH$_3$)$_3$), 72.2 (C_5), 71.4 (C_3), 67.0 (OCH$_2$), 66.2 (C_4), 62.6 (C_6), 49.9 (C_2), 37.3 (CH_2NH_2), 29.5 (CH$_2$*C*H$_2$CH$_2$), 28.3 (C(*C*H$_3$)$_3$), 22.9, 20.6, 20.5 (CO*C*H$_3$). ESI-MS *m/z*: calcd for $C_{22}H_{36}N_2O_{11}Na$ $[M + Na]^+$ 527.2, found 527.4.

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

N-(Boc)aminopropyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopyranoside (**10**) (0.24 g, 0.47 mmol) was dissolved in dry DCM (15 mL), then trifluoroacetic acid (1.27 mL, 16.45 mmol) was added dropwise. The reaction was allowed to stir at room temperature for 1 h, then it was quenched by the addition of Et_3N. The solvent was evaporated under reduced pressure and the desired compound was obtained as a yellow-orange oil in quantitative yield (0.19 g, 0.46 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.85 (d, J = 9.0 Hz, 1H, NHAc), 5.08 (t, J = 9.6 Hz, 1H, H_4), 4.99 (dd, $J_{3,2}$ = 4.6, $J_{3,4}$ = 9.6 Hz, 1H, H_3), 4.78–4.70 (m, 2H, H_1, H_2), 4.24 (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, OC*H*HCH$_2$), 3.81–3.74 (m, 2H, H_5, OC*H*HCH$_2$), 3.71–3.64 (m, 2H, CH$_2$C*H*$_2$NH$_2$), 2.07 (s, 3H, CH_3CO), 2.02 (s, 6H, CH_3CO), 1.99 (s, 3H, NHCOC*H*$_3$), 2.00–1.96 (m, 2H, CH$_2$C*H*$_2$CH$_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 161.5, 161.2, 160.4 (*C*OCH$_3$), 98.2 (C_1), 72.3 (C_5), 71.3 (C_3), 66.6 (OCH$_2$), 66.0 (C_4), 62.4 (C_6), 49.5 (C_2), 37.4 (CH_2NH_2), 26.3 (CH$_2$*C*H$_2$CH$_2$), 22.4, 20.4 (CO*C*H$_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{17}H_{28}N_2O_9Na$ $[M + Na]^+$ 427.1687, found 427.1658.

4.1.9. N-(tertbutoxycarbonyl)-3-amminopropyl (2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopyranosyl)-(1 → 4)-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1 → 2)-3,4-di-O-acetyl-L-rhamnopyranoside (15)

Compound **16** [37] (0.19 g, 0.147 mmol) and *N*-(benzyloxycarbonyl)-3-amminopropyl (0.12 g, 0.586 mmol), as previously described [38], were dissolved in dry CH_2Cl_2 (3 mL) and activated powder molecular sieves 4 Å (0.10 g) were added. The suspension was stirred under Ar atmosphere at room temperature for 15 min, then it was cooled to 0 °C and TMSOTf 0.1 M in dry CH_2Cl_2 (0.29 mL, 0.029 mmol) was added. After 15 min, the reaction was quenched by the addition of TEA, filtered over a Celite pad and the solvent evaporated under reduced pressure. Purification of the crude through flash chromatography (Hexane/Ethyl Acetate 6:4) afforded 0.48 g of the less polar α-anomer, 0.80 g of a mixture of the two anomers, and 0.63 g of the β-anomer (**17** overall yield: 96%). **17α** and **17β** were reacted separately in the next step. To a solution under Argon of compound **SP3** in MeOH (0.01 M), Boc_2O (3.5 eq.) and then $Pd(OH)_2$/C (1/1, w/$w_{substrate}$) were added. The mixture was stirred under hydrogen atmosphere for 3 h, and checked by TLC (hexane/AcOEt, 1/1) to confirm that the Z-amino protecting group has been exchanged with BOC. Then, one drop of HCl 1 N was added, and the reaction was stirred again under hydrogen atmosphere overnight. TLC (DCM/MeOH, 75/25) control showed that the

reaction was completed, then few drops of dry Py were added, and the reaction was filtered over filter paper. After evaporation of the solvent, the crude was dissolved in dry Py (0.03 M), and acetic anhydride was added (Ac_2O/Py, 1/2) together with a catalytic amount of DMAP. The reaction was stirred at room temperature for 19 h, diluted with MeOH, and then the solvent evaporated. Purification of the crude by flash chromatography (hexane/EtOAc, 1:9) gave compound **15** as an amorphous white solid.

15α: 0.32 g of **15α** were obtained starting from 0.48 g of **17α** (88% yield). $[\alpha]_D^{20} = +22.3$ ($c = 1$ in chloroform). ^{1}H NMR ($CDCl_3$): δ = 5.90 (d, 1H, $J_{2',NH}$ = 7.2 Hz, NH), 5.39 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.4 Hz, $H_{3'}$), 5.29–5.20 (m, 2H, $H_{1'}$,H_3), 5.15–5.03 (m, 2H, H_4,$H_{4'}$), 4.92 (dd, 1H, $J_{2'',3''}$ = 3.8 Hz, $J_{3'',4''}$ = 10.0 Hz, $H_{3''}$), 4.76–4.56 (m, 5H, H_1, $H_{1''}$, $H_{2'}$, $H_{2''}$ and NH), 4.36 (dd, 1H, $J_{5'',6a''}$ = 5.3 Hz, $J_{6a'',6b''}$ = 12.5 Hz, $H_{6a''}$), 4.30–4.22 (m, 2H, 2$H_{6'}$), 4.15–4.03 (m, 2H, $H_{5'}$, $H_{6a''}$), 4.02–3.98 (m, 1H, H_2), 3.84–3.69 (m, 3H, H_a, $H_{4''}$, H_5), 3.67–3.59 (m, 1H, $H_{5''}$), 3.49–3.40 (m, 1H, $H_{a'}$), 3.28–3.17 (m, 2H, 2H_c), 2.21–1.98 (9 s, 27H, 9 CH_3), 1.85–1.75 (m, 2H, 2H_b), 1.46 (s ,9H, $(CH_3)_3CO$), 1.20 (d, 3H, $J_{5,6}$ = 6.0 Hz ,3H_6). ^{13}C NMR ($CDCl_3$): δ = 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 ($C_{5''}$), 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 ($C_{4''}$), 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 (9C, CH_3CO), 17.7 (C_6). MS (ESI) *m/z* (%): 1045.3 (1 0 0) $[M + Na]^+$.

15β: 0.42 g of **15β** were obtained starting from 0.63 g of **17β** (85% yield). $[\alpha]_D^{20} = +55.4$ ($c = 1$ in chloroform). ^{1}H NMR ($CDCl_3$): δ = 5.94 (d, 1H, $J_{2',NH}$ = 7.2 Hz, NH), 5.62 (br d, 1H, $J_{1',2'}$ = 3.9 Hz, $H_{1'}$), 5.41 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, $H_{3'}$), 5.13–5.04 (m, 2H, H_4, $H_{4''}$), 4.98 (dd, 1H, $J_{2,3}$ = 3.0 Hz, $J_{3,4}$ = 10.0 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, $H_{2'}$), 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, $J_{6a'',6b''}$ = 12.4 Hz, $H_{6''}$), 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, $H_{5'}$, $H_{6b'}$), 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, $J_{3',4'} = J_{4',5'}$ = 9.5 Hz, $H_{4'}$), 3.65–3.61 (m, 1H, $H_{5''}$), 3.50–3.41 (m, 2H, H_a, H_5), 3.29–3.19 (m, 1H, H_c), 3.11–3.02 (m, 1H, H_c), 2.17–2.00 (9 s, 27H, 9 CH_3), 1.82–1.66 (m, 2H, 2H_b), 1.45 (s ,9H, $(CH_3)_3CO$), 1.26 (d, 3H, $J_{5,6}$ = 6.0 Hz, 3H_6). ^{13}C NMR ($CDCl_3$): δ = 172.1–169.5 (9C, C═O), 156.1 (C═O), 100.9 (C_1), 98.2 ($C_{1''}$), 94.3 ($C_{1'}$), 75.7 ($C_{4'}$), 72.7 ($C_{5''}$), 72.3 (C_2), 71.9 (2C, C_3, $C_{3''}$). 71.1 (C_4), 70.7 (C_5), 70.5 ($C_{3'}$), 70.3 ($C_{2'}$), 67.8 ($C_{5'}$), 67.6 (C_a), 65.8 ($C_{4''}$), 62.2 (2C, C_6, $C_{6''}$), 50.8 ($C_{2''}$), 37.3 (C_c), 31.6 (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 (1 0 0) $[M + Na]^+$.

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

In a two-neck round-bottom flask calixarene **4b** (0.05 g, 0.0609 mmol) was dissolved in dry DCM (6 mL) under N_2 atmosphere, then sugar **11** (0.123 g, 0.305 mmol) and Et_3N (255 μL, 1.83 mmol) were added. The mixture was stirred at rt for 24 h, after which half equivalents of sugar and Et_3N were added and the mixture stirred for additional 16 h. The solvent was removed under reduced pressure and the crude was purified via flash column chromatography (DCM/MeOH 20/1, v/v) yieldig *cone* derivative **5b** in 37% yield as a white solid (0.0547 g, 0.0225 mmol). Mp: dec > 130 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.36 (br, 4H, NHCS), 6.65 (br, 8H, ArH), 6.38 (br, 8H, CSN*H*CH_2, NHAc), 5.15–5.09 (m, 8H, H_3, H_4), 4.78–4.74 (m, 8H, H_2, H_1), 4.41 (d, J = 13.2 Hz, 4H, ArC*H*H_{ax}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, $J_{6b,6a}$ = 12.0 Hz, 4H, H_{6b}), 3.97–3.96 (m, 4H, OC*H*HCH_2CH_2), 3.84 (br, 12H, O*C*$H_2CH_2CH_3$, C*H*HNHCS), 3.70–3.69 (m, 4H, H_5), 3.57 (br s, 4H, OC*H*HCH_2CH_2), 3.49–3.48 (m, 4H, C*H*HNHCS), 3.14 (d, J = 13.2 Hz, 4H, ArC*H*H_{eq}Ar), 2.07 (s, 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, $OCH_2CH_2CH_3$, $CH_2CH_2CH_2$), 0.94 (t, J = 7.5 Hz, 12H, CH_2CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 180.4 (CS), 172.1 (NH*C*OCH_3), 170.7 (*C*OCH_3), 170.5 (*C*OCH_3), 169.8 (*C*OCH_3), 154.5 (C_{Ar} ipso), 136.2 (C_{Ar} ortho), 132.1 (C_{Ar} para), 124.8 (C_{Ar} meta), 99.1 (C_1), 77.3 ($OCH_2CH_2CH_3$) 72.4 (C_5), 71.4 (C_4), 68.7 ($OCH_2CH_2CH_2$), 66.0 (C_3), 62.5 (C_6), 50.3 (C_2), 43.0 (*C*H_2NHCS), 30.9 ($ArCH_2Ar$), 29.9 ($CH_2CH_2CH_2$), 28.9 ($OCH_2CH_2CH_3$), 23.4 (CO*C*H_3), 23.2 (CO*C*H_3), 20.8 (CO*C*H_3), 20.7 (CO*C*H_3), 10.2 ($CH_2CH_2CH_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{112}H_{156}N_{12}O_{40}S_4Na_2$ $[M + 2Na]^{2+}$ 1241.4590, found 1241.4586.

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)-propyl-thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (5d)

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 NEt_3 (273 μL, 1.96 mmol) were added and the mixture stirred at rt for 72 h. The solvent was removed under reduced pressure and the crude purified via flash column chromatography and preparative TLC (DCM/MeOH 94/6, v/v) to afford **5d** as a yellowish solid (0.0317 mg, 0.0091 mmol, 23%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.66 (br, 6H, NHCS), 6.92 (br, 12H, ArH), 6.57 (s, 12H, CSN*H*CH_2, NHAc), 5.18 (br, 6H, H_4), 5.03 (br, 6H, H_3), 4.77 (br, 6H, H_2), 4.71 (br, 6H, H_1), 4.29 (br, 6H, H_{6a}), 4.14 (br, 6H, H_{6b}), 4.03–3.17 (overlapped, 60H, OC*H*HCH_2CH_2, C*H*HNHCS, H_5, $ArCH_2Ar$, OCH_3), 2.10 (s, 18H, CH_3CO), 2.07 (s, 36H, CH_3CO), 2.03 (s, 18H, CH_3CO), 1.81 (br, 12H, $CH_2CH_2CH_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 180.6 (CS), 172.1 (*C*OCH_3), 170.7 (*C*OCH_3), 170.3 (*C*OCH_3), 169.7 (*C*OCH_3), 154.2 (C_{Ar} ipso), 134.8 (br, C_{Ar} ortho, C_{Ar} para), 125.3 (C_{Ar} meta), 99.9 (C_1), 72.5 (C_5), 71.3 (C_3), 68.6 ($OCH_2CH_2CH_2$), 66.1 (C_4), 62.6 (C_6), 61.0 (OCH_3), 50.4 (C_2), 42.9 (*C*H_2NHCS), 29.7 ($ArCH_2Ar$), 29.0 ($CH_2CH_2CH_2$), 23.4 (NHCO*C*H_3), 20.9 (CO*C*H_3), 20.8 (CO*C*H_3), 20.7 (CO*C*H_3). HRMS (ESI-TOF) *m/z*: calcd for $C_{156}H_{210}N_{18}O_{60}S_6Na_2$ $[M + 2Na]^{2+}$ 1766.6028, found 1766.6047.

4.1.12. N-[3-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-mannopiranosyloxy)-propyl-thioureido]-4-propoxybenzene (14)

To a solution of **13** (0.03 g, 0.155 mmol) in dry DCM (4 mL) under N_2 atmosphere, sugar **11** (0.0943 g, 0.233 mmol) and NEt_3 (324 μL, 2.33 mmol) were added and the mixture stirred for 12 h at rt. The solvent was removed under reduced pressure and the crude purified by flash column chromatography (DCM/MeOH 20/1, v/v) yielding **14** as an off-white solid (0.0490 g, 0.0821 mmol, 53%). Mp: 90–91 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 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, CH_2N*H*CS), 6.05 (d, J = 7.6 Hz, 1H, NHAc), 5.08 (t, J = 9.6 Hz, 1H, H_4), 4.99 (dd, $J_{3,2}$ = 4.0 Hz, $J_{3,4}$ = 10.0 Hz, 1H, H_3), 4.72 (d, J = 4.0 Hz, 1H, H_2), 4.63 (s, 1H, H_1), 4.26 (dd, $J_{6a,5}$ = 5.2 Hz, $J_{6a,6b}$ = 12.4 Hz, 1H, H_{6a}), 4.08 (d, J = 12.0 Hz, 1H, H_{6b}), 3.89–3.86 (m, 3H, OC*H*HCH_2CH_2, $OCH_2CH_2CH_3$), 3.76 (br, 1H, C*H*HNHCS), 3.67–3.56 (m, 2H, H_5, OC*H*HCH_2CH_2), 3.53 (br, 1H, C*H*HNHCS), 2.06 (s, 3H, $COCH_3$), 2.03 (s, 3H, $COCH_3$), 2.02(s, 3H, $COCH_3$), 1.97 (s, 3H, $COCH_3$), 1.75 (m, 4H, $OCH_2CH_2CH_3$, $CH_2CH_2CH_2$), 0.98 (t, J = 7.2 Hz, 3H, CH_2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 181.7 (CS), 171.7 (NH*C*OCH_3), 170.6 (*C*OCH_3), 170.1 (*C*OCH_3), 169.7 (*C*OCH_3), 157.9 (C_{Ar} ipso), 129.8 (C_{Ar} ortho), 127.3 (C_{Ar} para), 127.3, 125.6 (C_{Ar} meta), 99.1 (C_1), 72.5 (C_5), 71.0 (C_3), 69.8 ($OCH_2CH_2CH_3$), 68.5 ($OCH_2CH_2CH_2$), 66.0 (C_4), 62.4 (C_6), 50.3 (C_2), 43.1 (*C*H_2NHCS), 29.7 ($CH_2CH_2CH_2$), 23.4 (CO*C*H_3), 20.8 ($OCH_2CH_2CH_3$), 20.8, 20.7 (CO*C*H_3), 10.5 ($OCH_2CH_2CH_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{27}H_{39}N_3O_{10}SNa$ $[M + Na]^+$ 620.2254, found 620.2261.

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-(1 → 4)-2,3,6-tri-O-acetyl-α-D-glucopyranosyl-(1 → 2)-3,4-di-O-acetyl-α-L-rhamnopyranosyloxy)-propyl-thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (18a)

15α (22 mg, 0.0215 mmol) was dissolved in dry DCM (4 mL) under

Ar atmosphere. The temperature was decreased to 0 °C and 0.5 mL of trifluoroacetic were added. The reaction proceeded at room temperature for 1 h, after which the TLC showed the complete consumption of the reagent, thus the solvent was evaporated at reduced pressure. The obtained deprotected product was dissolved in dry DCM (2 mL) and 5,11,17,23,29,35-hexaisothiocyanate-37,38,39,40,41,42-hexamethoxycalix[6]arene (**4d**) (2.5 mg, 0.00239 mmol) and NEt_3 (17 μL, 0.120 mmol) were added under Ar atmopshere. The mixture was stirred for 48 h at room temperature, after which the reaction was quenched by removal of the solvent at reduced pressure. The desired product was obtained after column chromatography (DCM/MeOH 96:4 v/v) as a white solid in 86% yield (13.5 mg, 0.00205 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.01 (br, 12H, ArH), 5.91 (br, 6H, NHAc), 5.38 (t, $J_{3',4'} = J_{3',2'} = 9.5$ Hz, 6H, $H_{3'}$), 5.24 (br, 12H, H_3, $H_{1''}$), 5.14–5.03 (m, 12H, H_4, $H_{4'}$), 4.93 (br, 6H, $H_{3''}$), 4.77–4.67 (m, 12H, $H_{2'}$, $H_{1''}$), 4.62 (br, 12H, $H_{2''}$, H_1), 4.37 (dd, $J_{6a,5} = 5.1$, $J_{6a,6b} = 12.5$ Hz, 6H, $H_{6a''}$), 4.25 (br, 12H, $H_{6'}$), 4.12–4.03 (m, 12H, $H_{5'}$, $H_{6''}$), 4.00 (br, 6H, H_2), 3.84–3.70 (m, 18H, H_5, $H_{4'}$, OC*H*HCH$_2$CH$_2$), 3.67 (br, 12H, $H_{5''}$, OC*H*HCH$_2$CH$_2$), 3.45 (br, 12H, CH_2NHCS), 2.16 (s, 18H, CH_3CO), 2.13 (s, 18H, CH_3CO), 2.12 (s, 18H, CH_3CO), 2.11 (s, 18H, CH_3CO), 2.09 (s, 18H, CH_3CO), 2.06 (s, 36H, CH_3CO), 2.04 (s, 18H, CH_3CO), 2.02 (s, 18H, CH_3CO), 1.88 (br, 12H, CH$_2$CH_2CH$_2$), 1.19 (d, $J = 5.8$ Hz, 18H, H_6). HRMS (ESI-TOF) *m/z*: calcd for $C_{288}H_{390}N_{18}O_{144}S_6Na_4$ $[M + 4Na]^{4+}$ 1672.0388, found 1672.0396.

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-(1 → 4)-2,3,6-tri-O-acetyl-α-D-glucopyranosyl-(1 → 2)-3,4-di-O-acetyl-β-L-rhamnopyranosyloxy)-propyl-thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (6b)

15β (29 mg, 0.0283 mmol) was dissolved in dry DCM (4 mL) under Ar atmosphere. The temperature was decreased to 0 °C and 0.5 mL of trifluoroacetic were added dropwise. The reaction proceeded at room temperature for 1 h, after which the TLC showed the complete consumption of the reagent, thus the solvent was evaporated at reduced pressure. The crude was then dissolved in dry DCM (3 mL) and 5,11,17,23,29,35-hexaisothiocyanate-37,38,39,40,41,42-hexamethoxycalix[6]arene (**4d**) (3.3 mg, 0.00314 mmol) and NEt_3 (21 μL, 0.157 mmol) were added under Ar atmopshere. The mixture was stirred for 48 h at room temperature, after which the reaction was quenched by removal of the solvent at reduced pressure. The desired product was obtained after column chromatography (DCM/MeOH 95/5, v/v) as a white solid in 82% yield (17 mg, 0.00258 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.11 (br, 12H, ArH), 5.93 (d, $J = 7.3$ Hz, 6H, NHAc), 5.60 (br, 6H, $H_{1'}$), 5.41 (t, $J = 9.1$ Hz, 6H, $H_{3'}$) 5.17–5.02 (m, 12H, $H_{4''}$, H_4), 4.96 (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, $H_{3''}$) 4.74 (br, 6H, $H_{1''}$), 4.69 (br, 6H, $H_{2'}$), 4.62 (br, 6H, $H_{2''}$), 4.50 (br, 6H, H_1), 4.39 (dd, 6H, $H_{6a''}$) 4.28 (br, 6H, $H_{6a'}$), 4.13 (br, 18H, H_2, $H_{5'}$, $H_{6b'}$), 4.05 (d, $J = 12.0$ Hz, 6H, $H_{6b''}$), 3.86 (br, 12H, CH_2NHCS), 3.75 (br, 6H, $H_{4'}$), 3.66 (br, 6H, $H_{5''}$), 3.45 (br, 18H, H_5, 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, CH_3CO), 2.05 (s, 18H, CH_3CO), 2.03 (s, 18H, CH_3CO), 2.01 (s, 18H, CH_3CO), 1.75 (br, 12H, CH$_2$CH_2CH$_2$), 1.23 (d, $J = 5.8$ 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, 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, 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 for $C_{288}H_{390}N_{18}O_{144}S_6Na_4$ $[M + 4Na]^{4+}$ 1672.0388, found 1672.0399.

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

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

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

The crude was first purified by C18 reverse phase column chromatography (MeOH/H_2O 5.5/4.5, v/v), and then by size exclusion chromatography on Sephadex G-25 as stationary phase (MeOH/H_2O 1.5/8.5, v/v). The final purification step was performed via standard flash chromatography (*i*-PrOH/H_2O/Et_3N 8/2/0.5, v/v/v) to afford the mobile calix[4]arene **1a** in 40% yield (0.037 g, 0.0191 mmol). Mp: dec > 140 °C. ^{1}H NMR (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, 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, CH$_2$CH_2CH$_2$). ^{13}C NMR (100 MHz, MeOD, 55 °C) δ (ppm): 180.6 (CS), 173.6 (NHC*O*CH$_3$), 155.7 (C_{Ar} ipso), 135.32 (C_{Ar} ortho), 132.4 (C_{Ar} para), 124.4 (C_{Ar}), 99.5 (C_1), 77.0 (C_5), 73.0 (C_3), 67.2 (C_4, O*C*H$_2$CH$_2$CH$_2$), 61.0 (C_6, OCH_3), 53.6 (C_2), 42.1 (*C*H$_2$NHCS, ArCH$_2$Ar), 29.2 (CH$_2$*C*H$_2$CH$_2$), 22.2 (NHCO*C*H$_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{80}H_{116}N_{12}O_{28}S_4Na$ $[M + Na]^+$ 1843.6803, found 1843.6812.

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

The pure product **1b** was obtained after purification by C18 reverse phase column chromatography (MeOH/H_2O 4/1, v/v) in 78% yield as a white solid (0.034 g, 0.0176 mmol). Mp: dec > 178 °C. ^{1}H NMR (400 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, ArCHH_{ax}Ar), 3.98–3.86 (m, 24H, $H_{6a,b}$, OC*H*HCH$_2$CH$_2$, OCH_2CH$_2$CH$_3$), 3.71–3.63 (m, 16H, H_3, OC*H*HCH$_2$CH$_2$, C*H*HNHCS), 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, ArCHH_{eq}Ar), 2.08 (s, 12H, NHCOCH_3), 2.02–1.96 (m, 8H, OCH$_2$CH_2CH$_3$), 1.86 (br, 8H, CH$_2$CH_2CH$_2$), 1.06 (t, $J = 6.8$ Hz, 12H, OCH$_2$CH$_2$CH_3). ^{13}C NMR (100 MHz, MeOD) δ (ppm): 179 (CS), 173.6 (NH*C*OCH$_3$), 154.0 (C_{Ar} ipso), 135.32 (C_{Ar} 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 (O*C*H$_2$CH$_2$CH$_2$), 66.9 (C_4), 60.7 (O*C*H$_2$CH$_2$CH$_3$), 53.6 (C_2), 47.7 (*C*H$_2$NHCS), 42.2 (C_2), 30.5 (ArCH$_2$Ar), 28.7 (CH$_2$*C*H$_2$CH$_2$), 23.1 (OCH$_2$*C*H$_2$CH$_3$), 21.7 (NHCO*C*H$_3$), 9.5 (OCH$_2$CH$_2$*C*H$_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{88}H_{132}N_{12}O_{28}S_4Na$ $[M + Na]^+$ 1955.8055, found 1955.8069.

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

Compound **1c** was obtained after purification via C18 reverse phase column chromatography (MeOH/H_2O 3/2, v/v) as a white solid (0.023 g, 0.0119 mmol, 83%). Mp: dec > 176 °C. ^{1}H NMR (400 MHz, MeOD) δ (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, C*H*HNHCS), 3.87 (d, $J = 3.2$ Hz, 8H, H_{6ab}), 3.83–3.64 (m, 24H, H_3, OCH_2CH$_2$CH$_2$, C*H*HNHCS, OCH_2CH$_2$CH$_3$), 3.63–3.50 (m, 12H, ArCH$_2$Ar, H_4), 3.31–3.25 (m, 4H, H_5), 2.10 (s, 12H, NHCOCH_3), 2.01–1.81 (m, 8H, CH$_2$CH_2CH$_2$), 1.96–1.80 (m, 8H, OCH$_2$CH_2CH$_3$), 1.04 (t, $J = 7.6$ Hz, 12H, OCH$_2$CH$_2$CH_3). ^{13}C NMR (100 MHz, MeOD) δ (ppm): 180.7 (CS), 173.5 (NH*C*OCH$_3$), 153.9 (C_{Ar} ipso), 133.6 (C_{Ar} ortho), 131.6 (C_{Ar} para), 125.4 (C_{Ar}), 99.52 (C_1), 76.90 (C_5), 74.9 (O*C*H$_2$CH$_2$CH$_3$), 72.8 (C_3), 67.2 (*C*H$_2$NCS), 66.9 (C_4), 60.7 (C_6), 53.6 (C_2), 42.2 (O*C*H$_2$CH$_2$CH$_2$), 35.0 (ArCH$_2$Ar), 28.8 (CH$_2$*C*H$_2$CH$_2$), 23.6 (OCH$_2$*C*H$_2$CH$_3$), 21.6 (NHCO*C*H$_3$), 9.7 (OCH$_2$CH$_2$*C*H$_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{88}H_{132}N_{12}O_{28}S_4Na$ $[M + Na]^+$ 1955.8055, found 1955.8070.

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

Purification by C18 reverse phase column chromatography (MeOH/H_2O 64/36, v/v) afforded **1d** in 54% yield as a off-white solid (0.015 g, 0.00549 mmol). Mp: 186–187 °C. ^{1}H NMR (400 MHz, D_2O, 80 °C) δ (ppm): 7.45 (s, 12H, ArH), 5.20 (s, 6H, H_1), 4.97 (d, $J = 4.4$ Hz, 6H, H_2), 4.44 (br, 12H,

$ArCH_2Ar$), 4.40–4.23 (m, 24H, H_3, $H_{6a,b}$, $OCHHCH_2CH_2$), 4.18–4.11 (m, 6H, $OCHHCH_2CH_2$), 4.07–4.00 (m, 18H, CH_2NHCS, H_4), 3.84 (br, 6H, H_5), 3.77 (br, 18H, CH_3), 2.52 (s, 18H, $NHCOCH_3$), 2.31 (br, 12H, $CH_2CH_2CH_2$). ^{13}C NMR (100 MHz, D_2O, 80 °C) δ (ppm): 180.7 (CS), 175.4 ($NHCOCH_3$), 154.8 (C_{Ar} ipso), 135.6 (C_{Ar} ortho), 134.1 (C_{Ar} para), 126.8 (C_{Ar}), 99.9 (C_1), 77.2 (C_5), 75.0 (C_3), 67.8 (C_4), 67.7 ($OCH_2CH_2CH_2$), 61.2 (OCH_3), 59.9 (C_6), 53.8 (C_2), 42.5 (CH_2NHCS), 30.7 ($CH_2CH_2CH_2$), 29.2 ($ArCH_2Ar$), 22.8 ($NHCOCH_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{120}H_{174}N_{18}O_{42}S_6K_2$ $[M + 2K]^{2+}$ 1404.6177, found 1404.6204.

4.1.15.5. N-[3-(2-acetamido-2-deoxy-β-D-mannopiranosyloxy)-propyl-thioureido]-4-propoxybenzene (1-MON). The pure product was obtained after purification by C18 reverse phase column chromatography ($MeOH/H_2O$ 1/1, v/v) as a off-white solid (0.0272 g, 0.0577 mmol, 66%). Mp: dec > 76 °C. ^1H NMR (400 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, H_1), 4.48 (dd, $J_{2,1} = 1.5$, $J_{2,3} = 4.5$ Hz, 1H, H_2), 3.95 (t, $J = 6.5$ Hz, 2H, $OCH_2CH_2CH_3$), 3.93–3.89 (m, 1H, $OCHHCH_2CH_2$), 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, H_3), 3.61 (m, 2H, $OCHHCH_2CH_2$, $CHHNHCS$), 3.53 (t, $J = 9.6$ Hz, 1H, H_4), 3.29–3.23 (m, 1H, H_5), 2.04 (s, 3H, $NHCOCH_3$), 1.89–1.76 (m, 4H, $OCH_2CH_2CH_3$, $CH_2CH_2CH_2$), 1.06 (t, $J = 7.2$ Hz, 3H, $OCH_2CH_2CH_3$). ^{13}C NMR (100 MHz, MeOD) δ (ppm): 181.1 (CS), 173.5 ($NHCOCH_3$), 157.7 (C_{Ar} ipso), 127.3 (C_{Ar} para), 126.7, 114.5 (C_{Ar} meta), 99.4 (C_1), 76.9 (C_5), 76.8 ($OCH_2CH_2CH_3$), 69.4 (C_4, $OCH_2CH_2CH_2$), 60.6 (C_6), 53.5 (C_2), 41.9 (CH_2NHCS), 28.6 ($CH_2CH_2CH_2$), 22.3 ($OCH_2CH_2CH_3$), 21.4 ($NHCOCH_3$), 9.4 ($OCH_2CH_2CH_3$). HRMS (ESI-TOF) *m/z*: calcd for $C_{21}H_{33}N_3O_7SNa$ $[M + Na]^+$ 494.1937, found 494.1955.

4.1.16. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-2-deoxy-β-D-mannopyranosyl-(1 → 4)-α-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranosyloxy)-propyl-thioureido]-37,38,39,40,41,42-hexamethoxycalix[6]arene (2a)

The pure product was obtained after trituration in diethyl ether (7.5 mg, 0.00164 mmol, 80%). ^1H NMR (400 MHz, $MeOD/D_2O$ 75/25) δ (ppm): 8.49 (s, NH), 7.01 (br, 12H, ArH), 5.04 (br, OH), 4.94 (br, 6H, $H_{1'}$), 4.87 (br, 6H, $H_{1''}$), 4.85 (br, 6H, H_1), 4.54 (br, 6H, $H_{2''}$), 4.14–3.22 (ovelapped, 144H, $H_{3''}$, $H_{4''}$, $H_{5''}$, $H_{6a,b''}$, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{6a,b'}$, H_2, H_3, H_4, H_5, $OCH_2CH_2CH_2$, CH_2NHCS, OCH_3, $ArCH_2Ar$), 2.08 (s, 18H, $NHCOCH_3$), 1.86 (br, 12H, $CH_2CH_2CH_2$), 1.29 (br, 18H, H_6). ^{13}C NMR (100 MHz, $MeOD/D_2O$ 1:1) δ (ppm): 174.5, 99.4, 98.0, 97.7, 78.8, 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 (ESI-TOF) *m/z*: calcd for $C_{192}H_{294}N_{18}O_{96}S_6Na_3$ $[M + 3Na]^{3+}$ 1550.2248, found 1550.2276.

4.1.17. 5,11,17,23,29,35-Hexakis-N-[3-(2-acetamido-2-deoxy-β-D-mannopyranosyl-(1 → 4)-α-D-glucopyranosyl-(1 → 2)-β-L-rhamnopyranosyloxy)-propyl-thioureido]-37,38,39,40,41,42-hexamethoxy calix[6]arene (2b)

The pure product was obtained after trituration in diethyl ether (9.6 mg, 0.00209 mmol, 81%). ^1H NMR (400 MHz, $MeOD/D_2O$ 9/1) δ (ppm): 7.04 (br, 12H, ArH), 5.11 (br, 6H, $H_{1'}$), 4.83 (s, 6H, $H_{1''}$), 4.67 (br, 6H, H_1), 4.63 (br, OH), 4.53 (br, 6H, $H_{2''}$), 4.24–3.27 (overlapped, 144H, $H_{3''}$, $H_{4''}$, $H_{5''}$, $H_{6a,b''}$, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{6a,b'}$, H_2, H_3, H_4, H_5, $OCH_2CH_2CH_2$, CH_2NHCS, OCH_3, $ArCH_2Ar$), 2.07 (s, 18H, $NHCOCH_3$), 1.90 (br, 12H, $CH_2CH_2CH_2$), 1.34 (br, 18H, H_6). ^{13}C NMR (100 MHz, $MeOD/D_2O$ 9:1) δ (ppm): 173.4, 100.7, 100.4, 99.5, 78.9, 78.4, 77.0, 72.9, 72.6, 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 $C_{192}H_{294}N_{18}O_{96}S_6Na_3$ $[M + 3Na]^{3+}$ 1550.2248, found 1550.2273.

4.2. Competitive ELISA

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

Declaration of Competing interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103305.

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