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Clustering Zwitterionic Amino Acids at the Upper Rim of Cone Calix[4]arene Triggers the Selective Recognition of Gram-Negative Bacterial Envelope

Francesco Rispoli, Luca Moretti, Carlo Alberto Vezzoni, Eloisa Tosi, Linda Molteni, Carlotta Ciaramelli, Luciano Marchiò, Stefano Volpi, Laura Baldini, Francesco Sansone, Alessandro Palmioli, Cristina Airoldi, and Alessandro Casnati**

Eleven calix[4]arene ligands, bearing zwitterionic α-amino acids or charged ammonium or sulfonate/carboxylate groups, are synthesized and screened for the binding to the envelopes of three bacterial strain representatives of Gram-positive, Gram-negative, and mycobacteria. The binding is followed by on-cell Saturation Transfer Difference Nuclear Magnetic Resonance (NMR) experiments directly on alive cells. While the anionic tetrasulfonatocalixarene does not bind to any bacterial strains significantly and the cationic calixarenes strongly bind to both Gram-positive and Gram-negative bacteria, the zwitterionic tetraprolino- and tetraphenylalaninocalix[4]arene show a remarkable selectivity for Gram-negatives over Gram-positives and mycobacteria. The tetraprolinocalixarene binds to the lipopolysaccharides extracted from two Gram-negative bacteria (*Pseudomonas putida* or *Escherichia coli*), suggesting these biomacromolecules as possible targets in the recognition of their cell walls. The ligand binding epitope map demonstrates a deep involvement of the amino acids and calixarene aromatic nuclei in the interaction. In this study, for the first time, the ability of synthetic macrocycles to selectively recognize the envelope of Gram-negative bacteria is highlighted, and the way to the chemical modifications of the ligand structure is paved to develop devices for the detection or treatment of bacterial infections, thus allowing to add another string to the bow for the fight against antimicrobial resistance.

1. Introduction

Easy and rapid detection, characterization, and quantification of bacterial strains are essential goals in medicine. Apart from the need to monitor bacteria in air, water, and foods,[1–3] it lately became important to determine the composition of microbial populations in human bodies (also referred to as microbiota) since it was demonstrated that the homeostasis of human beings depends not only on nutrition and external factors but also on a proper balance between the different bacterial strains that populate, in a dynamic and mutualistic symbiosis, our gut.[4] Infections with pathogenic bacteria can alter the microbiota composition (dysbiosis) thus promoting chronic inflammations, autoimmune diseases,[5] or even pathologies of other organs which might eventually lead to cancer.[4,6] In addition, bacterial identification and quantification are becoming extremely urgent also in the fight against antimicrobial resistance (AMR), one of the major threats of the 21st century,[7] that is spreading around also because of an inappropriate use of broad-spectrum antibiotics, often imposed by the urgency of the treatment and the lack of rapid methods for pathogen identification.[8] More rapid molecular methods that simultaneously detect the presence of multiple pathogens on the same sample are, therefore, highly desirable. Although novel sequencing-based approaches to pathogen identification have shown considerable promise in research settings, to date, these techniques have been too slow, expensive, and labor intensive for clinical use,[9] so that the traditional use of techniques relying on agar plates are commonly used to isolate, cultivate, and count cells in biological samples.[10] An expeditious, cost-effective and selective method capable of rapidly identifying one or more pathogens is still lacking and this is strongly prompting an intense research activity in this field. A considerable attention has been paid to biosensors,[11,12] based on a biological recognition element such as antibodies, aptamers, peptides, or bacteriophages, but improvements should still be achieved in the stability and specificity of these

F. Rispoli, C. A. Vezzoni, E. Tosi, L. Marchiò, S. Volpi, L. Baldini, F. Sansone, A. Casnati
Department of Chemistry, Life Sciences and Environmental Sustainability
Università di Parma
Parco Area delle Scienze 17/A, 43124 Parma, Italy
E-mail: alessandro.casnati@unipr.it

L. Moretti, L. Molteni, C. Ciaramelli, A. Palmioli, C. Airoldi
BioOrg NMR Lab
Department of Biotechnology and Biosciences
University of Milano-Bicocca
Piazza della Scienza, 2, 20126 Milan, Italy
E-mail: cristina.airoldi@unimib.it

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devices, especially in polymicrobial environments. On the other side, using a supramolecular approach, several artificial systems have been used to detect bacteria through a molecular recognition approach.[13] It might be envisaged that recognition of bacteria may take place via specific recognition of targets (proteins, lectins, peptidoglycans, sugars, etc.) present on the cell membrane or via a specific electrostatic interactions with the bacterial surface. Macrocycles have been also used in the fights against bacteria but usually showing low selectivity and an associated bactericidal action mainly ascribed to a specific cell membrane disruption, biofilm disaggregation, and DNA damage or to their drug-delivery function.[14] Calixarenes[15–17] also showed similar activity but, apart from some multivalent glycosylated calixarenes,[18–20] which are very selective in the binding of specific carbohydrate recognition proteins (lectins) present on the membrane of some bacterial strains, and the macrobicyclic calixarenes showing a vancomycin-like activity,[21,22] to the best of our knowledge, there are no other examples in the literature of calixarenes able to selectively distinguish between different bacterial strains. We herein report that zwitterionic-upper-rim-functionalized amino acidic calix[4]arenes[23–26] show remarkable selectivity in the recognition of Gram-negative versus Gram-positive bacteria and mycobacteria. The binding of these ligands to bacteria was studied by using on-cell saturation transfer difference (STD) NMR experiments. STD NMR techniques rely on the rapid and efficient transfer of magnetization from a receptor to a ligand exchanging among the free and the bound state.[27,28] In particular, a 1D-STD NMR spectrum is achieved by subtracting a spectrum in which some receptor resonances are selectively saturated (on-resonance) from one without receptor saturation (off-resonance); the difference spectra contain only signals of the compounds that received saturation from the receptor through dipolar coupling and thus direct interaction.[27] STD NMR experiments allow the fast screening of putative ligands of a specific target receptor, and, at the same time, they afford the identification of the ligand binding epitope. Being based on the observation of ligand resonances, they require only small amounts of receptor (pM–μM concentrations) that does not strictly need to be isolated. This is the case of on-cell STD NMR,[20,29–33] allowing to study interactions involving components of the external envelope of cells, without their purification. Moreover, STD can afford accurate determinations of dissociation constants (K_D).[28,34–36] These techniques support both the screening and the rational structure-based design of new ligands with improved binding properties.[37–39]

2. Results and Discussion

In our efforts to select a simple, robust, and easily accessible macrocyclic ligand able to selectively recognize Gram-positive, Gram-negative, or mycobacteria, we synthesized a series of hydrophilic calixarene ligands bearing zwitterionic (**1**–**7**), anionic (**8**), or cationic (**9**) groups (**Figure 1**).

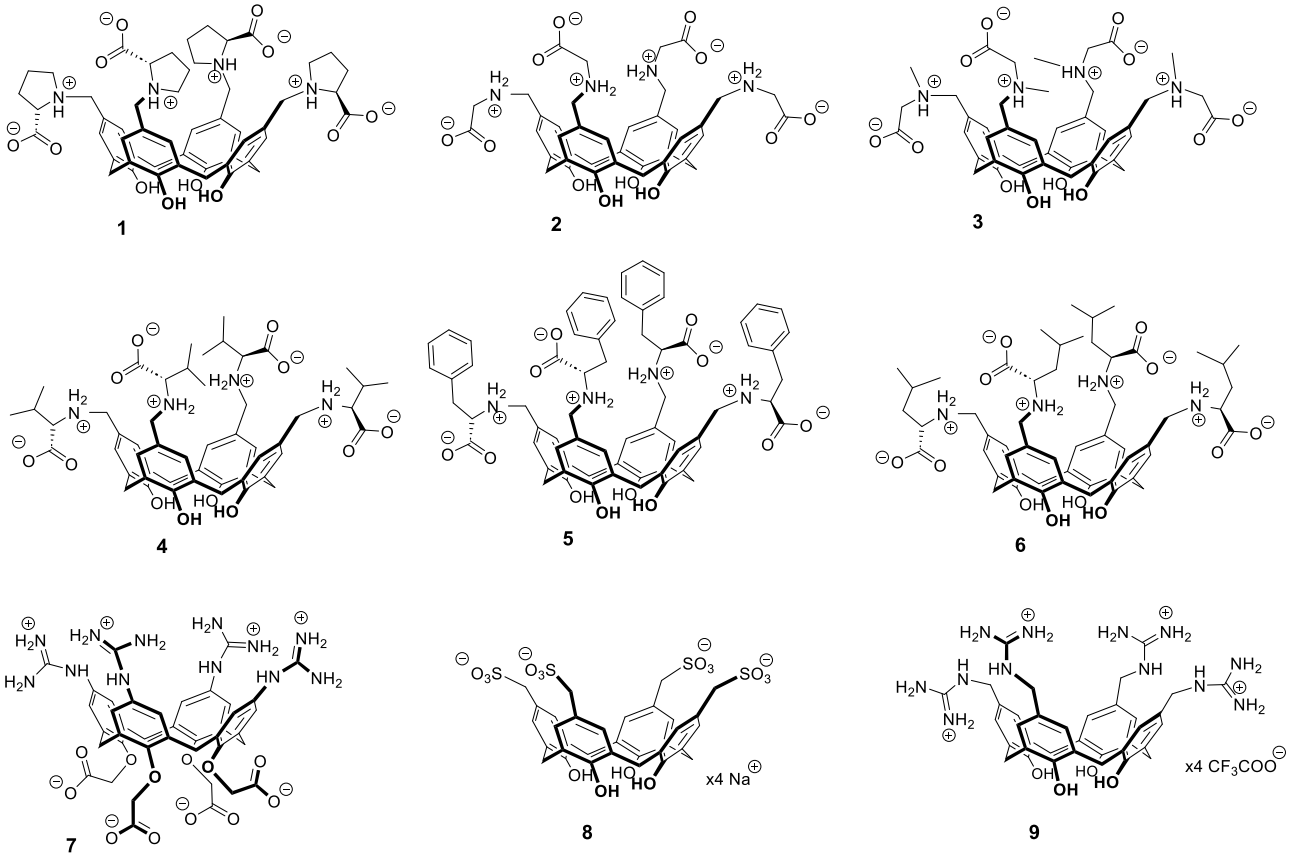


Figure 1. Zwitterionic (**1**–**7**), anionic (**8**), and cationic (**9**) calix[4]arenes studied in this work.

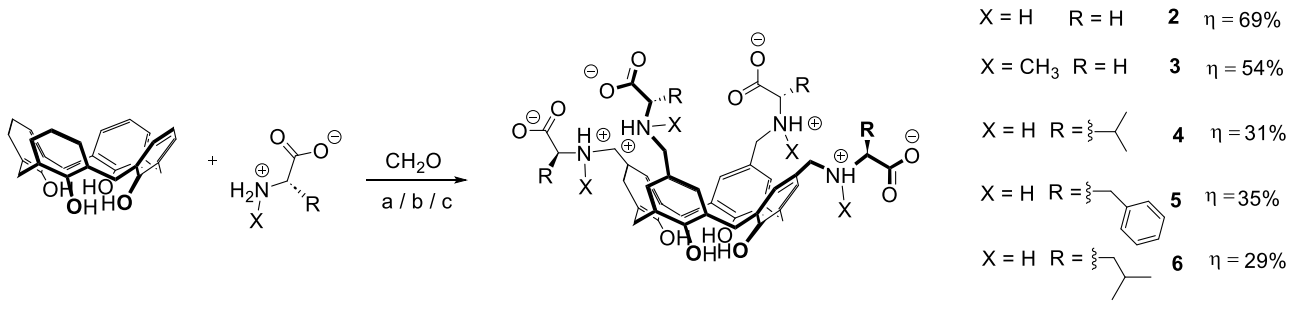
Apart from **7**, all these ligands bear the charged groups necessary to ensure water solubility at the upper rim of the calixarene and free OH groups at the lower rim. The reason for this design is twofold. From one side, the alkylation of the lower rim of charged calixarenes provides ligands possessing an amphiphilic character, even more marked as the length and lipophilicity of the chain increases. This amphiphilicity thus results in the self-assembling of the macrocycles into micellar or vesicular structures interesting for several biotechnological applications,[15,16,40] but that would have made our STD studies on bacteria cells (*vide infra*) even more challenging. On the other side, the presence of free OH groups at the lower rim contributes to the necessary water compatibility and, taking into consideration that at physiological pH at least one of the four hydroxyl groups is deprotonated,[41] this also allows to partly rigidify the cone structure thanks to intramolecular H bonds. Derivative **7** is the only ligand, in this study, bearing substituents (acetate groups) at the lower rim and was designed to have a zwitterionic macrocycle in the cone structure, as all the other calixarene ligands, but bearing cationic and anionic groups in separate regions. Guanidinium groups, present also in **9**, were chosen as cationic head groups since their acid–base properties ensure their complete protonation in a wide range of pH and, particularly, even at physiological pH, independently of the simultaneous presence of four of these groups in close proximity to each other.[17,42] Moreover, guanidinium groups have been often used in supramolecular and bioorganic chemistry for the efficient complexation of anionic units such as phosphates[43] or carboxylates[44,45] in biomolecules and therefore they could result important ligating units for the recognition of peculiar molecular patterns on the bacterial cell walls. Sulfonatomethylcalix[4]arene **8** was chosen as an anionic ligand due to its ease of preparation and isolation, and for the possibility to have four net negative charges at the upper rim[46] in a similar disposition as the positive charges of **9**. Moreover, sulfonatocalixarenes also show relevant biological properties and recognition ability of groups even on the surfaces of biomacromolecules.[47]

2.1. Syntheses and Structures of the Ligands

Zwitterionic calixarenes **1**–**6** were obtained by Mannich reaction of *p*-Hcalix[4]arene with formaldehyde and the proper L-amino acid. Compound **1** was obtained following the experimental procedure published by Mocerino and coll.,[24,26] in yields similar to those reported. For other amino acids (cfr. conditions a/b/c in **Scheme 1**) the procedure was slightly adapted by using different solvent mixtures to allow solubilization of the amino components.

Aminomethylation of phenols with primary and secondary amines preferably gives ortho functionalization, but the reaction of aminomethylation of simple phenols where amino acids are the amino components is seldom used and, to the best of our knowledge, very few reports are present in the literature.[48] We noticed that such reactions on calixarenes, probably also because the ortho positions are occupied in these macrocycles by the methylene bridges, generally require long reaction times and give low yield especially when amino acids with hydrophobic residues (Val, Phe, and Leu) are used. More satisfactory yields were obtained in the case of less hindered amino acids (Gly) or amino acids having secondary amino groups (Pro and N-Me-Gly). Compound **1** shows a fair solubility in water which allows to record its NMR spectra in pure D_2O. The methylene bridge protons ($ArCH_2Ar$), giving rise to a singlet (even if broad) around 3.88–3.80 ppm, are indicative of a relatively fast cone-to-cone exchange between conformations of the calixarene macrocycle.[49] Compound **6**, having Leu residues, is rather insoluble in D_2O preventing subsequent studies with bacteria, while compounds **2**–**5** need to be dissolved in a slightly basic solution, D_2O:NaOD (40% w/w) = 25:1, to register NMR spectra. This results in a slight freezing of the cone-to-cone interconversion also thanks to the partial deprotonation of the phenolic OH groups at the lower rim. Under these conditions, the methylene bridge protons appear as two broad signals around 4.10 and 3.15 ppm for the axial and equatorial protons, respectively. Variable temperature experiments for the glycine derivative **2** show (Figure S16, Supporting Information) the coalescence temperature around 50 °C (400 MHz) with the appearance of a broad signal for the $ArCH_2Ar$ between 4.0–3.5 ppm that becomes a sharper singlet at 3.63 ppm when heated to 80 °C. In calixarenes **1** and **4**–**6**, the $ArCH_2N$ methylene protons result diastereotopic due to the presence of the amino acid stereocenters and originate an AB system (two doublets) between 3.15–3.38 and 2.98–3.15 ppm, while in achiral compounds **2** and **3**, they appear as a sharp singlet around 3.25 ppm. We could also determine the X-Ray crystal structure of the glycine derivative **2** (**Figure 2** and S48–S50, Supporting Information) crystallized from a H_2O/MeOH/DMF mixture.

The asymmetric unit comprises a sodium cation, a disordered dimethylformamide molecule, and several water molecules of



Scheme 1. Conditions: **a**) toluene/EtOH/H_2O (5/1/1) reflux, 72 h (for compound **2**); **b**) THF/MeOH/CH_3COOH (10/3/2), reflux, 96 h (for compound **3**); and **c**) THF/H_2O/CH_3COOH (5/1/1), rt, 96 h (for compounds **4**, **5**, **6**) (THF is tetrahydrofuran).

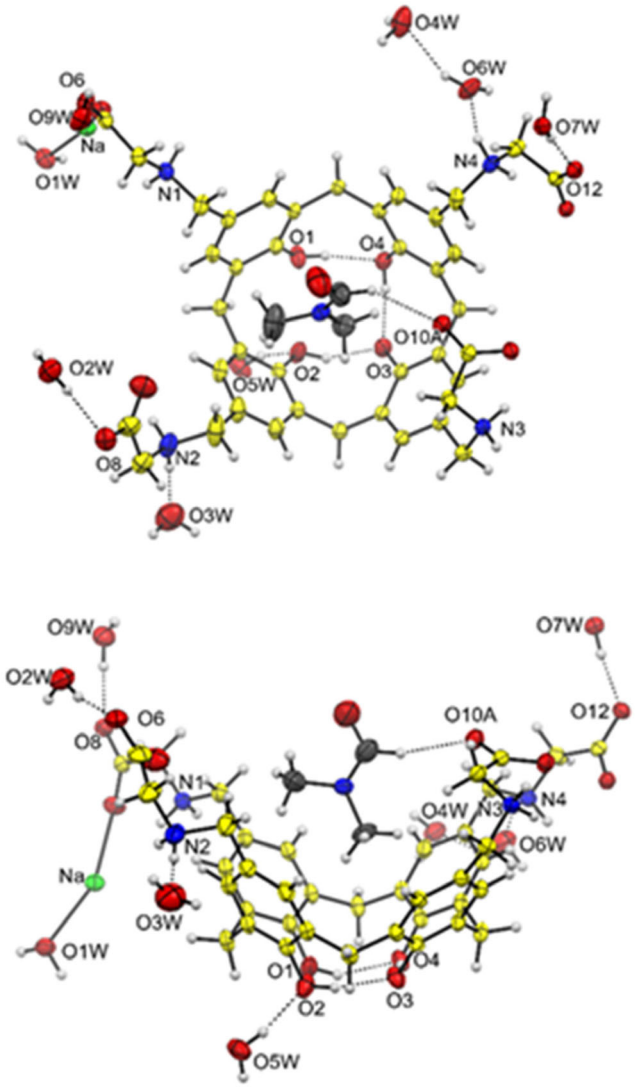


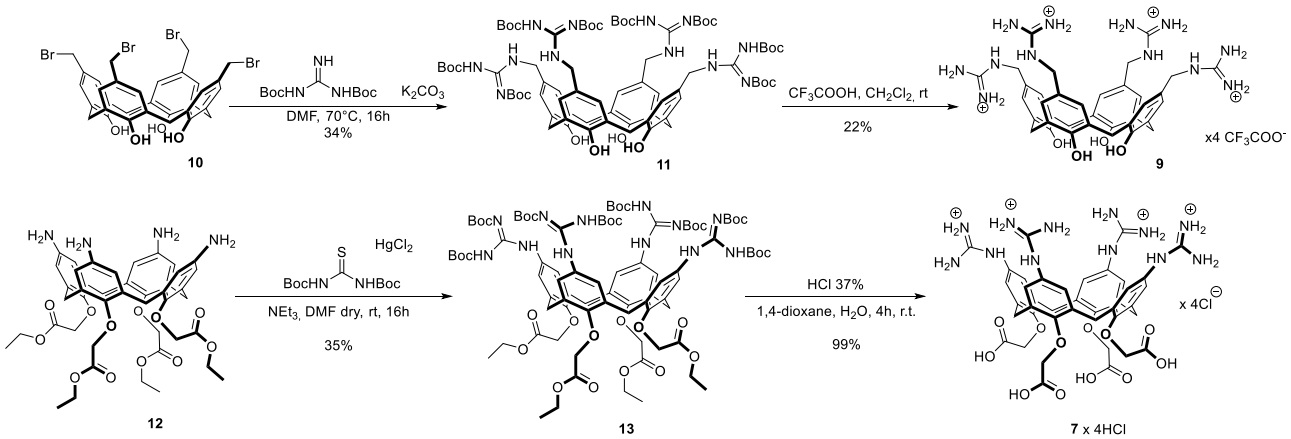
Figure 2. Two different views of the asymmetric unit of **2** with thermal ellipsoids depicted at the 30% probability level. The disordered fragments and some water molecules of crystallization were omitted for clarity. Apical (top) and lateral views (bottom).

crystallization. The upper rim of the calixarene exhibits four zwitterionic α amino acid residues, and three of the four carboxylate groups are directly bound to symmetry-related sodium atoms. The lower rim of the calixarene shows three protonated phenol rings (O1, O2, and O4) and a deprotonated one (O3), which acts as hydrogen bond acceptor toward O2 and O4 (O2···O3, 2.56 Å; O4···O3, 2.58 Å). The disordered dimethylformamide molecule is located inside the cavity of the calixarene with the methyl groups pointing toward the inner cavity and the oxygen atom toward the exterior. Several water molecules of crystallization are involved in hydrogen bonds with the protonated amino groups, the carboxylate residues, and the oxygen atoms of the lower rim of the calixarene.

In parallel to zwitterionic aminoacidic calixarenes **1**–**6**, we also synthesized, as control, two water-soluble calix[4]arenes (**8** and **9**) bearing hydroxy groups at the lower rim and anionic or cationic groups at the upper rim, respectively. The *p*-tetramethylensulfonatocalix[4]arene **8**[46] and the *p*-tetramethyleneguanidinium calix[4]arene **9** bear, at the upper rim, four permanent anionic charges and four permanent cationic charges, respectively. Sulfonato- and guanidinocalixarenes have been widely used in calixarene chemistry for the recognition of different classes of large biomolecules such as protein, glycosaminoglycans, and nucleic acids and could potentially be active also toward molecular patterns present on the bacterial cell walls. The tetramethyleneguanidiniumcalix[4]arene **9** has never been synthesized, since all of the *p*-guanidiniumcalixarenes used so far, mainly devised as gene delivery systems,[50–52] needed to have lipophilic groups at the lower rim to obtain amphiphilic molecules simultaneously able to complex nucleic acids through the ammonium groups and to maintain a sufficient lipophilicity to allow the lipoplexes to penetrate the cell membranes. Compound **9** was synthesized by bromomethylation of the *p*-Hcalix[4]arene to obtain compound **10**,[53] followed by reaction of the latter with bis-Boc-guanidine ((bis(N-(tert-butoxycarbonyl))guanidine)) to obtain the protected compound **11** which was then deprotected by using trifluoroacetic acid (TFA)- in dichloromethane (**Scheme 2**). Finally, we also synthesized, as a further control, the zwitterionic calix[4]arene ligand **7** (Scheme 2) having the opposite charges distant from each other, i.e., positive charges at the upper and negative charges at the lower rim. The tetramino-tetraethylester of calix[4]arene (**12**)[54] was guanidinylated to **13** by reaction with bis-Boc-thiourea and $HgCl_2$. The treatment of compound **13** with conc. HCl simultaneously cleaves the Boc protecting groups at the upper rim and hydrolyzes the esters at the lower rim. Contrary to all the other ligands **1**–**6** having free phenolic OH groups at the lower rim, compound **7** presents a fixed cone structure due to the bulky $-CH_2COO^-$ groups at the lower rim. In this case, the $ArCH_2Ar$ methylene groups give rise to a sharp AX system in the ^{1}H NMR spectrum, as supported by their signals at 4.75 and 3.45 ppm, and to a signal at 30.5 ppm in the ^{13}C NMR spectrum (Figure S33 and 34, Supporting Information).[49] Figures of the ^{1}H-NMR, ^{13}C-NMR, and MS spectra of all the newly synthesized calixarene ligands **1a**,**b**, **2**–**7**, and **9** and intermediates **11**, **13**, and **15** are reported in Figure S6–S47, Supporting Information.

2.2. Binding Studies by STD NMR Experiments

The ability of compounds **1**–**9** to recognize bacterial surfaces was investigated by NMR spectroscopy ligand–receptor interaction studies.[39] The bacterial envelope has a heterogeneous and highly variable composition that primarily reflects the class the microorganism belongs to (Gram-positives, Gram-negatives, and mycobacteria).[55] Their multicomponent composition led us to investigate the interaction of our putative ligands through a methodology allowing the use of entire bacterial cells presenting an intact external envelope. On-cell STD NMR, already used to study binding events involving the external surface of both bacterial[20,30] and mammalian[29,31–33] cells, was chosen for this purpose. Bacterial strains *Staphylococcus epidermidis*, *Pseudomonas putida*, and *Mycobacterium smegmatis* were selected as model organisms for Gram-positive, Gram-negative, and mycobacteria,



Scheme 2. Reaction scheme for the synthesis of **7** and **9**.

respectively, and compounds **1**–**9** were tested for the ability to bind each of them. Given the notable propensity of some bacterial strains to aggregate and precipitate too quickly for the event to be compatible with the acquisition of good quality in-solution STD NMR spectra, we exploited high-resolution magic angle spinning (HR-MAS) NMR.[33]

Cell integrity under HR-MAS experimental conditions, being the rotation at high spin rates imposed using the HR-MAS probe particularly demanding, was verified. To this aim, cell metabolite release was monitored overtime by ^{1}H NMR (Figure S51–S53, Supporting Information). Free metabolite concentration became significant, suggesting cell lysis, only after cells samples were rotated at 3 kHz and 37 °C, for at least 5 h (for the less resistant strain), indicating that experimental conditions are compatible with the acquisition of STD spectra which lasts ≈40 min.

NMR samples for binding studies were prepared suspending about 8 mg dry weight bacterial cells (see Experimental Section in Supporting Information for details) in deuterated phosphate buffer saline (d-PBS) and adding the tested compounds dissolved in deuterated dimethyl sulfoxide (d_6-DMSO), to reach a final concentration of 2 mM for each molecule and 20% v/v d_6-DMSO. d_6-DMSO was required to solubilize the less hydrophilic molecules and therefore used for all the ligands for uniformity reason in the experimental setup. Despite this, we did not obtain spectra of sufficient quality for compound **6**, too insoluble even under these conditions. After the acquisition of each STD NMR experiment, the retention of bacteria structural integrity upon the addition of d_6-DMSO and tested molecules was verified by the careful inspection of the off-resonance spectra. Moreover, an additional ^{1}H NMR experiment was acquired after each STD NMR experiment. No evidence of bacteria lysis was detected, showing that neither d_6-DMSO nor the compounds under investigation altered the bacteria structural integrity in the time frames required for the acquisition of NMR binding experiments.

STD spectra were acquired using three different on-resonance saturation frequencies (5.5, 0.5, and −1.0 ppm), to maximize the possibilities of magnetization transfer from the diverse biomolecules constituting the envelope of the three bacteria classes. For each frequency, a blank STD experiment was performed in the presence of the tested compounds alone. For each compound and bacterial strain pair, a representative STD spectrum obtained under the experimental conditions affording the signals with the highest intensities (if any) is reported in Figure 3, 4, and 6 and S54–S56, Supporting Information.

The NMR-based screening of the zwitterionic amino acidic calixarenes **1**–**5** afforded the identification of compound **1**, bearing proline moieties at the upper rim, and compound **5**, having phenylalanine substituents, as good and selective ligand of Gram-negative bacteria envelope. **Figure 3** reports the STD NMR spectra of the two compounds acquired in the presence of *S. epidermidis* (Figure 3D), *P. putida* (Figure 3F), and *S. smegmatis* (Figure 3H). Resonances of both the compounds appear in STD NMR spectra only when **1** and **5** were in the presence of *P. putida* (Figure 3F) as unequivocal evidence of their interaction with this bacterium. As further confirmation, STD NMR experiments were also carried out in the presence of another Gram-negative model strain, *E. coli* (Figure 3J), that confirmed the ability of **1** and **5** to interact with this class of bacteria.

With the substitution of proline and phenylalanine with glycine residues (compound **2**), a drastic reduction in the intensity of the STD signals of the ligand, and therefore in the binding to *P. putida*, occurred (Figure S54, Supporting Information). Compound **2** does not even bind *S. epidermidis* and *S. smegmatis*. Moreover, interaction with *P. putida* of sarcosine (**3**) and valine (**4**) derivatives was also negligible (data not shown).

To try to identify the structural features of the calixarene ligands which impart the ability to selectively bind Gram-negative bacteria, we also checked other water-soluble charged calixarenes. Compound **7**, having ammonium and carboxylate groups at the two different rims, showed a different behavior. As can be seen in **Figure 4**, the ^{1}H spectrum of the corresponding mixtures clearly demonstrated the binding of **7** to *S. epidermidis* (Figure 4B) and *P. putida* (Figure 4C), as a significant broadening of the macrocycle signals, compared to the spectra of the compounds alone (Figure 4A), can be observed. This broadening, with the consequent dramatic decrease of signal intensities in spectra B and C (Figure 4B and 4C), also suggests a high affinity for both Gram-positive and Gram-negative envelope. Vice versa, the line shape of its signals in the presence of *S. smegmatis* (Figure 4D) was the same as the free compound (Figure 4A).

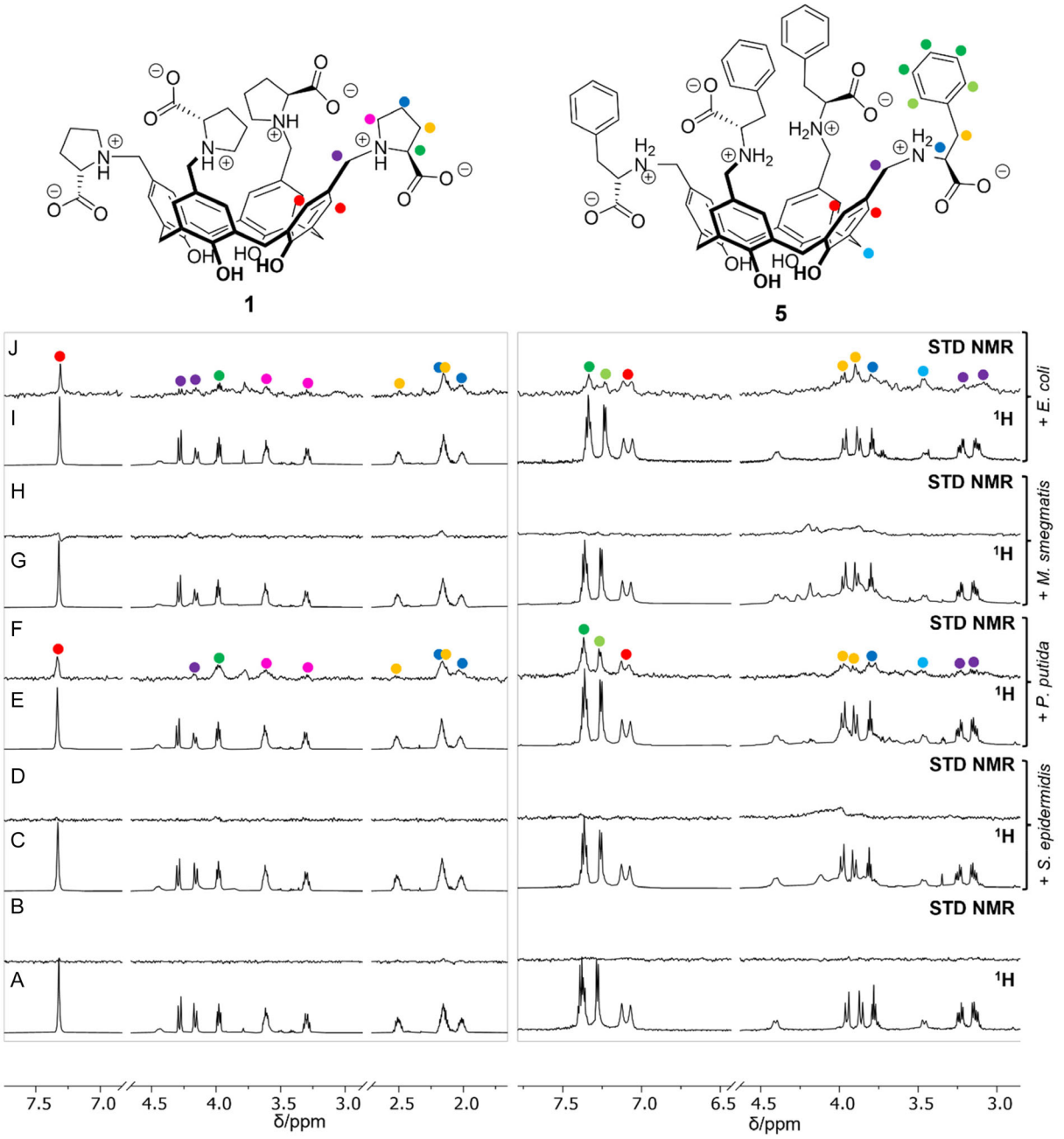


Figure 3. Representative STD NMR spectra acquired for compounds **1** and **5**. **Left column**: A) ^{1}H NMR and B) 1D STD NMR spectra of a sample containing compound **1** (2 mM), deuterated PBS, pH 7.4; C) ^{1}H NMR and D) 1D STD NMR spectra of a sample containing compound **1** (2 mM) and *S. epidermidis* cells (≈8 mg), deuterated PBS, pH 7.4; E) ^{1}H NMR and F) 1D STD NMR spectra of a sample containing compound **1** (2 mM) and *P. putida* cells (≈8 mg), deuterated PBS, pH 7.4; G) ^{1}H NMR and H) 1D STD NMR (H) spectra of a sample containing compound **1** (2 mM) and *M. smegmatis* cells (8 mg), deuterated PBS, pH 7.4; I) ^{1}H NMR and J) 1D STD NMR spectra of a sample containing compound **1** (2 mM) and *E. coli* cells (≈8 mg), deuterated PBS, pH 7.4. **Right column:** (A) ^{1}H NMR and (B) 1D STD NMR spectra of a sample containing compound **5** (2 mM), deuterated PBS, pH 7.4; (C) ^{1}H NMR and (D) 1D STD NMR spectra of a sample containing compound **5** (2 mM) and *S. epidermidis* cells (≈8 mg), deuterated PBS, pH 7.4; (E) ^{1}H NMR and (F) 1D STD NMR spectra of a sample containing compound **5** (2 mM) and *P. putida* cells (≈8 mg), deuterated PBS, pH 7.4; (G) ^{1}H NMR and (H) 1D STD NMR spectra of a sample containing compound **5** (2 mM) and *M. smegmatis* cells (≈8 mg), deuterated PBS, pH 7.4; (I) ^{1}H NMR and (J) 1D STD NMR spectra of a sample containing compound **5** (2 mM) and *E. coli* cells (≈8 mg), deuterated PBS, pH 7.4. ^{1}H spectra were acquired with 80 scans; STD NMR spectra were acquired with 256 scans and 3 s of saturation time, on-resonance frequency of 0.5 ppm, off-resonance frequency of 30 ppm; signals were enhanced 10 x. All spectra were recorded at 37 °C at 600 MHz.

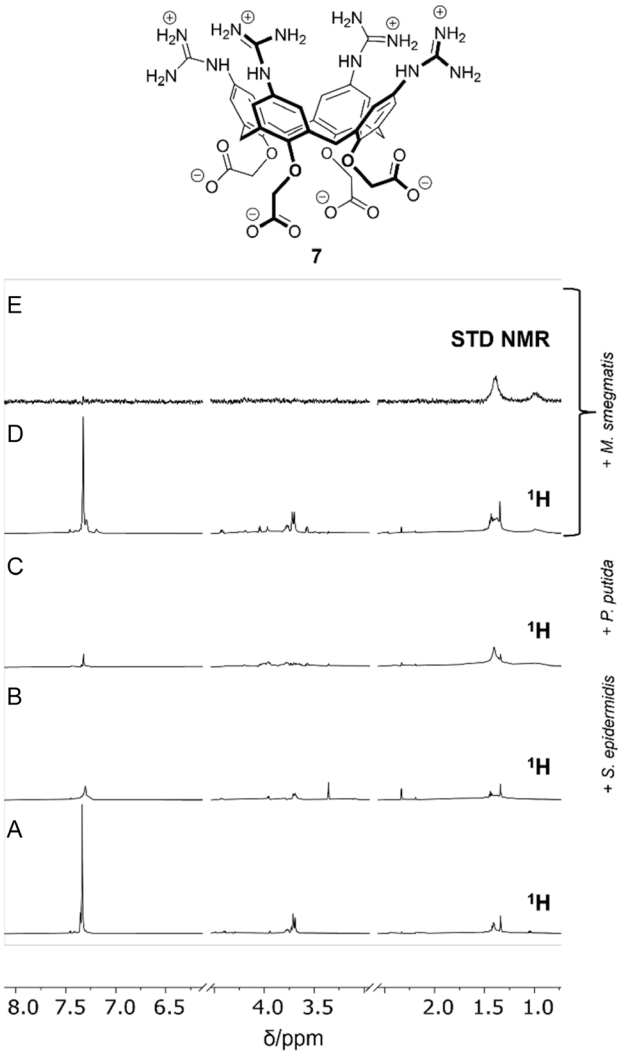


Figure 4. Representative ^{1}H and STD NMR spectra acquired for compound **7**. A) ^{1}H NMR spectrum of a sample containing compound **7** (2 mM), deuterated PBS, pH 7.4; 80 scans; B) ^{1}H spectrum of a sample containing compound **7** (2 mM) and *S. epidermidis* cells (≈8 mg), deuterated PBS, pH 7.4; 80 scans; C) ^{1}H spectrum of a sample containing compound **7** (2 mM) and *P. putida* cells (≈8 mg), deuterated PBS, pH 7.4; 80 scans; D) ^{1}H NMR and E) ^{1}D STD NMR spectra of a sample containing compound **7** (2 mM) and *M. smegmatis* cells (≈8 mg), deuterated PBS, pH 7.4. ^{1}H spectra were acquired with 80 scans; STD NMR spectra were acquired with 256 scans and 3 s of saturation time, on-resonance frequency of 0.5 ppm, off-resonance frequency of 30 ppm; signals were enhanced 10 x. All spectra were recorded at 37 °C at 600 MHz.

The STD NMR spectrum could therefore be acquired on the same sample (Figure 4E) assessing the lack of interaction with this model mycobacterium.

These results suggested that, although ligand **7** is zwitterionic like compounds **1** and **5**, the separation of charges between the upper rim and lower rim leads to the loss of the specificity for Gram-negative bacteria previously observed. A similar behavior was shown by the cationic compound **9** (data not shown). Collectively, data obtained for calixarenes **7** and **9** suggested that the presence of positive-charged groups at the upper rim allows the recognition of both Gram-positive and Gram-negative bacteria (but with the lack of the specificity shown by ligands **1** and **5**). In contrast, the anionic compound **8** did not bind to any of the bacterial strain models used in this study (Figure S55, Supporting Information), suggesting that the presence of only negative charges at the upper rim is not functional to bacteria recognition.

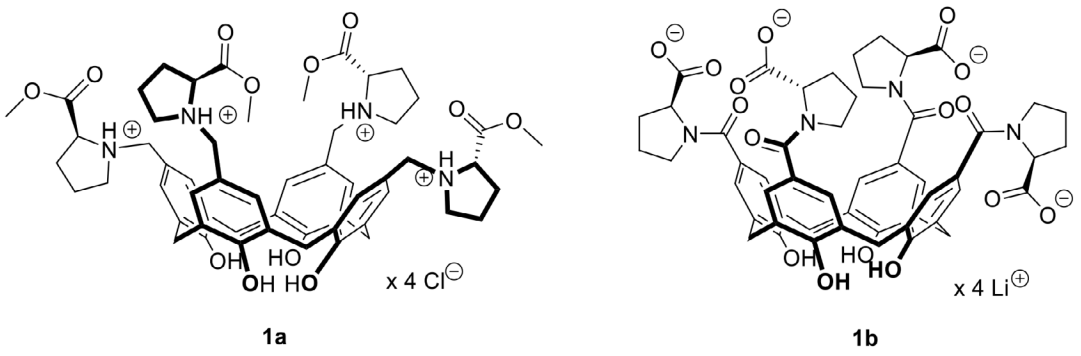
To have some hints of the groups most likely involved in recognition, we prepared two compounds sharing a structure similar to that of the tetraprolino **1** but having only protonable amino groups (the tetraprolino-tetramethyl ester **1a**) or only negatively charged carboxy units (the tetraprolinamide **1b**, tetralithium salt, **Figure 5**). The tetramethylester **1a** was simply obtained by esterification of **1** in methanol and $(COCl)_2$ (Figure S4, Supporting Information). In contrast, the tetraprolinamide **1b** was obtained by the reaction sequences depicted in Figure S5, Supporting Information, adapting the procedures used in literature for similar compounds.[56]

The interaction of compound **1a** and **1b** with *P. putida* was investigated by STD NMR (Figure S56 and S57, Supporting Information, respectively). In both he cases, the binding is quite negligible, suggesting that the zwitterionic structural motif, which in **1a** and **1b** is missing, is essential for the recognition process.

Being lipopolysaccharides (LPSs) a distinctive feature of Gram-negative bacteria outer membrane, we investigated their interaction with ligands **1** and **5**, showing a selective recognition of Gram-negative strains.

For this purpose, LPS produced by *P. putida* and *E. coli* were isolated, purified, and employed to carry out STD experiments in the presence of compounds **1** and **5**. Since *E. coli* MG1655 produced a R-type LPS (Lipooligosaccharide, LOS),[57] dry cells were treated according to PCP (90% phenol–chloroform–light petroleum ether (2:5:8 v/v/v)) extraction,[58] more suitable for LOS precipitation. During this procedure, cells are not lysed, allowing a more selective, even if often incomplete, isolation of LOS. In contrast, *P. putida* cells, producing both R- and S-type LPS,[59] were extracted according to the two subsequent procedures. The pellet resulting from the PCP extraction was treated with the hot phenol/water extraction protocol. This latter protocol is less selective and suitable for both R- and S-type LPS, as it causes cellular lysis (see Table S2, Supporting Information, for extraction yields and for further details). Figure S58, Supporting Information, clearly shows the isolation of an R-type LPS for *E. coli*, as only a low-molecular weight (MW) band is present in lane (b) of the sodium dodecyl sulfate - polyAcrylamide gel electrophoresis (SDS-PAGE) gel, while for *P. putida* both the extraction procedures afforded a mixture of R-type (low-MW bands) and S-type (high-BMW bands) LPS. Notably, more than one band appears around 30–50 kDa in lanes (a) and (c), suggesting the presence of *O*-antigens of different sizes, as typical of S-LPS. In fact, since the addition of *O*-antigen is not stoichiometric, the presence of molecules deriving from the addition of increasing repeating units of *O*-antigen is usually observed.

STD NMR experiments (**Figure 6**) provided clear evidence of compounds **1** and **5** binding to both *P. putida* (Figure 6B) and *E. coli* (Figure 6D) LPS, suggesting that the recognition of this component of the outer membrane of Gram-negative bacteria



1a 1b

Figure 5. Calix[4]arene-tetraprolino derivatives **1a** and **1b** used as control compounds.

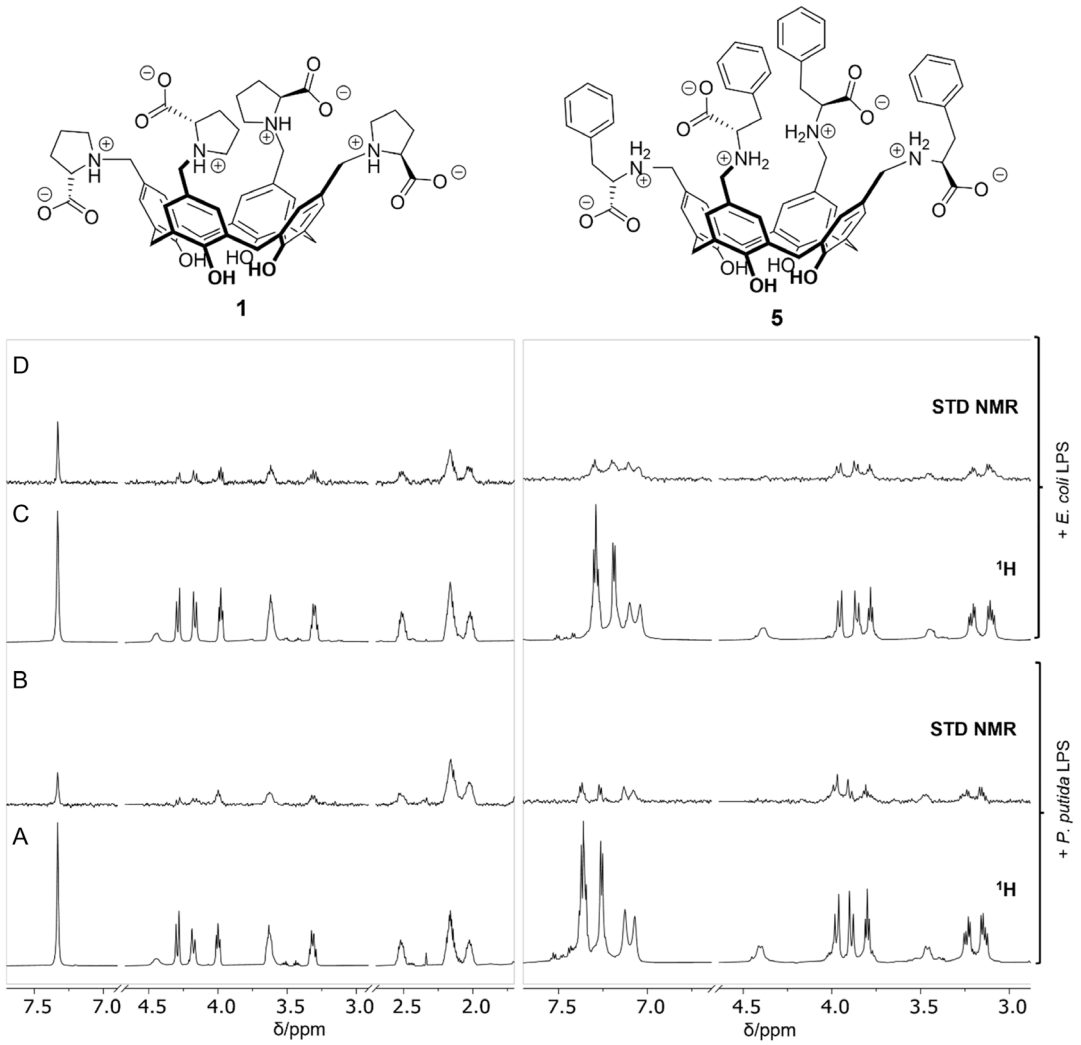


Figure 6. Representative STD NMR spectra acquired for compounds **1** and **5** in the presence of *P. putida* and *E. coli* LPSs. **Left column**: A) ^{1}H NMR and B) 1D STD NMR spectra of a sample containing compound **1** (2 mM) and *P. putida* LPS (0.4 mg mL^{-1}), deuterated PBS, pH 7.4; C) ^{1}H NMR and D) 1D STD NMR spectra of a sample containing compound **1** (2 mM) and *E. coli* LPS (0.4 mg mL^{-1}), deuterated PBS, pH 7.4. **Right column**: (A) ^{1}H NMR and (B) 1D STD NMR spectra of a sample containing compound **5** (2 mM) and *P. putida* LPS (0.4 mg ml^{-1}), deuterated PBS, pH 7.4; (C) ^{1}H NMR and (D) 1D STD NMR spectra of a sample containing compound **5** (2 mM) and *E. coli* LPS (0.4 mg mL^{-1}), deuterated PBS, pH 7.4. ^{1}H spectra were acquired with 80 scans; STD NMR spectra were acquired with 256 scans and 3 s of saturation time, on-resonance frequency of 0.5 ppm, off-resonance frequency of 30 ppm; signals were enhanced 10 x. All spectra were recorded at 37 °C at 600 MHz.

may play a pivotal role in the selective binding of **1** and **5** to bacteria envelopes.

Considering all the binding data collected and its greater water solubility (complete at a concentration of 10 mM), compound **1** is the best hit compound for the development of molecular probes for the rapid detection of Gram-negative pathogenic bacteria. Thus, its interaction with LPS was further investigated by recording STD spectra at different saturation times (Figure S59A, Supporting Information) to identify its binding epitope[27,30,60,61] that, in the case of *E. coli* LPS, is depicted in **Figure 7** and S59B, Supporting Information.

According to the STD relative intensities, both the proline ring and the calixarene aromatic moieties are pivotal for LPS recognition. Similar results were obtained for *P. putida* LPS.

Moreover, to simulate the recognition of LPS when bound to the outer membrane of Gram-negative bacteria, STD NMR experiments were repeated in the presence of d_{25}-SDS, a bacterial membrane[62] mimetic employed for NMR studies of membrane components. These experiments confirmed the ability of compound **1** to bind LPS when dispersed in SDS micelles (Figure S60, Supporting Information), suggesting that such recognition may also occur at the bacterial surface.

Finally, the K_D for compound **1** binding to *E. coli* LPS was determined applying the method reported by Angulo et al.[34] and already used by our group to obtain dissociation constants for different ligand/receptor pairs.[36,61] Briefly, a series of STD spectra was recorded at different **1**/LPS ratios. For each ligand concentration, STD amplification factor (STD-AF) values for Pro Hδ_b proton were obtained at different saturation times and fit by using the equation

$$\text{STD} - \text{AF}(t_{sat}) = \text{STD} - \text{AF}_{max}[1 - \exp(-k_{sat}t_{sat})] \quad (1)$$

where STD-AF is the saturation transfer difference amplification factor, t_{sat} is the saturation time, and k_{sat} is the saturation rate.

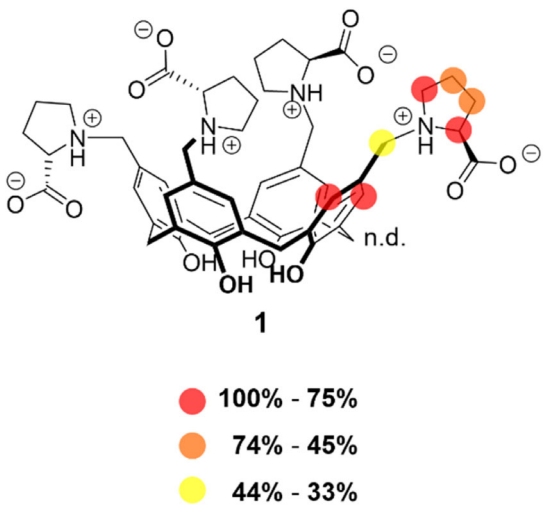


Figure 7. Ligand binding epitope of compound **1** to *E. coli* LPS as determined based on STD NMR spectra acquired with 0.75 s of saturation time, by normalizing the whole set of STD relative intensities against the highest value obtained. The results are shown in percentages. The relative STD effect of some protons was not determined (n.d.) due to peak overlapping or proximity to solvent resonance.

The initial slopes, STD–AF_0, calculated from STD–AF_0 = STD–$AF_{max}k_{sat}$, were then represented as a function of the ligand concentration, and the mathematical fit to a Langmuir isotherm ($y = B_{max}x/(K_D + x)$) afforded the K_D value of 12.74 ± 2.41 μM, at 25 °C. Details on the procedure and experimental data are reported in Supporting Information (Experimental Section and Figure S61, Supporting Information, respectively).

3. Conclusion

A general procedure for the insertion of α-amino acids at the upper-rim of lower-rim-unfunctionalized calix[4]arene by aminomethylation (Mannich reaction) has been devised, taking advantage of a previously reported synthesis of ligand **1**. The obtained ligands are present in the zwitterionic forms, as also stated by single-crystal X-ray diffraction studies on ligand **2**, and show a water solubility which depends on the hydrophilicity of the amino acid residues, from fair (**1**) to medium (**2–5**) to very low (**6**). Among the tested ligands, calix[4]arenes **1** and **5**, bearing L-proline and L-phenylalanine moieties, have a surprising and remarkable selectivity for Gram-negative bacteria (*P. putida* and *E. coli*) over Gram-positive bacteria (*S. epidermidis*) and mycobacteria (*M. smegmatis*), as determined by on-cell STD NMR studies driven under HR-MAS conditions. Comparative studies carried out using similar ionizable calix[4]arene ligands, synthesized on purpose as controls, allows to state that: 1) a calix[4]arene bearing only anionic sulfonate groups at the upper rim (**8**) is not able to bind any of the tested bacteria; 2) a calixarene bearing only ammonium groups at the upper rim (**9**) binds efficiently but unselectively both Gram-negative and Gram-positive bacteria (but not mycobacteria); 3) a zwitterionic calixarene (**7**) having ammonium and carboxylate groups separated onto the two different rims is also unselective; 4) when the zwitterionic motif of ligand **1** is removed either with loss of negative charges upon methylation of the carboxy groups (**1a**) or by blinding the basicity of the amino groups by transforming them into amide N-atoms, the binding to bacteria is quite negligible. All these data show not only that both carboxylate and ammonium groups are necessary for the selective recognition of Gram-negative bacteria but also that their relative disposition on the ligand structure is crucial. STD NMR experiments carried out on LPS aggregates, extracted from two different Gram-negative bacteria (*P. putida* and *E. coli*), nicely highlighted that these biomacromolecules are tightly bound to ligands **1** and **5**, suggesting that these can be the distinguished target elements present on the bacterial envelope responsible of their selective recognition. We determined the K_D for the interaction between ligand **1** and *E. coli* LPS achieving a value of about 13 μM, confirming its good affinity for this component of the Gram-negative bacteria envelope. Interestingly, binding is even observed when LPS is diluted in d_{25}-SDS micelles, in part mimicking the situation present on the Gram-negative bacteria envelope. The ligand binding epitope determination on ligand **1** allows to identify the α-CH and N-CH_2 groups of the amino acid, together with the ArH protons of the calixarene, as the groups more involved in the binding to LPS, suggesting that both electrostatic and dispersion forces might be involved in the recognition event.

In conclusion, this study discloses, for the first time, the possibility to use relatively simple, cheap, and versatile chemical systems for the fast and effective discrimination among different classes of bacteria, paving the way to the possibility of modifying the structure of these macrocycles to obtain sensor elements or selective drug-delivery systems to be used in the fight against AMR. Moreover, additional studies are in due course to understand if the upper rim zwitterionic motif, which endows the calixarenes with the ability to selectively bind Gram-negative bacteria, can also be transposed to other macrocycles or surfaces in the attempt to achieve other multivalent systems with similar or even improved discrimination ability.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Francesco Rispoli: data curation (equal); investigation (equal). **Luca Moretti**: data curation (equal); investigation (equal); methodology (equal). **Carlo Alberto Vezzoni**: investigation (supporting). **Eloisa Tosi**: investigation (supporting). **Linda Molteni**: data curation (supporting); investigation (supporting). **Carlotta Ciaramelli**: investigation (supporting). **Luciano Marchiò**: investigation (supporting). **Stefano Volpi**: investigation (supporting); methodology (supporting); supervision (supporting). **Laura Baldini**: investigation (supporting); methodology (supporting). **Francesco Sansone**: conceptualization (supporting); methodology (supporting); supervision (supporting). **Alessandro Palmioli**: investigation (supporting); methodology (supporting); supervision (supporting). **Cristina Airoldi**: conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); writing—original draft (equal); writing—review & editing (equal). **Alessandro Casnati**: conceptualization (equal); funding acquisition (equal); methodology (equal); supervision (equal); writing—original draft (equal). **Francesco Rispoli** and **Luca Moretti** contributed equally to this work.

Data Availability Statement

Research data are not shared.

Keywords

antimicrobial resistances, gram-negative bacteria, lipopolysaccharides, saturation transfer difference nuclear magnetic resonance, supramolecular chemistry

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