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Functional organisation and gain of activity: The case of the antibacterial tetra-*para*-guanidinoethyl-calix[4]arene

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Abstract—The antibacterial activities of the *para*-guanidinoethylphenol and of its cyclic tetramer, the tetra-*para*-guanidinoethyl-calix[4]arene, have been evaluated on reference Gram-positive and Gram-negative bacteria. Antibiotic disk diffusion assays completed by micromethod technique were employed to determine if a synergistic effect could be expected from the spatial organisation of the monomer into its cyclic tetrameric analogue. Disk diffusion assays and microdilution experiments revealed better properties for the calixarene species, with a real and important gain of activity with regards to the monomer.

The dramatic situation of mankind in face of the resistance of pathogenic microorganisms to present antibiotics requires developing research dedicated to the discovery of new drugs in this field.[1] In this sense, we have recently described the synthesis of new potent molecular drug dispensers, based on a calixarene platform displaying at the lower rim penicillin or quinolone moieties attached via a labile bound.[2,3] At the same time, we have focused on the development of calixarene derivatives displaying an intrinsic antimicrobial activity. Among the various approaches that are under evaluation in our group, one is dedicated to polycationic calixarene-based guanidinium podands. As pharmaceutical antimicrobial agents, the guanidinium derivatives have been modestly investigated in recent years, and essentially reported in the form of patents: for example, antibacterial, antiviral or antitumoural activities are described.[4] Most of the studied compounds are poly-guanidinium species derived from the old antidiabetic and trypanocidal Synthalin A or B,[5] in which guanidiniums are generally attached at the ends or along alkyl or polymeric chains, that could be considered as flexible linear organising templates.

More rigid, the calixarene species[6] are oligomeric phenolic macrocycles that have demonstrated their excellent organisational behaviour for a multitude of active functionalities. Very few reports, essentially under the form of patents, have focused on their therapeutical properties; some of them, hydrophilic, have shown interesting activities against bacteria,[7] fungi, cancerous cells and viruses,[8] enveloped viruses,[9] but also against thrombotic[10] or fibrotic diseases.[11] In the mid-1950s, the calixarene derivative 'Macrocyclon,'[12] and more recently some parent structures,[13] were studied in the treatment of tuberculosis and other mycobacterioses. The building of designed calixarenic mimics of vancomycin has also been studied as antimicrobial agents.[14]

Based on the fact that most bacteria are negatively charged, and on the aforementioned organising behaviour, the introduction of positive charges on the calixarene core leads to a constrained oligomeric polycation. Its high organisation could lead, with regard to the monomeric analogue, to an interesting synergistic effect in ionic interactions with the surface of bacteria, resulting in an antibacterial behaviour.

Among the rare organic cations available, the guanidinium was first chosen for its stability in a large range of pH values. A few calixarene guanidinium derivatives have been studied so far,[15] and some biological studies related to plasmid DNA binding and cytotoxicity evaluation have been reported by Ungaro and co-workers.[15a]

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To evaluate the potent synergistic effect mentioned above, we present here our preliminary results concerning the tetra-*para*-guanidino-ethyl calix[4] arene **3** and its monomer **4**. Compound **3** was synthesized according to Scheme 1; the process involved the addition of di-Boc-triflylguanidine to the tetra-ethylamino species **1**,[16] according to Baker et al.[17] The octa-Boc species **2** was finally treated with trifluoroacetic acid to give the tetra-guanidinium salt **3**. The overall yield was 52%. ^{1}H, ^{13}C NMR, elemental analyses and mass spectrometry were consistent with the proposed formulas for the new compounds **2** and **3**.[18] According to de Mendoza and co-workers,[19] ^{13}C NMR experiment carried out in D_2O suggested that **3** is in the cone conformation, with an Ar–CH_2–Ar resonance signal at 30.96 ppm. Nevertheless, the corresponding protons appear as a singlet at 3.82 ppm, expressing a mobile cone conformation.

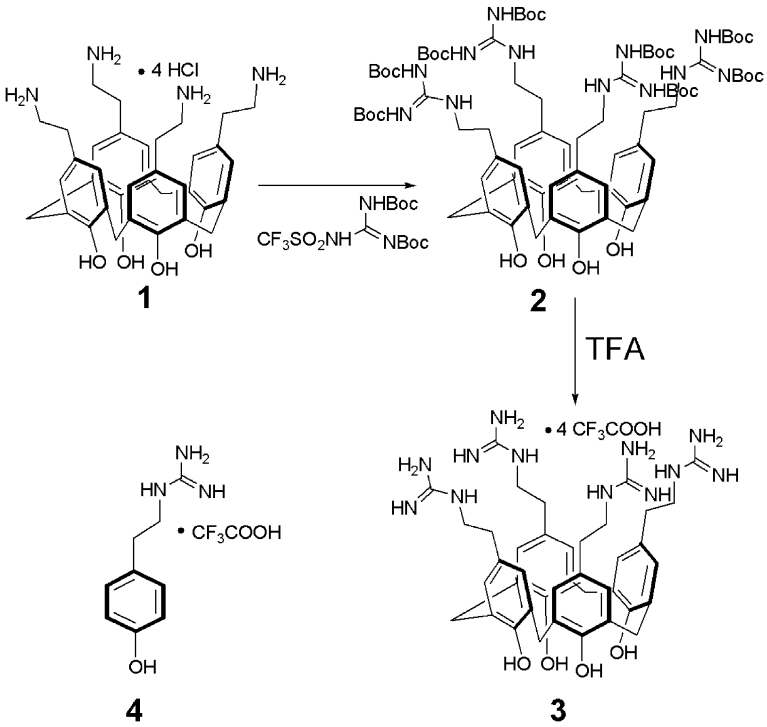
The *para*-guanidinoethylphenol **4**, monomer of **3**, has been evaluated as uptake inhibitor of prazosin by transport-P system,[20] for treating mitochondria-associated diseases,[21] non-insulin-dependent diabetes mellitus and obesity,[22] hypotension,[23] but no antibiotic activity has been described until now. **4** was prepared from tyramine, *N*,*N*-di(Boc)-*S*-methylisothiourea and $HgCl_2$.[24] This less expensive process was also employed for **3**, but resulted in the formation of multiple products not easy to separate, leading us to prefer the former procedure.

In the present study, microbiological tests were carried out with compounds **3** and **4** against various Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212) bacteria. Antibacterial activities were evaluated both in solid and liquid phases. For the former, antibiotic disk diffusion assays were performed on Mueller–Hinton agar with sterile 6 mm diameter disks impregnated with different quantities of compounds **3** and **4**. The Petri dishes were incubated at 37 °C, and the diameters of the zone inhibition were measured at 24 h of incubation, according to NCCLS and CA-SFM guidelines.[25] Controls made with reference antibiotics[26] were consistent with the values of the literature.[25] Results are given in Table 1 for compounds **3** and **4**. In order to compare **3** and **4** versus the number of guanidinium units, the quantities deposited on disks are given in mass, **3** being in this way considered as a simple tetramer of **4**.

According to Table 1, **3** displays a significant antibacterial activity, very interestingly both on Gram-positive and Gram-negative species, at different quantities, while **4** remains inactive, except for *E. coli* and *P. aeruginosa* at the highest deposit (256 μg). Comparing here the zone inhibition diameters showed that **3** is ca three times more potent than **4**.

Such solid-phase experiments being strongly dependent on drug diffusion process, and comparing here two compounds differentiated by a 1–4 mass ratio, we preferred to perform liquid-phase experiments that should provide a full contact between drug and cells.

Antibacterial activities of compounds **3** and **4** against *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis*, were thus carried out according to microdilution protocols performed in 96-well U-shaped microtitre plates described by NCCLS.[27] The minimum inhibitory concentration (MIC) of both compounds was determined against the four bacterial strains using this technique.



Scheme 1. Synthetic pathway to compound **3**, and representation of monomer *p*-guanidinoethylphenol **4**.

Table 1. Solid-phase antibacterial activities of compounds **3** and **4**

	3 270[a]	**4** 256	**3** 135	**4** 128	**3** 67	**4** 65	**3** 34	**4** 32
E. coli ATCC 25922	11.2 (±0.8)[b]	7.6 (±0.1)	9.3 (±0.6)	na	7.3 (±0.6)	na[c]	na	na
P. aeruginosa ATCC 27853	7.8 (±0.3)	na	na	na	na	na	na	na
S. aureus ATCC 25923	11.0 (±0.0)	7.8 (±0.3)	9.0 (±0.0)	na	8.0 (±0.0)	na	6.3 (±0.6)	na
E. faecalis ATCC 29212	10.0 (±0.0)	na	8.3 (±0.6)	na	6.6 (±0.6)	na	na	na

[a] Quantity (μg) deposited on a 6 mm diameter cellulose disk.
[b] Diameter of inhibition, values in millimeter, means of three experiments, standard deviation given in parentheses.
[c] na means no activity.

Table 2. Liquid-phase antibacterial activities and selectivity indexes of compounds **3** and **4**

	Minimum inhibitory concentration (MIC)				Selectivity index	
	3 in μg mL^{-1} (10^{-5} mol L^{-1})	**4** in μg mL^{-1} (10^{-5} mol L^{-1})	Mass ratio **4/3**[a]	Molar ratio **4/3**	Ratio IC_{50} 48 h/MIC **3**	Ratio IC_{50} 48 h/MIC **4**
E. coli ATCC 25922	16 (1.3)	512 (175)	32	135	43	0.48
S. aureus ATCC 25923	16 (1.3)	512 (175)	32	135	43	0.48
E. faecalis ATCC 29212	16 (1.3)	512 (175)	32	135	43	0.48
P. aeruginosa ATCC 27853	64 (5.1)	⩾512 (175)	8	34	11	⩽0.48

[a] Or molar ratio **4**/(**3** as 4 guanidiniums).

The results presented in Table 2 show that the calixarene species **3** is strongly active at 18 h since 16 μg mL^{-1} for *E. coli*, *S. aureus* or *E. faecalis*, and 64 μg mL^{-1} for *P. aeruginosa*, while the monomer **4** needs the higher concentrations of 512 μg mL^{-1} or more. Compound **3** appears thus incontestably more active than **4**, from 32 (mass ratio) and 135 (molar ratio) times for *E. coli*, *S. aureus* and *E. faecalis*, to 8 and 34 times, respectively, for *P. aeruginosa*.

In view of these interesting results, a necessary preliminary evaluation of selectivity indexes of **3** and **4** was done. This was performed via testing the viability effect of these molecules on eukaryotic cells using an MTT assay.[28]

MRC-5 cells (human embryonic lung fibroblasts) were plated on 96-well plates (10^4 cells/well) and different amounts of **3** and **4** were added. The relative number of metabolically active cells was assessed by reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). At 48 h, the observed viability IC_{50} values are 5.6×10^{-4} M for calixarene **3** and 8.5×10^{-4} M for monomer **4**. The resulting selectivity indexes IC_{50}/MIC were thus evaluated (Table 2) for **3** to 43 for *E. coli*, *S. aureus* or *E. faecalis*, and 11 for *P. aeruginosa*, while monomer **4** appears as toxic as active, with an index of 0.48.

In conclusion, the results obtained in this preliminary study show a remarkable gain of antibacterial properties, without decrease of eukaryotic cell viability, from the monomeric *para*-guanidinoethyl phenol **4** to its tetrameric cyclic isomer **3**. As one of the possible explanations, we can suspect, at this stage of the study, an important organisational role of the calixarene core of **3**, that tethers close together, and arrays at its upper rim four guanidinium groups, resulting in a synergistic effect in ionic interactions with the membrane targets. Improvement of these hypotheses requires the development of parent compounds and their non-conic conformers, as well as the linear oligomers, in order to obtain more pertinent structure–activity relationships. This is under current investigation.

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27. National Committee for Clinical Laboratory Standards. 2001. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; 5th ed. Approved standard. NCCLS document M7-A5. National Committee for Clinical Laboratory Standards. Wayne, PA, USA. Minimal inhibitory concentration was assessed by microdilution method which offers precise quantification of bacterial growth. Bacteria (e.g., *Staphylococcus aureus* ATCC 25923, *Enterococcus feacalis* ATTC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) were prepared in Mueller–Hinton broth (Difco, 275730) and inoculated in 96-well U-shaped microtitre plates. All wells were inoculated with bacteria to yield a final inoculum of 1×10^5 colony-forming units (CFU)/mL. Then various concentrations of the drugs were added. The cultures were grown for 18 h at 37 °C. The resulting bacterial growth was measured on Titertek Multiscan MCC/340 MK II apparatus (Labsystems, Helsinki, Finland) at a wavelength of 540 nm.
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