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FULL PAPER

Impact of tetracationic calix[4]arene conformation - from conic structure to expanded bolaform - on their antibacterial and antimycobacterial activities.

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Abstract: The four possible conformers of a new tetra-guanidino calix[4]arene thought to interact deleteriously with bacterial membrane were synthesized, characterized and evaluated for their in vitro cytotoxicity and antibacterial activity against various reference Gram negative and Gram positive bacteria as well as *M. tuberculosis*. It appears that the reversal of at least one phenolic unit allows a clear increase of the activities. This can be attributed to the evolution towards bolaform structures prone to interact deeper with the bacterial membrane. Indeed, the *1,3-alternate* conformer **16** exhibits the best antibacterial activity (MIC < 1.0 µg·mL^{-1} on *S. aureus*). Moreover, **16** displays very good antibacterial activities against isoniazid-resistant strain of *M. tuberculosis* (MIC = 1.2 µg·mL^{-1}), associated to the lowest cytotoxicity, making it the most potent compound of the series; this can open new ways of research in the field of anti-infective drugs development to face the huge current demand.

Introduction

The last thirty years have shown a growing interest for calixarene chemistry due to their excellent organizational behavior of various chemical functions that permits to address very diverse fields of application.[1] Even if they remain somewhat confidential, the applications of calixarenes in biology are constantly increasing in very different fields such as protein-surface recognition, cell transfection, DNA condensation….[2], as anti-infectives.[3] or as anti-infectives including intrinsically active species and prodrugs[3a], but also recent amphiphilic species cumulating both transfective and antibacterial properties.[3b]

Problems related to infectiology and bacterial resistance are now considered as a major public health concern,[4] bacterial resistance being for the WHO one of the "biggest threats to global health today".[5] Facing a possible therapeutic impasse due to the lake of new anti-infective agents, leads to turn to the search of new anti-infective compounds, aiming to cope with resistance mechanisms developed by bacteria, regarding the active compounds commonly used, or to try to face lake of new anti-infectious agents possessing an innovative mechanism of action. In this sense, it has been shown that some calixarene derivatives could be potent and promising antibacterial agents.[3] In this context, our team is conducting, a program dedicated to the search for new antibacterial compounds, notably based on polycationic calixarenes. Our mechanistic hypothesis is based on deleterious interactions that could occur between the negatively charged surfaces of bacteria, and the constrained positive charges introduced on a spatial organizer such as a calixarene macrocyclic platform. Our first studies were extremely encouraging with the development, among others, of conic calixarene structures exhibiting four guanidinium functions on the upper rim.[6]

Owing to the aforementioned organizing behavior, the introduction of positive charges on the calix[4]arene core results in an excellent antibacterial agent contrary to its monomeric constitutive phenolic analogue.[6-9] Afterward we also developed polycationic compounds organized around a benzene core (mono-, bis-, tetra- and hexa-guanidinium) [10] or at the ends of alkyl chains (bis-guanidinium) that can be considered as flexible linear organizing templates.[11] Many of these structures showed excellent activities against both sensitive or resistant bacteria sometimes with Minimum Inhibitory Concentrations (MIC) < 1 µg/mL.[5-9]

In parallel, we started to explore the activity of these compounds on a particular bacteria: *Mycobacterium tuberculosis*, responsible of the airborne and contagious pulmonary tuberculosis. The latter is estimated by WHO as the cause in 2016 of 1.7 million deathes over *ca*. 10 million new cases; immunodeficient patients (i.e. HIV-positive patients) and emergence of strains (multi)-resistant to the few drugs currently employed, darken the board.[12]

With the hope to propose new molecules facing resistance (and multiresistance) of tubercular bacilli to the active substances of first intention (*i.e.* isoniazide (INH) and rifampicin) we chosen to investigate the antimycobacterial properties of some of our compounds. *De facto*, we found that our cationic derivatives exhibited very interesting antimycobacterial activities. (*i.e.* <1 µg·mL^{-1} for some of them on INH-resistant MYC5165 *M. tuberculosis* strain).[12-14]

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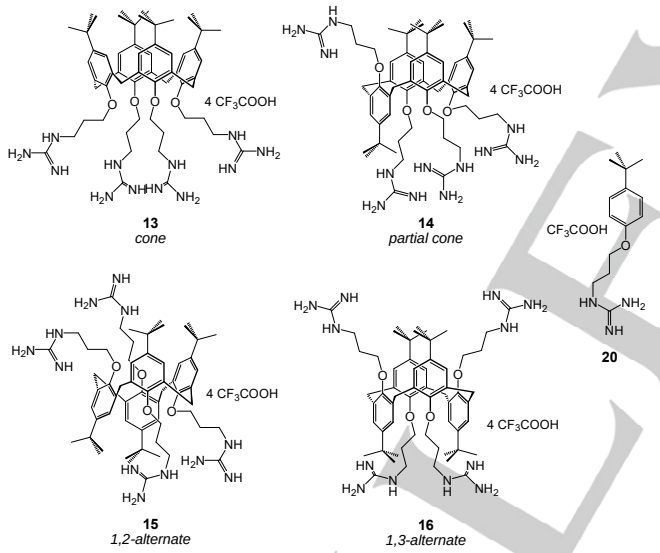
In the continuing search of new active compounds, we are trying to refine our data on the existence of any structure-activity relationships. In this sense, we present here the first study comparing the antibacterial activities of different conformers of *tetra*-(guanidinopropyloxy)-*tetra*-*tert*-butylcalix[4], *i.e.*: *cone* **13**, *partial cone* **14**, *1,2-alternate* **15** and *1,3-alternate* **16** presented in scheme 1 (respectively named: *cone*, *paco*, *1,2-alt* and 1,*3-alt*). Compared to our previous amphiphilic compounds where the cationic charges are tethered at the upper rim of the conic calix[4]arene *via* an ethylene linker, the guanidinium subunits are attached, in the case of the conic compound **13**, at the lower rim by means of longer propylene spacers. This difference in length and orientation of polarity could result in modifications of activities, but also could allow, by partial inversion of phenyl rings, the genesis of bolaamphiphiles displaying charges on both ends of the macrocycle. In the case of the *paco* **14**, *1,2-alternate* **15** and *1,3-alternate* **16**, an idealized stretched structure should approach a length of *ca.* 16-19 Å, near the thickness of the membrane lipidic bilayer (*ca.* 25-30 Å), prone to interactions of opposite cations with anions of both lipid layers.

As in our previous studies, we prepared for comparative biological studies, the formal monomeric building block of calixarenes, the *p*-tert-butyl-(guanidinopropyloxy)-benzene **20** (Scheme 1).



Scheme 1. Compounds of the study.

These five compounds were fully characterized and their *in vitro* antibacterial activities *i.e.* MIC against Gram positive and Gram negative reference bacteria and on *M. tuberculosis*, and cellular toxicity (IC_{50}) on non-cancerous human pulmonary embryonic fibroblasts (MRC-5 cell line) were assessed. They show, for most of them, very interesting antibacterial activities, combined to low- to modest- cytotoxicity. And we demonstrated that this is directly related to the conformation of platforms.

Results and Discussion

The cationic structures (especially guanidinium salts) extensively studied in our group have shown, for most of them, very interesting results in term of activity on a wide range of bacterial strains. The effect of active polycationic calix[4]arene was analyzed as the disruption of the bacterial membrane[15], associated to the modification of electrophoretic mobility[15] and increase of the membrane permeability.[16] Atomic Force Microscopy (AFM) investigations have enabled us to verify denaturation of the bacterial wall with loss of elasticity and holes formation in the outer membrane.[17] Combined to physicochemical investigations on various eukaryotic and prokariotic membrane models with Langmuir balance,[18] these first results have in fact validated our initial hypothesis, based on strong synergized electrostatic interactions between constrained positive charges of guanidinium and membrane anions representative of the global negative charge of bacterial surface. These results encouraged us to continue our research in this sense, *via* the introduction of hydrophobic groups on the cationic calixarenes, considering here a greater amphiphily in the aim to generate supplementary interactions with hydrophobic membrane constituents, in synergy with cations.

In this study, the *tetra*-*p*-tBu-calix[4]arene associated to the propyloxy arms will represent the lipophilic part. The cone conformer **13** should display an amphiphilic character, resulting in strong oriented and synergic electrostatic interactions of the tetracationic head with the anionic components of the bacteria surface, and orienting the additional functionalities externally or internally, with respect to the membrane structure, for further interactions. The declination in its various possible conformations (i.e. partial cone **14**, 1,2-alternate **15** and 1,3-alternate **16**) should allow us to control the amphiphilicity and open to bolaform structures by modulation of the ratio cation / *tert*-butyl on the calixarene rims. This should increase/strengthen the interactions with both parts of the lipid bilayer of the bacterial membrane and have a consequence on the antibacterial activity.

Synthesis

The general synthetic pathway leading to final guanidylated compounds **13**, **14**, **15**, **16** and **20** involved four steps from the commercially available calixarene **A** and *p*-tBu-phenol **C** (Scheme 2). For the synthesis of this family of conformers our starting point was based on a procedure adapted from Böhmer *et al.*[19] reporting the development of various *1,3-alternate* calix[4]arene selectively functionalized by amino groups. *Tetra*-*p*-tBu-calix[4]arene **A** was suspended in anhydrous THF in the presence of cesium carbonate. After addition of bromopropylphtalimide, the mixture was refluxed under Ar. After treatment, the crude material is, according to the authors, taken back one cycle of successive crystallization allowing each of them the isolation of one conformer (*1,3-alt*, *1,2-alt* and *paco*). A first crystallization in a CH_2Cl_2-MeOH mixture, afforded white crystals of *1,3-alternate* conformer **4**. If it is possible, from mother liquor, to obtain by crystallization the two other conformers, chromatographic purification (SiO_2, CH_2Cl_2) was found faster and more efficient. The three conformers *1,3-alt* (**4**), *1,2-alt* (**3**) and

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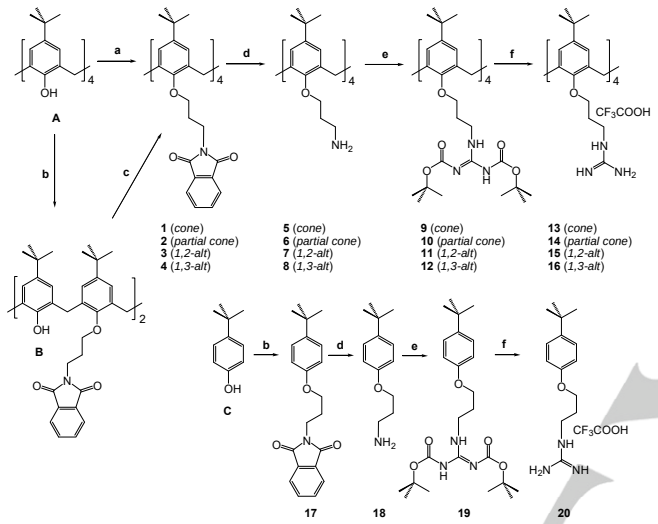
paco (**2**) were then obtained in the form of white crystalline powder with spectral analysis consistent with the literature data.[20] The cone conformer **1** was obtained in two steps with an overall yield of 60%, first by reaction of **A** with bromopropylphtalimide and K_2CO_3 in refluxing MeCN to give the diether **B** with a yield of 88%; **B** was then alkylated at the residual OH functions by reaction in toluene with bromopropylphtalimide using NaH as base, to give **1** with a yield of 70%. The formation of tetraethers can be achieved in one step from the native calixarene **A** with NaH in DMF [20-21], but the two steps process, as aforementioned, appears preferable according to our experience.



Scheme 2. Reagents and conditions: a) Bromopropylphtalimide, Cs_2CO_3, THF, Rflx, 7 days; b) Bromopropylphtalimide, K_2CO_3, CH_3CN, Rflx, 18h; c) Bromopropylphtalimide, NaH, toluene/CH_3CN, Rflx, 18h; d) H_2N-NH_2, H_2O, EtOH, Rflx, 6h; e) N_1,N_2-(di-Boc)-N_3-triflylguanidine, CH_2Cl_2/MeOH, RT, overnight; f) CH_2Cl_2, TFA, RT, overnight.

These four *tetra*-phtalimidopropyl conformers were then engaged in the phtalimide cleavage process, according to a standard procedure using hydrazine in refluxing ethanol. This afforded the *tetra*-aminopropyl derivatives, *cone*, *partial cone*, *1,2-alternate* and *1,3-alternate* conformers **5**, **6**, **7** and **8** respectively. The guanidine functions were generated by reaction of the latter with N_1,N_2-(di-Boc)-N_3-triflylguanidine according to our previous works.[8] The *octa*-boc derivatives **9**, **10**, **11** and **12** thus obtained were finally treated with trifluoroacetic acid to give the corresponding *tetra*-guanidinium salts **13**, **14**, **15** and **16** (*cone*, *partial cone*, *1,2-alternate* and *1,3-alternate* respectively). NMR analyses (^{1}H and ^{13}C) and mass spectrometry were consistent with the literature for **13** [19], and with the proposed formulas for the three new salts (Table 1). Elemental analyses were consistent with the presence of 2 or 3 water molecules. An equivalent protocol was used to obtain the monomeric derivative **20** from **C**.

NMR analysis for conformational determination

The NMR analyzes allow us to easily identify the different conformations of the derivatives **13** to **16**. For example, with ^{13}C resonance signals in the range around 28-32 ppm for the C-atom of the CH_2 bridge when the attached phenolic units are in the *syn*-orientation and in the range 37-40 ppm when they are in *anti*-orientation.

^{1}H-NMR and ^{13}C-NMR processed in D_6-DMSO were completed by 2D HSQC experiments in order to identify precisely the Ar-CH_2-Ar resonance signals. All NMR data enabling access to the different conformations are shown in Table 1. The shape of the resonance signal of the methylene bridges protons and of the aromatic protons also confirms the conformational structure. Thus the compound **13** presents a *cone* conformation with a ^{1}H-NMR Ar-CH_2-Ar resonance signal appearing as AX system around 3.15 and 4.24 ppm (4 H for each doublet) and as a ^{13}C-NMR singlet at 32.4 ppm. A singlet at 6.80 ppm integrating 8 protons for the aromatic hydrogens shows a *syn*-orientation of all phenols, and therefore an identical space environment for every Ar*H*. For compound **14**, the resonance signal corresponding to the methylene bridges is divided into an AX system at 3.04 and 3.99 ppm integrating 4 H, and a singlet at 3.64 ppm integrating 4 H, characteristic of at least one *anti*-orientation of two phenolic units. The ^{13}C-NMR signals at 30.20 (*syn*-orientation) and 37.67 (*anti*-orientation) confirms the *partial-cone* conformation. The presence of two singlets and two doublets for the aromatic H confirms also the inversion of one phenolic unit. Indeed, the 4 aromatic protons of phenolic units adjacent to the reversed cycle lose their equivalence in terms of chemical space environment. Thus there is formation of two doublets integrating 2 H, and resulting from a low ^{4}J coupling (2.1 Hz). We confirm the *1,2-alternate* conformation of the derivative **15** by the presence of an AX system (4 H) and a singlet (4 H) resonance signal for the methylene bridges protons, and two ^{13}C resonance signals at 28.6 and 37.8 (*syn*- and *anti*-orientation respectively). The disappearance of the singlets of Ar*H* to the benefit of two doublets integrating 4 H each, confirms the reversal of two adjacent cycles. Finally, the compound **16** exhibits a singlet resonance signal at 3.64 ppm for Ar-CH_2-Ar as well as a single signal at 37.3 ppm (*anti*-orientation) in ^{13}C-NMR, characteristic of the *1,3-alternate* conformation. The aromatic H, all equivalents, appear as one singlet at 6.99 ppm.

Table 1. Analytical data of the calixarene conformers **13** to **16**

	NMR					HR-MS	Elemental Analyses
	ArCH$_2$Ar			ArH		calcd for [M-4 TFA+H$^+$]$^+$ 1045.7437 *[M-4 TFA+2 H$^+$]$^{2+/2}$ 523.3755 found	(MW) g/mol
	^{1}H		^{13}C	^{1}H			
Compounds	δ (ppm)	*J* (Hz)	δ (ppm)	δ (ppm)	*J* (Hz)		
13 (*cone*)	3.15 (d, 4 H) 4.24 (d, 4 H)	12.4 12.4	32.46	6.80 (s)	-	*523.3762	$C_{68}H_{96}F_{12}N_{12}O_{12}$, 2 H_2O (1537.57)
14 (*paco*)	3.04 (d, 2 H) 3.99 (d, 2 H) 3.64 (s, 4 H)	12.5 12.4 -	30.20 37.64	6.56 (d, 2 H) 6.83 (d, 2 H) 7.13 (s, 2 H) 7.19 (s, 2 H)	2.1 2.1 - -	1045.7467 *523.3716	$C_{68}H_{96}F_{12}N_{12}O_{12}$, 2 H_2O (1537.57)
15 (*1,2-alt*)	3.14 (d, 2 H) 4.01 (d, 2 H) 3.89 (s, 4 H)	12.3 12.3 -	28.83 37.82	7.06 (d, 4 H) 7.18 (d, 4 H)	1.9 2.1	1045.7478 *523.3753	$C_{68}H_{96}F_{12}N_{12}O_{12}$, 3 H_2O (1555.59)
16 (*1,3-alt*)	3.64 (s, 8 H)	-	37.28	6,99 (s, 8 H)	-	1045.7456 *523.3745	$C_{68}H_{96}F_{12}N_{12}O_{12}$, 3 H_2O (1555.59)

Biological evaluations

All compounds were tested *in vitro* for their antibacterial activity (*i.e.* MIC determination) against various Gram positive (*E. faecalis* and *S. aureus*) and Gram negative (*E. coli* and *P. aeruginosa*) bacteria, as well as *M. tuberculosis*. In parallel, *in vitro* cellular toxicity (*i.e.* IC_{50} determination) was also evaluated on non-cancerous human pulmonary embryonic fibroblasts (MRC-5 cells), allowing us to access to the selectivity indexes (SI). All results of biological evaluations are presented in Tables 2-4.

The first two subsequent parts describe the results obtained for Gram positive and Gram negative bacterial strains and *M. tuberculosis*. As the cell wall structure/composition of *M. tuberculosis* is different from those of Gram positive and Gram negative bacteria, it appeared to us preferable to present them separately.

Finally, the last part confronts our results in order to establish a correlation between the antibacterial activity and the spatial organization of our compounds.

Part 1: Activities on reference Gram positive and Gram negative *bacteria*

The four new calixarenic coumpounds expressed overall very interesting activities on these five strains used in this study (Table 2). The amphiphilic derivative **13** exhibits the most lower activity of the series. Indeed, this cone conformer leaves appear MICs at 32, 64, and 128 µg·mL-1 on *E. coli*, *S. aureus* and *P. aeruginosa* respectively. On the other hand, the reversal of at least one phenolic unit allows a clear increase of the activities (factor 2 to 16 on all strains). The active concentration decreases for example for *partial-cone* conformer, to 2 or 8 µg·mL^{-1} on *S. aureus* and *P. aeruginosa*, against respectively 32 and 128 µg·mL^{-1} for compound **13**. The other conformers *1,2-alternate* and *1,3-alternate* (**15** and **16**) retain very good MICs, two times as active as **14**. The best activity is obtained for the *1,3-alternate* bolaamphiphile conformer **16** on *S. aureus* ATCC25923 with sub-micromolar MIC value. As for previous studies, [6,8] we compared these tetraguanidinium salts to the constitutive monomer **20** in order to verify, here also, the positive influence of macrocyclisation on the antibacterial activity. This monomeric species **20** has a modest activity on all reference strains, comprised between 32 and 128 µg·mL^{-1}. On one hand, these MIC values remain higher by a factor 8 to 32 with respect to those measured for calixarene analogues **14**, **15** and **16**. On the other hand, they are of same order than that of the *cone* conformer **13**, excepted for *E. faecalis* against which **13** is very active. Thus, a contrario to our previous studies, the monomer **20** is as active as its conic tetramer **13**. This equivalence of MIC values implies that there is no synergizing macrocyclic effect, except for *E. faecalis*, leading us to propose that the four guanidinium subunits of **13** are far enough one from each other to be considered as independent, thus moving closer to the monomer **20** in term of amphiphilicity.

The conic **13** is much less active than the three non-conic conformers **14**, **15** and **16**, excepted for *E. faecalis*. This differences should be due to the bolaform character of the latter, that spatially organize charges very differently than **13** and on a bigger length.

Cytotoxicity studies on eukaryotic MRC-5 cells (cell viability) of compounds **13**-**16** and **20** allowed the measurement of their 50% inhibitory concentration (IC_{50}) at 24, 48 and 168 h (7 days) presented in Table 3. These data enable us to determine, for each compound, a Selectivity Index (SI) *via* the ratio IC_{50} at 24h/MIC. Reported in Table 4, these SI values are used to identify compounds exhibiting the best potential, *i.e.* having a cytotoxicity away from the activity domain.

Table 2. MICs in $\mu g \cdot mL^{-1}$ (in µM) of guanidinium derivatives **13**, **14**, **15**, **16** and **20** against various bacteria of the study.

	Drugs	13 *cone*	14 *paco*	15 *1,2-Alt*	16 *1,3-Alt*	20
Reference Strains						
E. coli	ATCC 25922	64 (41.6)	8 (5.2)	8 (5.1)	**4 (2.6)**	64 (86)
P. aeruginosa	ATCC 27853	128 (83.2)	8 (5.2)	16 (10.3)	**16 (10.3)**	128 (172)
S. aureus	ATCC 25923	32 (20.8)	2 (1.3)	4 (2.6)	**< 1 (< 0.6)**	32 (43)
S. aureus	ATCC 29213	32 (20.8)	4 (2.6)	8 (5.1)	**8 (5.1)**	32 (43.0)
E. faecalis	ATCC 29212	8 (5.2)	4 (2.6)	8 (5.1)	**8 (5.1)**	128 (172)
Reference *M. tuberculosis*	$H_{37}Rv$	78 (50.7)	38 (24.7)	39 (25.1)	**5 (3.2)**	10 (13.4)
INH-resistant *M. tuberculosis*	MYC5165	9.5 (6.2)	2.4 (1.6)	3,8 (2.4)	**1,2 (0.8)**	37.2 (50)

Note: MIC INH: 0.16 and 20 $\mu g \cdot mL^{-1}$ against H34Rv and INH-resistant MYC5165, respectively.

The monomeric compound **20** appears as the most cytotoxic with an IC_{50} values of 6.1 $\mu g \cdot mL^{-1}$ at 24h, *i.e.* at concentration largely lower to MIC; its selectivity indexes calculated on all strains are close to zero. Regarding calixarene compounds, significant differences appear between the four conformers. The *partial cone* **14** and the *1,2-alternate* **15** are the most cytotoxic with IC_{50} at 24h of 68.9 and 57.6 $\mu g \cdot mL^{-1}$ respectively. Their good antibacterial activities confer them SI from 4 (for **15** against *P. aeruginosa*) to 35 (for **14** against S*. aureus* ATCC 25923). These two conformers thus have a variable interest depending on the strain but without presenting specific selectivity against Gram positive or Gram negative bacteria. The cone conformer **13** exhibits an IC_{50} at 140 $\mu g \cdot mL^{-1}$, about two times less cytotoxic than **14** and **15**. However, it still appears to be the less attractive of the conformer series owing to its higher active concentration, resulting in low SI from 1 to 4. Nevertheless,**13** displays, a selectivity against *E. faecalis* with a MIC of 8 $\mu g \cdot mL^{-1}$ resulting in a SI of 17. Faced to **13**, **14** and **15** the *1,3-alternate* **16** is found to be the less cytotoxic (IC_{50} > 256 $\mu g \cdot mL^{-1}$ at 24h) *i.e.* at concentration significantly away of activity domain. This allows to determine, for **16**, the best SI of the conformer series, from >16 against *P. aeruginosa* to >256 against *S. aureus* ATCC 25923. The conformer **16** can thus be considered as non-toxic while exhibiting an active concentration well below to the cytotoxicity domain.

Table 3. IC_{50} values in $\mu g \cdot mL^{-1}$ (in µM) obtained with viability assays (MTT) on MRC-5 cells.

Drugs	13	14	15	16	20
Time					
24h	139,3 (90.6)	69.8 (45.4)	57.6 (37.0)	**>256 (>164.6)**	6.1 (8.2)
48h	>256 (>166.5)	28.0 (18.2)	28.6 (18.4)	**201.1 (129.3)**	23.6 (31.7)
168h	>256 (>166.5)	9.7 (6.3)	7.9 (5.1)	**6.2 (4.0)**	1.5 (2.0)

Part 2: Activities on M. tuberculosis strains

These five cationic compounds were also evaluated on both isoniazid-sensitive ($H_{37}Rv$) and isoniazid-resistant (MYC5165) *Mycobacterium tuberculosis* strains. Their inhibitory activities are presented in Table 2.

Regarding the sensitive strain $H_{37}Rv$ on the one hand, clear differences appear between conformers of calixarene series. Effectively, the *1,3-alternate* **16** is, for example 16 times more active than the *cone* **13** (MIC at 5 $\mu g \cdot mL^{-1}$ for **16** against 78 $\mu g \cdot mL^{-1}$ for **13**). The *cone* conformer thus presents the weakest activity of the series against *M. tuberculosis*. Both *partial-cone* **14** and *1,2-alternate* **15** conformers exhibit a similar activity with MIC about 40 $\mu g \cdot mL^{-1}$, twice less than **13**. Surprisingly, the monomeric compound **20** exhibits a higher activity than the majority of the calixarenes of this series. Its MIC value of 10 $\mu g \cdot mL^{-1}$ makes it so about four to nine times more active than **14** (or **15**) and **13**. The SI on $H_{37}Rv$ calculated by IC_{50} 24h (table 3)/MIC are given in Table 4. Here again, their variations are very marked between the four spatial orientation adopted by the calixarenic skeleton. The compound that exhibit the best activities is the *1,3-alternate* **16** with an SI to about 50, 30 times greater than for conformers **13**, **14** and **15**. Indeed the *cone* conformer **13** although two times less toxic is likewise twice less active, bringing it *via* SI to the level of 1.*2-alternate* **15** and *partial-cone* **14** in terms of interest. The monomer **20**, active at 10 $\mu g \cdot mL^{-1}$ loses all its interest owing to its high toxicity measured at 6.1 $\mu g \cdot mL^{-1}$, resulting in a low SI at 0.7 on $H_{37}Rv$.

Our previous work has shown that our guanidinium compounds retained or improved their activities during the transition to INH-resistant strain. The results obtained on MYC5165 and presented in Table 2 show that the calixarene derivatives exhibit higher activities than against H37Rv. For example, MICs of 1.2 to 2.4 $\mu g \cdot mL^{-1}$ (for **16** and **14** respectively) confer to the INH-resistant strain a sensitivity 4 to 16 higher compared to H37Rv. The *cone* conformer **13** remains the less active of the series but, anyway, with a low MIC value of 9.5 $\mu g \cdot mL^{-1}$. The *1,3-alternate* **16** retains the forefront in term of activity. Eight times more active than **14**

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and **15** against $H_{37}Rv$ the factor decrease to 2 and 3 times respectively against MYC5165. **16** happens to be, moreover, 17 times more active than INH (1.2 and 20 $\mu g \cdot mL^{-1}$ respectively). With respect to the monomer **20,** it seems to lose its activity (MIC = 37.2 $\mu g \cdot mL^{-1}$). SI on MYC5164 presented in Table 4 show a moderate to high interest of these new calixarenes. Effectively, the 1*,3-alternate* derivative **16** exhibits an excellent SI of 213.
More modestly **13**, **14** and **15** (SI = 14.6, 29.0 and 15.1, respectively) seem, all the same, to be more interesting against this resistant strain.

Table 4. Selectivity indexes calculated (IC50 24h/MIC) for the five reference strains, reference H37Rv and INH-resistant MYC5165 *M. tuberculosis* strains, after 24 h exposure of MRC-5 cells to the compounds of the study.

	Drugs	**13** *cone*	**14** *paco*	**15** *1,2-Alt*	**16** *1,3-Alt*	**20**
Reference strains						
E. coli	ATCC 25922	2.2	8.7	7.2	**>64**	0.1
P. aeruginosa	ATCC 27853	1.1	8.7	3.6	**>16**	<0.1
S. aureus	ATCC 25923	4.4	34.9	14.4	**>256**	0.2
S. aureus	ATCC 29213	4.3	14.5	7.2	**>32**	0.2
E. faecalis	ATCC 29212	17.4	17.5	7.2	**>32**	<0.1
Reference *M. tuberculosis*	$H_{37}Rv$	1.8	1.8	1.5	**>51.5**	0.7
INH-resistant *M. tuberculosis*	MYC5165	14.6	29.0	15.1	**>213**	0.1

Part 3: Analysis of overall activity

Without trying to compare the Gram +/- bacteria with the tuberculosis bacillus, we can extract a general trend of these compounds, based on the relationship between their spatial geometry and overall activity that they demonstrate against the various microorganisms used in this study.
Whatever the strains studied, the trend is the same and compound **13** is the least active but is nevertheless low toxic. The reversal of at least one phenolic unit (*partial cone* **14** for example) generates a remarkable gain in activity, especially on reference strains (for example MIC from 64 to 8 μg/mL on *E. coli*). **14** and **15**, *partial cone* and *1,2-Alternate* respectively, exhibit a similar activity but also a cytotoxicity approximately 2.5 times greater than the cone conformer **13**. Across this study, the derivative **16** (*1,3-Alternate*) proves to be the most interesting with the best activity on all strains associated to the lowest cytotoxicity, as characterized by its high selectivity indexes.
These differences in behavior are probably due to the orientation of the different hydrophilic (guanidine part) and lipophilic (aromatic nuclei and propyl ether parts) regions, modifying the amphiphilic nature of the present calixarene species, from a normal amphiphilicity for **13** to a bolaamphiphilicity, partial for **14** and full for **15** and **16**. For these three last compounds, and particularly **15** and **16** which are very close in terms of global repartition of charges, the symmetry of the latter coupled to that of the lipophilic backbone may play a leading role face to the membrane symmetry, with regards to the efficiency.

Conclusions

The *cone*, *paco, 1,2-alternate* and *1,3-alternate* conformers of the tetra-guanidinopropyloxy-tetra-p-tBu-calix[4]arene, then their common constitutive monomer have been synthesized and fully characterized in view of evaluating the impact of their conformation on their anti-(myco)bacterial activities and on their cellular toxicities. In spite of possessing identical functionalities, the four calixarenes, owing to of their particular conformation, organize cationic and lipophilic subunits differently in space, that could generate some positive discriminations in biological activities. Evaluation of the latter, *i.e.* anti(myco)bacterial activity and cytotoxicity, show a significant variation of activity clearly related to the conformations. Except for *E. faecalis*, the amphiphilic cone conformer appears to have the lowest global antibacterial activity with Minimum Inhibitory Concentrations (MIC) above 32 μg/mL, associated to a low toxicity. The reversal of at least one phenolic unit of the calixarenic crown which generates bolaamphiphilic structures, results in a neat increase in antibacterial activity with a reduction of MIC to values below 10 μg/mL. Even if the latter are similar, the differentiation of these three bolaamphiphiles is done while evaluating the cytotoxicities, strongly to the benefit of the 1,3-alternate conformer which clearly stands out from this group. In addition, this derivative remains the most active against M. tuberculosis H37Rv with a MIC value similar to those observed for other bacteria, while the two other bolaforms become less active. Against the strain resistant to INH, MYC5165, the four conformers exhibit a high activity, with MIC < 10 μg/mL, *i.e.* < 6 μM; here also, the bolaamphiphiles are the most active, with the 1,3-alternate standing out.
This first study allows us to advance that the variation of the hydrophilic / hydrophobic balance of various functional groups grafted on the calixarene and their organization as bolaamphiphiles appears to modify the *in vitro* activity (bacterial inhibition and cellular toxicity). Moreover, the *1,3-alternate* conformer with highest symmetry, alternating guanidinium / p-tertbutyl groups on each rim of the calixarenic crown, seems to be the best compromise for a good activity and low toxicity. The target compounds, especially the latter, showed promising inhibition activity against seven tested strains, notably against *Mycobacterium tuberculosis* and particularly on INH-resistant strain.
On the assumption of a deleterious action of these cationic calixarene derivatives on the bacterial membrane integrity, the results obtained in this study show that the bolaamphiphilic polycationic calixarenes are interesting candidates for developing new antibacterial agents, notably for the 1,3-alternate conformer.

Deeper investigations related to the physico-chemical behavior of these derivatives, for example on model bacterial and eukaryotic membrane, will be useful to understand their possible mechanism of action.

Experimental Section

Chemistry

General Remarks:

Melting points (°C, uncorrected) were determined on an Electrothermal 9200 in Capillary apparatus. NMR spectra were recorded on a Bruker DRX 400 (operating at 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C nuclei) or DPX 250 (operating at 250 for ^{1}H nuclei and 62.9 MHz for ^{13}C nuclei). Chemical shifts are given in ppm relative to the solvent residual peak. The following abbreviations are used for multiplicity of NMR signals: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, br s = broad signal). High resolution mass spectrometry (HRMS) was carried out by electrospray ionization (ESI) on a Bruker MicrOTOFQ apparatus, at the Service Commun de Spectrométrie de Masse Organique, Nancy. Infrared measurements were performed on a Vector 22 Bruker FT apparatus (KBr, ν in cm-1) and UV spectra were recorded a SAFAS *UV mc²* apparatus, λmax in nm, ε in dm^3 mol^{-1} cm^{-1}. Elemental analyses were performed at the Service de Microanalyse, Nancy. Merck TLC plates were used for chromatography analysis (SiO_2, ref 1.05554; Al_2O_3, ref 1.05581) and compounds were visualized using UV light (254 nm). All commercially available products were used without further purification unless otherwise specified. All solvents were used as supplied without further purification.

Synthesis of *tetra*-[3-phtalimidopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *cone* (1): Step1: A suspension of *p*-tBu-calix[4]arene **A** (3.0 g, $4.62\cdot10^{-3}$ mol, 1 eq.) and K_2CO_3 (1.27 g, $9.26\cdot10^{-3}$ mol, 2.0 eq.) in CH_3CN (110 mL) was refluxed 30 min. under Ar before the addition of bromopropylphtalimide (2.78 g, $10.39\cdot10^{-3}$ mol, 2.25 eq.). After one night of reflux, the mixture was cooled then concentrated. The crude material was dissolved in CH_2Cl_2, filtered and organic phase was precipitated by adding an excess of MeOH. The precipitate was filtered, washed by MeOH and dried under vacuum. The expected bis-phtalimido-calix[4]arene **B** was obtained as a white powder (4.15 g, 88%). Step 2: a mixture of **B** (1.5 g, $1.46\cdot10^{-3}$ mol, 1 eq.) dissolved in toluene (30 mL) and NaH (0.1 g, $4.39\cdot10^{-3}$ mol, 3.0 eq.) was stirred at room temperature during 1h. A solution of bromopropylphtalimide (1.57 g, $5.86\cdot10^{-3}$ mol, 4 eq.) in CH_3CN (30 mL) was then added and the resulting mixture was refluxed overnight. After cooling and elimination of solvent under vacuum the crude material was dissolved in CH_2Cl_2 (40 mL) and washed by distilled water (40 mL). The organic phase was dried over Na_2SO_4 and filtered. The filtrate was then purified by chromatography (SiO_2, CH_2Cl_2/MeOH 99 : 1) to give **1** as a white powder (1.4 g, 70%). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.04 (s, 36 H, *tBu*), 2.40 (m, 8 H, OCH_2CH_2), 3.12 (d, *J* = 12.7 Hz, 4 H, $ArCH_2Ar$), 3.88 (t, *J* = 7.1 Hz, 8 H, CH_2N), 3.99 (t, *J* = 7.3 Hz, 8 H, OCH_2), 4.38 (d, *J* = 12.6 Hz, 4 H, $ArCH_2Ar$), 6.74 (s, 8 H, Ar*H*), 7.57-7.75 (m, 16 H, ArH_{phta}).

Synthesis of *tetra*-[3-phtalimidopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *partial-cone* 2, *1,2-alternate* 3 and *1,3-alternate* 4

According to the protocol described by Böhmer *et al.* [13]

***tetra*-[3-phtalimidopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *partial-cone* (2)**: ^{1}H-NMR (400 MHz, $CDCl_3$): 0.96 (s, 18 H, *tBu*), 1.27 (s, 9 H, *tBu*), 1.30 (s, 9 H, *tBu*), 1.88 (m, 2 H, OCH_2CH_2), 2.09 (m, 2 H, OCH_2CH_2), 2.23 (m, 4 H, OCH_2CH_2), 3.10 (d, *J* = 12.5 Hz, 2 H, $ArCH_2Ar$), 3.56-3.85 (m, 20 H, CH_2N, OCH_2, and $ArCH_2Ar$), 4.03 (d, *J* = 12.5 Hz, 2 H, $ArCH_2Ar$), 6.38 (d, *J* = 2.2 Hz, 2 H, Ar*H*), 6.80 (d, *J* = 2.4 Hz, 2 H, Ar*H*), 7.03 (s, 2 H, Ar*H*), 7.18 (s, 2 H, Ar*H*), 7.51-7.85 (m, 16 H, ArH_{phta}).

***tetra*-[3-phtalimidopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *1,2-alternate* (3)**: ^{1}H-NMR (400 MHz, $CDCl_3$): 1.05 (m, 4 H, OCH_2CH_2), 1.29 (s, 36 H, *tBu*), 1.63 (m, 4 H, OCH_2CH_2), 3.03 (d, *J* = 12.4 Hz, 2 H, $ArCH_2Ar$), 3.38-3.54 (m, 16 H, CH_2N, OCH_2), 3.90 (s, 4 H, $ArCH_2Ar$), 4.09 (d, *J* = 12.3 Hz, 2 H, $ArCH_2Ar$), 7.06 (d, *J* = 2.2 Hz, 4 H, Ar*H*), 7.11 (d, *J* = 2.4 Hz, 4 H, Ar*H*), 7.53-7.57 (m, 16 H, ArH_{phta}).

***tetra*-[3-phtalimidopropyloxy]-*tetra*-*tetra*-*p*-tBu-calix[4]arene *1,3-alternate* (4)**: ^{1}H-NMR (400 MHz, $CDCl_3$): 1.17 (s, 36 H, *tBu*), 1.68 (m, 8 H, OCH_2CH_2), 3.46 (t, *J* = 7.9 Hz, 8 H, CH_2N), 3.63 (t, *J* = 6.6 Hz, 8 H, OCH_2), 3.69 (s, 8 H, $ArCH_2Ar$), 6.95 (s, 8 H, Ar*H*), 7.68-7.83 (m, 16 H, ArH_{phta}).

General procedure for *tetra*-[3-aminopropyloxy]-*tetra*-*p*-tBu-calix[4]arene synthesis (5 to 8)

To a suspension of previous phtalimido derivative (**1** to **4**) in EtOH (*c.a.* 15mL/$0.2\cdot10^{-3}$ mol) hydrazine hydrate (5 mL/$0.2\cdot10^{-3}$ mol) was added. The solution was refluxed during 6h. After cooling, the resulting solution was concentrated under vacuum. The crude compound was dissolved in CH_2Cl_2 and washed with water. The organic phase was then dried over Na_2SO_4, filtered and evaporated to give the tetra-amino compound directly used without further purification.

According to the same protocol **5**, **6**, **7** and **8** were prepared and characterized.

***tetra*-[3-aminopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *cone* (5)**: From *cone* conformer **1**, slightly yellow solid, 84%. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.07 (s, 36 H, *tBu*), 2.17 (m, 8 H, OCH_2CH_2), 2.97 (t, *J* = 7.2 Hz, 8 H, CH_2N), 3.14 (4 H, d, 12.5 Hz, $ArCH_2Ar$), 3.91 (t, *J* = 7.1 Hz, 8 H, OCH_2), 4.34 (d, *J* = 12.4 Hz, 4 H, $ArCH_2Ar$), 6.79 (s, 8 H, Ar*H*).

***tetra*-[3-aminopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *partial-cone* (6)**: From *partial-cone* conformer **2**, white solid, quant. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.08 (s, 18 H, *tBu*), 1.16 (s, 9 H, *tBu*), 1.31 (s, 9 H, *tBu*), 1.77 (m, 2 H, OCH_2CH_2), 1.88 (m, 2 H, OCH_2CH_2), 2.22 (m, 4 H, OCH_2CH_2), 2.75-4.11 (m, 32 H, CH_2N, OCH_2, NH_2 and $ArCH_2Ar$), 6.82 (s, 4 H, Ar*H*), 6.96 (s, 2 H, Ar*H*), 7.11 (s, 2 H, Ar*H*).

***tetra*-[3-aminopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *1,2-alternate* (7)**: From *1,2-alternate* conformer **3**, white solid, 82%. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.08 (m, 4 H, OCH_2CH_2), 1.31 (s, 36 H, *tBu*), 1.39 (m, 4 H, OCH_2CH_2), 2.35-2.49 (m, 8 H, CH_2N), 3.17 (d, *J* = 12.4 Hz, 2 H, $ArCH_2Ar$), 3.50 (m, 8 H, OCH_2), 3.89 (s, 4 H, $ArCH_2Ar$), 4.16 (d, *J* = 12.3 Hz, 2 H, $ArCH_2Ar$), 7.03 (d, *J* = 2.4 Hz, 4 H, Ar*H*), 7.19 (d, *J* = 2.4 Hz, 4 H, Ar*H*).

***tetra*-[3-aminopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *1,3-alternate* (8)**: From *1,3-alternate* conformer **4**, white solid, 96%. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.24 (m, 8 H, OCH_2CH_2), 1.31 (s, 36 H, *tBu*), 2.54 (t, *J* = 7.0 Hz, 8 H, CH_2N), 3.49 (t, *J* = 7.3 Hz, 8 H, OCH_2), 3.84 (s, 8 H, $ArCH_2Ar$), 7.02 (s, 8 H, Ar*H*).

General procedure for *tetra*-[3-(N,N'-di-Boc)guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene synthesis (9 to 12)

A solution of *tetra*-[3-aminopropyloxy]-*tetra*-*p*-tBu-calix[4]arene (**5** to **8**) in a mixture of anhydrous CH_2Cl_2/MeOH (3:1, c.a. 40 mL/$0.3\cdot10^{-3}$ mol) was added by Et_3N (8 eq.) and N_1,N_2-(di-Boc)-N_3-triflylguanidine (4 eq.). After 24h at room temperature under argon the solvents were evaporated. The crude material was dissolved in CH_2Cl_2, washed with 2 M aqueous solution of $NaHSO_4$ then with 10% aqueous solution of K_2CO_3. The organic phase was then dried over Na_2SO_4, filtered and the filtrate was purified by chromatography.

***tetra*-[3-(N,N'-di-Boc)guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *cone* (9)**: From *cone* conformer **5**, white powder, 74% (Al_2O_3, CH_2Cl_2). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.07 (s, 36 H, *tBu*), 1.46 (s, 36 H, *tBu*), 1.47 (s, 36 H, *tBu*), 2.27 (m, 8 H, OCH_2CH_2), 3.14 (d, *J* = 12.7 Hz, 4 H, $ArCH_2Ar$), 3.58 (m, 8 H, CH_2N), 3.94 (t, *J* = 7.1 Hz, 8 H, OCH_2), 4.33 (d, *J* = 12.5 Hz, 4 H, $ArCH_2Ar$), 6.76 (s, 8 H, Ar*H*), 8.39 (m, 4 H, N*H*), 11.50 (s, 4 H, N*H*). These analyzes were consistent with that of the literature (Sansone et al., Organic Letters, 2008, 10, 3953-3956).

***tetra*-[3-(N,N'-di-Boc)guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *partial-cone* (10)**: From *partial-cone* conformer **6**, white powder, 79%, (SiO_2, CH_2Cl_2). ^{1}H-NMR (400 MHz, $CDCl_3$): 0.97 (s, 18 H, *tBu*), 1.24 (s, 9

H, *tBu*), 1.30 (s, 9 H, *tBu*), 1.41 (m, 72 H, *tBu*), 1.78 (m, 4 H, OCH_2CH_2), 2.04 (m, 4 H, OCH_2CH_2), 3.00 (d, *J* = 12.5 Hz, 2 H, $ArCH_2Ar$), 3.32-3.75 (m, 20 H, $ArCH_2Ar$, CH_2N et OCH_2), 3.97 (d, *J* = 12.5 Hz, 2 H, $ArCH_2Ar$), 6.56 (d, *J* = 2.3 Hz, 2 H, Ar*H*), 6.78 (d, *J* = 2.4 Hz, 2 H, Ar*H*), 6.99 (s, 2 H, ArH), 7.13 (s, 2 H, ArH), 8.14 (t, *J* = 4.8 Hz, 1 H, CH_2NH), 8.34 (t, *J* = 5.0 Hz, 2 H, CH_2NH), 8.38 (t, *J* = 4.9 Hz, 1 H, CH_2NH), 11.40 (br s, 4 H, N*H*).

***tetra*-[3-(N,N'-di-Boc)guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *1,2-alternate* (11)**: From *1,2-alternate* conformer **7**, white powder, 91%, (Al_2O_3, CH_2Cl_2/Hex 7:3). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.27 (m, 44 H, *tBu* and OCH_2CH_2), 1.46 (s, 36 H, *tBu*), 1.47 (s, 36 H, *tBu*), 3.14 (d, *J* = 12.5 Hz, 2 H, $ArCH_2Ar$), 3.22 (m, 8 H, CH_2N), 3.47 (m, 8 H, 8 OCH_2), 3.90 (s, 4 H, $ArCH_2Ar$), 4.09 (d, *J* = 12.3 Hz, 2 H, $ArCH_2Ar$), 7.08 (d, *J* = 2.3 Hz, 4 H, Ar*H*), 7.16 (d, *J* = 2.3 Hz, 4 H, Ar*H*), 8.18 (t, *J* = 4.9 Hz, 4 H, CH_2NH), 11.44 (s, 4 H, N*H*).

***tetra*-[3-(N,N'-di-Boc)guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *1,3-alternate* (12)**: From *1,3-alternate* conformer **8**, white powder, 72%, (SiO_2, CH_2Cl_2/Hex 8:2). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.24 (m, 44 H, *tBu* and OCH_2CH_2), 1.49 (s, 36 H, *tBu*), 1.5 (s, 36 H, *tBu*), 3.26 (m, 8 H, CH_2N or OCH_2), 3.43 (m, 8 H, CH_2N or OCH_2), 3.85 (s, 8 H, $ArCH_2Ar$), 7.04 (s, 8 H, Ar*H*), 8.34 (br s, 4 H, CH_2NH), 11.47 (br s, 4 H, N*H*).

General procedure for *tetra*-[3-guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *tetra*-trifluoroacetate synthesis (13 to 16)

A solution of *tetra*-[3-(N,N'-di-Boc)guanidinopropyloxy]- *tetra*-*p*-tBu-calix[4]arene (**9** to **12**) in a mixture of CH_2Cl_2 and TFA (80:20, *c.a.* 7mL/0.05·10^{-3} mol) was stirred at room temperature under Ar during 12 h. The solvent was evaporated under vacuum and the residual acid was eliminated by multiple dissolution/co-evaporation cycles in CH_2Cl_2 until formation of a solid. The latter was treated by trituration with dry Et_2O, until pH of the filtrate remained neutral. The crude material was dissolved in H_2O and lyophilized to give the desired tetra-trifluoroacetate salt.

All the designed *tetra*-[3-guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *tetra*-trifluoroacetate were synthesized using this general procedure and were fully characterized spectroscopically.

***tetra*-[3-guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *tetra*-trifluoroacetate *cone* (13)**: From *cone* conformer **9**, white solid, 88%, F°: 180-182°C; IR (ATR): 3352 (=NH), 3163 (-NH_3+), 2950 (-CH_2-), 1645 (NH_2); UV-Vis (H_2O): 286 (3884); ^{1}H-NMR (400 MHz, D_6-DMSO): 1.04 (s, 36 H, *tBu*), 2.14 (t, *J* = 7.1 Hz, 8 H, $OCH_2CH_2CH_2N$), 3.15 (d, *J* = 12.4 Hz, 4 H, $ArCH_2Ar$), 3.28 (m, 8 H, $OCH_2CH_2CH_2N$), 3.85 (t, *J* = 7.5 Hz, 8 H, $OCH_2CH_2CH_2N$), 4.24 (d, *J* = 12.4 Hz, 4 H, $ArCH_2Ar$), 6.81 (s, 8 H, Ar*H*), 7.22 (br s, 12 H, N*H*), 7.77 (t, *J* = 5.4, 4 H, N*H*); ^{13}C-NMR (100 MHz, D_6-DMSO): 31.0 ($OCH_2CH_2CH_2N$), 32.5 ($ArCH_2Ar$), 33.2 (CMe_3), 35.6 (CMe_3), 40.0 (CH_2N), 74.2 (OCH_2), 126.8 (C_m), 135.3 (C_o), 146.0 (C_p), 155.0 (C_{ipso}), 159.1 (C_{gua}), 161.6 (q, *J* = 32. Hz, CF_3COO^-); Anal. calcd for $C_{68}H_{96}F_{12}N_{12}O_{12}$, 2 H_2O (1537.57): C: 53.12; H: 6.56; N: 10.93; found: C: 53.05; H: 6.52; N: 11.04; HR-MS (ES+): calcd for $C_{60}H_{94}N_{12}O_4$ [M – 4 TFA + 2 H^+]$^{2+/2}$ 523.3755, found 523.3762.

***tetra*-[3-guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *tetra*-trifluoroacetate *partial-cone* (14)**: From *partial-cone* conformer **10**, white solid, 87%, F°: 132-134°C; IR (ATR): 3332 (=NH), 3165 (-NH_3+), 2958 (-CH_2-), 1660 (NH_2); UV-Vis (H_2O): 281 (3458); ^{1}H-NMR (400 MHz, D_6-DMSO): 0.99 (s, 18 H, *tBu*), 1.30 (s, 9 H, *tBu*) 1.35 (s, 9 H, *tBu*) 1.70 (m, 2 H, $OCH_2CH_2CH_2N$), 1.91 (m, 2 H, $OCH_2CH_2CH_2N$), 2.03 (m, 4 H, $OCH_2CH_2CH_2N$), 3.04 (d, *J* = 12.5 Hz, 4 H, $ArCH_2Ar$ and $OCH_2CH_2CH_2N$), 3.17-3.29 (m, 6 H, $OCH_2CH_2CH_2N$), 3.64 (s, 4 H, $ArCH_2Ar$), 3.50-3.58 (m, 8 H, $OCH_2CH_2CH_2N$), 3.96 (d, *J* = 12.3 Hz, 2 H, $ArCH_2Ar$), 6.56 (d, *J* = 2.3 Hz, 2 H, Ar*H*), 6.83 (d, *J* = 2.1 Hz, 2 H, Ar*H*), 7.14 (s, 2 H, Ar*H*), 7.20 (s, 2 H, Ar*H*), 7.22 (br s, 12 H, N*H*), 7.57 (t, *J* = 5.1 Hz, 1 H, N*H*), 7.71 (t, *J* = 5.5 Hz, 1 H, N*H*), 7.81 (t, *J* = 5.2 Hz, 2 H, N*H*); ^{13}C-NMR (100 MHz, D_6-DMSO): 28.1, 29.3, 29.8 ($OCH_2CH_2CH_2N$), 30.2 ($ArCH_2Ar$), 31.1, 31.4, 31.6 (CMe_3), 33.3, 33.6, 33.7 (CMe_3), 37.1, 37.8, 38.2 (CH_2N), 37.6 ($ArCH_2Ar$), 69.5, 70.9, 71.4 (OCH_2), 117.0 (q, *J* = 298.6 Hz, CF_3COO^-), 125.1, 125.8, 127.2 (C_m), 131.4, 131.7, 132.5, 135.1 (C_o), 142.4, 142.8, 144.7 (C_p), 153.0, 153.3, 154.9 (C_{ipso}), 156.9, 156.9, 157.0 (C_{gua}), 159.2 (q, *J* = 31.5 Hz, CF_3COO^-); Anal. calcd for $C_{68}H_{96}F_{12}N_{12}O_{12}$, 2 H_2O (1537.57): C: 53.12; H: 6.56; N: 10.93; found: C: 53.01; H: 6.64; N: 11.10; HR-MS (ES+): calcd for $C_{60}H_{93}N_{12}O_4$ [M – 4 TFA + H^+]$^+$ 1045.7437, found 1045.7367 ; calcd for $C_{60}H_{94}N_{12}O_4$ [M – 4 TFA + 2 H^+]$^{2+/2}$ 523.3755, found 523.3716.

***tetra*-[3-guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *tetra*-trifluoroacetate *1,2-alternate* (15)**: From *1,2-alternate* conformer **11**, white solid, 83%; F°: 204-206°C; IR (ATR): 3352 (=NH), 3167 (-NH_3+), 2960 (-CH_2-), 1666 (NH_2); UV-Vis (H_2O): 279 (4011); ^{1}H-NMR (400 MHz, D_6-DMSO): 1.00 (m, 4 H, $OCH_2CH_2CH_2N$), 1.26 (s, 36 H, *tBu*), 1.34 (m, 4 H, $OCH_2CH_2CH_2N$), 2.88 (m, *J* = 6.4 Hz, 4 H, $OCH_2CH_2CH_2N$), 2.94 (m, *J* = 6.6 Hz, 4 H, $OCH_2CH_2CH_2N$), 3.14 (d, *J* = 12.3 Hz, 2 H, $ArCH_2Ar$), 3.30 (m, 4 H, $OCH_2CH_2CH_2N$), 3.42 (m, 4 H, $OCH_2CH_2CH_2N$), 3.89 (s, 4 H, $ArCH_2Ar$), 4.01 (d, *J* = 12.3 Hz, 2 H, $ArCH_2Ar$), 7.06 (d, *J* = 1.9 Hz, 4 H, Ar*H*), 7.16 (br s, 12 H, N*H*), 7.18 (d, *J* = 2.1 Hz, 4 H, Ar*H*) 7.33 (br s, 4 H, CH_2NH); ^{13}C-NMR (100 MHz, D_6-DMSO): 28.1 ($OCH_2CH_2CH_2N$), 28.6 ($ArCH_2Ar$), 31.4 (CMe_3), 33.6 (CMe_3), 37.8 ($ArCH_2Ar$ and CH_2N), 69.7 (OCH_2), 117.0 (q, *J* = 298.6 Hz, CF_3COO^-), 125.1, 125.4 (C_m), 131.8, 133,21 (C_o), 143.6 (C_p), 153.3 (C_{ipso}), 156.9 (C_{gua}), 159.1 (q, *J* = 31.5 Hz, CF_3COO^-); Anal. calcd for $C_{68}H_{96}F_{12}N_{12}O_{12}$, 3 H_2O (1555.59): C: 52.50; H: 6.61; N: 10.80; found: C: 52.47; H: 6.55; N: 10.83; HR-MS (ES+): calcd for $C_{60}H_{93}N_{12}O_4$ [M – 4 TFA + H^+]$^+$ 1045.7437, found 1045.7478 ; calcd for $C_{60}H_{94}N_{12}O_4$ [M – 4 TFA + 2 H^+]$^{2+/2}$ 523.3755, found 523.3753.

***tetra*-[3-guanidinopropyloxy]-*tetra*-*p*-tBu-calix[4]arene *tetra*-trifluoroacetate *1,3-alternate* (16)**: From *1,3-alternate* conformer **12**, white solid, 92%; F°: 152-154°C; IR (ATR): 3352 (=NH), 3167 (-NH_3+), 2960 (-CH_2-), 1666 (NH_2);UV-Vis (H_2O): 273 (4659); ^{1}H-NMR (400 MHz, D_6-DMSO): 1.20 (s, 36 H, *tBu*), 1.71 (m, *J* = 7.0 Hz, 8 H, $OCH_2CH_2CH_2N$), 3.17 (m, *J* = 6.0 Hz, 8 H, $OCH_2CH_2CH_2N$), 3.49 (t, *J* = 7.8 Hz, 8 H, $OCH_2CH_2CH_2N$), 3.64 (s, 8 H, $ArCH_2Ar$), 6.98 (s, 8 H, Ar*H*), 7.23 (br s, 12 H, N*H*), 7.63 (t, *J* = 5.6 Hz, 4 H, CH_2NH); ^{13}C-NMR (100 MHz, D_6-DMSO): 29.0 ($OCH_2CH_2CH_2N$), 31.4 (CMe_3), 33.5 (CMe_3), 37.3 ($ArCH_2Ar$), 37.8 (CH_2N), 68.3 (OCH_2), 117.0 (q, *J* = 298.6 Hz, CF_3COO^-), 126.3 (C_m), 132.8 (C_o), 142.7 (C_p), 154.0 (C_{ipso}), 156.9 (C_{gua}), 159.1 (q, *J* = 31.5 Hz, CF_3COO^-); Anal. calcd for $C_{68}H_{96}F_{12}N_{12}O_{12}$, 3 H_2O (1555.59): C: 52.50; H: 6.61; N: 10.80; found: C: 52.39; H: 6.48; N: 10.97; HR-MS (ES+): calcd for $C_{60}H_{93}N_{12}O_4$ [M – 4 TFA + H^+]$^+$ 1045.7437, found 1045.56 ; calc for $C_{60}H_{94}N_{12}O_4$ [M – 4 TFA + 2 H^+]$^{2+/2}$ 523.3755, found 523.3762.

(3-Phtalimidopropyloxy)-*p*-tBu-benzene (17): A mixture of *p*-tertiobutylphenol **C** (2.0 g, 13.31·10^{-3} mol, 1 eq.) and K_2CO_3 (1.84 g, 13.31·10^{-3} mol, 1 eq.) in CH_3CN (50 mL) was added refluxed 1h before the addition of bromopropylphtalimide (3.56 g, 13.31·10^{-3} mol, 1 eq.). After one night of reflux, the mixture was cooled then concentrated. The crude material was dissolved in CH_2Cl_2 and the insoluble materials were filtered off. The concentrated filtrate was chromatographed (SiO_2, CH_2Cl_2) to give the desired compound **17** as a white powder (3.19 g, 72%); ^{1}H-NMR (400 MHz, $CDCl_3$): 1.28 (s, 9 H, *tBu*), 2.17 (t, *J* = 6.5 Hz, 2 H, $OCH_2CH_2CH_2N$), 3.90 (t, *J* = 6.9 Hz, 2 H, $OCH_2CH_2CH_2N$), 4,01 (t, *J* = 6.1 Hz, 8 H, $OCH_2CH_2CH_2N$), 6.76 (m, 2 H, Ar*H*), 7.25 (m, 2 H, Ar*H*), 7.70 (m, 2 H, Ar*H* phta), 7.85 (m, 2 H, Ar*H* phta).

(3-Aminopropyloxy)-*p*-tBu-benzene (18): To the solution of **17** (1.57 g, 4.67·10^{-3} mol, 1 eq.) in a mixture of EtOH/THF (30:30 mL), hydrazine hydrate (9 mL) was added. The solution was refluxed during the night. After cooling, the solvent was evaporated. The crude compound was dissolved in $CHCl_3$ and washed with water (20 mL). The organic phase was then dried over Na_2SO_4, filtered and evaporated to give a white solid (0.5 g, 53%). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.30 (s, 9 H, *tBu*), 1.91 (q, *J* = 6.3 Hz, 2 H, OCH_2CH_2), 2.90 (t, *J* = 6,6 Hz, 2 H, CH_2N), 4.00 (t, *J* = 6.0 Hz, 2 H, OCH_2), 6.84 (d, *J* = 11.6 Hz, 2 H, Ar*H*), 7.29 (d, *J* = 8.7 Hz, 2 H, Ar*H*).

(3-(N,N'-di-Boc)guanidinopropyloxy)-*p*-tBu-benzene (19): The amino derivative **18** (1.57 g, 4.67·10^{-3} mol, 1 eq.) was dissolved in 10 mL of anhydrous CH_2Cl_2 and 5 mL of MeOH, N_1,N_2-(di-Boc)-N_3-triflylguanidine (0.44 g, 1.13·10^{-3} mol, 12 eq.) was added and Et_3N (0.23 mL, 2.26·10^{-3} mol, 2 eq.). The solution stirred under Ar at room temperature overnight. After evaporation of the solvent the residue was solubilized in CH_2Cl_2 (20 mL) and washed with 2M aqueous solution of $NaHSO_4$ (10 mL), then by 10% aqueous solution of K_2CO_3 (10 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness. The resulting solid was chromatographed (Al_2O_3, Cyclohex/AcOEt 9:1) to give the desired compound **19** as a white solid (0.3 g, 75%); ^{1}H-NMR (400 MHz, $CDCl_3$): 1.29 (s, 9 H, *tBu*), 1.50 (m, 18 H, *tBu*), 1.91 (m, 2 H, OCH_2CH_2), 3.63 (m, 2 H, CH_2N), 4.04 (t, *J* = 5.7 Hz, 2 H, OCH_2), 6.88 (d, *J* = 8.7 Hz, 2 H, Ar*H*), 7.28 (d, *J* = 10.2 Hz, 2 H, Ar*H*), 8.64 (br s, 1 H, CH_2NH), 11.49 (s, 1 H, NH).

D3: A solution of **19** in a mixture of anhydrous CH_2Cl_2 (5 mL) and TFA (1 mL) was stirred at room temperature under Ar during the night. The solvent

was evaporated under vacuum and the residual acid was eliminated by multiple dissolution/co-evaporation cycles in CH_2Cl_2 until formation of an oil. The latter was treated by trituration with dry Et_2O, until pH of the filtrate remained neutral. The crude material was dissolved in H_2O and lyophilized to give the desired trifluoroacetate salt (0.08 g, 92%); F°: 126-128°C; IR (ATR): 3346 (=NH), 1646 (-NH_2); UV-Vis (H_2O) 260 (2336); ^{1}H-NMR (400 MHz, D_6-DMSO): 1.25 (s, 9 H, *tBu*), 1.91 (q, *J* = 6.5 Hz, 2 H, OCH_2CH_2), 3.28 (m, 2 H, CH_2N), 3.98 (t, *J* = 6.1 Hz, 2 H, OCH_2), 6.86 (d, *J* = 8.9 Hz, 2 H, Ar*H*), 7.13 (m, 4 H, N*H*), 7,29 (d, *J* = 8.8 Hz, 2 H, Ar*H*), 7.61 (t, J = 5.3 Hz, 1 H, CH_2NH); ^{13}C-NMR (60 MHz, D_6-DMSO): 28.92 (OCH_2CH_2), 32.03 (CMe_3), 34.45 (CMe_3), 38.60 (CH_2N), 65.32 (OCH_2), 114.66 (*Co* or C_m), 122.49 (q, *J* = 290.0 Hz, CF_3COOH), 126.74 (*Co* or C_m), 134.53 (C_p), 156.86, 157.69 (C_{gua} and C_{ipso}), 159.48 (q, *J* = 31.2 Hz, CF_3COOH); Anal. calcd for $C_{16}H_{24}F_3N_3O_3$, 0.5 H_2O (744.36): C: 51.61; H: 6.77; N: 11.28; found: C: 51.67; H: 6.70; N: 11.35; HR-MS (ES+): 250.1912 [M + H^+]$^+$.

Biology

References Bacterial strains and MIC determination

According to Clinical and Laboratory Standards Institute guidelines (CLSI)[22], and of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) [23] five reference bacterial strains were used in this study: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 & ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853. Strains were grown on Mueller-Hinton agar (Difco, 225250) or Mueller-Hinton broth (Difco, 275730) at 35°C. Purity of isolates was checked at the time of every test by examination of colony morphology and Gram staining.

MICs were determined using the broth microdilution method recommended by the CLSI [22], and as described previously.[6-9] Results are expressed as mean values of three independent determinations.

Inhibition of mycobacterial growth

The susceptibility of the two *M. tuberculosis* strains H37Rv and MYC5165 to all synthesized guanidino compounds was evaluated by determining the minimum inhibitory concentration (MIC) and the 50% inhibitory concentration (IC_{50}). We used a colorimetric microassay based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) to formazan by metabolically active cells.[24-27] Briefly, serial twofold dilutions of each drug were prepared in 7H9 broth (Middlebrook 7H9 broth base (Difco)) using 96-well microtiter plates and 100 mL of bacterial suspension in 7H9 broth were added to each well. After 6 days of incubation, MTT was added (50 μL, 1 mg/mL). After one day of incubation, solubilisation buffer was added to each well. The optical densities were measured at 570 nm. The MIC was determined as the lowest concentration of drug that inhibited bacterial growth (absorbance from untreated bacilli was taken as a control for growth). The IC_{50} were determined by using the Graph Pad Prism 5.0 software. The reported MICs are an average of at least three individual measurements.

Cell viability

MRC-5 cells (Non-cancerous human pulmonary embryonic fibroblasts) were obtained from BioMerieux (France). The cells were maintained in modified Eagle's medium (MEM, Invitrogen 41090) supplemented with 10% decomplemented fetal bovine serum (FBS, Invitrogen 10270, batch 40Q5150K) without antibiotics at 37°C, 5% CO_2, under a humid atmosphere. Cells were plated at 10^4 cells/well in 96-well plates (Sarstedt 831835). Forty-eight hours after plating, the growth medium was removed and replaced with the test solutions (100 μL). Viability tests were performed using a commercially available cell proliferation reagent MTT [3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma 1350380), as described previously.[28] The assay is based on cleavage of the tetrazolium salt MTT by active mitochondria to produce an insoluble purple formazan salt. As this conversion only occurs with viable cells, it directly correlates with cell count. After 24, 48 and 168 h of exposure, 10 μL of a 5 mg/mL MTT solution was added to each well and plates were incubated at 37°C for 4 h. Then, insoluble purple formazan was dissolved by adding 100 μL of SDS to each well. The absorbance A_{540} was measured with a reference wavelength of A_{690}, using an ELISA reader (Multiskan EX, Thermo Electron Corporation). The results were expressed as the per cent absorbance of treated versus untreated control cultures. Eight wells per dose and time point were counted in 3 different experiments.

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Keywords: calixarene • conformers • guanidinium • antibacterial • cytotoxicity

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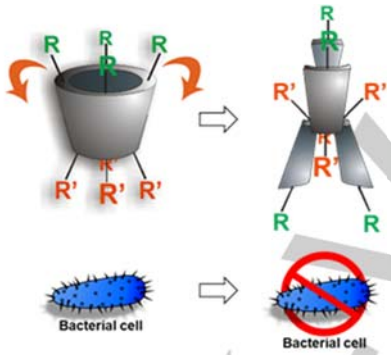

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Impact of tetracationic calix[4]arene conformation - from conic structure to expanded bolaform- on their antibacterial and antimycobacterial activities.