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Anti-mycobacterial activities of some cationic and anionic calix[4]arene derivatives

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ABSTRACT

Various polycharged calix[4]arenes were assayed as anti-mycobacterial agents against *Mycobacterium tuberculosis*, H_{37}Rv strain. The sulfonate, carboxylate and phosphonate anionic species displayed no activity. Cationic derivatives integrating four aminoethyl groups at the upper rim and two 6,6′-dimethyl-2,2′-bipyridyl- or 4,4′-dimethyl-2,2′-bithiazolyl subunits at the lower rim were also found inactive against *M. tuberculosis*, while the unsubstituted and the 5,5′-dimethyl-2,2′-bipyridyl–analogues exhibited MIC values of 3.2 and 0.8 μM respectively. Introduction of guanidinoethyl groups at the upper rim resulted, except for the 6,6′-dimethyl-2,2′-bipyridyl-derivative, in high anti-mycobacterial activities for the unsubstituted, the 5,5′-dimethyl-2,2′-bipyridyl– and the 4,4′-dimethyl-2,2′-bithiazolyl analogues, with MIC values of 0.8, 0.8 and 1.6 μM, respectively, similar to those of current commercial anti-tuberculosis agents. The five more active substances were also evaluated against the isoniazid-resistant strain MYC5165, resulting in highly interesting micromolar or sub-micromolar MIC and IC_{50}, ca. 4–125 times more active than isoniazid. These preliminary results are attractive for the development of new anti-TB agents.

1. Introduction

Data from WHO concerning tuberculosis (TB), lead to an estimation of 8.8 million new cases in 2010, with 1.4 million deaths (0.35 million HIV-positive people).[1] This airborne infectious disease that is preventable and curable is thus the cause of the largest number of human death due to a single etiologic agent. Approaches to fight TB are related to hygienic strategies, to vaccination and to drug development.[2] Some very recent reviews have focused on the past, present and future of anti-tuberculosis drugs.[3–5]

In the mid 50s, Cornforth et al. described a water-soluble synthetic non-ionic surface active agent able to suppress experimental tuberculosis in mice.[6] Apparently conceived as structurally related analogues of Triton A-20 or Triton WR-1339, a *para*-isooctylpolyoxyethylenephenol/formaldehyde polymer that was shown to be active over tubercle bacilli,[7] various derivatives were prepared by condensation of ethylene oxide under alkaline conditions with crystalline substances, HOC (High melting Octyl Compound) and HBC (High melting Butyl Compound). HOC and HBC were previously obtained from condensation of corresponding phenols and formaldehyde. More precisely, the condensation product of HOC with 45–50 equiv of ethylene oxide afforded a water-soluble compound, non-toxic and exhibiting anti-tuberculosis activity at higher level than streptomycin. This compound, named Macrocyclon, was initially thought to be a cyclic tetramer of corresponding phenol, the tetra-*para*-iso-octyl-calix[4]arene substituted at the lower rim by PEG 10.0–12.5 units.[8] Evidence of a calix[8]arene structure was given later by means of mass spectrometry and X-ray crystallography,[9] then, by NMR studies, evidence was made of a mixture of oligomers in which the calix[8]arene derivative is the major one.[10]

Since this period, Macrocyclon has been investigated as an anti-infective agent, some reports dealing with leprosy,[11] and tuberculosis.[12,13] More recently, Macrocyclon and pure *p*-tert-butylcalix[8]- and [4]arenes analogues were investigated again in vitro and in vivo in view of developing therapeutical alternatives against MDR-TB and some new derivatives displaying a controlled PEG-chain length have been reported.[14–16] At the very beginning of 2011, Hailes and co-workers reported the synthesis and in vivo anti-mycobacterial activities of new derivatives of Macrocyclon, incorporating notably at the upper rim *t*-Butyl-, phenyl- and sulfonate groups, the latter displaying promising properties.[17]

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In addition to the above-mentioned literature, and as recently reviewed by de Fatima et al.[18] Kalchenko and co-workers[19] and Perret and Coleman[20] very few reports, essentially under the form of patents, describe therapeutical activities of calixarenes and derivatives, mainly as anti-infectious, anti-cancerous, anti-thrombic or anti-fibrotic agents. Some of them, hydrophilic, have shown interesting activities against bacteria;[21,22] calixarene-based mimics of vancomycin,[23] and, during correction of this manuscript, hydrophobic calix[4]arene heterocyclic podands displaying antibacterial and anti-mycobacterial activities have also been described.[24] In this field, our contribution has been devoted to the design of new anti-HIV[25] and, with regards to spreading resistances of pathogenic microorganisms against actual antibiotics,[26] to new anti-bacterial agents.[27–33]

As part of a research programme dedicated to new families of anti-infectious agents, we have developed water-soluble calix[4]-arene derivatives that display constrained and highly organised ionic functions thought to improve water-solubility and to interact with the envelopes of viruses or the surface of bacteria.

Some anionic species, integrating phosphonate, sulfonate and carboxylate groups displayed anti-HIV activities.[25]

Among other positively charged calixarenes, the tetra-*para*-(guanidinoethyl)calix[4]arene **14** exhibited, contrary to its single phenol analogue, interesting anti-bacterial properties over Gram positive (*S. aureus*, *E. faecalis*) and Gram negative (*E. coli*, *P. aeruginosa*) reference bacterial strains,[27,28] susceptible and resistant strains,[29] and multi-resistant strains.[33] According to microelectrophoresis and Atomic Force Microscopy experiments,[30] reinforced by fluorescence studies,[32] an impact of this molecule on bacterial cell wall integrity seems to be confirmed at this time. Evaluation of its cell toxicity resulted in an interesting Selectivity Index.[31]

Other polycationic derivatives integrating at the lower rim N-containing biheterocyclic subunits that could interact with metal cations of biological importance,[34] have also been developed, and showed similar anti-bacterial activities, nevertheless coupled to a higher cell toxicity.[31]

In front of these results, and in accordance with the urgent need of new anti-tubercular drugs, we found obvious and exciting to evaluate the properties of these poly-ionic calixarene derivatives over *Mycobacterium tuberculosis*.

2. Chemistry

Most of the compounds studied here (scheme 1) arise from controlled multi-step synthetic processes and have been previously described: **1**[35,36]; **3**, **4, 6, 7, 9**[25]; **5**[37]; **10**, **11**, **12**, **13**, **15**, **16**, **17**[31]; **14**[27,31]

The bis-(β-bipyridyl) sulfonate derivative **2** (Scheme 2) was prepared by reaction of chlorosulfonic acid on the 25,27-bis (6-methyleneoxy-6′-methyl-2,2′-bipyridyl)-26,28-dihydroxycalix [4]arene **A**[38] in CH_2Cl_2, followed by hydrolysis of the chlorosulfonic groups by pyridine in acetone/water mixture. The resulting raw pyridinium salt was treated with aqueous NaOH under a stream of argon to remove the pyridine. The resulting sodium salt was purified by dialysis (Float-A-Lyser, cellulose acetate, MWCO 100 D) and lyophilisation to give **2** with a yield of 69%. 1D and 2D ^{1}H NMR analyses showed that **2** was a mixture of two conformers, involving four AB resonance signals of different spreading for the Ar–C*H*$_2$–Ar groups, and two strong AB systems for the OC*H*$_2$bpy. Integration values of the latter led us to evaluate the ratio of the two conformers to 3:2. HSQC experiment showed that ^{13}C resonance signals of Ar–CH_2–AR groups appeared as groups at ca. 30.6 and 37.0 ppm, confirming, according to de Mendoza and co-workers[39,40] the multi-conformational nature of **2**. The aromatic part appears as an overlapping of sharp resonance signals unsolvable by 2D techniques, except for two pyridyl *d-t-d* triads. We were unable to separate or to transform this mixture into the single cone conformer, as it was possible for the bis-bithiazolyl analogue.[25] Elemental analysis was consistent with the presence of 3.5 Na_2SO_4 and 5 H_2O molecules associated to the ligand. Electrospray mass spectrometry showed the presence of dicharged species issued from the loss of Na ions compensed by protonation, then from the loss of bipyridyl fragments.

The synthesis of compound **8** (Scheme 3) was previously described without procedures and full analyses;[41] the synthetic pathway, starting from tetra-*para*-chloromethylcalix[4]arene, was similar to the one given for its bithiazolyl analogue **9**.[25]

The anionic calix[4]arenes of the present study belong to the *p*-sulfonate, *p*-methylcarboxylate and *p*-methylphosphonate series; each group contains three derivatives, the first one without substituent at the lower rim (**1**, **4** and **7**, respectively), the second one incorporating two chelating α-bipyridyl (or 6-[methylene-6′-methyl-2,2′-bipyridine]-yl) groups in alternate position of the lower rim (**2**, **5** and **8**, respectively), the third one incorporating two 4-[methylene-4′-methyl-2,2′-bithiazole]-yl groups in alternate position of the lower rim (**3**, **6** and **9**, respectively).

The cationic calixarenes belong to the *p*-aminoethyl and *p*-guanidinoethyl series; as for anionic species, each group contains three derivatives, the first one without substituent at the lower rim (**10** and **14,** respectively), the two other ones incorporating two bi-heterocycles (α-bipyridyl, **11** and **15**, respectively; bithiazolyl, **12** and **16**, respectively). Two other derivatives have been introduced in this family, incorporating two β-bipyridyl (or 5-[methylene-5′-methyl-2,2′-bipyridine]-yl) pendant arms in alternate positions at the lower rim (**13** and **17**, respectively).

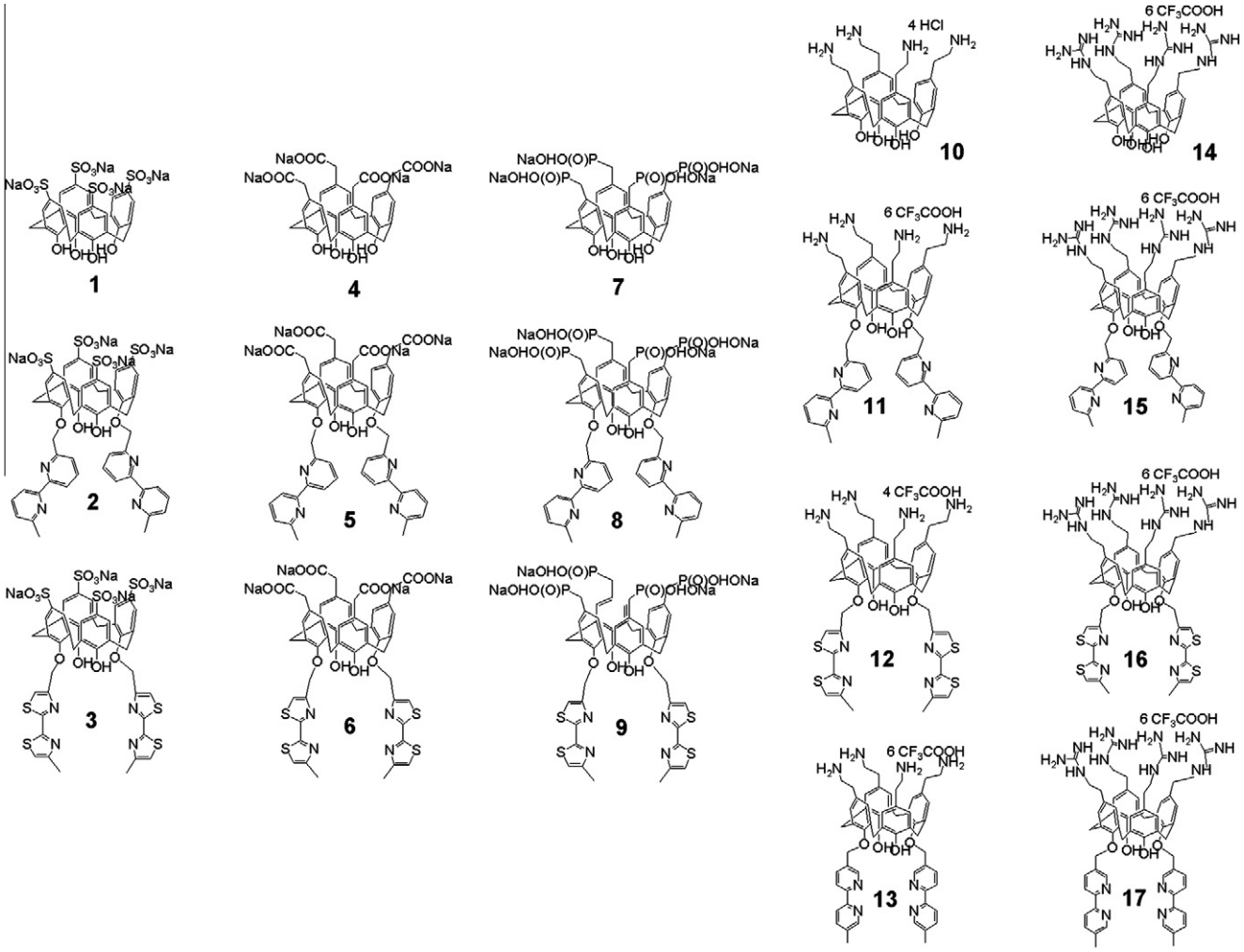
3. Results and discussion

A preliminary screen at 10 and 1 μM was first carried out against the *M. tuberculosis* H_{37}Rv reference strain, with some of the anionic (**1**, **4** and **7)** and all the cationic compounds. While the anionic species tested **1**, **4** and **7** were all inactive, the cationic amino derivatives **10**, **11**, **12** and **13**, then guanidine derivatives **14**, **15**, **16** and **17** displayed from no activity to very high activity. (Table 1)

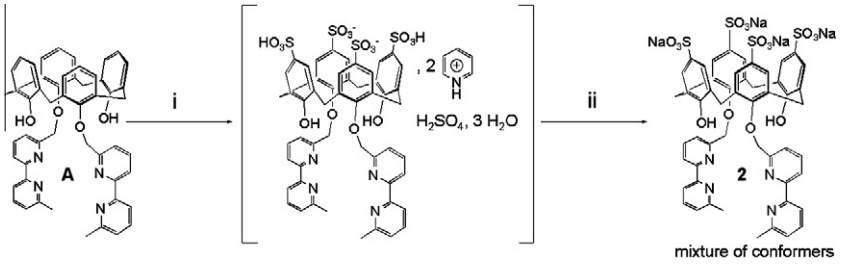
In the amino family, the α-bipyridyl derivative **11** was qualified as non active, with 0% and 7% inhibitory activities at 10 and 1 μM concentrations, respectively; the bithiazolyl derivative **12** displayed a better but also weak activity with 19 and 25% inhibitory activities at same concentrations. The unsubstituted derivative **10** displayed an interesting inhibitory activity, that is, 76% inhibition at 10 μM concentration and 27% inhibition at 1 μM, while the β-bipyridyl derivative **13** exhibited high inhibitory properties at both concentrations, evolving from ca. 81% at 10 μM to ca. 63% at 1 μM.

In the guanidine family, a similar behaviour was observed for all compounds, with a lack of activity for the α-bipyridyl derivative **15**, which displays 10% and 7% inhibitory activities at 10 and 1 μM concentrations, respectively. The unsubstituted derivative **14**, the bis-(bithiazolyl) **16** and the bis-(β-bipyridyl) **17** display all a high activity at 10 μM concentration, with ca. 90% to 98% inhibitory activities but also at 1 μM concentration with 54 to 73% inhibition. As a preliminary conclusion, the negatively charged species **1**, **4** and **7**, the α-bipyridyl amino- and guanidino- derivatives **11** and **15**, and the bithiazolyl amino-derivative **12** were qualified as non active over *M. tuberculosis* H_{37}Rv, while the other amino- and guanidino-derivatives **10**, **13**, **14**, **16** and **17** were qualified as active to very active species.

This first selection led us to study more precisely this last group, with the evaluation of their Minimum Inhibitory Concentrations (MIC) and their 50% Inhibitory Concentrations (IC_{50}).



Scheme 1. Anionic and cationic calixarene derivatives of the study.



Scheme 2. Reagents and conditions: (i) (a) $ClSO_3H$, CH_2Cl_2, rt, (b) pyridine, Me_2CO, H_2O, reflux, 90%; (ii) NaOH, H_2O, 100%.

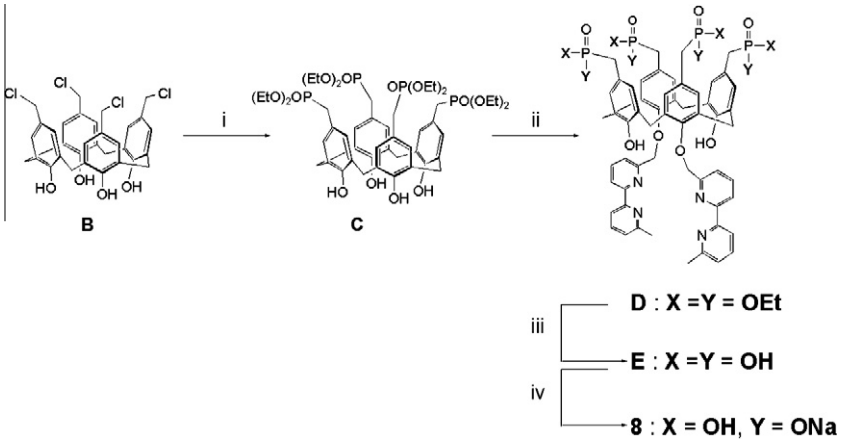
In addition, α-bipyridyl and bithiazolyl derivatives of **1**, **4** and **7**, that is, anionic compounds **2**, **3**, **5**, **6** and **8**, **9**, respectively, were also evaluated. This group of anionic derivatives exhibited a lack of activity with MIC values superior or equal to 50 μM; their IC_{50} values were thus not evaluated.

The amino derivatives **10** and more particularly **13** displayed interesting activities with MIC values of 3.2 and 0.8 μM, corresponding to massic concentrations of 2.6 and 1.3 μg/mL, respectively. Their IC_{50} were 0.4 and 0.2 μM, corresponding to massic concentrations of 0.32 and 0.33 μg/mL.

The introduction of the guanidine groups at the upper rim in derivatives **14, 16** and **17** resulted, particularly for **17**, in more interesting activities with MIC values of 0.8, 1.6 and 0.8 μM (1.0, 2.7 and 1.5 μg/mL, respectively), and IC_{50} of 0.25, 0.40 and 0.05 μM, (0.31, 0.67 and 0.10 μg/mL).

Comparison with the MIC values we obtained for commercial anti-*M. tuberculosis* products (isoniazid 0.6 μM (0.08 μg/mL); ethambutol 9.8 μM (2 μg/mL); streptomycin 0.7 μM (0.4 μg/mL)) or described elsewere (rifampicin 0.24 μM (0.2 μg/mL); ethionamid 9 μM (1.5 μg/mL)), shows that the five cationic substances presented above display better molar activities than ethambutol and ethionamid, that the β-bipyridyl amino- and guanidino-derivatives **13** and **17**, then the unsubstituted guanidino derivative **14** are at least as active as streptomycin and reach the domain of active concentrations for isoniazid and rifampicin.

The β-bipyridyl subunit seems to carry an important part of the activities observed for **13** and **17**, as well as the guanidino groups. It is interesting to note that the anti-mycobacterial streptomycin and dihydrostreptomycin, that contain two guanidine units, could be representative of the impact of this group on the high activities



Scheme 3. Reagents and condition: (i) $POEt_3$, CH_2Cl_2, reflux, 98%; (ii) $BrCH_2$bpy(Me), MeCN, K_2CO_3, reflux, 71%; (iii) (a) BTMS, rt, (b) HCl, 85%; (iv) NaOH, H_2O, 100%.

Table 1
Anti-mycobacterial (*M. tuberculosis* H37Rv strain) activities of anionic and cationic calixarene species

Compound (mol W)	% inhib.		MIC μM	IC_{50} μM
	10 μM	1 μM	(μg/mL)	(μg/mL)
1 (1016.2)	0	0	ND	ND
2 (1784.3)			>50	ND
3 (1435.3)			>50	ND
4 (932.6)	7	0	ND	ND
5 (1315.9)			>50	ND
6 (1438.9)			>50	ND
7 (1138.5)	5	0	ND	ND
8 (1422.5)			50 (70)	12.4 (17)
9 (1376.1)			50(63.8)	ND
10 (805.6)	76	27	3.2 (2.58)	0.4 (0.32)
11 (1717.4)	0	7	ND	ND
12 (1512.5)	19	25	ND	ND
13 (1671.5)	81	63	0.8 (1.34)	0.2 (0.33)
14 (1239.0)	97	73	0.8 (1.0)	0.25 (0.31)
15 (1867.5)	10	7	ND	ND
16 (1695.5)	90	54	1.6 (2.69)	0.4 (0.67)
17 (1885.5)	98	62	0.8 (1.51)	0.05(0.10)
EMB	65		9.8 (2)	ND
Ciprofloxacin			2.5 (0.8)	ND
INH			0.6 (0.08)	ND
Streptocycin			0.7 (0.4)	ND

ND, not determined.

observed for **14**, **16** and **17**. The positive role of these guanidino groups can be demonstrated when comparing the inactive amino- and active guanidino-bithiazolyl derivatives **12** and **16**.

The relatively homogeneous MIC values—between 4 and 32 μg/mL—obtained previously[27,31] with guanidino derivatives **14**, **15, 16** and **17** against Gram negative and Gram positive reference strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and 29213, *E. faecalis* ATCC 29212) revealed, contrary to the present work, no discrimination for the α-bipyridyl analogue **15**.

Here, with respect to these observations, the α-bipyridyl groups could support alone the absence of activity of derivatives **11** and **15** against *M. tuberculosis*. The structural difference with **13** and **17** is the position of the terminal methyl and methylene groups, with regards to pyridyl *N*-atoms. Interesting data presented by do Nascimento et al.[42], show that 2,2′-bipyridine and 4,4′-dimethyl-2,2′-bipyridine exhibit MIC values of 160.1 μM (25 μg/mL) and 135.7 μM (25 μg/mL), and were thus considered as inactive species. Nevertheless, some examples of 2,2′-bipyridyl or bipyridyl-like-containing metal (copper, ruthenium) complexes have been described as effective anti-mycobacterial agents,[43–46] involving no methyl groups in α-positions to N-chelating atoms.

Table 2
Anti-mycobacterial activities of selected cationic calixarene species against the INH-resistant strain MYC5165

Compound (mol. W)	MYC5165	
	MIC μM (μg/mL)	IC_{50} μM (μg/mL)
10 (805.6)	3.2 (2.6)	0.7 (0.56)
13 (1671.5)	1.6 (2.7)	0.5 (0.83)
14 (1239.0)	0.8 (1.0)	0.1 (0.12)
16 (1695.5)	0.1 (0.17)	<0.1 (0.19)
17 (1885.5)	0.4 (0.75)	0.1 (0.19)
INH	12.5 (1.7)	ND
Ciprofloxacin	2.5 (0.8)	ND

ND: not determined.

In addition to the fact that water-soluble calix[4]arene bipyridyl ligand have been shown to complex copper(I) in water, even in the presence of seric proteins,[41] these informations can help us in defining future complementary studies related to the abilities of our ligands to complex metal ions of biological importance in the metabolism of *M. tuberculosis*. Nevertheless, the absence of heterocycles in **14** correlated to its low MIC value shows that a metal-chelating ability is not compulsory to generate an anti-mycobacterial activity in this family.

The emergence of multi- (MDR—isoniazid/INH and rifampicin) and extensively-drug resistant *Mycobacterium tuberculosis* strains becomes a serious problem. For this reason, we decided to test the more active derivatives **10**, **13**, **14**, **16** and **17** against the INH-resistant strain MYC5165 (*M. tuberculosis* strain mutated in InhA).[47,48] Here also, these five compounds displayed highly interesting low MIC and IC_{50} values, most of them sub-micromolar, while INH was active at 12.5 μM. (Table 2).

Compound **16**, integrating 2,2′-bithiazolyl units at the lower rim, showed 125 fold better in vitro activity against MYC5165 strain (**16**; MIC = 0.1 μM) as compared to INH (MIC = 12.5 μM).

Compounds **14**, unsubstituted at the lower rim, and **17**, integrating 5,5′-bipyridyl units, showed them 15–30-fold better in vitro activities than INH against MYC5165 strain (**14**, **17**; MIC = 0.8 and 0.4 μM respectively). Compounds **10** and **13** also showed, but in a lesser extent, activities higher than INH.

Comparison of micromolar MIC and IC_{50} values of species **10**, **13**, **14**, **16** and **17** when tested against *M. tuberculosis* H37Rv (Fig. 1) showed that the differences were maximal for **10** ($\Delta MIC-IC_{50} = 2.80$), intermediate for **16** ($\Delta MIC-IC_{50} = 1.2$) and minimal for **13** ($\Delta MIC-IC_{50} = 0.60$), **14** ($\Delta MIC-IC_{50} = 0.55$), and **17** ($\Delta MIC-IC_{50} = 0.75$), giving for the latter a stronger anti-infectious interest.

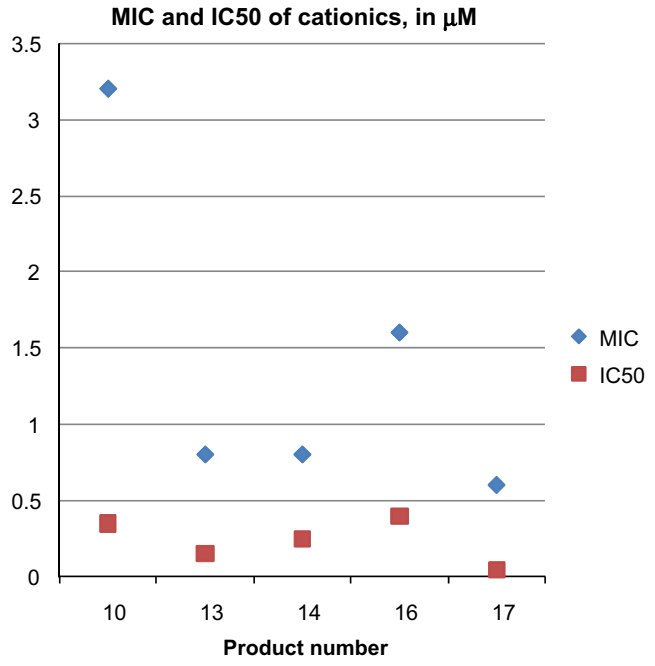


Figure 1. Comparison of MIC and IC_{50} values in µM for amino derivatives **10** and **13**, and guanidino derivatives **14**, **16** and **17** when tested against *M. tuberculosis* H37Rv.

Comparing the MIC values of guanidine derivatives **14**, **16** and **17** to their already described cytotoxic concentrations (CC_{50} = 256, 16–22 and 128 µg/mL, respectively; MRC-5 cells)[31] resulted in selectivity indexes (SI = CC_{50}/MIC) of 256, 6–12 and 85, reinforcing the interest of compounds **14** and **17** for further development.

4. Conclusion

In this investigation, seventeen calix[4]arene derivatives, incorporating at the upper rim four sulfonate, methylphosphonate or methylcarboxylate anions, or aminoethyl or guanidinoethyl cations, and, at the lower rim four hydroxyl groups or two hydroxyl groups alternated with two α-bipyridyl, β-bipyridyl or bithiazolyl units, were evaluated against INH- sensitive and resistant *M. tuberculosis* strains. The nine anionic species, the unsubstituted-, α-bipyridyl- and bithiazolyl- aminoethyl derivatives, as well as the α-bipyridyl guanidinoethyl analogue did not exhibit interesting activities. Five amino and guanidino derivatives incorporating for some of them two β-bipyridyl or bithiazolyl units at the lower rim exhibited, notably against the INH resistant strain, highly interesting activities convenient to the development of anti-mycobacterial agents. Very preliminary structure–activity relationships study suggested an important role of the guanidine groups and of the β-bipyridyl units, calling for deeper investigations related to guanidine pre-organisation, as well as to metal complexation properties of bi-heterocyclic derivatives.

5. Experimental section

5.1. Chemistry

5.1.1. 5,11,17,23-*Tetra*-(sulfonic acid)-25,27-bis(6-methyleneoxy-6′-methyl-2,2′-bipyridyl)-26,28-dihydroxycalix[4]arene, tetra sodium salt (2)

The 25,27-bis(6-methyleneoxy-6′-methyl-2,2′-bipyridyl)-26,28-dihydroxycalix[4]arene **A**[54] (0.55 g, 0.69 10^{-3} mol) was solubilised in anhydrous CH_2Cl_2 (70 mL) and cooled to 0 °C. Chlorosulfonic acid (0.9 mL, 13.94 mmol) was then added and the mixture was stirred under argon for ca. 5 h (TLC monitoring; SiO_2, CH_2Cl_2/MeOH, 95:5). The solvent was evaporated and the residue was dissolved in a mixture of H_2O (3 mL), acetone (30 mL) and pyridine (30 mL). This solution was stirred at reflux under Ar for 20 h, then the solvent were evaporated. The resulting oily material was dissolved in MeOH (1–2 mL) and addition of EtOH in excess followed by cold evaporation under rotavapor resulted in the formation of a white precipitate. The latter was collected by filtration, rinsed with cold EtOH, then dried. This solid material was dissolved in H_2O (100 mL) and 0.1 M NaOH was carefully added under bubbling of Ar (pH-metre monitoring; from 2.12 to 9.15). The resulting aqueous phase was concentrated, dialysed (Float-A-Lyser, cellulose acetate, MWCO 100D), then lyophilised to afford **2** (0.83 g, 69%). White cotton. Mp: >230 °C. IR (KBr): 3449.2 (OH), 1188.0 (SO_3^-). ^{1}H NMR (400 MHz; D_2O): 2. 18 (s, *Me*bpy, minor); 2.47 (s, *Me*bpy, major); 3.22,3.92 (AB, J_{AB} = 13.5 Hz, Ar–C*H*$_2$–Ar); 3.24,4.58 (AB, J_{AB} = 12.7 Hz, Ar–C*H*$_2$–Ar); 3.34,4.22 (AB, J_{AB} = 15.8 Hz, Ar–C*H*$_2$–Ar); 3.88,4.41 (AB, J_{AB} = 15.0 Hz, Ar–C*H*$_2$–Ar); 4.44,5.10 (AB, J_{AB} = 11.9 Hz, Ar-OC*H*$_2$bpy, minor); 5.03,5.17 (AB, J_{AB} = 11.9 Hz, Ar-OC*H*$_2$bpy, major); 6.71 (d, J = 7.5 Hz, H(3) or H(5) py$_a$), 7.10 (t, J = 7.7 Hz, H(4) py$_a$); 7.18 (d, H(5) or H(3) py$_a$); 6.9–2.75 (m, ArH and other H of py); 7.78 (d, J = 7.5 Hz, H(3) or H(5) py$_b$), 7.89 (d, J = 7.8 Hz, H(5) or H(3) py$_b$); 8.07 (t, J = 7.9 Hz, H(4) py$_b$). ^{13}C NMR (100 MHz, DMSO-d_6): not interpretable. Anal. Calcd for $C_{52}H_{40}O_{16}N_4S_4Na_4$, 3.5 Na_2SO_4, 5 H_2O (1784.34): C 35.00; H 2.82; N 3.14; found: C 35.31; H 2.95; N 2.67. ES–MS (ES^+): 575.03 [M–2 Na]$^{2-/2}$; 564.09 [M–3 Na+H$^+$]$^{2-/2}$; 553.10 [M–4 Na+2 H$^+$]$^{2-/2}$; 462.06 [M–4 Na–MebpyCH$_2$+2H$^+$]$^{2-/2}$; 371.03 [M–4 Na–2 MebpyCH$_2$+2H$^+$]$^{2-/2}$.

5.1.2. 5,11,17,23-Tetra-[(diethylphosphono)methyl]-25,27,26,28-tetrahydroxycalix[4]arene (C)

A solution of tetra-*para*-chloromethyl-calix[4]arene **B** (1 g; 1.62 10^{-3} mol) and P(OEt)$_3$ (15 mL) in CH_2Cl_2 (25 mL) was heated at reflux under argon for 6 h. (TLC monitoring; SiO_2, CH_2Cl_2/CH_3OH 95:5). The mixture was then evaporated to dryness at 50 °C and under high vacuum. The oily residue was dissolved in the minimum of CH_2Cl_2 and cyclohexane was added to give a suspension. The latter was frozen then lyophilised to give the tetra-phosphonoester **C**. White gummy powder (1.53 g; 98%). Mp: 131–236 °C. IR (KBr): 1026.5 (P–O–R); 1246.4 (P=O); 3329.8(Ar–OH). UV–vis (CH_2Cl_2): 231 (21200); 280 (8600). ^{1}H NMR ($CDCl_3$): 1.23 (t, J = 7.1 Hz, 24H, C*H*$_3$); 2.92 (d, J = 21.2 Hz, 8H, C*H*$_2$P); 3.52–2.21 (br AB, 8H, Ar–C*H*$_2$–Ar); 4.00 (m, 16H, C*H*$_2$OP); 6.99 (d, J = 2.5 Hz, 8H, Ar*H*); 10.07 (s, 4H, ArO*H*). ^{13}C NMR ($CDCl_3$): 16.8 (d, J = 5.8 Hz, *C*H$_3$CH$_2$O); 32.0 (Ar–C*H*$_2$–Ar); 33.1 (d, J = 139.2 Hz, Ar–CH$_2$–P); 62.5 (d, J = 6.9 Hz, CH$_3$CH$_2$O); 125.4 (d, J = 9.1 Hz), 128.6 (d, J = 2.5 Hz), 130.7 (d, J = 6.2 Hz), 148.2 (d, J = 3.6 Hz), (C_{ipso}, C_p, C_o, C_m of Ar). Anal. calcd for $C_{48}H_{68}O_{16}P_4$ (1024,94): C 56.25; H 6.69; found: C 56.13; H 6.69. ES–MS (pos. mode): 1047.4 (M+Na$^+$)$^+$, 1042.5 (M–C$_2$H$_5$+2 Na$^+$)$^+$, 532.4 (M–C$_2$H$_5$+3 Na$^+$)$^{2+/2}$. ES–MS (neg. mode): 1023.5 (M–H$^+$)$^-$, 873.4 (M–CH$_2$P(O)(OC$_2$H$_5$)$_2$–H$^+$)$^-$, 723.4 (M–2CH$_2$P(O)(OC$_2$H$_5$)$_2$–H$^+$)$^-$.

5.1.3. 5,11,17,23-Tetra-[(diethylphosphono)methyl]-25,27-Bis(6-methyleneoxy-6′-methyl-2,2′-bipyridyl)-26,28-dihydroxycalix[4]arene (D)

A solution of **C** (3.1 g; 3.20 10^{-3} mol) in MeCN (100 mL) was heated at reflux under argon for 5 min. in the presence of K_2CO_3 (0.446 g; 3.23 10^{-3} mol). 6-bromomethyl-6′-methyl-2,2′-bipyridine (1,87 g; 7.12 10^{-3} mol) was then added and reflux was continued for 3.5 h. (TLC monitoring: SiO_2 CH_2Cl_2/CH_3OH 96:4). The solvent was evaporated to dryness and the residue dissolved in CH_2Cl_2 then filtered to remove insoluble salts. The resulting filtrate was concentrated then chromatographed (SiO_2, CH_2Cl_2/CH_3OH

96:4). The fractions containing the desired product were evaporated to dryness, dissolved in CH_2Cl_2 and added to excess cyclohexane. The resulting suspension was frozen then solvent were lyophylised to give **D**. White powder. (3.5 g; 71%). Mp: 63–27 °C. IR: 1026.9 (P–O–R); 1242.8 (P═O); 1739.7(ArO–CH_2); 3407.4(Ar–OH). UV (CH_2Cl_2): 231 (36700); 290 (31100).^{1}H NMR ($CDCl_3$): 1.03 (t, J = 7.0 Hz, 12H, CH_3CH$_2$); 1.23 (t, J = 7.1 Hz, 12H, CH_3CH$_2$); 2.65 (s, 6H, *Me*bpy); 2.83 (d, J = 21.4 Hz, 4H, ArCH_2P); 3.06 (d, J = 21.0 Hz, 4H, ArCH_2P); 3.43–2.41 ('q', AB, J_{AB}: 13.2 Hz, 8H, Ar–CH_2–Ar); 3.83 (m, 8H, CH$_3$CH_2); 4.00 (m, 8H, CH$_3$CH_2); 5.21 (s, 4H, OCH_2bpy); 6.89 (d, J = 2.5 Hz, 4H, Ar*H*); 7.06 (d, J = 2.5 Hz, 4H, Ar*H*); 7.15 (d, J = 7.8 Hz, 2H, H(5′) bpy); 7.62 (m, 4H, H(4) and H(4′) bpy); 8.03 (s, 4H, ArO*H*); 8.05 (d, J = 7.8 Hz, 2H, H(5) bpy); 8.20 (d, J = 7.8 Hz, H(3′) bpy); 8.38 (d, J = 7.6 Hz, 2H, H(3) bpy). ^{13}C NMR ($CDCl_3$): 16.4, 16.5, 16.8, 16.9 (*C*H$_3$CH$_2$); 25.0 (*C*H$_3$ bpy); 31.8 (Ar–*C*H$_2$–Ar); 33.1 (d, J = 138.8 Hz, Ar–*C*H$_2$–P); 33.5 (d, J = 138.0 Hz, Ar–*C*H$_2$–P); 62.4, 62.5 (CH$_3$*C*H$_2$); 79.2 (O*C*H$_2$bpy); 118.6, 120.5, 121.3, 123.7, 137.4, 138.5 (C(3), C(3′), C(4), C(4′), C(5), C(5′) of bpy); 122.0 (d, J_{PC} = 9 Hz, C_p of Ar); 128.7 (d, J_{PC} = 9 Hz, C_p of Ar); 128.0, 133.6 (C_o of Ar); 130.4 (d, J_{PC} = 6.0 Hz, C_m of Ar); 131.0 (d, J_{PC} = 6.0 Hz, C_m of Ar); 151.6, 152.8, 155.6, 156.4, 156.5, 158.2 (C(2), C(2′), C(6), C(6′) of bpy, C_{ipso} of Ar). Anal. calcd for $C_{72}H_{88}N_4O_{16}P_4$, 0.2 $(C_2H_5O)_3P$ (1422.21): C 61.80; H 6.45; N 3.94; found: C 62.00; H 6.64; N 3.50. ES–MS (pos. mode): 1411.5 (M+Na$^+$)$^+$, 1406.5 (M–C_2H_5+H+2 Na$^+$)$^+$, 1229.5 (M–CH$_3$bpyCH$_2$+H+Na$^+$)$^+$, 1224. 5 (M–CH$_3$bpyCH$_2$–C_2H_5+2H+Na$^+$)$^+$, 717.5 (M+2 Na$^+$)$^{2+/2}$, 714.5 (M–C_2H_5+3 Na$^+$)$^{2+/2}$, 706.5 (M+Na$^+$+H$^+$)$^{2+/2}$, 695.5 (M+2H$^+$)$^{2+/2}$.

5.1.4. 5,11,17,23-Tetra-[(phosphono)methyl]-25,27-bis(6-methyleneoxy-6′-methyl-2,2′-bipyridyl)-26,28-dihydroxycalix[4]arene (E)

A solution of **D** (0.66 g; 5.53 10^{-4} mol) and bromotrimethylsilane (2 mL) in dry CH_2Cl_2 (10 mL) was stirred at rt under Ar for 20 h. The solvent were evaporated to dryness and the solid residue triturated with water, filtered, rinsed with water (3 × 10 mL), MeOH (3 × 10 mL) and CH_2Cl_2 (2 × 10 mL) to give **E**. (0.55 g; 81%). White powder. Mp: 263 °C (dec.). IR: 984.7 (P–OH); 1233.5 (P═O); 3384.9 (Ar–OH). UV (THF): 290 (11500). ^{1}H NMR (400 MHz, DMSO-d_6): 2.54 (s, 6H, *Me*bpy); 2.72 (d, 4H, CH_2P); 2.78 (d, J = 22.7 Hz, 4H, CH_2P); 3.36–2.32 ('q', AB, J_{AB} = 12.7 Hz, 8H, Ar–CH_2–Ar); 5.14 (s, 4H, OCH_2bpy); 7.00 (d, J = 2 Hz, 4H, Ar *H*); 7.02 (d, J J = 2 Hz, 4H, Ar *H*); 7.25 (d, J = 7.6 Hz, 2H, H(3′) or H(5′) bpy); 7.67 (t, J = 7.6 Hz, 2H, H(4′) bpy); 7.69 (t, J = 7.6 Hz, 2H, H(4) bpy); 7.82 (d, J = 7.2 Hz, 2H, H(3) or H(5) bpy); 8.23 (d, J = 7.8 Hz, 2H, H(5′) or H(3′) bpy); 8.32 (d, J = 8.3 Hz, 2H, H(5) or H(5) bpy); 8.35 (s, 2H, ArO*H*). Anal. calcd. for $C_{56}H_{56}N_4O_{16}P_4$, 0.25 H_3PO_4, 2.5 H_2O (1234.49): C 54.48; H 5.04; N 4.54; found: C 54.37; H 4.77; N 4.26. ES–MS (neg. mode): 1163.3 (M–H$^+$)$^-$, 581.4 (M–2H$^+$)$^{2-/2}$, 387.4 (M–3H$^+$)$^{3-/3}$.

5.1.5. 5,11,17,23-Tetra-[(phosphono)methyl]-25,27-bis(6-methyleneoxy-6′-methyl-2,2′-bipyridyl)-26,28-dihydroxycalix[4]arene, tetra sodium salt (8)

A suspension of **E** (0.3 g; 2.43 10^{-4} mol) in water (40 mL) was treated with 1 M NaOH until pH 7 was raised, while solubilisation occured at pH 4. The resulting neutral solution was evaporated to dryness, the residue was treated with MeOH then dried again to give **8**. (0.35 g; 100%). White powder. Mp: 271–280 °C (dec.). IR: 910.8 (P–OH); 1229.8 (P═O); 3385.0 (Ar–OH). UV (H_2O): 288 (26100). ^{1}H NMR (400 MHz, CD_3OD): 2.60 (s, 6H, *Me*bpy); 2.76 (d, J = 20.6 Hz, 4H, CH_2P); 2.79 (d, J = 20.2 Hz, 4H, CH_2P); 3.42–2.44 ('q', AB, J_{AB} = 12.8 Hz, 8H, Ar–CH_2–Ar); 5.17 (s, 4H, OCH_2bpy); 7.07 (d, J = 2.0 Hz, 4H, Ar*H*); 7.16 (d, J = 2.0 Hz, 4H, Ar*H*); 7.22 (H(3′) or H(5′) bpy); 7.66 (m, 4H, H(4) and H(4′) bpy); 7.90 (H(3) or H(5) bpy); 8.25 (m, 4H, H(3) or H(5), and H(3′) or H(5′) bpy). ^{13}C NMR (400 MHz, D_2O): 23.3 (*C*H$_3$ bpy); 31.0 (Ar–CH_2–Ar); 34.6, 34.8, 36.0 (Ar–*C*H$_2$–P); 78.1 (O*C*H$_2$); 120.4, 121.8, 123.0, 124.6 (C(3), C(3′), C(5), C(5′) of bpy); 127.1, 127.2, 128.1, 133.5, 150.2, 150.8 (C_p, C_o, C_{ipso} of Ar); 130.8, 130.5 (C_m of Ar); 137.4, 138.5 (C(4), C(4′) of bpy); 154.7, 155.5, 155.7, 158.4 (C(2), C(2′), C(6), C(6′) of bpy). Anal. calc. for $C_{56}H_{52}N_4O_{16}Na_4P_4$, 0.25 Na_2HPO_4, 7.5 H_2O (1422.76): C 47.25, H 4.76, N: 3.94; found: C 47.29, H 4.20, N 3.58. ES–MS (pos. mode): 1275.7 (M+Na$^+$)$^+$, 858.0 (2 M+3 Na$^+$)$^{3+/3}$, 850.7 (2 M+2 Na$^+$+H$^+$)$^{3+/3}$, 843.4 (2 M+Na$^+$+2H$^+$)$^{3+/3}$, 836.0 (2 M+3H$^+$)$^{3+/3}$, 649.2 (M+2 Na$^+$)$^{2+/2}$, 638.1 (M+Na+H$^+$)$^{2+/2}$, 627.2 (M+2H$^+$)$^{2+/2}$, 616.2 (M–Na$^+$+3H$^+$)$^{2+/2}$. ES–MS (neg. mode): 1229.7 (M–Na$^+$)$^-$, 1207.2 (M–2 Na$^+$+H$^+$)$^-$, 812.7 (2 M–3 Na$^+$)$^{3-/3}$, 603.3 (M–2 Na$^+$)$^{2-/2}$, 592.3 (M–3 Na$^+$+H$^+$)$^{2-/2}$, 581.3 (M–4 Na$^+$+2H$^+$)$^{2-/2}$, 512.2 (M–CH$_3$bpyCH$_2$–2 Na$^+$)$^{2-/2}$, 501.2 (M–CH$_3$bpyCH$_2$–3 Na$^+$+H$^+$)$^{2-/2}$, 490.3 (M–CH$_3$bpyCH$_2$–4 Na$^+$+2H$^+$)$^{2-/2}$, 394.7 (M–3 Na$^+$)$^{3-/3}$, 387.4 (M–4 Na$^+$+H$^+$)$^{3-/3}$.

5.2. Biology

5.2.1. Inhibition of mycobacterial growth

The susceptibility of the two *M. tuberculosis* strains H_{37}Rv and MYC5165 to all synthesized 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.[49,50] Briefly, serial twofold dilutions of each drug were prepared in 7H9 broth (Middlebrook 7H9 broth base (Difco)) using 96- well microtiter plates and 100 μL 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.

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