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Research Article

Benzocaine Complexation with *p*-Sulfonic Acid Calix[*n*]arene: Experimental (^{1}H-NMR) and Theoretical Approaches

Lucas M. Arantes[1], Eduardo V. V. Varejão[1], Karin J. Pelizzaro-Rocha[2], Cíntia M. S. Cereda[2], Eneida de Paula[2], Maicon P. Lourenço[3], Hélio A. Duarte[3] and Sergio A. Fernandes[1,*]

[1]Grupo de Química Supramolecular e Biomimética (GQSB), Departamento de Química, Universidade Federal de Viçosa (UFV), Viçosa, Brazil 36570-000
[2]Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil 13083-970
[3]Grupo de Pesquisa em Química Inorgânica Teórica (GPQIT), Departamento de Química, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil 31270-901
**Corresponding author: Sergio A. Fernandes, santonio@ufv.br or sefernandes@gmail.com*

The aim of this work was to study the interaction between the local anesthetic benzocaine and *p*-sulfonic acid calix[*n*]arenes using NMR and theoretical calculations and to assess the effects of complexation on cytotoxicity of benzocaine. The architectures of the complexes were proposed according to ^{1}H NMR data (Job plot, binding constants, and ROESY) indicating details on the insertion of benzocaine in the cavity of the calix[*n*]arenes. The proposed inclusion compounds were optimized using the PM3 semiempirical method, and the electronic plus nuclear repulsion energy contributions were performed at the DFT level using the PBE exchange/correlation functional and the 6-311G(d) basis set. The remarkable agreement between experimental and theoretical approaches adds support to their use in the structural characterization of the inclusion complexes. *In vitro* cytotoxic tests showed that complexation intensifies the intrinsic toxicity of benzocaine, possibly by increasing the water solubility of the anesthetic and favoring its partitioning inside of biomembranes.

Key words: benzocaine, calix[*n*]arenes, density functional theory, inclusion compounds, NMR, semiempirical methods, supramolecular chemistry

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There are two classes of anesthetic compounds: general and local anesthetics. General anesthetics are drugs that act on the synaptic transmission and are preferable administered by intravenous or inhalation routes (1). In the case of local anesthetics (LA), their action is over excitable membranes, inhibiting the conduction of nerve stimulation, when applied locally or regionally, at high concentrations (2).

Benzocaine (BZC) is an ester-type local anesthetic mostly used in topical, dermal, and mucous formulations. It was present in the first drug delivery system employing a local anesthetic for topical use, patented in 1940, and up to the 70s, benzocaine was the only LA mentioned in such patents (3). Its anesthetic action is characterized by non-dose-dependent blockage with a rapid but brief effect, compared with the potential duration of pain (4). Moreover, the toxic effect of ester-type local anesthetics, related to their systemic absorption, has been reported (5) for benzocaine.

Therefore, the development of a new effective topical delivery system intended to suitably modulate the release rate of BZC, extending its anesthetic effect, and thus reducing problems such as systemic toxicity, limited water solubility, low chemical stability, or fast plasma clearance could be particularly advisable (3,6–9). Encapsulation of therapeutic agents is one of the most attractive areas in fashionable supramolecular host–guest chemistry and shows great potential in drug delivery. Materials used in encapsulation include liposomes (10,11), peptides (12), nanoparticles (13), chitosan (14,15), functional polymers (16), and host molecules such as cyclodextrins (17,18) and calix[*n*]arenes (6,19–23).

In that context, the aim of this study was primarily to investigate the capacity of *p*-sulfonic acid calix[4]arene (**2**) and *p*-sulfonic acid calix[6]arene (**3**) to form complexes with the local anesthetic benzocaine (**1**) (Figure 1). We have analyzed the degree of complexation between *p*-sulfonic acid calix[4]arene (**2**) and *p*-sulfonic acid calix[6]arene (**3**) with benzocaine (**1**) in an aqueous medium to obtain details on the stoichiometry, binding constants, and geometry of complexation, as determined by different ^{1}H-NMR methodologies. Based on the obtained results, a

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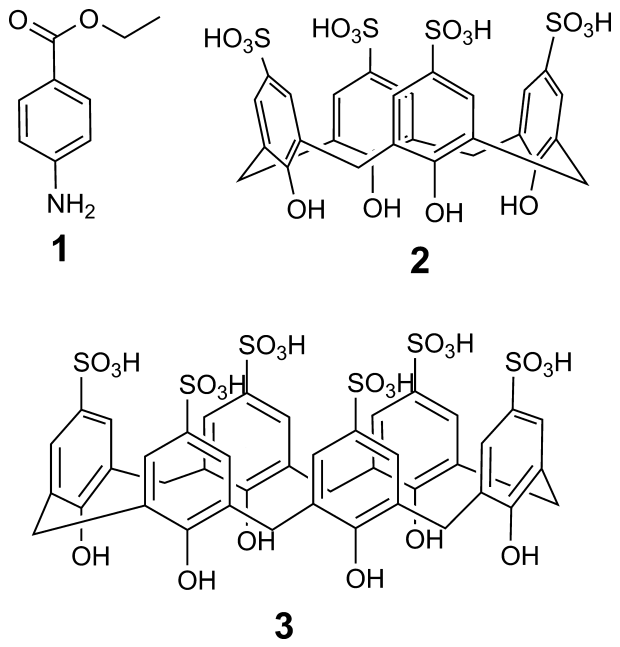

Figure 1: Structure of benzocaine (**1**), *p*-sulfonic acid calix[4]arene (**2**), and *p*-sulfonic acid calix[6]arene (**3**).

model for the topology of the formed complexes structure was proposed. In addition, PM3 semiempirical calculations were carried out to obtain the optimized inclusion complex geometries. The electronic plus nuclear repulsion energy contributions were performed at the PBE/6-311G(d) level of theory. The solvent effects were included using polarized continuum model (PCM) calculations.

Methods and Materials

Chemicals and reagents

Benzocaine (**1**) (99%) and D_2O (99.75%) were purchased from Aldrich (Minas Gerais, Brasil) and Merck (Minas Gerais), respectively. All other reagents were of analytical grade. *p*-Sulfonic acid calix[4]arene (**2**) and *p*-sulfonic acid calix[6]arene (**3**) were synthesized in our laboratory following the literature procedures (24–26).

Preparation of solid inclusion complexes

Inclusion complexes (**1/2** or **1/3**) at 1:1 M ratio were obtained by mixing 2 mM aqueous solutions of compounds (**2**) or (**3**) with aqueous solution of 2 mM benzocaine (**1**). Each system was stirred for 48 h at room temperature, a period of time considered as the optimum to reach the equilibrium. Each solution was frozen-dried in a Labconco Freeze-dry System (Freezone 4.5) and stored at 253 K until further use.

NMR Spectroscopy

All experiments were performed at 298 K in D_2O.

Routine 1D ^{1}H NMR

^{1}H NMR spectra were acquired with an Mercury-300 Varian spectrometer operating at 300.069 MHz for ^{1}H (64 k data points, 30° excitation pulse with duration of 2.2 μseconds, spectral width of 6 kHz, acquisition time of 3.3 seconds, and relaxation delay of 10 mseconds) in a 5-mm probe with direct detection mode at room temperature unless stated otherwise.

Determination of the stoichiometry of complexation

Job plots (27) have been prepared with 2 mM stock solutions of compounds (**1**), (**2**), and (**3**).

Determination of the binding constants

The binding constants were determined using the well-known y-reciprocal plot or Scott's plot (28). The complexation-induced chemical shifts for the (**1**) hydrogens were obtained in the presence of (**2**) and (**3**) by keeping the concentration of (**1**) fixed at 2 mM while increasing the concentration of (**2**) and (**3**) from 2 to 12 mM. The plots of ^{1}H NMR chemical shift change data for H-**2** of the guest in the form of [host]/$\Delta\delta_{H\text{-}2}$ versus [host] gave linear fits, confirming the 1:1 stoichiometry for both the complexes. The binding constants were determined according to the Scott's equation:

$$[host]/\Delta\delta_{obs} = [host]/\Delta\delta_{max} + \Delta\delta_{max}/K_a$$

where the slope of the plot is equal to [host]/$\Delta\delta_{max}$ and the intercept with the vertical axis to $\Delta\delta_{max}/K_a$.

ROE measurements

The ROESY 1D experiments were obtained with a selective 180° and a non-selective 90° pulse, and a mixing time of 0.5 seconds was used during the spin-lock. The selective pulses were generated by a waveform generator, which automatically attenuates the shape, power, and pulse duration to obtain the required selectivity. The subtraction of the on- and off-resonance acquisition furnished the ROESY 1D experiments. All spectra were acquired with a 5-mm inverse probe at 298 K in 5 mm tubes.

Computational details

As it has been shown elsewhere (29,30), the cone conformation is the most stable and has been used as starting point for geometry optimization of calix[*n*]arenes. Benzocaine was inserted into the *p*-sulfonic acid calix[4]arene macrocyclic ring, as follows: form α, when benzocaine is inserted into the *p*-sulfonic acid calix[4]arene (**2**) with the amine group out of the ring (α**1/2**); form β, when benzocaine (**1**) is included into the *p*-sulfonic acid calix[4]arene with its amine group into the ring (β**1/2**). In

a similar way, the two orientations proposed for benzocaine interaction with *p*-sulfonic acid calix[6]arene (**3**) were form α, when benzocaine is inserted into the *p*-sulfonic acid calix[6]arene with its amine group out of the ring (α**1/3**) and form β, when the amine group of the benzocaine is inserted into the *p*-sulfonic acid calix[6] arene (β**1/3**).

The methodology presented in this work was based on the work of Sousa *et al.* (31) and Nascimento *et al.* (32,33) where semiempirical and DFT approaches were used. Different geometry arrangements were fully optimized at the PM3 level (34). Harmonic frequency calculations at the PM3 level were performed to assure the equilibrium structures are in their potential energy surface minima because all frequencies were real. The electronic plus nuclear repulsion energy contributions were performed at the DFT level using the generalized gradient approximation PBE exchange/correlation functional developed by Perdew *et al.* (35) with the Pople's 6-311G (d) basis set (36).

Solvent effects were considered within the polarized continuum model (PCM) approach at the PBE/6-311(d)//PM3 level theory (37). The dielectric constant, ε, equal to 78.39 was used for water. All calculations were carried out using the Gaussian 2003 package (38).

In vitro *cytotoxicity (cell viability) tests*

Experiments were performed as described in reference (39) using BALB/c mouse 3T3 fibroblast cells, maintained under continuous culture in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 μL/mL of penicillin, and 100 μL of streptomycin sulfate, at pH 7.4 and 300 K, under a humid atmosphere containing 5% CO_2. Around 1×10^4 viable cells were inoculated into 96-well plates and incubated for 24 h. The cells were then exposed for 24 h to either benzocaine alone (**1**), free *p*-sulfonic acid calix[4]arene (**2**), and *p*-sulfonic acid calix[6]arene (**3**) or *p*-sulfonic acid calix[4]arene-benzocaine and *p*-sulfonic acid calix[6] arene-benzocaine complexes (**1/2** and **1/3**, respectively) at concentrations in the range 0.1–2 mM. Cell viability was evaluated by reduction in tetrazolium [the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test], with the cells being incubated in the presence of MTT (0.5 mg/mL) for 3 h at 300 K. The number of viable cells was determined by measuring the amount of MTT converted to insoluble formazan by mitochondrial dehydrogenases. The formazan crystals formed were dissolved in ethanol, with shaking for 30 min at room temperature. Non-treated cells were taken as 100% of viability, and three independent experiments were performed. The results were analyzed using one-way analysis of variance (ANOVA) with the Tukey's *post hoc* test. Statistical significance was defined as $p < 0.05$.

Results and Discussion

Characterization of inclusion complexes

NMR techniques were employed to obtain detailed information about the interactions between the guest benzocaine (**1**) and the hosts *p*-sulfonic acid calix[4]arene (**2**) and *p*-sulfonic acid calix[6]arene (**3**) in aqueous solution. The ^{1}H NMR spectra for benzocaine (**1**) in D_2O in the presence or absence of (**2**) or (**3**) were obtained. Benzocaine hydrogens were observed as a single resonance because of fast exchange between free and complexed guest molecules on the NMR time scale (Figure 2). The numbers corresponding to the NMR signals of benzocaine (**1**), *p*-sulfonic acid calix[4]arene (**2**), and *p*-sulfonic acid calix[6]arene (**3**) are shown in Table 1.

We started our investigation by analyzing the complexation-induced hydrogen chemical shifts ($\Delta\delta$) in the (**1/2**) and (**1/3**) complexes, in comparison with those of free benzocaine (**1**). Complexation between benzocaine (**1**) and *p*-sulfonic acid calix[4]arene (**2**) or *p*-sulfonic acid calix[6]arene (**3**) induced large shielding effects in all hydrogens of (**1**), mainly H-**3**, H-3', and H-**4'** $\Delta\delta$ −0.60; 0.55; and 0.88 (**1/2**) and −0.52; 0.12; and 0.20 (**1/3**), respectively (Figure 2 and Table 1), indicating interactions between the guest and both hosts. We have also observed variation in the chemical shifts of *p*-sulfonic acid calix[*n*]arene hydrogens (Ar-H and CH_2) (Table 1 and Figure 2).

The molecular interactions taking place between guest (**1**) and hosts (**2** or **3**) were further investigated by monitoring the chemical shifts of the H-**3** hydrogens of benzocaine (**1**). Job plots were conducted for determining the stoichiometry of the complexation reactions. These plots (Figure 3A and B) showed a maximum at 0.5 guest–host molar ratio, thus indicating that 1:1 complexes (**1/2** and **1/3**) are formed in aqueous solution (27).

The binding constants (K_a) of the (**1/2**) and (**1/3**) complexes were determined using the Scott's method (28), which is a modification of Benesi–Hildebrand equation (40). Typical Scott's plots for the obtained complexes are shown in Figure 4. The binding constants for **1/2** and **1/3** were determined as 835 and 1393 per M, respectively, showing that a stronger association takes place between benzocaine and *p*-sulfonic acid calix[6]arene.

To gain more insight into the inclusion geometries of the (**1/2**) and (**1/3**) complexes, we have performed 1D ROESY NMR experiments, which are usually suited to measure rOes in complexes with $\omega.\tau_c$ close to 1 (41). Specific rOe signals were not observed between guest (**1**) and the host (**2**) (data not shown). We therefore suggest the benzocaine (**1**) and *p*-sulfonic acid calix[4]arene (**2**) do not form an inclusion compound but rather a complex (19).

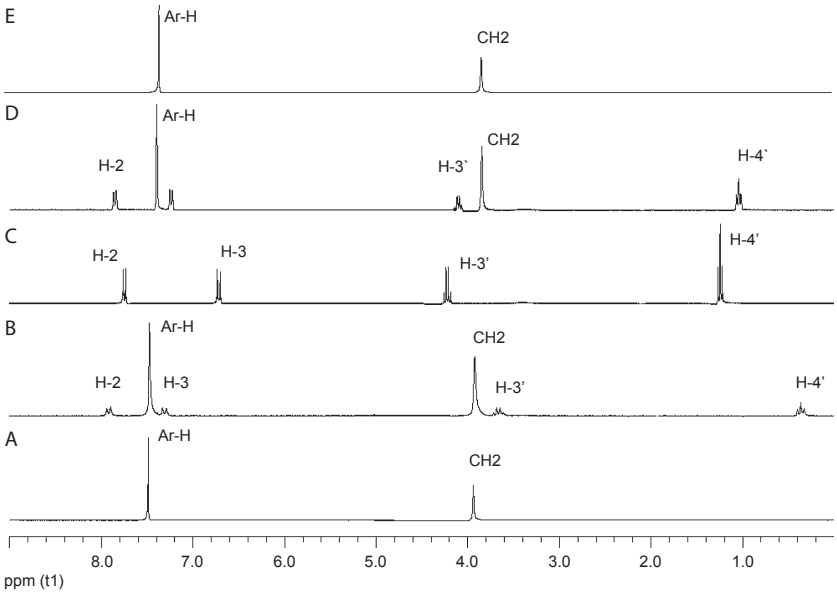

Figure 2: ^{1}H NMR spectra (300.069 MHz; D_2O; δ_{HDO} 4.67; 298 K, 2 mM each) of (A) *p*-sulfonic acid calix[4]arene (**2**); (B) benzocaine-*p*-sulfonic acid calix[4] arene complex (**1/2**); (C) benzocaine (**1**); (D) benzocaine-*p*-sulfonic acid calix[6]arene complex (**1/3**); and (E) *p*-sulfonic acid calix[6]arene (**3**).

Table 1: ^{1}H NMR: Chemical shifts (δ) and chemical shift differences ($\Delta\delta = \delta_{1\ free} - \delta_{1\ complex}$) of pure (**1**) and its complexes with *p*-sulfonic acid calix[4]arene (**1/2**) or *p*-sulfonic acid calix[6]arene (**1/3**) (2 mM samples, 298 K)

Hydrogens	1 δ	2 δ	1/2 δ	1/2 $\Delta\delta = \delta_{free} - \delta_{complex}$	3 δ	1/3 δ	1/3 $\Delta\delta = \delta_{free} - \delta_{complex}$
H-**4'**	1.25	–	0.37	0.88	–	1.05	0.20
H-**3'**	4.23	–	3.68	0.55	–	4.11	0.12
H-**2**	7.75	–	7.92	−0.17	–	7.85	−0.10
H-**3**	6.72	–	7.32	−0.60	–	7.24	−0.52
CH_2	–	3.94	3.93	0.01	3.81	3.86	−0.05
Ar**H**	–	7.48	7.47	0.01	7.31	7.39	−0.08

The observed rOe increments between hydrogen H-**2** and H-**3** of benzocaine (**1**) and H-**3** of *p*-sulfonic acid calix[6] arene (**3**) evidenced that the aromatic group of (**1**) is inside of the *p*-sulfonic acid calix[6]arene, while the amine group is turned to the larger opening border of the cone (data not shown). This is in accordance with the fact that the inner side border of the cone is hydrophobic. Furthermore, the presence of the amine group closer to the larger opening border of the cone favors its interaction with the water solvent.

Computational analysis

The calix[*n*]arenes can undergo different conformations. Structural modifications normally favor the cone conformation (42,43). This is an indicative of the ability to form inclusion compounds because the conformation of the calix[6]arene can, in principle, be modeled. The sulfonic groups at the *p*-sulfonic acid calix[*n*]arenes can form a hydrogen bound network enhancing the stability of the cone conformation. It has been pointed out that most of the inclusion mechanism is entropically driven and, therefore, the solvent effect has an important role in it. The mechanism involves displacement of the included water molecules and of the solvated water molecules surrounding the guest plus the consequent inclusion and solvation of the supramolecule. It is clear that the type of intermolecular interaction between the guest and host is very important to define whether the inclusion compound will be formed or not and how the guest will be included. For a host such as *p*-sulfonic acid calix[6] arene, important conformational changes can undergo to facilitate the inclusion of the guest. The cone conformation has the aromatic rings forming the wall in such way that one can easily infer that the inner side of the cone is hydrophobic. The *p*-sulfonic acid calix[4]arene forms a cone with a diameter of about 10.0 Å (Figure 5), while the aromatic ring of the benzocaine is *ca*. 2.80 Å wide. Furthermore, it is expected that in the smaller *p*-sulfonic acid calix[*n*]arene, the cone is more rigid because the angles in the methylenes are more strengthened. Therefore, it is not expected that benzocaine stays included in the *p*-sulfonic acid calix[4]arene. However, the *p*-sulfonic acid calix[6]arene forms a cone with a diameter of 11.9 Å, in which the guest molecule can be easily placed.

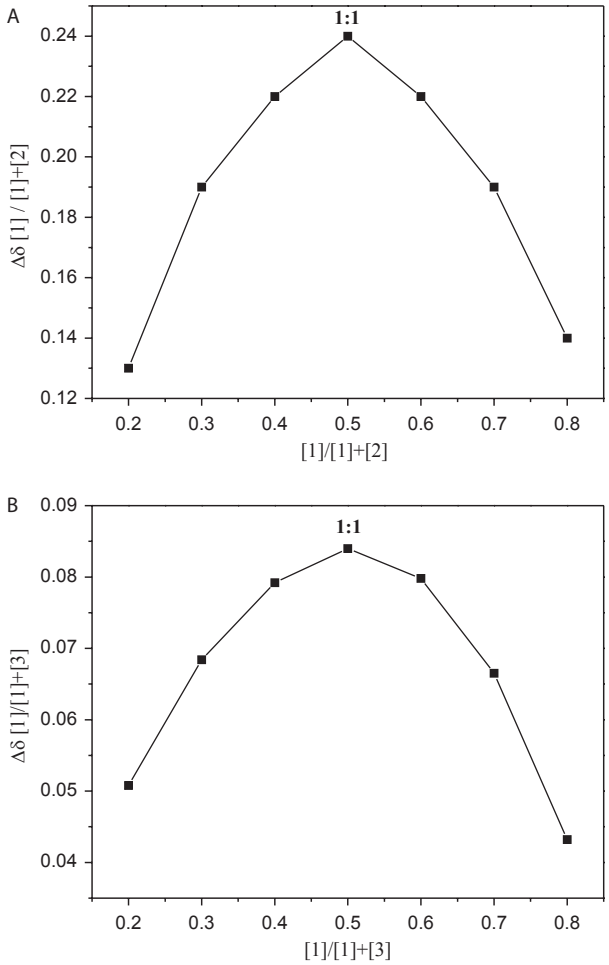

Figure 3: Job plot for the complexes formed between (A) benzocaine-*p*-sulfonic acid calix[4]arene (**1/2**) and (B) benzocaine-*p*-sulfonic acid calix[6]arene (**1/3**).

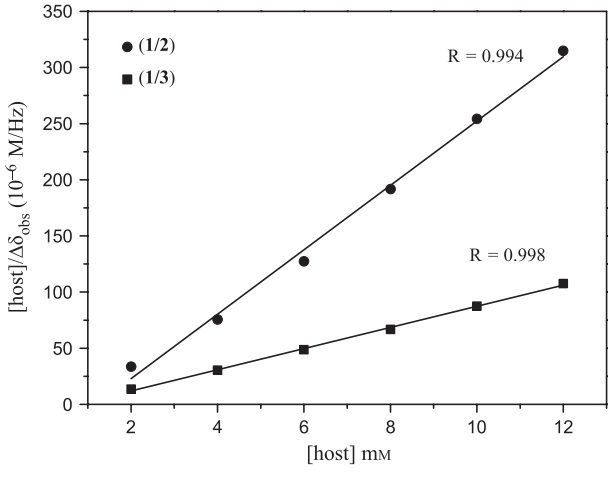

Figure 4: Typical Scott's plots for (**1/2**) and (**1/3**) inclusion complexes.

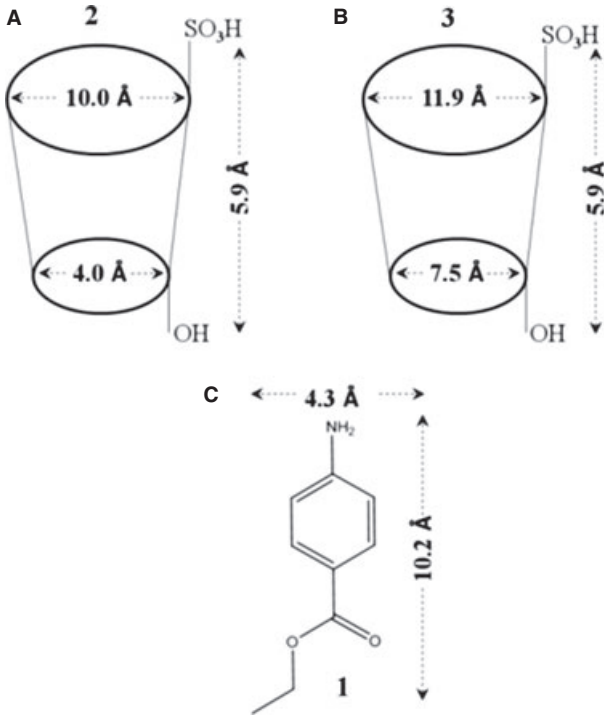

Figure 5: Molecular representation and dimensions of (A) *p*-sulfonic acid calix[4]arene; (B) *p*-sulfonic acid calix[6]arene; and (C) benzocaine.

Table 2: Relative interaction energy (kcal/mol) of the configurations α and β of the inclusion complexes **1/2** and **1/3** evaluated at the PBE/6-311G(d)//PM3 level of theory

		PBE/6-311G(d)//PM3	
Inclusion complex	PM3	Gas phase	Aqueous phase
α**1/2**	6.6	11.8	3.5
β**1/2**	0.0	0.0	0.0
α**1/3**	15.3	34.3	20.1
β**1/3**	0.0	0.0	0.0

PM3 semiempirical calculations have been performed to elucidate the molecular structure of **1/2** and **1/3** complexes. The benzocaine was carefully placed inside of the *p*-sulfonic acid calix[*n*]arene (n = 4, 6) and its structure was fully optimized. Following, single-point calculations at the PBE/6-311G(d) level of theory were performed. Table 2 presents the relative energies between the two possible modes of inclusion (α and β) for both (**1/2** and **1/3**) complexes. The α mode has the ester group of (**1**) inside of the host molecule, and the β mode has the ester group turned to the larger opening border of the host. It is important to highlight that the relative energies of the complexes **1/2** and **1/3** present in Table 2 were calculated by the difference in energy between the configurations (α or β). The entropy and the hydrogen bonding between water molecules and the inclusion compound are not being taken into

account. In face of that, the relative energies at Table 2 have to be analyzed keeping in mind those important solvent effects are being neglected. However, one can argue that as the two systems (α and β) are very similar, the entropy and solvent effects are canceled and the interaction energy term prevails, favoring one of the conformations. For all complexes, the results of the calculations in gas as well as in aqueous phase (PCM) show that the β configuration is favored.

The α**1/2** and β**1/2** optimized structures at the PM3 level of theory are depicted in Figure 6. The most stable structure, β**1/2**, shows an external interaction with the benzocaine amine group near the *p*-sulfonic acid calix[4]arene cone. The structure α**1/2** presents an external interaction between *p*-sulfonic acid calix[4]arene and aromatic hydrogens of benzocaine, whose amine group is far from the cone. In spite of different tries to find a minima with the benzocaine included in the *p*-sulfonic acid calix[4]arene, all calculations converged to the structures shown in Figure 6. One can argue that this is not an inclusion compound, but rather a complex. The steric hindrance due to the smaller inner diameter of the *p*-sulfonic acid calix[4]arene is probably the explanation for the fact that guest (**1**) does not remain included.

In addition, benzocaine interacts with the *p*-sulfonic acid calix[6]arene externally and internally. The α**1/3** and β**1/3** optimized structures are presented in Figure 7. The β**1/3** complex, which is the most favorable (right side of Figure 7), has the benzocaine located out of the ring with the amine group facing the larger opening of the cone. However, the α**1/3** presents the benzocaine inserted completely within the *p*-sulfonic acid calix[6]arene.

It can be seen that the distances between the H-**2** and H-**3** in the aromatic ring of benzocaine and the H-**3** of the *p*-sulfonic acid calix[6]arene are in good agreement with the ROESY experiments (Table 3) specially for the **1/3** complex. The structures shown in Figures 6 and 7 are reasonable and explain the NMR experiments. The theoretical calculations show that *p*-sulfonic acid calix[6]arene forms an inclusion compound with benzocaine which has its ester group facing the smaller opening of the cone. The results point out to a non-inclusion complex formation between benzocaine (**1**) and the *p*-sulfonic acid calix[4]arene (**2**).

In vitro *cytotoxic tests*

Cytotoxic tests were performed as described previously, using mouse 3T3 fibroblasts incubated with benzocaine

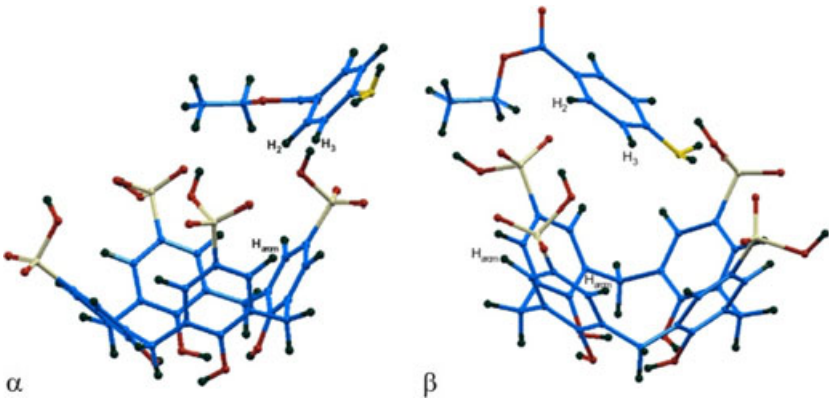

Figure 6: α**1/2** and β**1/2** optimized structures, respectively, at the PM3 level of theory.

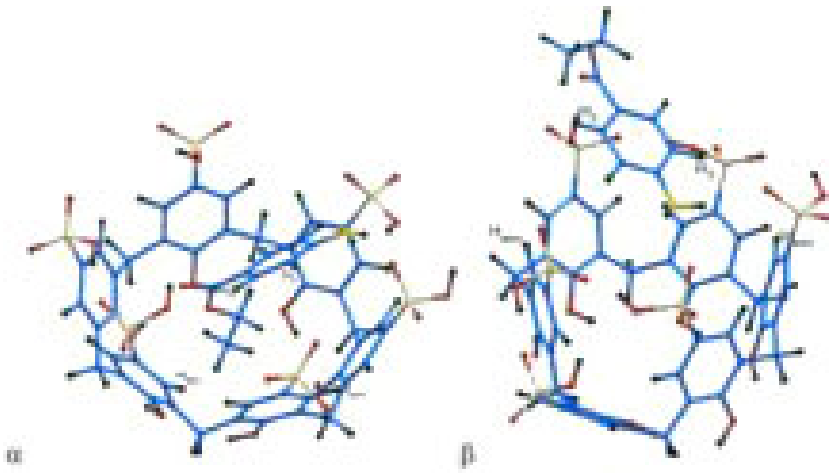

Figure 7: α**1/3** and β**1/3** optimized structures, respectively, at the PM3 level of theory.

Table 3: Intermolecular distances (Å) between the hydrogens of the benzocaine and of the *p*-sulfonic acid calix[*n*]arenes calculated at the PBE/6-311G(d)//PM3 level of theory

		Benzocaine		
	p-sulfonic acid calix[*n*]arene	H-**2**	H-**3**	H-**4'**
α**1/2**	H-**3**	4.56	6.14	5.47
	CH_2	6.68	7.98	7.83
β**1/2**	H-**3**	5.87	5.00	4.75
	CH_2	8.01	6.84	6.54
α**1/3**	H-**3**	3.82	4.04	5.51
	CH_2	5.26	6.30	3.61
β**1/3**	H-**3**	5.30	4.58	6.85
	CH_2	7.25	7.15	8.67

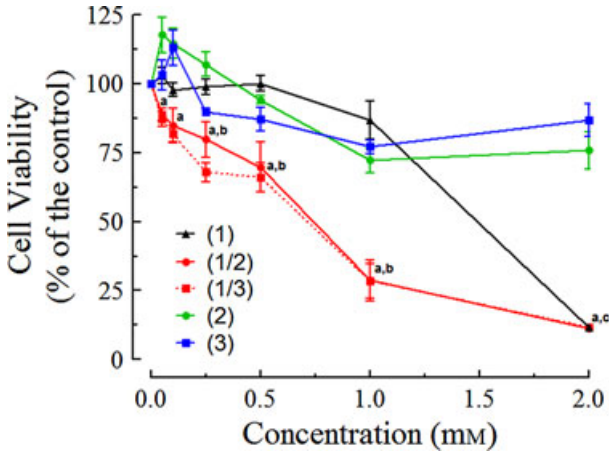

Figure 8: Cell viability of 3T3 cells treated with benzocaine (**1**), *p*-sulfonic acid calix[4]arene (**2**), *p*-sulfonic acid calix[6]arene (**3**), and their inclusion complexes (**1/2** and **1/3**). 3T3 cells were treated with different concentrations of the compounds for 24 h, and cell viability was measured by MTT assay. Non-treated cells were considered as 100% viability. Values are represented as mean ± SEM (n = 6). Statistically significant differences in each concentration were assessed by ANOVA followed by Tukey's test: (A) $p < 0.05$, (**1/2**) and (**1/3**) versus (**2**) and (**3**); (B) $p < 0.05$, (**1/2**) and (**1/3**) versus (**1**); and (C) $p < 0.05$, (**1**) versus (**2**) and (**3**).

alone (**1**), *p*-sulfonic acid calix[4]arene and *p*-sulfonic acid calix[6]arene-benzocaine complexes (**1/2** and **1/3**, respectively), and free *p*-sulfonic acid calix[4]arene (**2**) and *p*-sulfonic acid calix[6]arene (**3**). Calix[*n*]arenes showed low toxic effects even at the higher concentrations tested (Figure 8). The low toxicity of calix[*n*]arenes has also been proved in previous studies (44,45), demonstrating their safety for use as drug carriers. Both *p*-sulfonic acid calix[*n*]arene-benzocaine complexes were more cytotoxic than the free benzocaine at almost all concentrations tested (Figure 8). As both therapeutic (1,46) and cytotoxic effects (47,48) of local anesthetics are related to their ability to diffuse across biomembranes, these results indicate that the anesthetic effect of benzocaine may also be improved by its complexation with *p*-sulfonic acid calix[*n*]arenes.

Local anesthetics act by binding to the sodium channel protein of neuronal membranes, inhibiting Na^+ uptake and blocking the nervous impulse (46,49,50). Their ability to diffuse across the lipid-rich nerve membrane and access target receptors is greatly influenced by their lipophilicity: the higher lipophilicity, the greater partitioning into the membrane (51,52). On the other hand, the poor water solubility strongly influences the bioavailability of local anesthetics. In an electron paramagnetic resonance study conducted with nine ionizable local anesthetics, de Paula and Schreier (49) showed that the amount of neutral form species bound to the membrane (and thus their maximum fluidizing effect) was restrained by low water solubility of the anesthetics in such a way that when aqueous concentration corresponding to the drug solubility was reached, such saturation limited also the partitioning into the membrane (49). As pure *p*-sulfonic acid calix[4]arene and *p*-sulfonic acid calix[6]arene presented very low cytotoxic effect (Figure 8), the higher cytotoxicity of the complexes may be due to increased water solubility of benzocaine by its complexation with calix[*n*]arenes. In fact, increasing the solubility of poorly water soluble drugs by means of their complexation with water soluble *p*-sulfonic acid calix[*n*]arenes and *p*-sulfonate calix[*n*]arenes has been widely demonstrated (20–23).

In practice, for local anesthetics which possess ionizable terminal amino groups in their chemical structure, the bioavailability is improved using the drugs in the protonated and more hydrosoluble form (51,52). Benzocaine, which does not possess such ionizable amino group, can exist at physiological conditions only as a neutral, poor water soluble molecule. Thus, the clinical use of benzocaine is limited to topical formulations, because its low solubility in water hinders its use by parenteral route (53).

Such drawback may be overcome through the development of carrier systems. Improvement in bioavailability and therapeutic efficacy of benzocaine by its encapsulation in β-cyclodextrin and polymeric nanocapsules has been demonstrated (53,54). In the present work, we showed that *p*-sulfonic acid calix[*n*]arenes can form stable inclusion complexes with benzocaine. Also, results from *in vitro* cytotoxic tests showed that complexation with water soluble calix[*n*]arenes may improve the ability of benzocaine to diffuse across biomembranes. Together, these data indicate that *p*-sulfonic acid calix[*n*]arenes may also constitute a promising alternative for the development of new carrier systems for benzocaine.

Conclusions

The host–guest complexes formed between benzocaine (**1**) and *p*-sulfonic acid calix[4]arene (**2**) or *p*-sulfonic acid calix[6]arene (**3**) were correctly predicted by both NMR measurements and PBE/6-311(d)//PM3 calculations. ^{1}H NMR was the technique used to experimentally evaluate

the supramolecular complex (host–guest) formation which proved to be an effective tool to elucidate various aspects of the complexes formed in solution. Employing ^{1}H NMR and the Job plot method, the stoichiometry of the complexes were determined to be 1:1, for benzocaine in both hosts. The binding constants for **1/2** and **1/3** were determined as 835 and 1393 per M, respectively, showing a stronger association between benzocaine and *p*-sulfonic acid calix[6]arene. These finds were corroborated by data from ^{1}H NMR ROESY spectroscopy, which showed that only *p*-sulfonic acid calix[6]arene (**3**) is able to form inclusion complex with benzocaine. The topology of the complexes obtained by ROESY experiments was confirmed by theoretical calculations. In an attempt to rationalize these data, it can be concluded that both NMR data and theoretical calculations were found to be reliable for the determination of the preferred topology of the host–guest complexation. *In vitro* cytotoxic tests showed that complexation with *p*-sulfonic acid calix[*n*]arene improves the bioavailability of benzocaine, possibly by enhancing its water solubility and partitioning into 3T3 fibroblast cell membranes. Altogether, these results show that calix[*n*]arenes may constitute promising alternatives for the development of new pharmaceutical formulations for benzocaine.

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Conflict of Interest

The authors declare no financial/commercial conflict of interest.

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