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Complexes of Fluconazole with Sodium *p*-Sulfonatocalix[*n*]arenes: Characterization, Solubility and Antifungal Activity

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Aiming at providing new formulations capable of improving the biopharmaceutical properties of fluconazole, we studied the formation of host-guest complexes of this antifungal agent with the water-soluble sodium *p*-sulfonatocalix[4]arene and sodium *p*-sulfonatocalix[6]arene. The formation of inclusion complexes was first investigated using ^{1}H NMR spectroscopy and TGA experiments. The complexes' stoichiometry, apparent binding constants (*K*a) and the complexed populations (%p_{bound}), were determined, using ^{1}H NMR data. The topology of the complexes were assessed by 1D ROESY experiments, and the data were corroborated by semi-empirical and DFT calculations. The activity of fluconazole-calix[*n*]arene complexes against clinically relevant fungal species was investigated. Finally, the effect of complexation on the solubility of fluconazole was investigated using the phase-solubility method of Higuchi and Connors.

Introduction

The last two decades have witnessed a dramatic increase in the incidence of fungal infections, mainly in the case of patients with AIDS and patients receiving immunosuppressant therapies, such as cancer patients and transplant recipients. In spite of these facts, fungi have remained some of the most neglected pathogens in terms of new drug discovery.[1,2]

For nearly 30 years, the high nephrotoxic antifungal amphotericin B was the only drug available for treating serious fungal infections. In the late 1980s and early 1990s, the distribution of azole antifungals, first ketoconazole and later the first generation of triazole compounds, fluconazole (**1**) (Fig. 1) and itraconazole, constituted a great advance in the treatment of invasive fungal infections.[3,4]

Azoles inhibit two cytochrome P450 (CYP) enzymes, lanosterol 14 α-demethylase and 22 Δ-desaturase, involved in the biosynthesis of ergosterol, the major lipid component of the fungal cell membrane.[2] The broad spectrum of antifungal activity and the high safety profile of these triazole compounds, particularly fluconazole, have led to their extensive clinical application. Currently, five classes of antifungal agents for treating fungal infections in human are available, and the azoles remain the most widely used.[5,6]

Fluconazole presents fewer side effects and is the first-line drug for the treatment of a range of fungal infections, as well as for prophylaxis in immunosuppressed patients.[5] However, the low aqueous solubility of fluconazole may limit its bioavailability and consequently its therapeutic efficacy.[8,9]

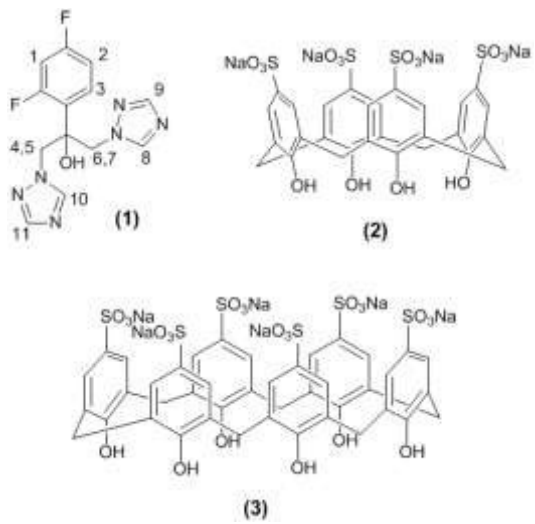

Fig 1 Chemical structure of fluconazole (**1**), sodium *p*-sulfonatocalix[4]arene (**2**) and sodium *p*-sulfonatocalix[6]arene (**3**).

One alternative to overcoming the poor water solubility of drugs is the development of supramolecular carrier systems using complexing agents capable of forming hydrosoluble inclusion complexes. In this context, cyclodextrins and calix[*n*]arenes have been the most studied carriers, and the ability of such supramolecules to form inclusion complexes capable of increasing the water solubility of a variety of drugs has been well documented.[10-15] The ability of *β*-cyclodextrin to

increase the water solubility of fluconazole by inclusion complex formation has been demonstrated.[16] However, no attempts were made, until now, to investigate the effects of water-soluble calix[*n*]arenes on the dissolution of this antifungal agent.

Calix[*n*]arenes are macrocyclic cavity-shaped molecules consisting of phenolic units linked by methylene or sulfur groups at the 2,6-positions, with defined upper and lower rims and a central annulus. Due to their variable conformation and cavity size, calix[*n*]arenes can act as hosts to form inclusion complexes with a variety of ions and neutral molecules. Additionally, the ease of introduction of different functional groups at both the upper and lower rims allows for the synthesis of a great variety of calix[*n*]arenes derivatives. Among them, water-soluble sodium *p*-sulfonatocalix[*n*]arenes (Fig. 1) have been widely studied as platforms for improving drug solubility and efficacy.[11,15] *p*-Sulfonatocalix[*n*]arenes can be promptly synthesized in large amounts and are more hydrosoluble than cyclodextrins which makes theses macrocycles promising candidates for the development of drug carries for low water soluble drugs.[17-19] In addition, a series of studies have shown that calix[*n*]arenes derivatives, including the sulfonated calix[*n*]arenes, present very low toxic effects, which is an important prerequisite for novel therapeutic agents.[20,23]

The aim of the present work was to investigate the formation of inclusion complexes of **1** with sodium *p*-sulfonatocalix[4]arene (**2**) and sodium *p*-sulfonatocalix[6]arene (**3**) (Fig. 1) and to determine the effects of complexation on drug solubility. Using ^{1}H NMR Job plot and diffusion-ordered spectroscopy (DOSY) methods, we determined the stoichiometry, the apparent binding constants (*K*a) and complexed populations (%p_{bound}). Information about the topology of the complexes was obtained by ^{1}H NMR ROESY 1D, and the effect of the sodium *p*-sulfonatocalix[*n*]arenes on the solubility of fluconazole was investigated.

Results and discussion

^{1}H NMR Experiments

We started our investigation by analysing the complexation-induced hydrogen chemical shifts (Δδ) in the **1/2** and **1/3** complexes and comparing these values with those for free **1** (Fig. 1). Small chemical shift differences were observed in the ^{1}H NMR spectra of fluconazole (Table 1 and Fig. 2), preliminarily indicating the formation of complexes.

Complexation with both **2** and **3** induced similar shielding effects, mainly in H-2 and H-1,3 of **1**, suggesting that the aromatic portion of fluconazole is inserted in the cavities of the hosts. Fluconazole hydrogen atoms were observed as a single resonance because of the fast exchange between free and complexed guest molecules on the NMR time scale (Fig. 2).

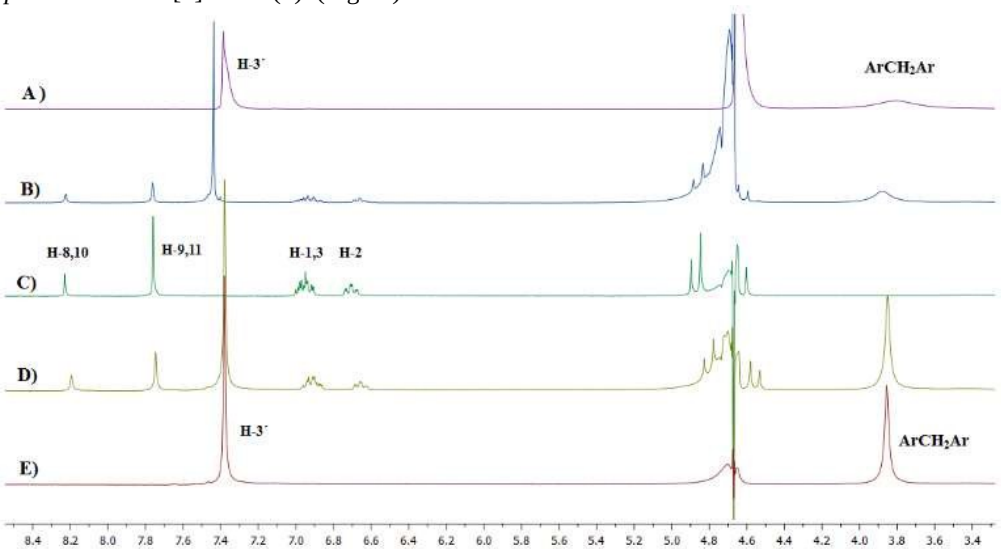

Fig. 2 ^{1}H NMR spectra (300.069 MHz; D_2O; δ_{HDO} 4.67; 298 K , 4 mmol L^{-1} each) of **A)** sodium *p*-sulfonatocalix[4]arene (**2**); **B)** fluconazole/sodium *p*-sulfonatocalix[4]arene (**1/2**); **C)** fluconazole (**1**); **D)** fluconazole/sodium *p*-sulfonatocalix[6]arene (**1/3**); and **E)** sodium *p*-sulfonatocalix[6]arene (**3**).

Table 1 ^{1}H NMR chemical shifts (δ) and chemical shift differences ($\Delta\delta = \delta_{\mathbf{1}free} - \delta_{\mathbf{1}complex}$) of the pure **1** and its complexes with **1/2** and **1/3***

Hydrogens of **1****	**1**	**1/2**	**1/3**	**1/2**	**1/3**
	δ	δ	δ	$\Delta\delta = \delta_{\mathbf{1}free} - \delta_{\mathbf{1}complex}$	$\Delta\delta = \delta_{\mathbf{1}free} - \delta_{\mathbf{1}complex}$
H-2	6.71	6.66	6.66	0.05	0.05
H-1,3	6.95	6.93	6.91	0.02	0.04
H-9,11	7.76	7.76	7.75	0.00	0.01
H-8,10	8.23	8.22	8.20	0.01	0.03

* Concentration of fluconazole and calix[*n*]arenes = 4 mmol L^{-1} each.
** The chemical shift value of hydrogens H-4,5 and H-6,7 could not be determined because of the overlap between their signal and that from HDO.

The complexes stoichiometry was than determined by means of the Job plot method.[24] The Job's curves showed a maximum at a molar fraction of 0.5 (Figs. 3; panels A and B), evidencing a 1:1 stoichiometry for both the complexes.[24]

Further studies employing ^{1}H NMR DOSY experiments[25,26] provided additional evidence for complexation of **1** with both **2** and **3**. The DOSY technique separates the ^{1}H NMR signals of different compounds within a mixture according to their diffusion coefficients. Thus, comparing the diffusion coefficients of the compounds in the mixture with those of the pure species allows for determining the molar fractions of the free and complexed forms.[27,28] The DOSY spectra are presented as supplementary information (Fig. S1-S5). The results showed reduction in the diffusion rate of **1** in the presence of both **2** and

3 (Table 2), indicating the formation of complexes. Taking into account that the studied system is under fast equilibrium on the NMR scale, both chemical shifts and diffusion coefficients for **1** are the average values of the free and bound species. From these diffusion coefficients and applying a well-established methodology[29], the complexed population (%p_{bound}) and apparent binding constants (*K*a) of the complexes were calculated (Table 2). The values of *K*a showed that, although a weak association takes place between **1** and both **2** and **3** (Table 2), the **1/3** complex is slightly more stable than the **1/2**.

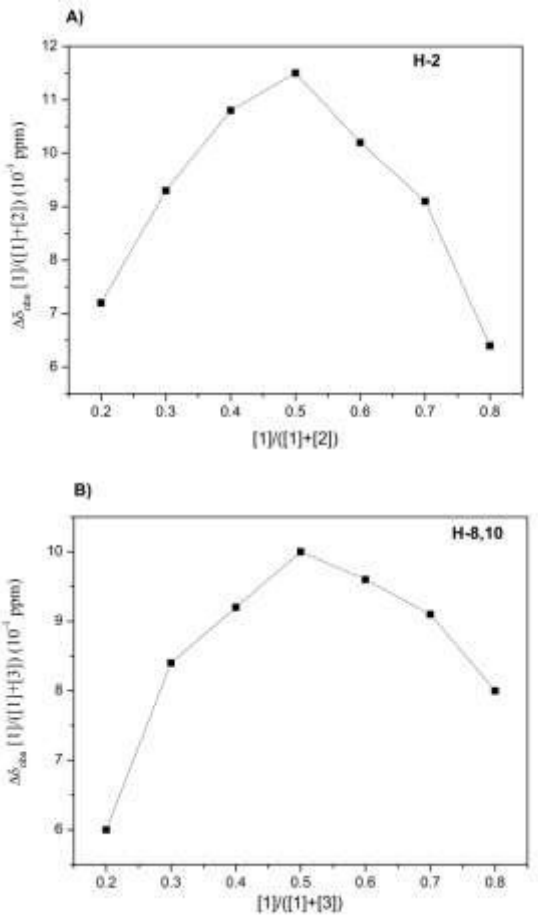

Fig. 3 Job plots for the complexes formed. **A) 1/2** complex; **B) 1/3** complex. Experiments performed at 25 °C in D_2O.

These data indicates that the inclusion complexation of **1** with **2** and **3** may be influenced by the cavity diameter of the hosts. Formation of **1/2** inclusion complex may be hindered by the small cavity diameter of **2** (3.0 Å).[30] Contrarily, the diameter of the cavity of **3** (7.6 Å)[30] may be large enough to allow for an effective inclusion of **1** and the formation of the **1/3** host-guest complex. Thus, the **1/3** complex can be stabilized by π-π interactions between the aromatic rings of both host and guest, resulting in a higher association constant for the **1/3** complex. Thus, the inclusion complex of **1** with **3** could also be stabilized by hydrogen bounds between fluorine atoms of **1** and non-dissociated hydroxyl groups of **3**. This is also supported by the preliminary ^{1}H NMR results, which indicated that the benzene ring of fluconazole is inserted into the cavity of **3** (Fig. 2). It is worth mentioning that the *K*a value of the **1/3** complex (240 M^{-1}) is similar to those obtained in previous works for the 1:1 inclusion complexes of fluconazole with *β*-cyclodextrin (132 M^{-1}) and hydroxypropyl-*β*-cyclodextrin (34.6 M^{-1}).[16,31,32] Also, the geometry of the **1/3** complex was found to be very similar to those found for the complexes between the *β*-cyclodextrins and fluconazole. Indeed, ^{19}F NMR[31], and ^{1}H NMR[16] and 2D ROESY studies indicated that the difluorophenyl ring of **1** is included into the cavity of cyclodextrins, allowing for hydrogen-bonding between fluorine atoms and hydroxyl groups at both ends of the hosts. A series of studies have shown that different *β*-cyclodextrin derivatives may constitutes useful platforms for the controlled release of fluconazole.[31,32] In this way, the similarity between the stability and geometries of the *p*-sulfonatocalix[6]arene- and cyclodextrins-fluconazole complexes is a preliminary indicative that water soluble calix[*n*]arenes may also constitute promising carries for delivery of fluconazole.[16]

Such "host-size selectivity" has been demonstrated in the inclusion complexes formation of calix[*n*]arenes and cyclodextrins.[30,33-36] Both are cavity-shaped macromolecules presenting an hydrophobic central annulus with hydrophilic groups at the edges,[30] capable of forming host-guest by means of non-covalent intermolecular interactions such as a hydrogen bond, ion pairing, and hydrophobic interactions.[11,12,37-39] As example of such "host-size selectivity", Stone and co-workers[36] found that complexes of 20 natural aminoacids with derivatized calix[4]arenes were weaker than those with the corresponding calix[6]arene derivatives, mainly because formation of hydrogen bonds between the ammonium group of the protonated amino acid and the phenolic groups of the hosts is hindered by the small cavity size of the derivatized calix[4]arenes.

Table 2 Diffusion coefficients of pure **1**, **2**, **3** and **1/2** and **1/3** complexes in D_2O (4 mmol L^{-1} samples, 298 K)

Complex	Compound	D (10^{-10} m^2 s^{-1})	D/D_{H2O}	%p	Ka (M^{-1})
	1	5.35	2.93		
	2	2.42	1.41		
	3	2.12	1.23		
1/2	**1**	4.21	2.45	31.5	167
	2	2.43	1.41		
1/3	**1**	3.97	2.30	37.5	240
	3	2.15	1.25		

^{1}H NMR 1D ROESY

Rotating frame Overhauser effect spectroscopy experiments (ROESY) is one of the most powerful tools to study intermolecular interactions. This technique was used to map interactions host-guest between **1/2** and **1/3** complexes to establish its geometry in solution. The experiments were performed according to the literature.[14,40-44]

Although we observed small chemical shift differences of the guest **1** in the complex **1/2**, no rOe increment was observed. These results indicated the formation of inclusion compound but rather an association complex.[14,43] The observed rOe increments between hydrogen H-1 and H-2 of **1** and aromatic hydrogens of **3** demonstrated that the aromatic group of **1** occurred inside of **3** (Fig. S6). This findings is in accordance with our previous supposition that the **1/3** complex is stabilized by both π-π interactions and hydrogen bonding resulting from transferring the poor water soluble fluconazole from the polar D_2O environment to the hydrophobic cavity of **3**.

Thermal Analyses

The thermogravimetric curves obtained for pure **1** and **2**, physical mixture **1+2**, complex **1/2**, pure **3**, physical mixture **1+3**, **1/3** are shown in Fig. 4. Pure fluconazole shows thermal decomposition starting at 190 °C, with a total mass loss in 287 °C. Pure **2** (Fig. 4A) and **3** (Fig. 4B) exhibit higher thermal stability, with less than 20% weight loss, up to 400 °C.

A comparison of the TGA curves obtained for the complexes **1/2** (Fig. 4A) and **1/3** (Fig. 4B) with those of the corresponding physical mixtures **1+2** and **1+3** shows that the complexes exhibit a different profile of thermal decomposition, confirming the complexation. Additionally, it is possible to verify that the formation of complexes with sodium *p*-sulfonatocalix[*n*]arenes provides a gain in thermal stability for fluconazole.

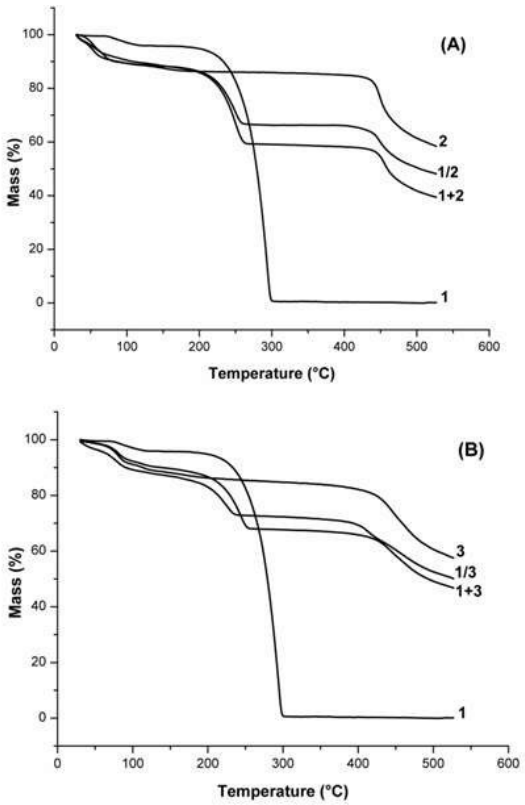

Fig. 4 Thermogravimetric analyses for fluconazole (**1**), sodium *p*-sulfonatocalix[4]arene (**2**), fluconazole/sodium *p*-sulfonatocalix[4]arene physical mixture (**1+2**), fluconazole/sodium *p*-sulfonatocalix[4]arene complex (**1/2**), sodium *p*-sulfonatocalix[6]arene (**3**), fluconazole/sodium *p*-sulfonatocalix[6]arene physical mixture (**1+3**) and fluconazole/sodium *p*-sulfonatocalix[6]arene complex (**1/3**). Experiments performed with ~ 2.00 mg of sample under a dynamic N_2 atmosphere (100.0 mL min^{-1}) at a heating rate of 10.0 ºC min^{-1} in the temperature range of 30.0-525.0 ºC.

Theoretical Results

In the present work, theoretical calculations were carried out in order to investigate the formation process of inclusion complexes formed by **2** and **3** with **1**. Our main objective was to design the structure and energetic properties of the inclusion compounds, which could be useful on the prediction of the most favourable **1/2** and **1/3** complexes. A detailed understanding of the intermolecular interactions between the host and guest species at a molecular level can be very important to support the elucidation of experimental findings.

The structures of the inclusion complexes **1/2** and **1/3** optimized at the PM3 level of theory are shown in Figs. 5 and 6. Table 3 contains the interaction energies (ΔE) calculated at the BLYP/6-31G(d, p)//PM3 level for the inclusion process. As can be seen, the most energetic favourable inclusion complex was the **1/3**, being in both gas and aqueous phase more stabilized due to intermolecular interactions than the **1/2** complex by a large amount. The considerable difference in stability can be attributed to four main reasons: (i) the depth of inclusion, (ii) the cavity size of calix[*n*]arene, (iii) the formation of hydrogen bonds between host and guest species and (iv) the formation of T-shaped π-stacking intermolecular interaction between benzene rings of host and guest molecules.

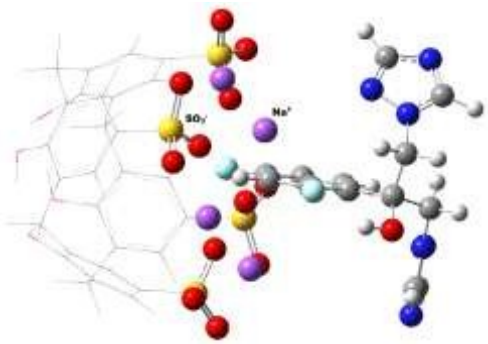

Fig. 5 PM3 fully optimized geometry of the 1:1 inclusion complex of **1** and **2**.

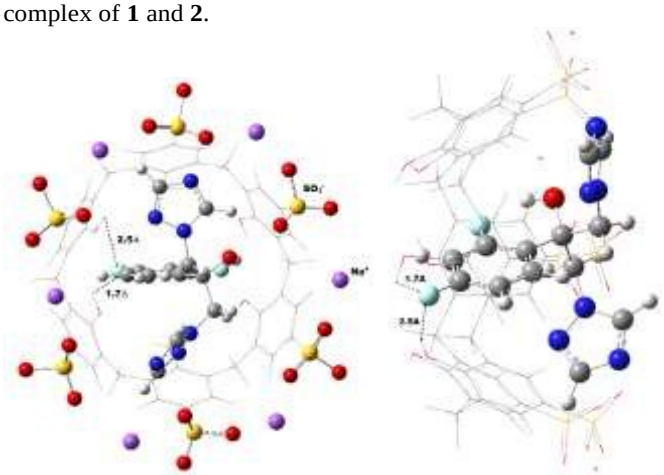

Fig. 6 PM3 fully optimized geometry of the 1:1 inclusion complex of 1 and 3 in both front and side view respectively. The hydrogen bond distances are quoted, and dotted lines were drawn to ease the visualization of the H-bonds in front and side view, respectively.

Table 3 DFT/BLYP interaction energies (ΔE) for the formation of the (**1/2**) and (**1/3**) complexes

Complexes	ΔE (kcal.mol-1)	
	Gas phase	Aqueous phase
(**1/2**)	-7.39	-9.43
(**1/3**)	-22.65	-25.91

Fig. 5 clearly shows that fluconazole does not form an inclusion complex **2**, but association or adsorption like complex. We believe that due to the small size of the cavity of **2** and the high electronic and steric repulsions experienced by the guest in the

cavity, the **1** molecule prefers to remain outside **2**, thereby minimizing such repulsions and forming a type of association complex. This preference may partly explain the fact that this complex does not appear to exhibit increased solubility, as indicated by experimental data.

On the other hand, Fig. 6 shows that fluconazole forms an inclusion complex with the **3**. Because **3** possesses a larger cavity than **2**, the molecule **1** can be spatially accommodated inside the cavity without steric repulsions forming strong intermolecular interactions, such as H-···F hydrogen bonds, and this placement stabilizes the complex (see magnified image in Fig. 6). Moreover, as shown in Fig. 7, this complex is also stabilized by T-shaped π-stacking intermolecular interactions between benzene rings of guest and host molecules. The benzene rings of fluconazole is perpendicular to two benzene rings of sodium **3**, characterizing this interaction as a T-shaped π-stacking interaction.[36] These configurations may explain why the **1/3** complex presents increased solubility, as observed experimentally, because at the molecular level, fluconazole 1 is fully enclosed in the cavity of **3**.

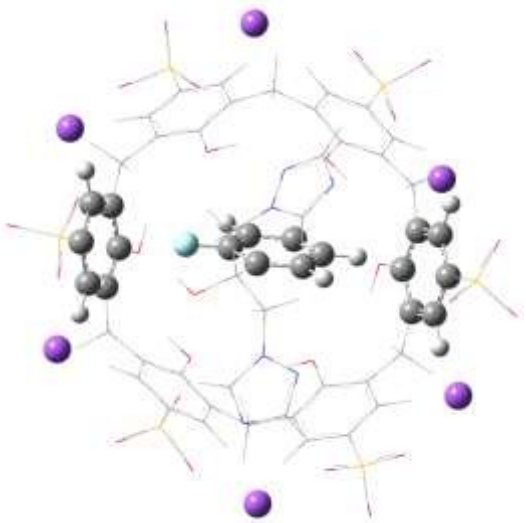

Fig. 7 PM3 fully optimized geometry of the 1:1 inclusion complex **1/3** showing the T-shaped π-stacking intermolecular interaction. The benzene rings of host and guest are highlighted to ease visualization.

The solvent effect was also taking to account using the PCM method.[37] The results confirms the previous conclusion observed in the gas phase regarding the energetic stability. As shown in Table 2, the presence of the solvent environment (water) increases the stabilization of the 1:1 **1/2** and **1/3** complexes by approximately 3.0 kcal mol^{-1} relative to that indicated by the gas phase results.

It should be also considered that experimental ^{1}H NMR and small K_a value data indicate weak intermolecular interactions that would be in line with an association mode of complexation. In the present work an extensive search for minimum energy structures on the potential energy surface (PES) for the **1/3** complex, which would locate an adsorption like structure similar to **1/2** molecule, have not been attempted, since it is a very demanding computational task. A comprehensive search for local minimum would only improve quantitatively the agreement between experimental and theoretical predictions, but the main conclusion would be unaltered.

In this theoretical study, we reported the energetic properties for the formation of the **1/2** and **1/3** complexes at a molecular level, which corroborate with the experimental data. Besides, a meticulous structural analysis pointed out that strong hydrogen bonds and π-stacking intermolecular interactions of fluconazole with the hydroxyl groups of **3**, which can explain the stability of fluconazole and, consequently, its increase in solubility detected experimentally.

It should also be stated that the model employed in this work considers calix[*n*]arenes as empty receptors, as has been successfully used in our previous work on the interaction with isoniazid, a first line antituberculosis drug, with sodium *p*-sulfonatocalix[4]arene and sodium *p*-sulfonatocalix[6]arene,[43] adding strong confidence to use a similar model in the present study. However, as have been shown very recently by Francisco and co-workers[64], in an experimental investigations on the effect of the alkali and transition metal cations on the formation of host-guest complexes with the water-soluble sodium *p*-sulfonatocalix[4]arene, reporting the formation of ternary 1:1:1 complexes, taking into account the mobility and explicit interactions of the cation would result in an improved theoretical model to describe the inclusion compound formation. We recognize the relevance of this approach, but such theoretical modeling is far beyond the scope of the present work.

Phase Solubility Studies

Phase solubility profiles of **1** in the presence of increasing concentration of **2** and **3** are shown in Fig. 8. Only **3** was capable of increasing the solubility of **1**, demonstrating that the effect of the calix[*n*]arenes on the solubility of fluconazole depends on the formation of inclusion complex, and thus on size of the calix[*n*]arene cavity. The linear increase in solubility of **1** as a function of increasing the concentration of **3** is also indicative of the formation of 1:1 soluble complexes,[47] thus confirming the stoichiometry of the complex determined by ^{1}H NMR.

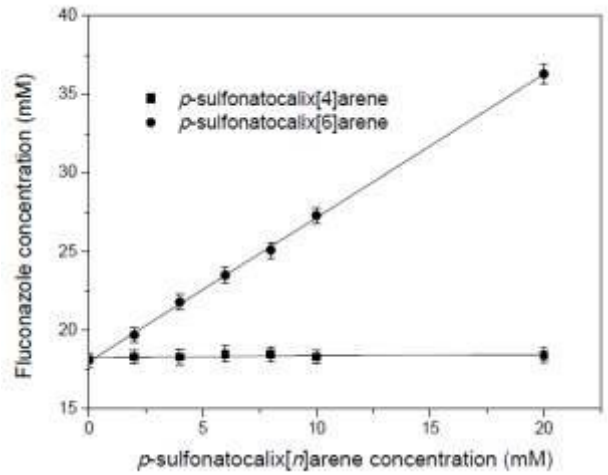

Fig. 8 Phase solubility profile of fluconazole in the presence of sodium *p*-sulfonatocalix[*n*]arenes at 30 ºC.

The effect of the cavity size of water soluble calix[*n*]arenes on the solubilization of poor water soluble drugs has already been demonstrated. Yang and de Villier found that the effect of *p*-sulfonic acid calix[*n*]arenes (*n* = 4, 6 and 8) on the solubilization of nifedipine, furosemide and niclosamide was also influenced by the calix[*n*]arenes' cavity size.[33-35] For furosemide[33] and niclosamide,[35] **3** produced higher solubilizing effects than *p*-sulfonic acid calix[4]arene at acidic aqueous media. However, these authors found that not only the cavity

size of the calix[*n*]arenes, but also pH and conformational changes in the calix[*n*]arenes, influenced the solubilization of the drugs. As we stated in the present work, Yang and de Villiers[33-35] also argued that hydrophobic interactions and hydrogen bonding was the main driving forces in the complexes formation of *p*-sulfonic acid calix[*n*]arenes with nifedipine, niclosamide and furosemide.[33-35]

The effect of **3** on the solubility of fluconazole was also found to be comparable to those produced by both *β*-cyclodextrin and hydroxypropyl-*β*-cyclodextrin.[32] Linear AL-type diagrams were obtained for all these systems, indicating the formation of soluble complexes. Again, these finds the suggestion that, as cyclodextrins, sodium *p*-sulfonatecalix[6]rene is a promising tool for the development of carriers for fluconazole.

Antifungal Activity

It has been regarded that the dissolution and therapeutic efficacy of **1** can be negatively influenced by its poor water solubility.[8-11] To investigate the possible influence of the solubilizing effect of **3** in the in vitro antifungal activity to the drug, free **1**, pure hosts **2** and **3**, and complexes **1/2** and **1/3** we tested against various clinically relevant fungi. Their Minimal Inhibitory Concentration (MIC), defined as the lowest concentration necessary to inhibit fungal growth by 50%, are presented in Table 4.

No antifungal activity was observed for the pure hosts **2** and **3**, while the complexes exhibited antifungal activity very similar to that of pure **1** for almost all tested fungi. Thus, complexation did not improve significantly the efficacy of the drug. Although these tests must be regarded as a preliminary evaluation, two suppositions can be done: a) the *in vitro* activity of **1** may not be limited by its water solubility, and b) despite the solubilizing effect, complexation with **3** did not improve significantly the efficacy of **1**, possibly due to the low *K*a and complexed population (%*p*bound) values. However, the hydrosoluble **1/3** complex may still be a good candidate for *in vivo* tests where the water solubility may be a more important factor for the biological activity of fluconazole.

Table 4 Minimal Inhibitory Concentration (MIC[a], μg mL^{-1}) of fluconazole (**1**), sodium *p*-sulfonatocalix[4]arene (**2**), sodium *p*-sulfonatocalix[6]arene (**3**) and their complexes (**1/2**) and (**1/3**) against clinically relevant fungi

Fungal species	Pure compounds			Complexes	
	1	**2**	**3**	**1/2**	**1/3**
Candida albicans ATCC18804	> 128	> 128	> 128	> 128	> 128
Candida krusei ATCC20298	64	> 128	> 128	64	32
Candida glabrata ATCC90030	32	> 128	> 128	16	32
Candida dubliniensis MYA-646	2	> 128	> 128	2	2
Cryptococcus neoformans H99	8	> 128	> 128	4	8
Cryptococcus gattii ATCC32608	8	> 128	> 128	8	8

[a]MIC values were determined after incubating for 72 h (*Cryptococcus* species) and 48 h (*Candida* species).

Experimental

Chemicals and Reagents. Fluconazole (**1**) (99.5%) and D_2O (99.75%) were purchased from Aldrich. All other reagents were of analytical grade. Sodium *p*-sulfonatocalix[4]arene (**2**) and sodium *p*-sulfonatocalix[6]arene (**3**) were synthesized in our laboratory following procedures reported in the literature.[48-50]

Preparation of Solid Inclusion Complexes. Inclusion complexes **1/2** or **1/3** at a 1:1 M ratio were obtained by mixing 4 mmol L^{-1} aqueous solution of compound **2** or **3** with an aqueous solution of 4 mmol L^{-1} fluconazole. Each system was stirred for 48 h at room temperature, a period of time considered optimal for reaching equilibrium. Each solution was freeze-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, using the signal of residual HDO (δ 4.67 ppm) as reference. Routine 1D ^1H NMR spectra were acquired with a Bruker or Varian spectrometer operating at 400.200 or 300.069 MHz respectively for ^1H (64 k data points, 90o excitation pulse with a duration of 9.75 μs, 128 pulse acquisition, spectral width of 8 kHz, acquisition time of 3.9 s and relaxation delay of 10 ms) in a 5-mm probe under the direct detection mode at room temperature unless stated otherwise.

Determination of Complexation Stoichiometry. The stoichiometry of the complexes was determined by using the Job Plots method.[24] 4 mmol L^{-1} stock solutions of **1**, **2** and **3** were used to prepare a series of solutions containing increasing molar fraction of **1** in the presence of **2** or **3**, while keeping the total concentration constant.

Determination of Association Constants. Association constants were determined by HR-DOSY experiments[25,26] conducted according to the experimental conditions already reported in the literature.[14]

ROE Measurements. The 1D ROESY experiments[31] (Bruker 400 spectrometer) were obtained using a 90° pulse, with a cw spin-lock mixing time of 0.4 s. The pulse sequence used was 1D ROESY. All spectra were acquired with a 5-mm inverse probe at 298 K in 5-mm tubes.

Thermogravimetric Analyses (TGA). Thermogravimetric analyses (TGA) were performed in a STA409EP (Netzsch) thermoanalyser using alumina (Al_2O_3) crucibles with ~ 2.00 mg of sample under a dynamic N_2 atmosphere (100.0 mL min^{-1}) at a heating rate of 10.0 ºC min^{-1} in the temperature range of 30.0-525.0 ºC.

Theoretical Details. A sequential methodology based on Semiempirical (PM3) and Density Functional Theory (DFT)[51] calculations with the BLYP[52] functional and 6-31G(d,p) basis set[53], were performed in order to obtain reliable structures and energetic parameters for the **1/2** and **1/3** complexes. The stoichiometry of the inclusion complexes was assumed, based on previous experimental findings, to be 1:1, which indicates one fluconazole molecule included in one calix[*n*]arene cavity.

The initial guess geometries for the complexes were fully optimized at the semiempirical PM3[54] level of theory, which has been shown to yield very plausible geometrical parameters for organic compounds.[55] PM3 harmonic frequency calculations were also carried out for the equilibrium structures, characterizing them as true minima on the potential energy surface (SEP). Posteriorly, the electronic plus nuclear repulsion energy contribution (ΔE) was estimated by single point BLYP/6-31G(d, p)/PM3 calculations using the fully optimized PM3 geometries. This sequential methodology has been successfully used for cyclodextrins supramolecular complexes.[56-59] Based on quantum mechanical formalism (BLYP/6-31G(d,p)/PM3) the solvent effect was considered using the polarized continuum model (IEFPCM).[60] In the aqueous phase, the solvent environment is replaced by its dielectric constant (for water ε = 78.39). The solute is set in a cavity of appropriated shape to enclose the entire molecule, which is instantly covered in the continuum dielectric. All theoretical calculations were performed on Gaussian 2009 quantum mechanical package.[46]

HPLC Analysis. HPLC analysis of **1** was performed using the methodology developed by Sadasivudu and co-workers (2009).[61] The analysis was carried out with an automated high-performance liquid chromatography (AS1000 autosampler and P2000 pump, Thermo Separation Products, Waltham, MA) with a UV detector (UV3000 detector) set at 263 nm. **1** was separated on a C18 reverse-phase column (RP-18 Li-Chrospher, 5 μm, 150 × 4.6 mm, Supelco, Bellefontaine, PA., USA) using water-acetonitrile (65:35; v/v) as the mobile phase. The column temperature was maintained at 30 ºC, the flow rate was 1 mL per min, and injection volume was 20 μL.

Phase Solubility Studies. The effect of **2** and **3** on the solubility of **1** was investigated according to the phase solubility technique.[47] The aqueous solubility of **1** is 1 mg mL^{-1}.[62] Thus, excess amounts of fluconazole (100 mg) were added to either distilled water or 5 mL of aqueous solutions containing increasing concentrations of the calix[*n*]arenes (0.0-20.0 mM). The suspensions were rotated at 60 rpm at 25 ºC until no further increase in the solubility of fluconazole was observed. Then, aliquots were filtered through a 0.45-μm membrane filter and analyzed by HPLC. Phase solubility diagrams were obtained by plotting the molar concentration of dissolved **1** versus the molar concentration of **2** and **3**.

***In vitro* Antifungal Susceptibility Assay. *Micro-organisms*:** *Candida albicans* ATCC 18804, *Candida krusei* ATCC 20298, *Candida glabrata* ATCC 90030, *Candida dubliniensis* MYA-646, *Cryptococcus neoformans* ATCC H99 and *Cryptococcus gattii* ATCC 32608 were obtained from the American Type Culture Collection (Manassas, VA, USA). All strains are maintained in the Laboratory of Mycology of the Institute of Biological Sciences at the Universidade Federal de Minas Gerais (Brazil) on slants of Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, MI, USA) and subcultured every 2 months. ***Fungal Susceptibility Assay*:** An antifungal assay was carried out as described in Martins and co-workers (2009).[63] Pure **1**, **2** and **3** and complexes **1/2** and **1/3** were tested at concentrations ranging from 128 to 2 μg mL^{-1}. The minimal inhibitory concentration (MIC) endpoint was defined as the lowest tested concentration that significantly reduced (by approximately 50%) fungal growth relative to the growth control for 48 h (*Candida* species) and 72 h (*Cryptococcus* species).

Conclusions

In view of the potential of sodium *p*-sulfonatocalix[4]arene (**2**) and sodium *p*-sulfonatocalix[6]arene (**3**) to increase the solubility and bioavailability of poorly water-soluble drugs, the formation of host-guest complexes between these cone-shaped macrocycles with fluconazole (**1**) was investigated. The formation of the complexes was confirmed by ^{1}H NMR and TGA experiments, and Job plots showed a 1:1 stoichiometry for both complexes. ^{1}H DOSY NMR experiments showed that both **2** and **3** formed weak but stable complexes with **1**, and the apparent binding constants for the **1/3** complex was higher than that for the **1/2** complex.

These results indicate a slightly more stable association between fluconazole **1** and **3**, suggesting that the inclusion complex formation depends on the calix[*n*]arene cavity size. These findings were corroborated both by experimental ^{1}H NMR ROESY data and theoretical calculations, which showed that only **3** is able to form an inclusion complex with **1**, in which the benzene ring of **1** is inserted into the cavity of **3**. The proposed topology of the **1/3** complex indicate that both π-π interactions and hydrogen bonds may be involved in the formation of the complex. These finds were corroborated by phase solubility studies, which showed that only **3** was capable of improving the solubility of **1**. Data from biological assays showed that complexation with **3** did not improve the efficacy of **1**, which implies that the antifungal efficacy of **1** may not be limited by its poor hydrosolubility or the **1/3** complex is not stable enough to influence the efficacy of **1**. Despite this, the **1/3** complex may still be a good candidate for *in vivo* tests, where the water solubility may be a more important factor for the biological activity. Furthermore, *p*-sulfonatocalix[6]arene, which can be easily derivatized, may constitute a promising platform for the development of more efficient hosts for fluconazole and other poor water soluble drugs.

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