European Journal of Pharmaceutical Sciences 47 (2012) 539–548

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Experimental and theoretical NMR determination of isoniazid and sodium *p*-sulfonatocalix[*n*]arenes inclusion complexes

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ARTICLE INFO

Article history:
Received 24 February 2012
Received in revised form 13 July 2012
Accepted 17 July 2012
Available online 25 July 2012

Keywords:
Isoniazid
Calix[*n*]arenes
NMR
Supramolecular chemistry
In vitro evaluation
Tuberculosis

ABSTRACT

In this work the inclusion complex formation of isoniazid with sodium *p*-sulfonatocalix[*n*]arenes is reported aiming to improve the physicochemical and biopharmaceutical properties of isoniazid a first line antibuberculosis drug. The architectures of the complexes were proposed according to NMR data Job plot indicating details on the insertion of the isoniazid in the calix[*n*]arenes cavities. DFT theoretical NMR calculations were also performed for sodium *p*-sulfonatocalix[4]arene complex with isoniazid, with various modes of complexation being considered, to provide support for the experimental proposal. A comparison between experimental and theoretical ^{1}H NMR chemical shifts profiles allowed for the inclusion complex characterization confirming the isoniazid inclusion mode which is preferentially through the hydrazide moiety. The remarkable agreement between experimental and theoretical NMR profiles adds support to their use in the structural characterization of inclusion compounds. Antibacterial activity was evaluated and the results indicated the inclusion complexes as a potential strategy for tuberculosis treatment.

1. Introduction

Tuberculosis (TBs) is an infectious deadly disease caused by the bacillus *Mycobacterium tuberculosis (MTb)*. It has been rising international health concern since TB is responsible for the death of about 2 million people yearly worldwide (Balganesh et al., 2008; Ekins et al., 2011; http://www.who.int/research/en/) and almost 9 million new cases in 2010 according to recent data of the World Health Organization (http://www.who.int/research/en/). Isonicotinic hydrazide (INH, isoniazid), first synthesized by Meyer and Mally in 1912 (Meyer and Mally, 1912), has been used since the 1950s as a first-line antituberculosis drug, characterized by an outstanding minimal inhibitory concentration in the human body (0.1–0.17 mol dm^{-3}) and having a high specificity toward TB bacteria. However, this drug is often administered together with other ones, as currently recommended by international guidelines, once tuberculosis is still resistant, regardless of the existence of multiple classes of antibiotics. Moreover, since it is a neglected disease there is no new approved drug to tuberculosis treatment (Ekins et al., 2011; Meyer and Mally, 1912).

In this scenario, however, new pharmaceutical preparations of isoniazid with prolonged activity and improved properties are very welcome (Terekhova and Kumeev, 2010). Inclusion complexes of therapeutic agents are one of the most attractive areas in fashionable host–guest chemistry and still show great potential in drug delivery. Supramolecular species that can be used in encapsulation include liposomes and host molecules (Arantes et al., 2009; Cabeça et al., 2008; Da Silva et al., 2011; De Araújo et al., 2008; De Fátima et al., 2009; Fernandes et al., 2007; Moraes et al., 2007; Rodrigues et al., 2011), such as crown ethers, cryptates, cyclodextrins and calix[*n*]arenes. It should be noted that, to the best of the authors knowledge, no data on interaction of calix[*n*]arenes with isoniazid exist, while its complexation with β-cyclodextrins derivatives has not been comprehensively and sufficiently investigated (Terekhova and Kumeev, 2010). In that context, the aim of this study was primarily to investigate the capacity of the sodium *p*-sulfonatocalix[4]arene and sodium *p*-sulfonatocalix[6]arene to form complexes with the isoniazid (Fig. 1). We have analyzed the degree of complex formation in an aqueous medium to obtain details on the

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http://dx.doi.org/10.1016/j.ejps.2012.07.015

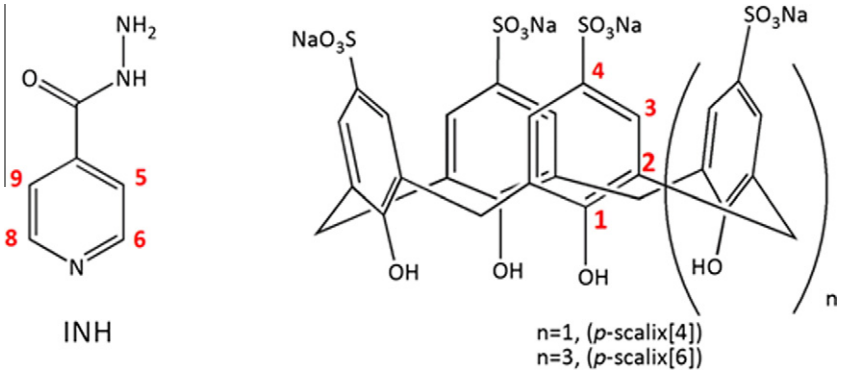

Fig. 1. Chemical structures of isoniazid (INH), sodium *p*-sulfonatocalix[4]arene (*p*-scalix[4]) and sodium *p*-sulfonatocalix[6]arene (*p*-scalix[6]).

stoichiometry and geometry, as determined by different ^{1}H NMR methodologies, and also thermodynamics of the complexation using theoretical calculations.

Supramolecular systems are structured by intermolecular interactions which are the major responsible for the spatial arrangements for the complexes. Therefore none covalent bond is broken or created in these processes and the final result are often a small universe of inclusion complex possibilities (Passos et al., 2011; Schatz, 2004). Hence, the characterization without single-crystal analysis which could provide undoubtedly data about the compound structure often depends on the chemical interpretation of the spectroscopic results as NMR experiments. Due to these features, theoretical studies (Passos et al., 2011; Rodrigues et al., 2011) can be very helpful since a molecular point of view can be accessed, and therefore, a better understanding of the inclusion complexes structures is provided. In the present work, well established quantum chemical methods of calculation are used for the determination of the molecular structure and NMR chemical shifts, in water solution, aiming to a precise characterization of the experimentally obtained inclusion compounds.

The inclusion complexes preparation and also the structural characterization were designed to protect the isoniazid pharmacological activity avoiding its reactions before the desired biological target. Subsequently for the complexes obtained in this study, the Minimum Inhibitory Concentration (MIC) was evaluated against *MTb* bacteria to indicate their potential as pharmaceutical preparations to the TB treatment.

2. Materials and methods

2.1. Chemicals and reagents

Isoniazid (INH) (99%), and D_2O (99.75%) were purchased from Aldrich and Merck, respectively. All other reagents were of analytical grade. Sodium *p*-sulfonatocalix[4]arene (*p*-scalix[4]) and sodium *p*-sulfonatocalix[6]arene (*p*-scalix[6]) were synthesized in our laboratory following literature procedures (Casnati et al., 2004; Gutsche and Bauer, 1985; Shinkai et al., 1987).

2.2. Preparation of solid inclusion complexes

Inclusion complexes (*p*-scalix[4]@INH and *p*-scalix[6]@INH) at 1:1 M ratio were obtained by mixing 10 mmol L^{-1} aqueous solution of calix[*n*]arene hosts with a aqueous solution of 10 mmol L^{-1} isoniazid (INH). 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.

2.3. NMR spectroscopy

All experiments were performed at 298 K in D_2O. Routine 1D: ^{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 μs, spectral width of 6 kHz, acquisition time of 3.3 s and relaxation delay of 10 ms) in a 5-mm probe with direct detection mode at room temperature unless stated otherwise.

2.4. Determination the stoichiometry of complexation

Job plots have been prepared with 10 mmol L^{-1} stock solutions of compounds (INH), *p*-scalix[4] and *p*-scalix[6] (Loukas et al., 1998).

2.5. NOE measurements

The ROESY 1D experiments were obtained with a selective 180° and a non-selective 90° pulse, a mixing time of 0.5 s 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 experiment. All spectra were acquired with a 5 mm inverse probe at 25 °C in 5 mm tubes.

2.6. Differential scanning calorimetry (DSC)

The samples (10 mg) were placed in aluminum pans and the experiments were carried out in a calorimeter (Universal V2.3D TA Instruments) at a 10 °C/min heating rate over a wide range (0–250 °C). An empty pan served as reference and indium was used to calibrate the temperature.

2.7. Antitubercular activity

The antimycobacterial activities of the complexes obtained here were assessed against *M. tuberculosis* ATTC 27294 (Canetti et al., 1963) using the micro plate Alamar Blue assay (MABA) (Franzblau et al., 1998). This methodology is non-toxic, uses thermally-stable reagents and shows good correlation with proportional and BACTEC radiometric methods (Vanitha and Paramasivan, 2004), (Reis et al., 2004). Briefly, 200 μL of sterile deionized water was added to all outer-perimeter wells of sterile 96 well plates (Falcon, 3072: Becton Dickinson, Lincoln Park NJ) to minimize evaporation of the medium in the test wells during incubation. The 96 plates received 100 μL of the Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) and a serial dilution of the compounds was made

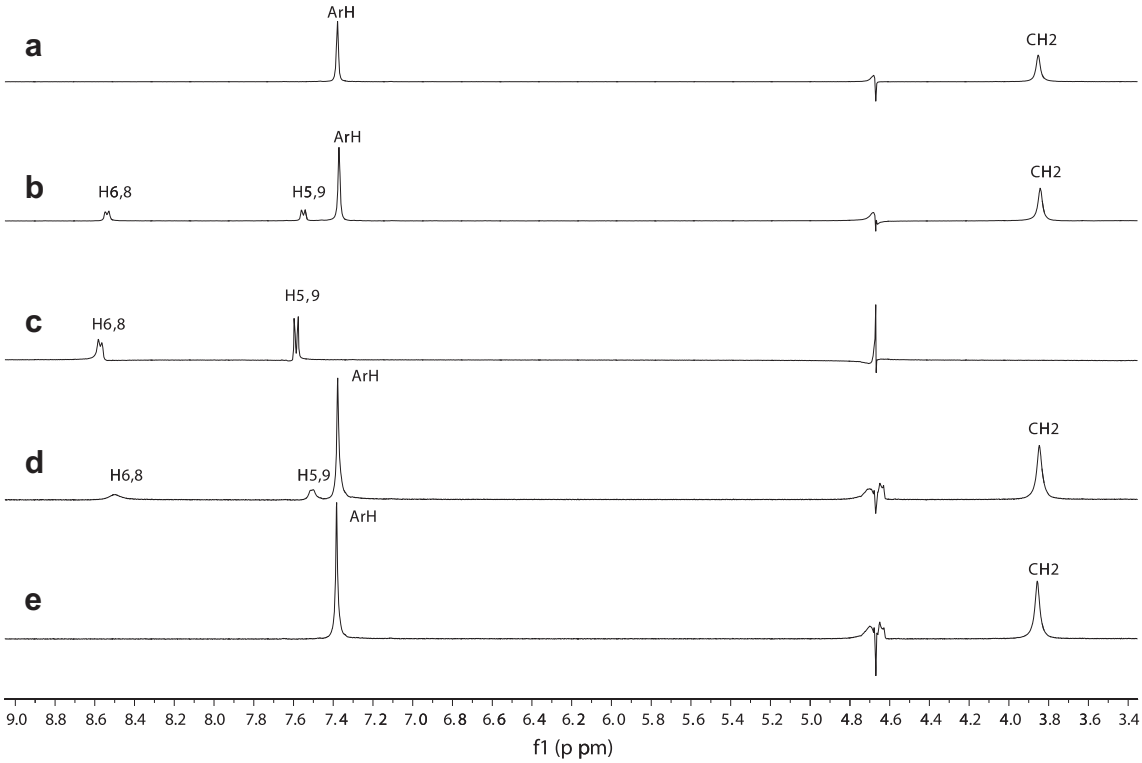

Fig. 2. ^{1}H NMR spectra (300.069 MHz; D_2O; δ_{HDO} 4.67; 298 K, 10 mmol L^{-1} each) of: (a) sodium *p*-sulfonatocalix[4]arene; (b) isoniazid/sodium *p*-sulfonatocalix[4]arene complex; (c) isoniazid; (d) isoniazid/sodium *p*-sulfonatocalix[6]arene complex and (e) sodium *p*-sulfonatocalix[6]arene.

Table 1
^{1}H NMR: chemical shifts (δ/ppm) and chemical shift differences ($\Delta\delta = \delta_{1free} - \delta_{1complex}$) of free isoniazid (INH), sodium *p*-sulfonatocalix[4]arene (*p*-scalix[4]) and sodium *p*-sulfonatocalix[6]arene (*p*-scalix[6]), and also the respective inclusion complexes with INH (*p*-scalix[4]@INH and *p*-scalix[6]@INH) (10 mmol L^{-1} samples, 298 K).

	Hydrogen	H-5,9	H-6,8	CH_2	Ar–H
INH	δ	7.59	8.57	–	–
p-scalix[4]	δ	–	–	3.89	7.45
p-scalix[4]@INH	δ	7.54	7.47	3.84	7.37
	$\Delta\delta$	0.05	0.10	0.05	0.08
p-scalix[6]	δ	–	–	3.86	7.38
p-scalix[6]@INH	δ	7.50	8.50	3.85	7.37
	$\Delta\delta$	0.09	0.07	0.01	0.01

directly on the plate. The final drug concentrations tested were 0.01–10.0 μL/mL. Plates were covered and sealed with parafilm and incubated at 37 °C for five days. After this time, 25 μL of a freshly prepared 1:1 mixture of Alamar Blue (Accumed International, Westlake Ohio) reagent and 10% Tween 80 were added to the plate and incubated for 24 h. A blue[1] color in the well was interpreted as absence of bacterial growth and a pink color was scored as growth.

2.8. Calculations

All the spectroscopic calculations were carried out using Density Functional Theory (DFT) (Parr and Yang, 1989) with the PBE1PBE functional (Perdew, 1986; Perdew et al., 1996) and 6-31G(d,p) (Hariharan and Pople, 1973; Hehre et al., 1972) basis set to optimize geometries and perform frequency analysis to characterize the equilibrium structures as true minima (none imaginary frequencies). All calculations were carried using the Gaussian 03 quantum mechanical package (Frisch et al., 2004). The gauge-independent atomic orbital method, GIAO, (Wolinski et al., 1990) was applied for all calculation of the ^{1}H magnetic shielding constants. The solvent effect (water: $\varepsilon = 78.39$) was accounted for using the polarizable continuum model by the integral equation formalism (IEFPCM) (Cances et al., 1997), in single point calculations on the fully optimized geometries in the vacuum, without the inclusion of explicit solvent molecules to evaluate the magnetic shielding tensor. Recent results reported for epoxide quebrachitol derivatives (de Almeida et al., 2010) provide support to this approach.

[1] For interpretation of color in Figs. 1, 4, 6–8, the reader is referred to the web version of this article.

3. Results and discussion

NMR techniques were employed to obtain detailed information about the interactions between the guest isoniazid (INH) and the calix[*n*]arenes hosts sodium *p*-sulfonatocalix[4]arene (*p*-scalix[4]) and sodium *p*-sulfonatocalix[6]arene (*p*-scalix[6]) in aqueous solution. The ^{1}H NMR spectra for isoniazid in D_2O in the presence or absence of calix[*n*]arenes were obtained and are presented in Fig. 2. Isoniazid hydrogens were observed as a single resonance because of fast exchange between free and complexed guest molecules on the NMR time scale (Fig. 2). The numbers corresponding to the NMR signals of isoniazid, sodium *p*-sulfonatocalix[4]arene and sodium *p*-sulfonatocalix[6]arene are shown in Table 1 and also in Fig. 1.

We started our investigation by analyzing the complexation-induced hydrogen chemical shifts ($\Delta\delta$) on the *p*-scalix[4]@INH and *p*-scalix[6]@INH complexes, in comparison to those of free INH. Complexation between isoniazid and *p*-scalix[4] or *p*-scalix[6] induced shielding effects in all the hydrogen atoms of INH, H-**5**,**9** and H-**6**,**8** ($\Delta\delta$ 0.08; 0.06 (*p*-scalix[4]@INH) and 0.09; 0.07 (*p*-sca-

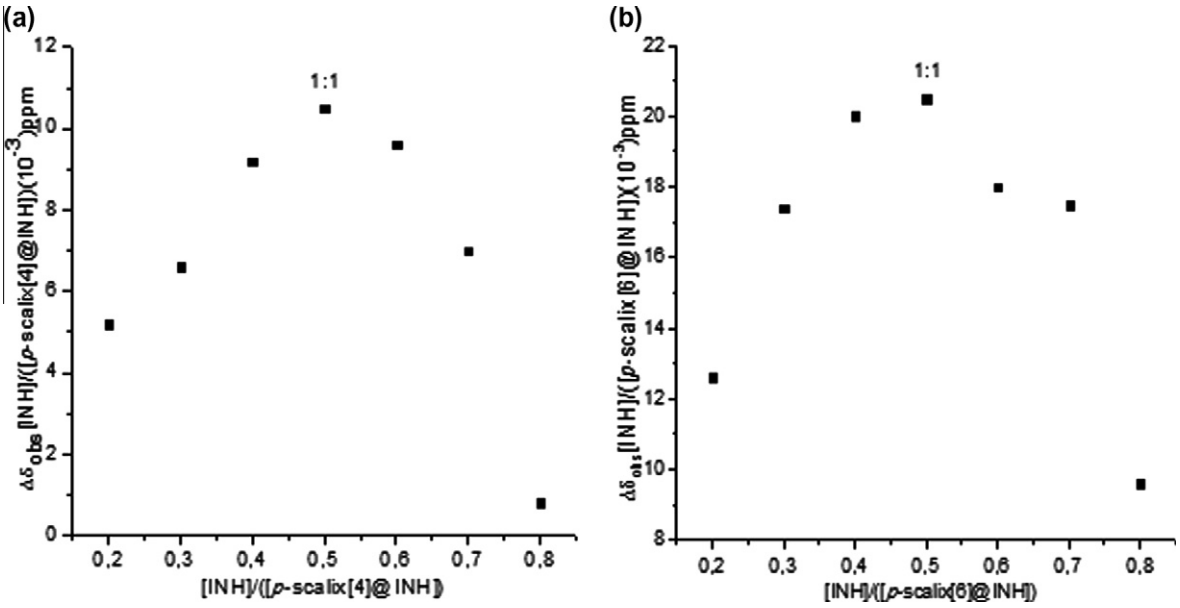

Fig. 3. Job plots for the inclusion complexes. (a) *p*-scalix[4]@INH and (b) *p*-scalix[6]@INH.

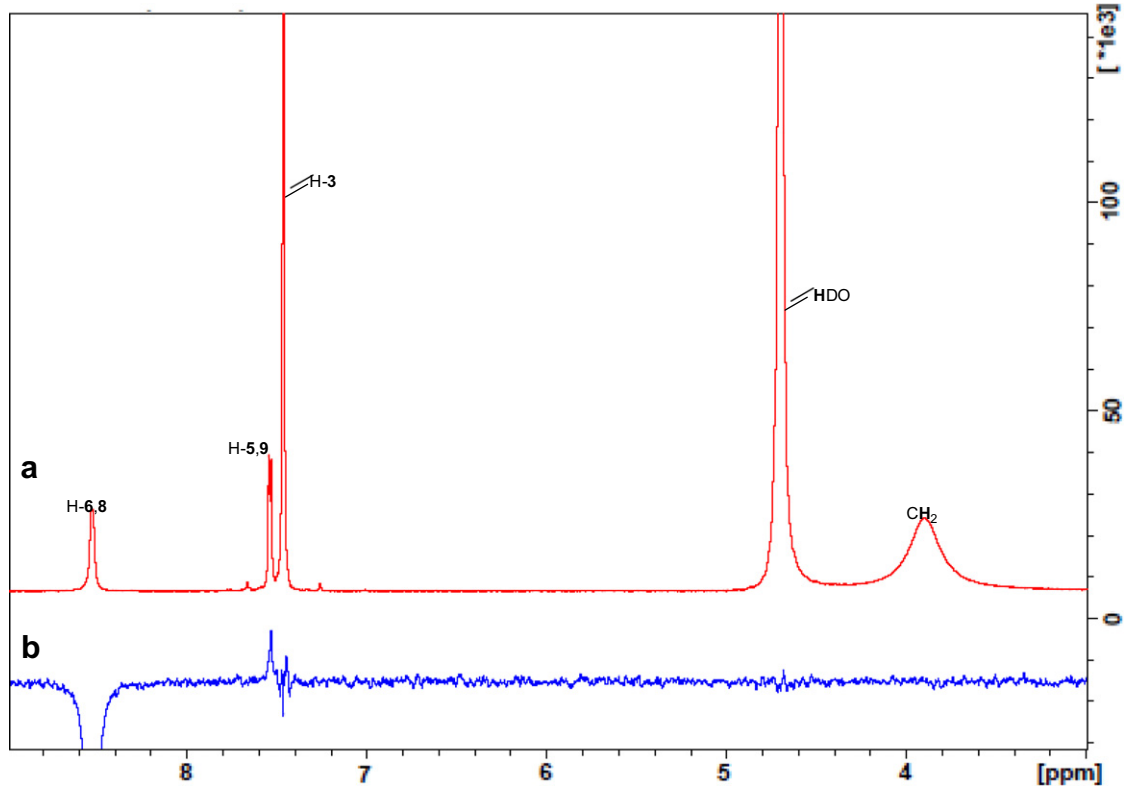

Fig. 4. (a) ^{1}H NMR spectra (400 MHz; D_2O; δ_{HDO} 4.67; 298 K) of the isoniazid/sodium *p*-sulfonatocalix[4]arene complex and (b) representative 1D-ROESY spectra of irradiated H-**6**,**8** of isoniazid.

lix[6]@INH) respectively, Table 1 and Fig. 2), indicating possibly interactions between the hydrazide group of INH with the SO_3Na group of *p*-scalix[4] or *p*-scalix[6]. We have also observed a variation in the chemical shifts of sodium *p*-sulfonatocalix[4]arene hydrogens (Ar–H and CH_2) (Table 1 and Fig. 2).

The molecular interactions taking place between INH and free calix[*n*]arenes were further investigated by monitoring the chemical shifts of the H-**5**,**9** hydrogen atoms of isoniazid. Job plots were conducted for determining the stoichiometry of the complexation reactions in aqueous solution (Fielding, 2000; Job, 1928). The graphs presented in Fig. 3 clearly show the 1:1 stoichiometry for the two inclusion complexes prepared.

1D ROESY NMR experiments were carried out to obtain complementary information on the inclusion geometries of *p*-scalix[4]@INH and *p*-scalix[6]@INH complexes. Unlike complexation-induced shifts, ROESY crosspeaks are indicative of specific proximity rela-

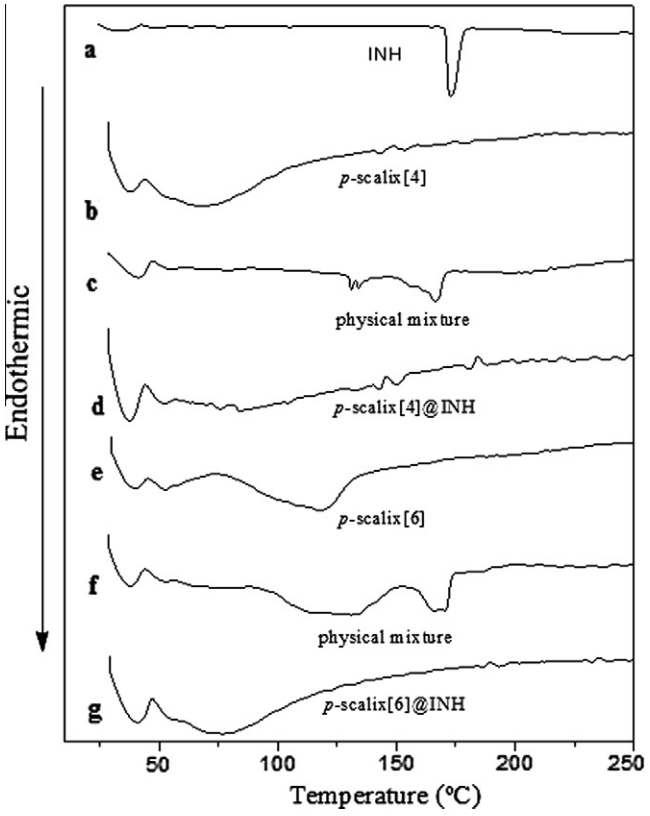

Fig. 5. DSC thermograms of: (a) isoniazid pure; (b) *p*-scalix[4]; (c) *p*-scalix[4]@INH physical mixture; (d) solid complex *p*-scalix[4]@INH; (e) *p*-scalix[6]; (f) *p*-scalix[6]@INH physical mixture; and (g) solid complex *p*-scalix[6]@INH.

tionships between guest and host hydrogens (generally 4 Å or less). To gain more insight into the topological aspects of these complexes the ^{1}H-ROESY NMR experiments have been performed, which are usually suited to measure NOEs in complexes with $\omega\tau_c$ close to 1 (Gunther, 1994).

According to the ROESY spectrum, the lack of rOe increments were observed between hydrogens H-**5,9** and H-**6,8** of isoniazid and hydrogens H-**3** and CH_2 of sodium *p*-sulfonatocalix[4]arene or sodium *p*-sulfonatocalix[6]arene (Fig. 4).

Differential scanning calorimetry (DSC) thermograms were obtained analyzing the rate of heat absorbed by INH, *p*-scalix[4], *p*-scalix[6], physical mixture (*p*-scalix[4]@INH and *p*-scalix[6]@INH) and inclusion complexes (*p*-scalix[4]@INH and *p*-scalix[6]@INH) (1:1 M ratio). These analyses gave supporting evidences for the complexation of isoniazid (INH) with sodium *p*-sulfonatocalix[4]arene (*p*-scalix[4]) and sodium *p*-sulfonatocalix[6]arene (*p*-scalix[6]).

Fig. 5 shows thermograms for isoniazid (Fig. 5a), *p*-scalix[4] (Fig. 5b), *p*-scalix[4]@INH physical mixture (Fig. 5c), solid complex *p*-scalix[4]@INH (Fig. 5d), *p*-scalix[6] (Fig. 5e), *p*-scalix[6]@INH physical mixture (Fig. 5f), solid complex solid complex (Fig. 5g). *p*-scalix[4] and *p*-scalix[6], it was also observed a peak corresponding to water loss (50–120 °C). A typical DSC curve for a crystalline anhydrous substance, with a sharp fusion endotherm (*T* peak = 168 °C), was obtained for isoniazid (Fig. 5a). The similar peak can be observed by analyzing physical mixtures thermal behavior (Fig. 5b and c). The disappearance as well as the shift of endo or exothermic peaks of drugs is a clear indication of the complexation phenomenon (Loukas et al., 1998) explaining the absence of the fusion peak of pure isoniazid (168 °C) in the thermogram showed in Fig. 5d and g. This result is an evidence of the inclusion of the isoniazid molecule into the calix[*n*]arenes hydrophobic cavity.

3.1. Theoretical calculations

In addition to the calculation of geometrical parameters, NMR theoretical data were obtained by electronic structure calculations. These results can assist the experimental characterization of this kind of inclusion compounds which is not so easily achieved. The variation of spectroscopic properties of the guest and host molecules on complexation is commonly used as an evidence or even confirmation of the inclusion complex formation. With this aim in mind, we carried out theoretical calculations of nuclear magnetic resonance (^{1}H NMR) spectra for the free species and inclusion complexes for *p*-scalix[4]@INH using the PBE1PBE/6-31G(d,p) level of calculation and the GIAO method (Wolinski et al., 1990) for the calculation of the chemical shifts (δ) for selected atoms obtained on the δ-scale relative to the TMS at the same theoretical level, as previously described in a spectroscopic determinations reported by our group (Venâncio et al., 2011).

Theoretical ^{1}H NMR spectra for the free isoniazid (INH), in its three distinct conformations, along with the fully optimized molecular structures, are shown in Fig. 6, with the experimental data being also given. The three conformers studied were named as INH-I, INH-II and INH-III. Observing the conformers spectra, it can be seen that the experimental NMR profile is only reproduced by structure INH-III, which has the hydrazide moiety placed orthogonally to the pyridine ring. This arrangement is different from that the reader can expect by simply looking to the proposed qualitative 2D structure given in Fig. 5, what can be easily rationalized on the basis that only such spatial arrangement would have the pair of hydrogen atoms H-**5**,**9** and H-**6**,**8** exhibiting the same NMR signal. The reason for the presence of structure INH-III in the experimental sample can be attributed to a fast interchange of two identical conformers like INH-I, so an averaged structure (INH-III) is observed in the NMR experiment. The agreement between the theoretical ^{1}H NMR chemical shifts for structure INH-III, calculated using the PCM model to simulate the solvent effects (water), and experimental data, measured in D_2O, can be also considered as very good. The small discrepancies relative to the absolute position of the experimental peaks are inherent in any comparison between theory and experiment.

A similar comparison between theoretical and experimental chemical shifts is reported in Fig. 7 for the free sodium p-sulfonatocalix[4]arene. The different conformations of calix[*n*]arenes, not contemplated by the calculations, were not considered here due to the fact that the cone conformation should be the major one for the sodium *p*-sulfonatocalix[4]arene based on the results published by Shinkai and co-workers (Shinkai et al., 1990) and also on the more recent X-ray experiments confirming the cone conformation for the isolated sodium *p*-sulfonatocalix[4]arene by Fucke et al. (2011). In addition, it is commonly accepted that the cone conformation should be predominant in solution based on hydrogen bond interactions argument. Therefore, a large conformational search, which is computationally prohibited, was avoided.

It can also be seen from Fig. 7 a very good agreement with experimental ^{1}H NMR data for *p*-scalix[4], with the splitting for the CH_2 groups observed in the theoretical spectra being assigned to a fast hydrogen interchange and so, in the scale time of the NMR experiment only one signal is detected. Also the calculated NMR data shows very detailed information about the chemical shifts allowing us to identify each hydrogen atom. The optimized structure for the *p*-scalix[4], shows the CH_2 on the bridge without symmetry through its bisection plane, which is detected by the theoretical spectrum. The same analysis can be done to evaluate the aromatic hydrogen chemical shifts. Therefore, having established that the PBE1PBE/6-31G(d,p)//IEFPCM-UFF level is sufficient for the prediction of the NMR chemical shift for the free monomers, one can used it to describe the NMR spectrum of the inclusion

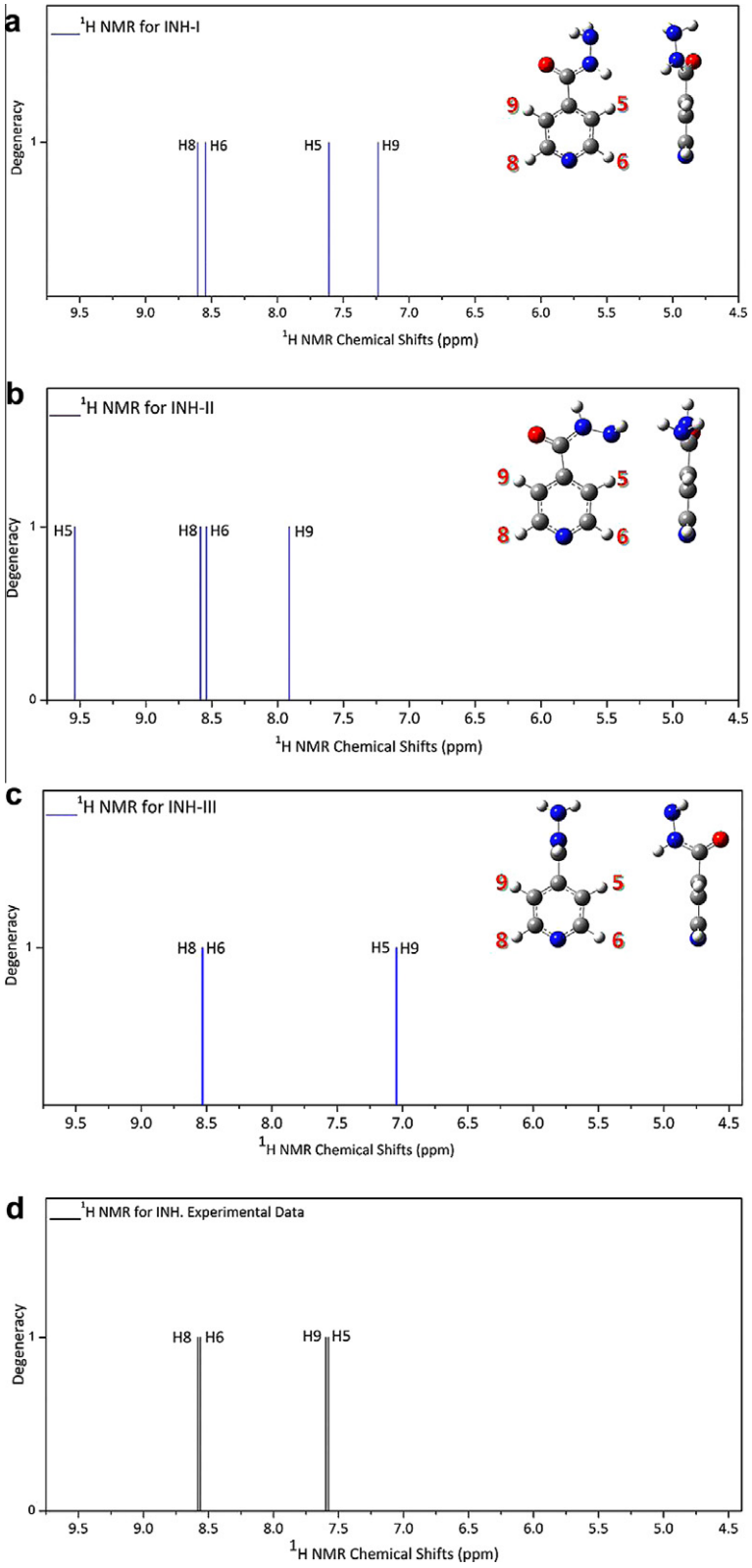

Fig. 6. ^{1}H NMR chemical shifts for Isoniazid. Theoretical spectra at PBE1PBE/6-31G(d,p)//IEFPCM-UFF for: (a) INH-I; (b) INH-II and (c) INH-III; d) The experimental data were also plotted at the same scale of experimental results.

compound and trust the best match between theoretical and experimental NMR profiles to elucidate the molecular structure of the *p*-scalix[4]@INH inclusion complex.

Six trial structures were chosen for the *p*-scalix[4]@INH complex, varying the position of the hydrazide moiety relative to the sodium *p*-sulfonatocalix[4]arene. The PBE1PBE/6-31G(d,p) fully optimized

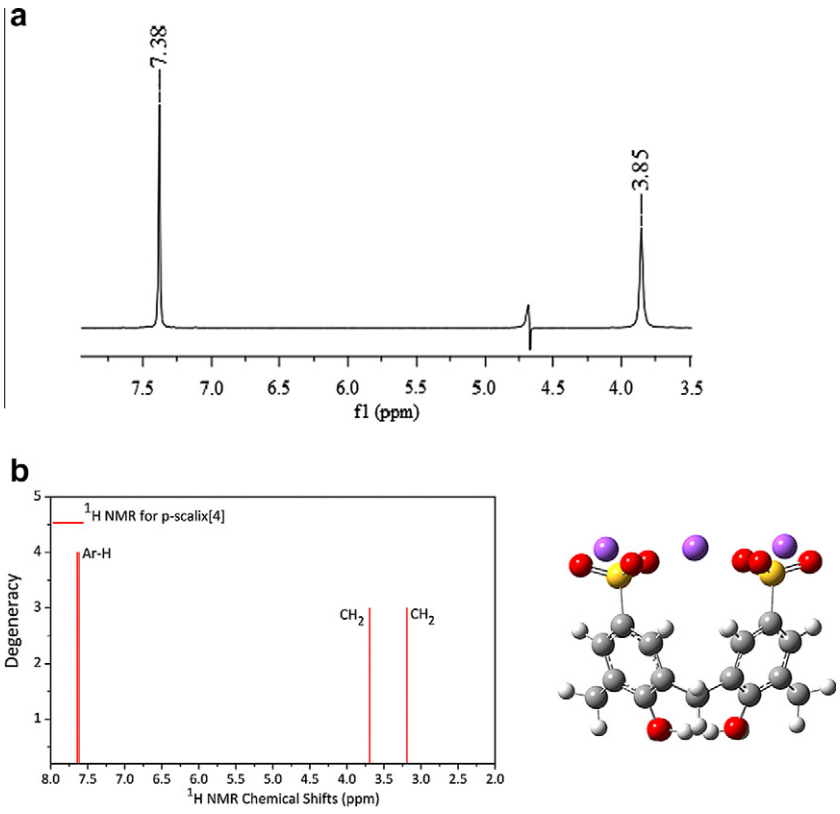

Fig. 7. ^{1}H NMR chemical shifts for the free sodium *p*-sulfonatocalix[4]arene. (a) Experimental and (b) calculated at PBE1PBE/6-31G(d,p)//IEFPCM-UFF.

structures (named *p*-scalix[4]@INH-I to *p*-scalix[4]@INH-VI) and the corresponding ^{1}H NMR spectra are shown in Fig. 8. Aiming to clarify the spectra analysis among the inclusion modes studied, just the isoniazid hydrogens are shown, and it is very clear how the inclusion process can change the ^{1}H NMR chemical shift due to the effect of the calix[*n*]arene cavity environment when compared with free INH spectra. Chemical shifts for all hydrogen atoms are reported for the *p*-scalix[4]@INH-III which exhibits the best agreement with the experimental data (also placed below it on purpose). The sensitivity of the calculated NMR spectra to structural changes can be promptly seen. For this inclusion complex, one can attest the splitting for all eight aromatic hydrogen in the light of the theoretical model used. Even slight changes on the symmetry around those atoms will be evaluated and they will appear as unique lines at the spectra. However, these differences among them are not substantial being 0.2 ppm at most, so one single peak with a spectral width should be observed. Again, the CH_2 sign shows a split into two sets with four hydrogens line each. The reason is the same explained for the free calix[*n*]arene, nevertheless these results are in excellent agreement with the experimental NMR shown in Fig. 8d. The outstanding best agreement between experimental and theoretical ^{1}H NMR profiles for the *p*-scalix[4]@INH-III inclusion complex leaves no doubt that this is the most populated molecular structure present in the sample handled in the NMR experiment.

As explained before, the theoretical spectrum (line spectrum) has more details and the intensity distribution should be interpreted by the molecular symmetry to allow us the degeneracy aspects of the NMR calculated chemical shifts. However, it can be clearly seen that the NMR pattern is correctly reproduced only by structure *p*-scalix[4]@INH-III, confirming the formation of an inclusion compound. This is a very interesting result, being an example where the use of a combined theoretical/experimental spectroscopic analysis is of practical value.

The calculated PBE1PBE/6-31G(d,p) thermodynamic properties for the inclusion complex formation model reaction (INH + *p*-scalix[4] → *p*-scalix[4]@INH) is given in Table 2 with the six varied orientations of the host–guest interactions. Observing Table 2, one can see that solvent effect using the continuum model causes a destabilization on the complex formation bringing a positive contribution to the interaction energy (ΔE). This could be associated to the PCM cavity scheme, which does not allowed the solvent polarization inside the calix[*n*]arene cavity, so the internal host–guest interactions are not solvent accessible and so, at this instance, continuum models may not be suitable to treat the solution medium in order to provide reliable interaction energy values including solvent effects. Intermolecular forces are the major responsible to the inclusion complexes stabilization. Therefore a complex equilibrium can be expected among the probable species. In this sense, the solvent will play a very important role and maybe, the theoretical model should have explicit molecules or even solvation shells. In other hand, this kind of approach would bring an excessive higher computational cost and also the thermodynamic explanation is not the major focus here. As a first thought, it seems that the analysis of theoretical NMR profiles can be more helpful than the evaluation of enthalpy and Gibbs free energy values aiming at the determination of the molecular structure of inclusion compounds.

It should also be mentioned that we have calculated the entropy contribution to the formation process of the *p*-scalix[4]@INH-III complex and the Gibbs free energy (ΔG) value at room temperature. The $T\Delta S$ term is -15.0 kcal mol^{-1} yielding a very small ΔG value of 1.4 kcal mol^{-1}, including the solvent effect (IEFPCM model) at the PBE1PBE/6-31G(d,p) level of calculation. Improving the description of electron correlation effects, using a post-Hartree–

Fock method, will raise the ΔG to just a few kcal mol^{-1} spontaneous value. The corresponding Hartree–Fock (HF) ΔG value is 7.6 kcal mol^{-1}. This higher HF value confirms that DFT calculations can partially recover the electron correlation effects. This ΔG result (PBE1PBE) shows that indeed the inclusion complex is weakly bound what makes difficult the experimental determination of the equilibrium constant through NMR experiment in aqueous solution.

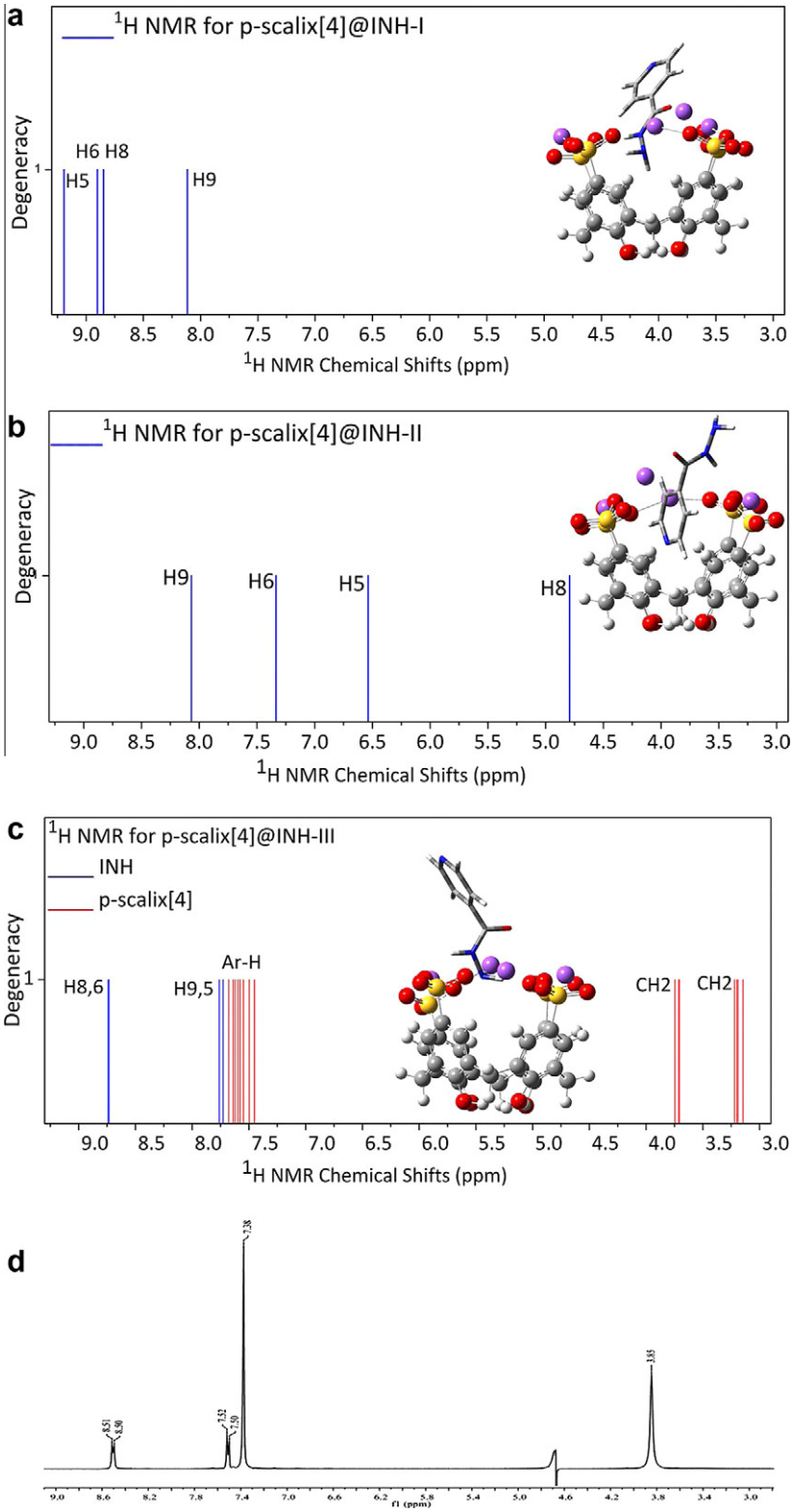

Fig. 8. ^{1}H NMR chemical shifts for the inclusion complexes *p*-scalix[4]@INH (a) I, (b) II, (c) III, all calculated at PBE1PBE/6-31G(d,p)//IEFPCM-UFF and (d) experimental spectrum for comparison continuation ^{1}H NMR chemical shifts for the inclusion complexes *p*-scalix[4]@INH (e) IV, (f) V, and (g) VI, all calculated at PBE1PBE/6-31G(d,p)//IEFPCM-UFF.

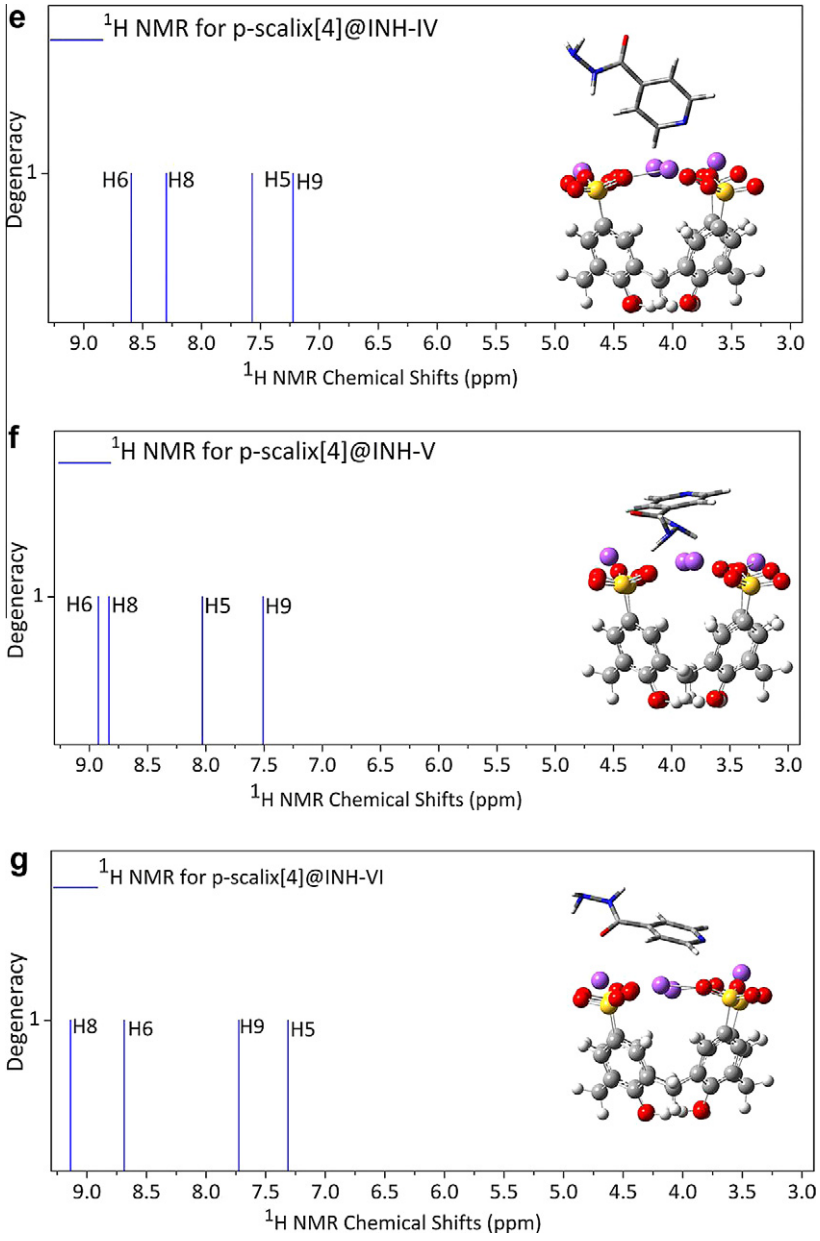

Fig. 8 (*continued*)

Table 2
Energy of complex formation in kcal mol^{-1} for the following model reaction: INH + *p*-scalix[4] → *p*-scalix[4]@INH.

	I	II	III	IV	V	VI
$\Delta E_{elec-nuc}^{gasphase}$	12.5	−15.1	−25.2	−14.7	−27.8	−24.3
$\Delta E_{elec-nuc}^{Solvation}$ [a]	3.9	−10.6	−14.4	−4.1	−12.3	−9.8
$\Delta H_{elec-nuc}^{Solvation}$	4.5	−9.3	−13.6	−3.8	−12.3	−8.9

[a] $\Delta E_{elec-nuc}^{Solvation} = \Delta E_{elec-nuc}^{gasphase} + \Delta E_{elec-nuc}^{Solution}$.

4. Antitubercular activity

The inclusion processes aimed to keep the isoniazid biological activity and also circumvent some possible side effects and parallel reactions. Therefore the antimycobacterial activities of the complexes obtained were evaluated against *M. tuberculosis* ATTC 27294 (Canetti et al., 1963) with the results presented in Table 3. The Minimal Inhibition Concentration (MIC) was defined as the lowest drug concentration to give 90% inhibition of bacterial growth, which prevented a color change from blue to pink. Table 3 shows the MIC of the inclusion complexes against *M. tuberculosis*. The results showed that the inclusion complexes exhibit biological activity next first line drugs such as isoniazid (INH), and could be a good starting point for further studies as well as find new lead compounds.

Table 3
MICs (mmol L^{-1}) of INH and inclusion complexes against *M. tuberculosis* H37Rv strain (ATCC 27294, susceptible both to rifampicin and isoniazid).

Compound	Samples	MIC_{90} (mmol L^{-1})
1	INH	1.5
2	*p*-scalix[4]@INH	2.6
3	*p*-scalix[6]@INH	1.8

5. Conclusion

In this paper NMR experimental and theoretical techniques were used in a complementary way to describe the inclusion complexes structures as well as the energetic stability of the multi-equilibrium structures in the inclusion processes in aqueous solution. Theoretical calculations were applied to the *p*-calix[4]@INH due to its smaller size which allowed the improvement of theoretical level but the conclusions can be extended to higher calix[*n*]arenes. As presented here, computational methods can be used as a support for one dimension NMR to indicate the structures for supramolecular arrangements. Also, the Minimal Inhibition Concentrations for the inclusion complexes were evaluated and the initial results showed that they exhibit biological activity very close to known drugs as isoniazid (INH), given some expectation in using inclusion compounds as isoniazid new formulations.

Acknowledgments

The authors would like to thank to Dr. Anita Jocelyne Marsaioli for helping on NMR experiments. This work was supported by FAPEMIG, FUNARBE and CAPES. This work is part of a collaboration research project of members of the Rede Mineira de Química (RQ-MG) supported by FAPEMIG (Project: REDE-113/10).

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