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ORIGINAL ARTICLE

NMR studies of inclusion complexation of the pyrrolizidine alkaloid retronecine and *p*-sulfonic acid calix[6]arene

Daniel Leite da Silva · Eder do Couto Tavares · Leila de Souza Conegero · Ângelo de Fátima · Ronaldo Aloise Pilli · Sergio Antonio Fernandes

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Abstract Accidental or intentional drug toxicity in humans and animals is a major concern and the search for detoxificant agents is a challenge. Pyrrolizidine-producing forages are a threat not only to livestock, but also to humans as a consequence of food contamination. Supramolecular systems are promising as detoxificant agents by decreasing the bioavailability of toxic compounds in biological environment. Cyclodextrin and calix[*n*]arenes are well known hosts for a variety of molecules and/or ions. Surprisingly, only few studies describe the potential of calix[*n*]arenes as host for toxic molecules. This study focused on the use of NMR techniques as tools for the investigation of the interactions between *p*-sulfonic acid calix[6]arene and retronecine, a toxic pyrrolizidine alkaloid.

Keywords Pyrrolizidines alkaloids · Calixarenes · NMR · Detoxification

D. L. da Silva · E. do Couto Tavares · S. A. Fernandes (✉)
Grupo de Química Supramolecular e Biomimética (GQSB), Departamento de Química, Universidade Federal de Viçosa (UFV), Campus Universitário, Avenida P.H. Rolfs, s/n, Viçosa, MG 36570-000, Brazil
e-mail: santonio@ufv.br

L. de Souza Conegero · R. A. Pilli
Departamento de Química Orgânica, Instituto de Química, Universidade Estadual de Campinas (UNICAMP), CP 6154, Campinas, SP 13084-971, Brazil

D. L. da Silva · Â. de Fátima
Grupo de Estudos em Química Orgânica e Biológica (GEQOB), Departamento de Química, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Pampulha, MG 31270-901, Brazil

Introduction

Pyrrolizidines alkaloids (PAs) occur naturally in a variety of plant species such as Asteraceae (especially in tribes Eupatorieae and Senecioneae), Boraginaceae and Leguminosae [1]. Pyrrolizidines alkaloid-containing plants are widely distributed in many geographic regions in the world [2]. *Senecio braziliensis* is widely distributed in cold and humid environments of Southeast Brazil and was reported to produce PAs [3, 4]. PAs have a vast array of biological functions such as antifungal [5, 6], antibiotic [5], and cytotoxic activities [7, 8] besides their role as messenger molecules in ecological interactions [9, 10]. In 1968 Culvenor found that PAs exhibit antitumor activity [11]. However, these compounds were never used in clinical trials due to their hepatotoxicity associated with the presence of a necine base in the basic structure. Hepatotoxic PAs are indeed a threat to domestic animals and human beings [12, 13]. Plants containing these hepatotoxins are among a range of livestock forage produced in the Northwest United States where PAs accounted for an economic loss of over $20 million in the 1990s [14]. Seneciosis is a disease caused by poisoning plants that trigger liver degeneration and necrosis, being the major problem of livestock forage in South Africa. Five to 15% of the cases in that country are originated from PAs-producing forage [15–17]. Additionally, PAs were found to contaminate human food sources such as wheat, milk, honey, herbal medicines and teas, which may potentially cause worldwide human health problems [18–21]. So far, no specific pharmaceutical antidotes have been developed to combat the toxic effects of certain PAs. Successful detoxification requires the selective and rapid adsorption of toxins from the affected organism. Recently, therapies using nanoparticulate systems including organic and inorganic nanoparticles,

hydrogels, nanocapsules, solid nanoparticles, oligochitosans, and microemulsions have been described [22, 23]. In 1994, Anderton and coworkers [24] reported the use of cyclodextrins as PAs' quenchers. Cyclodextrins (CDs) are some of the first molecular receptors whose ability to bind organic molecules was recognized and extensively studied by various experimental techniques [25–27]. CDs have pharmaceutical applications as high performance biomaterials in drug-delivery systems due to their action in the physical, chemical, and/or biological properties of guest molecules through the formation of inclusion complexes [28–36]. Surprisingly, calix[*n*]arenes, organic macrocyclic host molecules formed by the *ortho*-condensation of *para*-substituted phenols and formaldehyde [37], are well investigated in supramolecular chemistry but less studied with respect to their pharmacological properties [38, 39]. Moreover, only few studies exploring the use of calix[*n*]arenes (Fig. 1) as detoxificant agents are reported [40].

Therefore, the aim of this study is primarily to investigate the capacity of *p*-sulfonic acid calix[6]arene (**4**) to form complexes with retronecine (**1**), which would be useful for the detoxification of animals and humans poisoned by ingestion of harmful alkaloids.

Analysis of the degree of complexation between *β*-cyclodextrin (b-CD, **2**), 2-hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD, **3**) or *p*-sulfonic acid calix[6]arene (**4**) and retronecine (**1**) (Fig. 2) in an aqueous medium was performed and stoichiometry, complexed population ($\%p_{bound}$), formation/dissociation constant (K_a) and topology were determined by pulsed field gradient spin-echo (PGSE) experiments.

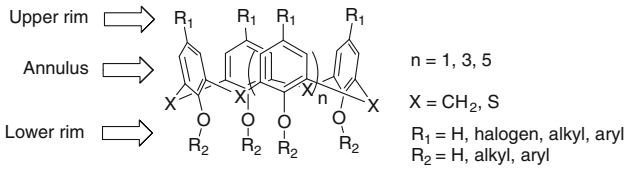

Fig. 1 Basic structure of calix[*n*]arenes

Results and discussion

Retronecine (**1**), the object of our studies, is a very toxic pyrrolizidine alkaloid synthesized by plants from the genus *Senecio*. We have isolated retronecine (**1**) from *Senecio braziliensis* shoots with an overall yield of 4% in relation to the total alkaloid content.

NMR techniques were employed to obtain detailed information about the interactions between retronecine (**1**) and *β*-CD (**2**), HP-*β*-CD (**3**) and *p*-sulfonic acid calix[6]-arene (**4**) in aqueous solution. The ^{1}H NMR and 2D NMR spectra for retronecine (**1**) in D_2O in the presence or absence of **2**, **3** or **4** were obtained. The guest hydrogens are observed as a single resonance because of fast exchange between a free guest and a complexed one on the NMR time scale.

We started our investigation by analyzing the complexation-induced hydrogen chemical shifts ($\Delta\delta$) in the **1/2**, **1/3** and **1/4** complexes and comparing these values with those for free retronecine (**1**).

Preliminary analysis revealed that the hydrogens of alkaloid (**1**) had small variation of chemical shifts ($\leq$0.02 ppm) in the presence of cyclodextrins suggesting very small interaction between compound **1** and *β*-CD (**2**) or HP-*β*-CD (**3**) (data not shown). This can be rationalized by the great solubility of retronecine (**1**) in water avoiding its migration to the hydrophobic cavity of *β*-CD (**2**) or HP-*β*-CD (**3**). These results support the findings obtained by Anderton and coworkers [24] where the inclusion of pyrrolizidine alkaloid (**1**) in *α*- or *β*-CD was investigated.

Complexation of **1**–**4** lead to large shielding effects on all hydrogen of compound **1**, in special H-**2a**, H-**2b**, H-**3a,** H-**3b** and H-**6** (Table 1, Fig. 3). This result indicates the formation of a inclusion complex between retronecine (**1**) and *p*-sulfonic acid calix[6]arene (**4**) where the apolar tail of the former is inserted into the cavity of the latter. Job plot's methods provided evidence for a 1:1 stoichiometry for **1/4** complex (Fig. 4) [41, 42]. Similar results were obtained when *p*-sulfonic acid calix[6]arene (**4**) was

Fig. 2 Structure of retronecine (**1**), *β*-CD (**2**), HP-*β*-CD (**3**) and *p*-sulfonic acid calix[6]arene (**4**)

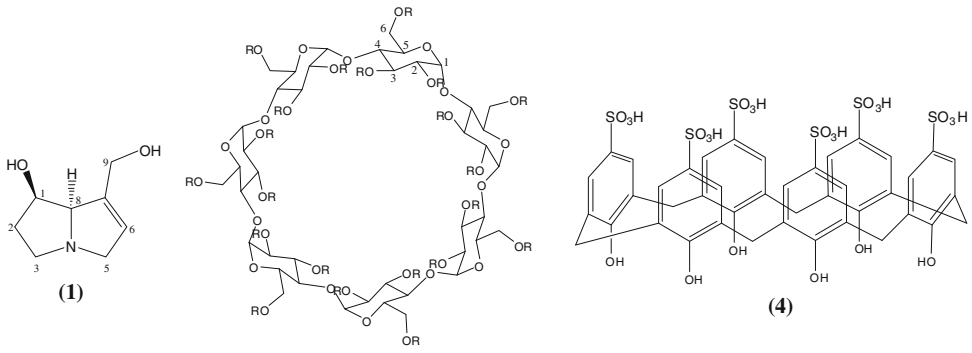

Table 1 ^{1}H NMR chemical shifts and chemical shift differences for retronecine (**1**) by itself and its complex with compound **4** (2 mmol L^{-1} samples, 298 K)

Hydrogen	**1** δ	**1/4** δ	**1/4** $\Delta\delta = \delta\mathbf{1}_{liver} - \delta\mathbf{1}_{complex}$
H-**2a**	1.91	1.05	0.86
H-**2b**	1.96	1.43	0.53
H-**3a**	2.79	2.49	0.30
H-**3b**	3.35	2.82	0.53
H-**5a**	3.46	3.25	0.21
H-**5b**	3.92	3.65	0.27
H-**9**	4.14	3.93	0.21
H-**8***	4.32	–[a]	–[a]
H-**1**	4.38	4.16	0.22
H-**6**	5.66	5.28	0.38

[a] The chemical shift for H-**8** in the complex **1/4** could not be determined due to its overlap with the signals from retronecine (**1**) and methylene hydrogens of the *p*-sulfonic acid calix[6]arene (**4**) as well

substituted for the corresponding sodium salt suggesting that the shielding effects do not result from ammonium salt of **4** and **1** (data not shown). Additionally, no variation in chemical shift was observed when retronecine (**1**) was mixed with *p*-toluenesulfonic acid in equimolar amounts. Taken together, these results unequivocally show that ammonium salt did not contribute for the observed chemical shifts.

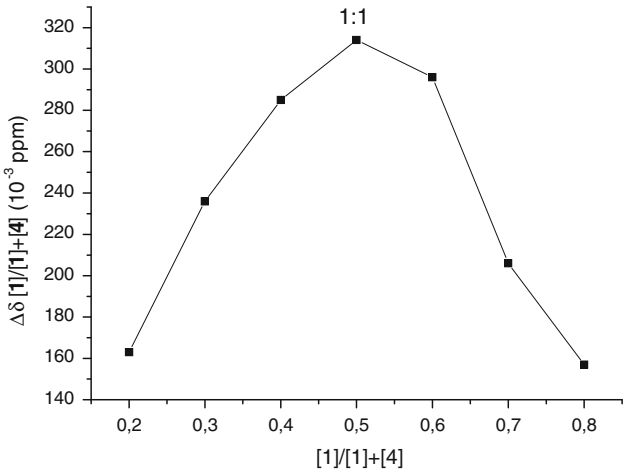

Fig. 4 Job plots for the complex formed between retronecine (**1**) and *p*-sulfonic acid calix[6]arene (**4**)

Additional information about the supramolecular structure of the complex **1/4** was obtained by using diffusion-ordered spectroscopy (DOSY) NMR experiments. This technique unequivocally confirmed the formation of a stable complex [33–35, 43–48]. Representative spectra are given in Figs. 5 and 6 for free retronecine (**1**) and for **1/4** complex (2 mmol L^{-1} samples, 298 K), respectively. The diffusion coefficients for free retronecine (**1**) and the host molecules **2**, **3** and **4** were first determined (Table 2). No significant reduction of the diffusion rate was observed for

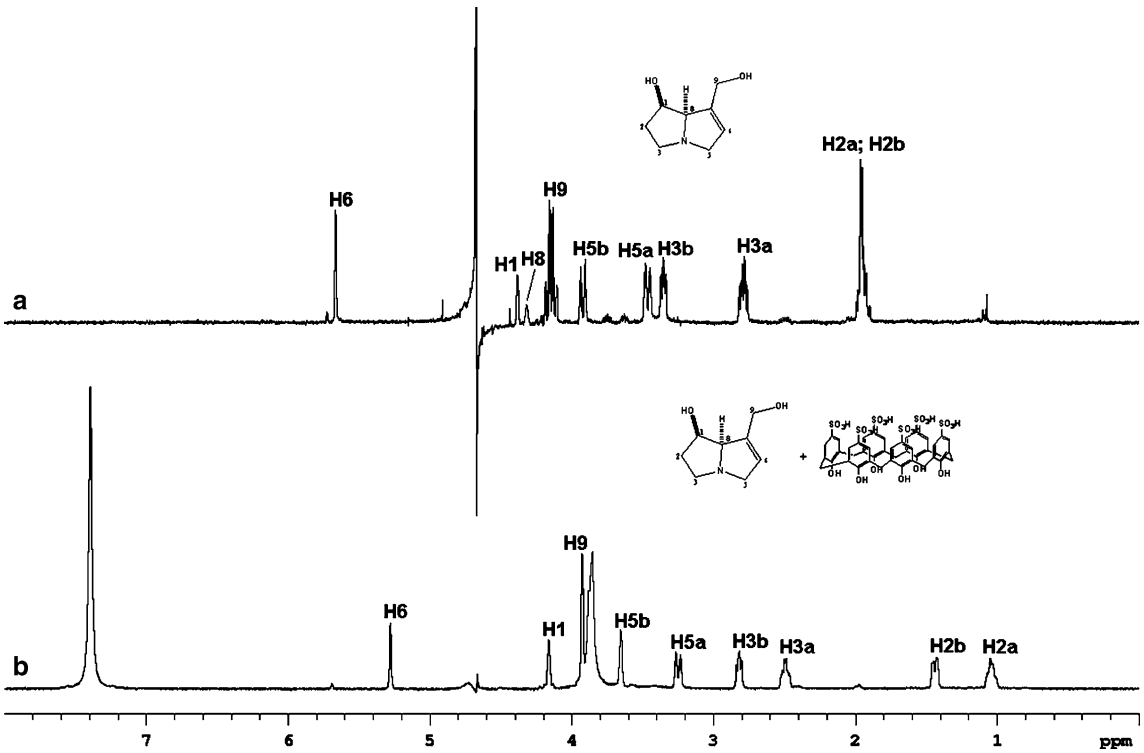

Fig. 3 ^{1}H NMR spectra (499.885 MHz, D_2O, 298 K, 2 mmol L^{-1} each). **a** Compound, **b** complex **1/4**

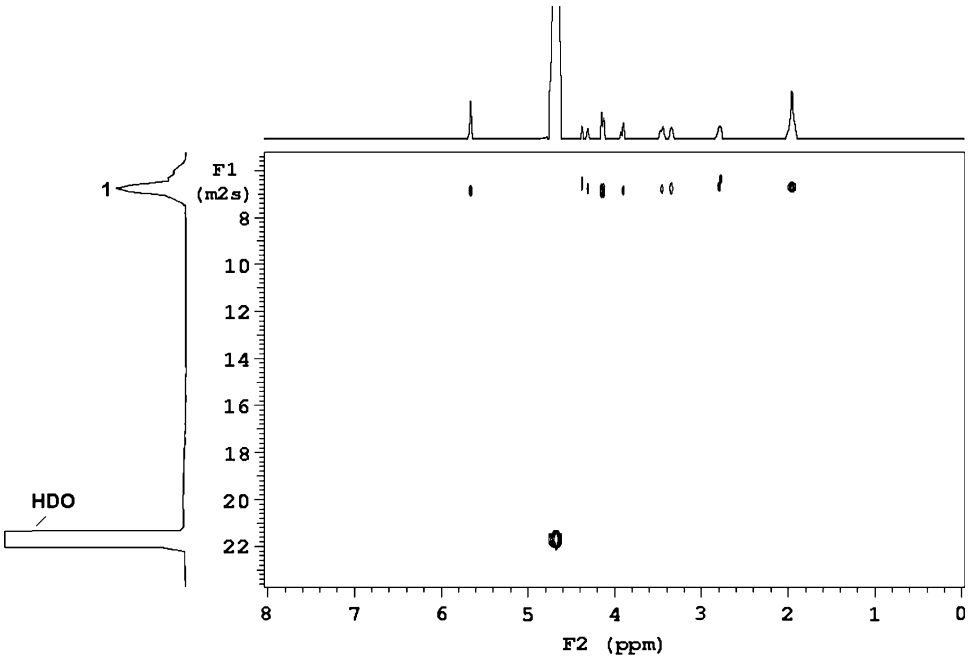

Fig. 5 ^{1}H DOSY NMR spectrum (499.885 MHz, D_2O, 298 K, 2 mmol L^{-1}) for retronecine (**1**)

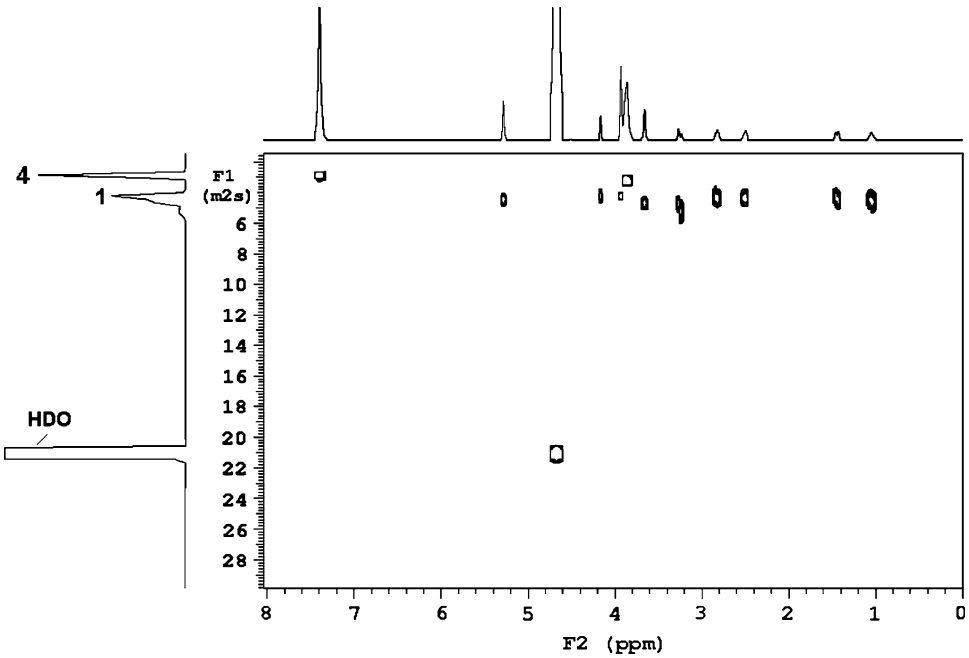

Fig. 6 ^{1}H DOSY NMR spectrum (499.885 MHz, D_2O, 298 K, 2 mmol L^{-1}) for complex **1/4**

retronecine (**1**) when in the presence of β-CD (**2**) or HP-β-CD (**3**) (Table 2) confirming that compound **1** does not form *host–guest* complex with either compounds **2** or **3**. In contrast, retronecine (**1**) showed a significant decrease in the diffusion rate ($D_{1/4} = 4.52 \times 10^{-10}$ m^2 s^{-1}) in the presence of *p*-sulfonic acid calix[6]arene (**4**) (Table 2, Fig. 6) indicating the formation of complex **1/4**. Taking into account that the studied system is under fast equilibrium on NMR scale, both chemical shifts and diffusion coefficients for compound **1** are the average values of the free and bound species. Further studies employing DOSY technique [48, 49] provided additional evidence that compounds **1** and **4** formed a stable complex. The complexed population ($\%p_{bound}$) and the apparent binding constants (K_a; Table 2) for complex **1/4** were calculated according to methods described elsewhere [48, 49]. Complex **1/4** exhibited a p_{bound} value of 56.3% and a K_a value of 1446.3 M^{-1}, confirming that a strong association took place between **1** and **4**.

To probe the **1/4** topological aspects, we used NOESY experiments. Specific NOE signals were observed between

Table 2 Diffusion coefficients of free compounds **1**–**4** and their respective complexes **1/2**, **1/3** and **1/4** in D_2O at 2 mmol L^{-1} and 298 K

Complex	Compounds	D (10^{-10} m^2 s^{-1})	D/D^{D_2O}	$\%p_{bound}$	K_a (M^{-1})
–	**1**	6.47 ± 0.21	0.30	–	–
–	**2**	3.16 ± 0.02	0.15	–	–
–	**3**	3.01 ± 0.02	0.14	–	–
–	**4**	3.05 ± 0.02	0.14	–	–
1/2	**1**	6.45 ± 0.27	0.30	[a]	[a]
	2	3.13 ± 0.07	0.15		
1/3	**1**	6.49 ± 0.14	0.30	[a]	[a]
	3	3.03 ± 0.03	0.14		
1/4	**1**	4.52 ± 0.20	0.21	56.3	1446.3
	4	3.06 ± 0.03	0.14		

[a] $\%p_{bound}$ and K_a values could not be determined because retronecine (**1**) did not present variation in the diffusion coefficient in the presence of β-CD or HP-β-CD

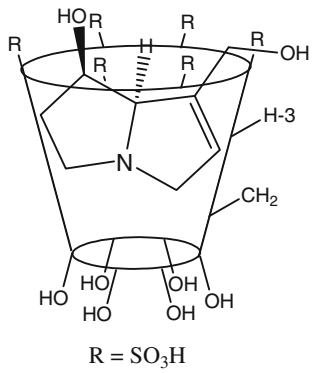

Fig. 7 Proposed topology for complex **1/4**

H-**5** and H-**6** of retronecine (**1**) with $–CH_2–$ and H-**3** of *p*-sulfonic acid calix[6]arene (**4**). We therefore suggest that one moiety of retronecine (**1**) was inside the *p*-sulfonic acid calix[6]arene (**4**). The proposed **1/4** complex topology is shown in Fig. 7.

Conclusion

The NMR techniques employed in this study were crucial for the elucidation of the supramolecular structure of complex **1/4**. A strong association between **1** and **4** was observed as a result of interactions established between the amine and/or hydroxyl groups of the alkaloid retronecine (**1**) and the SO_3H group of compound **4**. Our results point out the *p*-sulfonic acid calix [6]arene (**4**) as a potential host molecule for retronecine (**1**). The formation of complex **1/4** open a window for the development of *p*-sulfonic acid calix[6]arene as a detoxificant agent, particularly in cases of poisoning by ingestion of pyrrolizidine alkaloids.

Materials and methods

Chemicals and reagents

All reagents were of analytical grade. D_2O (99.75%) was purchased from Aldrich and *p*-sulfonic acid calix[6]arene (**4**) was synthesized according to procedures previously described [50–52].

Plant material

Senecio braziliensis (Asteraceae) was collected in December 2001, at Serra de Extrema, Minas Gerais, Brazil. About 1 kg of plant shoot (leaves, flowers and small stems) was homogenized in 8 L of ethanol. After 21 days of incubation at room temperature the solvent was removed by filtration. The same plant material was extracted twice with ethanol. The ethanol was eliminated under reduced pressure at 313 K, yielding 72 g of crude material. A solution of $HCl/CHCl_3$ (1 mol L^{-1}) was added to the crude material in a 1:1 w/v ratio. The acidic portion was then extracted four-times with 500 mL of $CHCl_3$. The organic phase was set aside and the acid phase (pH 1–2) was treated with an excess of zinc powder for 5 h in order to reduce alkaloid *N*-oxides formation. The sample was treated with an excess of an NH_4OH solution (25%) to adjust the extract pH to 11–12. After that the extract was treated three-times with $CHCl_3$/MeOH (4:1). The organic phases were combined, dried out with anhydrous Na_2SO_4, and concentrated under reduced pressure, which yielded 15 g of total alkaloids. Retronecine (**1**) was obtained by refluxing the alkaloid crude sample with $Ba(OH)_2{\cdot}8H_2O_{(aq)}$ (ratio of 1:1.2) for 2 h. Treatment of such mixture with dry ice provided the precipitation of barium carbonate. The mixture was filtrated and the solution evaporated until obtaining a yellow solid which was applied to a flash column containing basic alumina. Purification was performed by using the solvent mixture $CHCl_3$/MeOH/NH_4OH (42.5:7.5:1), which yielded 3 g of retronecine (**1**).

Preparation of solid inclusion complexes

Inclusion complexes (**1/2**, **1/3** or **1/4**) at 1:1 M ratio were attempted obtained by mixing a-2 mmol L^{-1} aqueous solution of compounds **2**, **3** or **4** with a solution of 2 mmol L^{-1} compound **1**. Each system was stirred for 12 h at room temperature, a period of time determined as the optimum to reach the equilibrium (data not shown). 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 ^1H spectra were acquired in an INOVA-500 Varian spectrometer operating at 499.885 MHz for ^1H (64 k data points, 30° excitation pulse 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 inverse detection mode at room temperature unless stated otherwise.

Determination of complexation stoichiometry

Job plots have been prepared with 2 mmol L^{-1} stock solutions of compounds **1**, **2**, **3** and **4** [50].

HR-DOSY experiments were carried out by carefully choosing the pulse sequence and gradients. The measurements were made using: (a) a 5-mm inverse probe with *z*-gradient coil; (b) a GCSTESL (Gradient Compensated Stimulated Echo Spin Lock) HR-DOSY sequence; (c) an amplitude of gradient pulses in the range of 0.000685–0.003427 T cm^{-1}, where an approximately 90–95% decrease in the resonance intensity was achieved at the largest gradient amplitude. A total of 25 different gradient amplitudes were used in these experiments. The baselines of all arrayed spectra were corrected prior to the data processing. The processing program (DOSY macro, Varian Instrument) involved the determination of the peak heights of all signals above a pre-established threshold and the fitting of the decay curve for each peak to an exponential decay. The DOSY macro was run with data transformed using fn = 64 K. Very crowded spectra were processed in sections due to the limitation of handling (512 lines at a time only). The DOSY results are pseudo two-dimensional spectra with NMR chemical shifts along one axis and calculated diffusion coefficients ($m^2\ s^{-1} \times 10^{-10}$) along with the other.

NOESY—Solutions for 2D NMR analysis were prepared with 20 mM of retronecine (**1**) and 40 mM of the *p*-sulfonic acid calix[6]arene, with ensured over 99% complex formation between the guest and host. The 2D NMR spectra were recorded by using a NOESY sequence from Varian and a mixing time of 400 ms.

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