

# Investigation of tetracaine complexation with beta-cyclodextrins and *p*-sulphonic acid calix[6]arenes by nOe and PGSE NMR

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**Abstract** Cyclodextrins (CD) and calixarenes are complexing agents that have been successfully used as pharmaceutical drug carriers, to improve the bioavailability of medicines. The aim of this work was to investigate the complexation of the local anesthetic tetracaine **1** with  $\beta$ -cyclodextrin **2**, as well as with *p*-sulphonic acid calix[6]-arene **3**.  $^1\text{H}$  NMR experiments were carried out in  $\text{D}_2\text{O}$ , i.e., with the charged tetracaine species **1**. HR-DOSY  $^1\text{H}$  NMR allowed determination of the fraction of complexed population ( $\%p_{\text{bound}} = 55\%$  and  $70\%$ ) and the apparent association constants ( $K_a = 1358$  and  $3889 \text{ M}^{-1}$ ), respectively, for **1/2** and **1/3**. These results confirm that a strong association takes place between **1** and **2**, while the **1/3** complex is even more stable, due to the negatively charged sulphonic groups of **3**. Studies conducted at pH 10 revealed that the association of the uncharged form of **1** with **3** is considerably weaker, while that with **2** increased

significantly ( $K_a = 6597 \text{ M}^{-1}$ ), protecting the anesthetic against alkaline hydrolysis.  $^1\text{H}$ -ROESY 1D NMR experiments allowed determination of the host-guest relative positions, revealing that the proposed topologies for the **1/2** and **1/3** complexes were quite different. The complexation of **1** with either **2** or **3** is being investigated in view of its potential use in new therapeutic formulations, designed to increase the bioavailability and/or to decrease the systemic toxicity of tetracaine, in anesthesia procedures.

**Keywords** Tetracaine · Cyclodextrin · Calixarene · Nuclear magnetic resonance

## Introduction

Tetracaine belongs to the aminoester family of local anesthetics (LA) an important class of nonceceptive agents whose action involves blockage of nervous impulse transmission. It is believed that both the cationic and the uncharged species of LA—that coexist at physiologic pH—bind to the  $\text{Na}^+$  channels of the nerve membranes, stabilizing its inactivated state and thus blocking the initiation and propagation of nervous impulses. However, LA show a relatively short duration of action (1–4 h) and may have adverse side effects such as cardiac and CNS toxicity, accompanied sometimes by allergic reactions [1].

The use of cyclodextrins (CD), calixarenes, liposomes and polymers as drug carrier systems has become an increasingly successful method to improve the potency of many therapeutic molecules whose bioavailability is threatened by problems such as limited (water/membrane) solubility, low chemical stability, fast serum clearance, etc. [2].

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Among the available complexing agents, CD are the most widely used in drug formulations [3], while calixarenes belong to a new class of cyclooligomers, formed via phenol-formaldehyde reactions [4]. Calixarenes and CD share common features since they are macrocyclic molecules with a repeating unit, and both are cage-like molecules with a hydrophobic cavity. The natural cyclodextrins ( $\alpha$ -,  $\beta$ - and  $\gamma$ -CD) have inner-cavity diameters of 5.7, 7.8, and 9.5 Å, respectively [2a]. In contrast, the inner cavity diameters of calix[4]arene, calix[6]arene, and calix[8]arene are 3.0, 7.6, and 11.7 Å, respectively [5]. Thus, the inner-cavity diameter of calix[6]arene is comparable to that of  $\beta$ -CD. Just like micelles, CD and sulphonate-calix[n]arenes can provide hydrophobic environments to guest molecules (through the glycoside and benzene rings, respectively), and hydrophilic outer surfaces (OH and SO<sub>3</sub><sup>-</sup>, respectively) [6]. However, there is a significant difference imposed by the oligosaccharide units of CD and the phenol units of calixarenes: whereas CD are quite rigid molecules, calixarenes are highly flexible molecules, possessing the ability to undergo complete ring inversion [7].

The characterization of the delivery systems formed by complexation of charged tetracaine **1** in  $\beta$ -cyclodextrins **2** or *p*-sulphonic acid calix[6]arene **3** (Scheme 1) will provide important information to optimize their future performance, which requires a better knowledge of their molecular properties [8]. Two pieces of information are crucial for the characterization of these complexes: the formation/dissociation constant and the relative positioning of the carrier/guest inclusion complex, both of which can be obtained from PGSE (pulsed field gradient spin-echo) [9] and NOE (nuclear Overhauser effect) [10] experiments, respectively. Such NMR techniques have been successfully applied to other supramolecular *host-guest* structures [11]. The aim of the present paper is to describe tetracaine **1** complexation with **2** and **3** (Scheme 1), by application of PGSE and NOE NMR methodologies.

## Results and discussion

Sulphonate-calix[n]arenes have been used for the encapsulation of commercial steroids, furosemide,

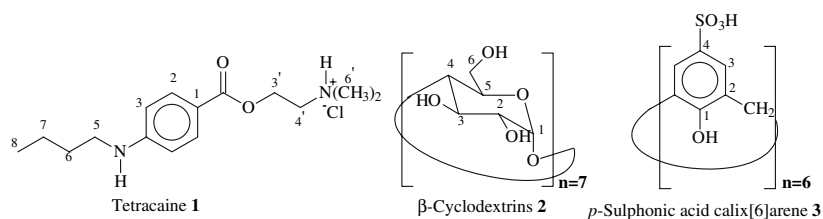
nifedipine and nicosamine [12] and the literature contains plenty of examples of CD based pharmaceutical formulations [2a, 13], most of them involving LA [14].

The association of **1** with **2** or **3** was first evaluated by complexation-induced hydrogen chemical shifts ( $\Delta\delta$ ) in the **1/2** and **1/3** complexes, relative to free **1**. Interestingly, chemical shift differences in the <sup>1</sup>H NMR spectra of **1**—free or in **1/2** complex—were mainly observed for H-3 hydrogens (Table 1). Complexation between **1** and **3** induced large shielding effects in all the hydrogens of **1**, mainly H-3', H-4' and H-6' ( $\Delta\delta$  0.43, 0.72 and 0.63, respectively, Table 1), indicating interactions between the ammonium group of **1** with the SO<sub>3</sub><sup>-</sup> group of **3** [15].

Diffusion-ordered spectroscopy (DOSY) NMR experiments were pivotal to demonstrate that **1** and **2** or **1** and **3** form stable complexes. It serves also to distinguish compounds or complexes by their differences in diffusion coefficients [9a]. The diffusion coefficients of pure **1**, **2** and **3** ( $D_1 = 5.58 \times 10^{-10}$ ,  $D_2 = 3.27 \times 10^{-10}$  and  $D_3 = 3.05 \times 10^{-10}$  m<sup>2</sup> s<sup>-1</sup>, respectively) were first obtained (Table 2). In the presence of **2** or **3**, compound **1** showed a significant reduction of the diffusion rate ( $D_{1/2} = 4.24 \times 10^{-10}$  and  $D_{1/3} = 3.70 \times 10^{-10}$  m<sup>2</sup> s<sup>-1</sup>, respectively) (Table 2, representative Figs. 1 and 2), indicating that **1** formed *host-guest* complexes with either **2** or **3**. Moreover, the diffusion rate values (Table 2) of **1** in the **1/3** complex are in agreement with the assumption of a strong association. Taking into account that we are dealing with a rapidly equilibrating system, both the chemical shifts and the diffusion coefficients are weight-averaged NMR values between free and bound **1** species. From these diffusion coefficients and applying a well established methodology [16], we have calculated the complexed population (%*p*<sub>bound</sub>) and apparent binding constants ( $K_a$ ) of the complexes. In D<sub>2</sub>O %*p*<sub>bound</sub> was found to be 55% and 70% for **1/2** and **1/3**, respectively. The values of  $K_a$  (1358 M<sup>-1</sup> and 3889 M<sup>-1</sup> for **1/2** and **1/3**, respectively) confirmed that a stronger association took place between **1** and **3** than between **1** and **2**, due to the negatively charged sulphonic groups of calix[6]arene.

At pH 8.2 there is an equilibrium between the protonated and non protonated form of **1** [17]. Studies

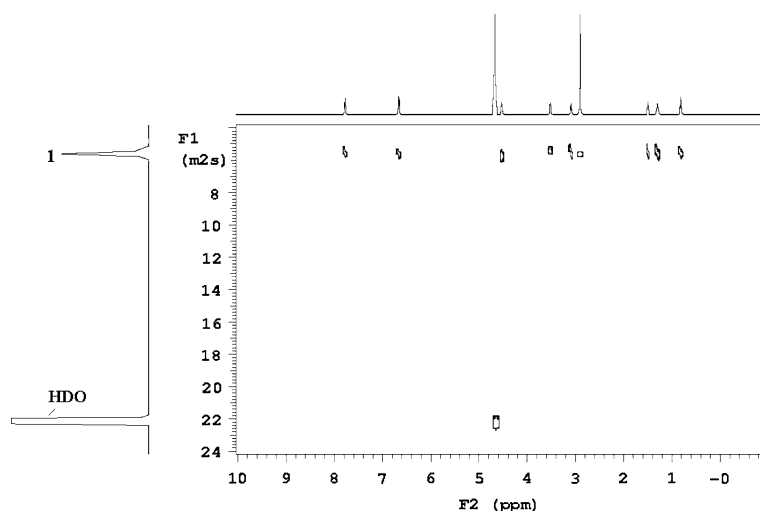
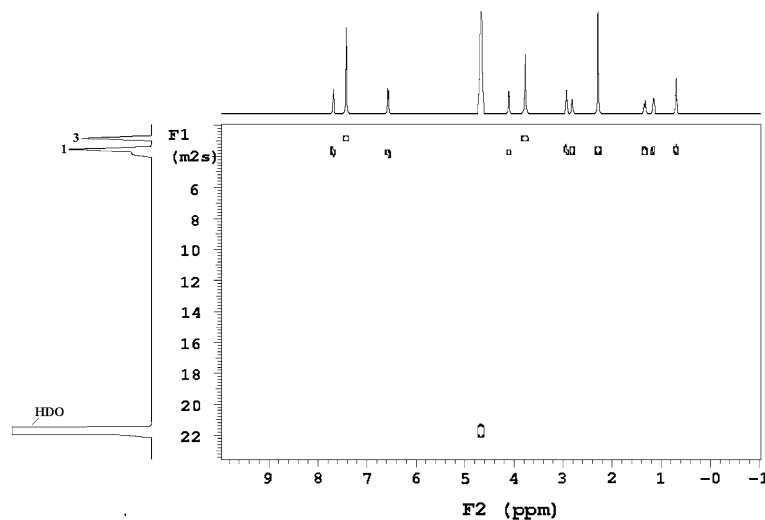
### Scheme 1



**Table 1** The values of the chemical shifts  $^1\text{H}$  NMR of pure **1** in  $\text{D}_2\text{O}$ , complexed **1/2**, **1/3** and chemical shift differences ( $\Delta\delta = \delta_{\text{1 free}} - \delta_{\text{1 complex}}$ )

Hydrogens of <b>1</b>	<b>1</b>	<b>1/2</b>	<b>1/3</b>	<b>1/2</b>	<b>1/3</b>
	$\delta$	$\delta$	$\delta$	$\Delta\delta = \delta_{\text{1 free}} - \delta_{\text{1 complex}}$	$\Delta\delta = \delta_{\text{1 free}} - \delta_{\text{1 complex}}$
H-2	7.78	7.76	7.69	0.02	0.09
H-3	6.66	6.54	6.56	0.12	0.09
H-5	3.08	3.09	2.93	-0.01	0.15
H-6	1.50	1.49	1.34	0.01	0.15
H-7	1.29	1.29	1.16	0.00	0.14
H-8	0.82	0.83	0.70	-0.01	0.12
H-3'	4.53	4.55	4.10	-0.02	0.43
H-4'	3.52	–	2.79	–	0.72
H-6'	2.90	2.90	2.27	0.00	0.63

\* The chemical shift value of **1/2** could not be determined because of the overlap between signals from H-4' and CD

**Fig. 1** Representative  $^1\text{H}$  DOSY NMR experiment (499.885 MHz,  $\text{D}_2\text{O}$ , 298 K, 2 mmol  $\text{l}^{-1}$ ) of pure **1****Fig. 2**  $^1\text{H}$  DOSY NMR experiment (499.885 MHz,  $\text{D}_2\text{O}$ , 298 K, 2 mmol  $\text{l}^{-1}$ ) of the **1/3** complex

conducted at pH 10.0 revealed an increase in the association between uncharged tetracaine **1** and **2** ( $K_a = 6597 \text{ M}^{-1}$ ). The association constant between uncharged **1** and **3** was not determined due to the

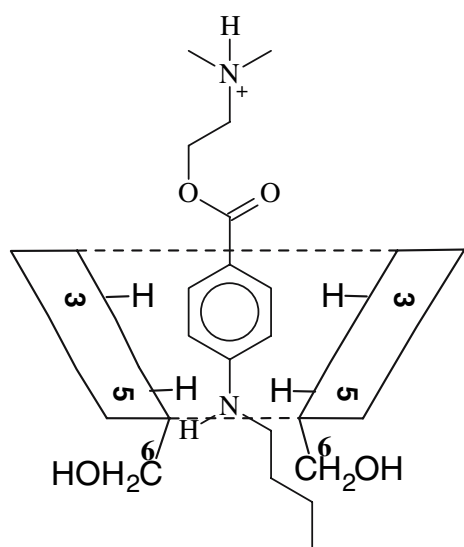
alkaline hydrolysis of the ester bond of **1** ( $t_{1/2} < 0.1 \text{ h}$ ). Fortunately alkaline hydrolysis was prevented ( $t_{1/2} > 16 \text{ h}$ ) by the strong association of **1** and **2** at pH 10.0, allowing the DOSY experiments to be carried out.

**Table 2** Diffusion coefficients of pure **1**, **2**, **3** and in the **1/2** and **1/3** complexes, in D<sub>2</sub>O (2 mmol l<sup>-1</sup> samples, 298 K)

Complex	Compounds	$D$ (10 <sup>-10</sup> m <sup>2</sup> s <sup>-1</sup> )	% $p$	$K_a$ (M <sup>-1</sup> )
–	<b>1</b>	5.58 ± 0.05	–	–
–	<b>2</b>	3.27 ± 0.03	–	–
–	<b>3</b>	3.05 ± 0.02	–	–
<b>1/2</b>	<b>1</b>	4.24 ± 0.02	55%	1358
	<b>2</b>	3.13 ± 0.02	–	–
<b>1/3</b>	<b>1</b>	3.70 ± 0.04	70%	3889
	<b>3</b>	2.90 ± 0.02	–	–
<b>1/2*</b> (pH 10)	<b>1</b>	3.66 ± 0.03	76%	6597
	<b>2</b>	3.04 ± 0.01	–	–

We further established the stoichiometry for the **1/2** and **1/3** complexes, using the Job plot method [18]. The plots obtained from the NMR analysis indicated the predominant formation of 1:1 complexes, both between **1** and **2** and with **1** and **3** (Figures not shown).

To gain more insight into the topological aspects of these two complexes (**1/2** and **1/3**) we have performed <sup>1</sup>H-ROESY NMR experiments, which are usually suited to measure NOEs in complexes with  $\omega\tau_c$  close to 1 [19]. Specific ROE signals were observed between H-**2** and H-**3** of **1** with H-**3** (enhancement of 0.46% and 0.45%, respectively) and H-**5** (1.01% and 0.15% of signal enhancement respectively) of  $\beta$ -CD **2**. We therefore suggest that the aromatic moiety of tetracaine **1** was inside the CD cavity. We have also addressed the association issue however no dipolar interaction between the H-**1**, H-**2** and H-**4** of the  $\beta$ -CD **2** was observed with the tetracaine **1**, thus the **1/2** complex' topology was proposed and is depicted in Fig. 3.

**Fig. 3** Proposed topology for the **1/2** complex, based on <sup>1</sup>H NMR evidences

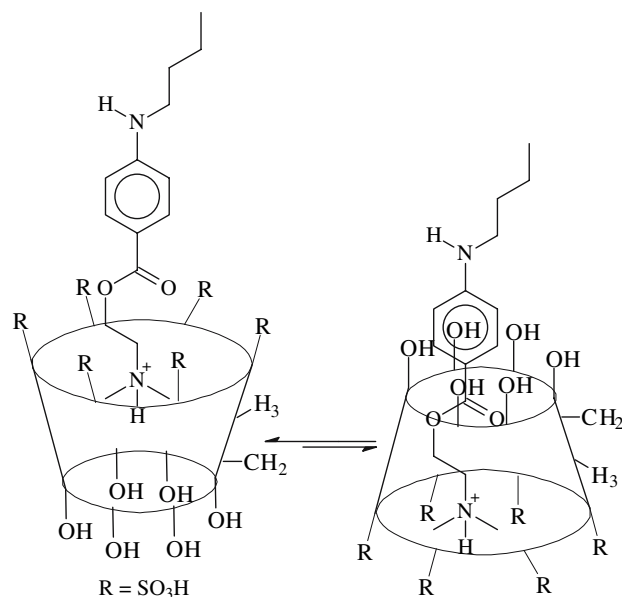
The signal enhancements in **1/3** (ROE) between H-**6'** of the tetracaine **1** and H-**3** (1.27% signal enhancement) of the *p*-sulphonic acid calix[6]arene **3** indicate that the ammonium group of **1** can be included in the cavity of **3**, as suggested in Fig. 4. The lack of more ROE signal is probably due to the conformational behavior of the *p*-sulphonic acid calix[6]arene **3** which will be better investigated in the near future.

## Conclusions

The combined use of PGSE and ROE techniques has helped the determination of the *host-guest* structure, kinetic stability and degree of guest encapsulation in solution, for the **1/2** and **1/3** complexes.

The complexation of **1** with either **2** or **3** has been investigated in view of its potential use for the preparation of new therapeutic formulations, to increase the bioavailability and to decrease the systemic toxicity of tetracaine **1** in anesthesia procedures. In water, we determined a strong association between **1** and **2**. The interaction between **1** and **3** was found to be even more stable, due to the binding of the ammonium group of **1** and the SO<sub>3</sub><sup>-</sup> group of **3**. This was confirmed by experiments performed at pH 10.0, which revealed that the association between **1** and **3** decreased in relation to that in D<sub>2</sub>O, while that of **1** and **2** increased ( $K_a = 6597$  M<sup>-1</sup>), preventing the alkaline hydrolysis of **1**.

The proposed topologies of the **1/2** and **1/3** complexes were established using ROESY 1D and they are

**Fig. 4** Proposed topology for the **1/3** complex, in fast equilibrium, according to <sup>1</sup>H NMR evidences

intrinsically distinct. In **1/2**, **1** is almost totally inserted into the cyclodextrin **2** cavity while in **1/3** it is located at the sulphonic rim so that we can say that **1/2** complexation is governed by hydrophobic interactions while complexation of **1/3** is governed by ion pair interactions. A topic of major interest in the development of drug delivery system is to understand the specific features that determine the interaction between *host-guest* molecules and the study of the molecular aspects involved in it is continuing in our laboratory.

## Experimental

### Chemicals and reagents

Tetracaine **1** (99%),  $\beta$ -CD **2** (99%), and D<sub>2</sub>O (99.75%) were purchased from Aldrich, Acros Organics and Merck, respectively. All other reagents were of analytical grade. *p*-sulphonic acid calix[6]arene **3** was synthesized in our laboratory following literature procedures [20].

### Preparation of solid inclusion complexes

Inclusion complexes (**1/2** or **1/3**) with 1:1 molar ratios were prepared by shaking appropriate amounts of **1** and **2** or **3**, e.g. 2 mmol l<sup>-1</sup>, in deionized water at room temperature (298 ± 1 K) for 1 h. Kinetic experiments revealed that equilibrium was reached after 40 min incubation (data not shown).

After reaching equilibrium, the 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<sub>2</sub>O. For the experiments with uncharged **1**, the pH value of the solutions was adjusted by addition of 0.02 mol l<sup>-1</sup> carbonate buffer, prepared in D<sub>2</sub>O.

Routine 1D <sup>1</sup>H experiments were acquired with an INOVA-500 Varian spectrometer operating at 499.885 MHz for <sup>1</sup>H (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.

*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 298 K in 5 mm tubes.

*HR-DOSY* experiments were carried out by carefully choosing the correct pulse sequence and gradients for the experiments. The measurements were made using: (a) 5 mm inverse probe with Z-gradient coil; (b) the GCSTESL (Gradient Compensated Stimulated Echo Spin Lock) HR-DOSY sequence; (c) amplitudes of the gradient pulses ranging from 0.000685 to 0.003427 T cm<sup>-1</sup>, where an approximately 90%–95% decrease in the resonance intensity was achieved at the largest gradient amplitudes. For all experiments, 25 different gradient amplitudes were used. The baselines of all arrayed spectra were corrected prior to processing the data. The processing program (the DOSY macro in a Varian instrument) involves 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 only 512 lines at a time. The results of the DOSY method of analysis are pseudo two-dimensional spectra with NMR chemical shifts along one axis and calculated diffusion coefficients (m<sup>2</sup> s<sup>-1</sup> × 10<sup>-10</sup>) along the other.

### Determination the stoichiometry of complexation

Job plots have been prepared with 2 mmol l<sup>-1</sup> stock solutions of **1** and **2** or **1** and **3** [21].

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