Supramolecular interaction of procaine with *p*-sulfonated calix[6]arene

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ABSTRACT

The inclusion interaction between procaine (PC) and *p*-sulfonated calix[6]arene (SC6A) was investigated via fluorescence and ^{1}H NMR spectroscopy. The effects of pH, temperature, ionic strength, and SC6A concentration were examined in detail. In a Britton–Robinson solution with a pH of 7.5, the fluorescence of PC significantly quenched upon the addition of SC6A, which revealed the formation of inclusion complexes between PC and SC6A. The stoichiometric ratio of 1:1 was obtained via the continuous variation method. The inclusion constant of PC–SC6A complexes was 1.29×10^4 L/mol, which was obtained via the nonlinear curve fitting method. The ^{1}H NMR spectra verified that the aliphatic chain of PC may be partially penetrated into the hydrophobic cavity of SC6A. This finding was confirmed by density functional theory calculations.
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INTRODUCTION

In recent years, non-covalent intermolecular interactions have a major function in biological chemistry as well as in supramolecular chemistry[1,2]. Many researchers in the field of molecular recognition processes focused their studies on a number of therapeutic molecules, whose bioavailability is often affected by problems such as limited solubility and stability. Molecular complexation with calix[*n*]arenes may be a potential application among the different methods proposed to improve the development of drug delivery systems[3]. Calixarenes[4], which are composed of phenolic units linked by methylene groups, represent one of the most widely studied classes of organic supramolecular hosts.

They are described as "macrocycles with (almost) unlimited possibilities" because of their capacity for facile modification. *p*-Sulfonated calixarenes, a typical class of water-soluble calixarene derivatives, with flexible and appropriate cavities have been the focus of many studies because of their high water solubility, less toxicity, and potential biological and pharmaceutical activities[5-8]. Given the 3D, flexible, ð-rich cavities of *p*-sulfonated calix[*n*]arenes (*n* = 4, 6, and 8), they are capable of versatile complexation with a range of guest molecules. Many research papers on the inclusion of various guests, including inorganic cations[9,10], organic ammonium cations[11-14], neutral molecules[15,16], dyes[17,18], native amino acids[19], and even biological or pharmaceutical molecules[20], have been recently published. Furthermore,

_p_-sulfonated calix[_n_]arenes have been applied to improve the solubility and stability of drugs and enzyme mimics[21-24].

Procaine (PC, Figure 1a) is a local anesthetic that is widely used for injections and local application to mucous membranes. PC inhibits the axonal voltage-gated sodium channels responsible for depolarization and thus prevents or diminishes the conduction of sensory nerve impulses near the site of their application. However, PC often shows a short duration of action and adverse side effects, such as cardiac and neurological toxicities, which are sometimes accompanied by allergic reactions. To mask or abolish all or several of these undesirable effects, Li et al.[25] investigated the determination of the inclusion constant of PC hydrochloride to b-cyclodextrin by using frequency doubling scattering and second-order scattering technology. To our knowledge, the supramolecular interactions between _p_-sulfonated calix[_n_]arenes and PC as determined via spectrofluorimetry have not been reported compared with other macrocycles, such as cyclodextrin[26] and cucurbituril[27]. Thus, the formulation of PC as a microencapsulate with _p_-sulfonated calixarenes is expected to show better bioavailability, with all or several of these undesirable effects masked or abolished.
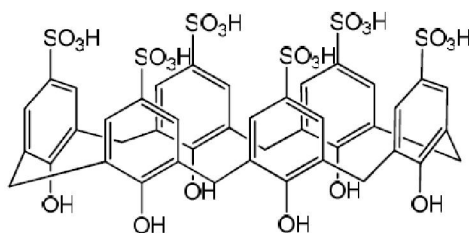
This paper used _p_-sulfonated calix[6]arene (SC6A, Figure 1b) as the host molecule to investigate the inclusion interaction with PC. The various factors affecting the inclusion interaction and the possible inclusion model are discussed. This research provides useful information for improving the bioavailability of PC.

MATERIAL AND METHODS

Apparatus
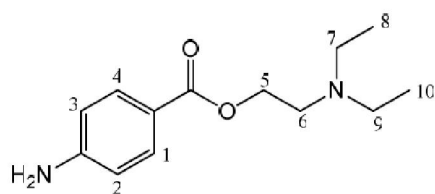
Fluorescence spectra were measured with a Agilent Technologies Cary Eclipse Fluorescence spectrofluorometer equipped with a pulsed lamp. The slit width of both the excitation and emission monochromators was set at 5 nm. The fluorescence spectra were recorded at a scan rate of 600 nm min^{-1}. The pH values was measured with a pHS-3TC digital precision pH meter (Shanghai, China). In the experiment of effect of temperature, the temperatures were controlled by using a thermostated cell holder and a thermostatically controlled water bath. All other measurements were performed in a standard 10 mm path–length quartz cell set to a temperature of 25.0 ± 0.5 °C. ^{1}H NMR spectra were recorded using a Bruker DRX-600MHz spectrometer (Switzerland) in D$_2$O. Molecular modeling calculations were optimized at the B3LYP/6-31G(d) level of density functional theory with the Gaussian 03 program.

Reagents

All chemicals were of analytical reagent grade, and double-distilled water was used throughout the procedures. SC6A was synthesized according to the literature[28] and identified by IR, ^{1}H NMR and element analysis. PC was purchased from Chinese Academy of Food and Drug Testing (content > 99.8%). The stock solution of 1.0×10^{-3} mol/L PC and 1.0×10^{-3} mol/L SC6A were prepared by directly dissolving in double-distilled water. Working solutions were obtained by dilution of the stock solution. A Britton-Robinson buffer solutions were prepared using 0.04 mol/L boric acid, acetic acid and phosphoric acid, then was adjusted to accurate values by using 0.2 mol/L sodium hydroxide.

Experimental procedure

A total of 1.0 mL of 1.0×10^{-4} mol/L PC solution was transferred into a 10 mL volumetric flask, and

Figure 1 : The structures of (a) PC and (b) SC6A
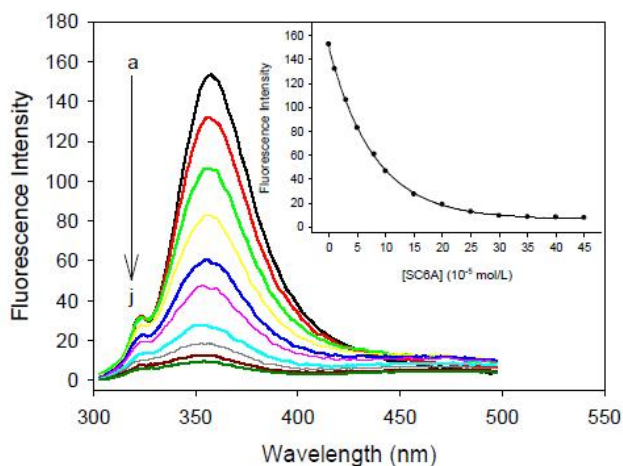
Figure 2 : Fluorescence spectra of PC (1.0×10⁻⁵ mol/L) in the absence and presence of SC6A at pH 7.5. The concentration of SC6A (×10⁻⁵ mol/L) is (a) 0, (b) 1.0, (c) 3.0, (d) 5.0, (e) 8.0, (f) 10, (g) 15, (h) 20, (i) 25, (j) 30. Insert: SC6A concentration dependence of the fluorescence intensity at 356 nm (●); the line represents the result of the nonlinear least-square fit. Slit widths: 5 nm

an appropriate amount of $1.0×10^{-4}$ mol/L SC6A was added. The pH was controlled by 1.0 mL of Britton-Robinson buffer solutions. The mixed solution was diluted to the final volume with distilled water and shaken thoroughly, then equilibrated for 15 min at room temperature. The fluorescence intensity values of the experimental ($F_{PC-SC6A}$) and blank solutions (F_{PC}) were measured at 356 nm using an excitation wavelength of 290 nm.

RESULTS AND DISCUSSION

The formation process of the complex

PC itself could emit strong fluorescence in the Britton-Robinson buffer solution at pH 7.5 with excitation and emission wavelengths at 290 and 356 nm, respectively. When an appropriate amount of $1.0×10^{-4}$ mol/L SC6A was added, the fluorescence intensity of PC decreased dramatically. As shown in Figure 2, the marked fluorescence quenching and blue shifts observed prove the formation of PC–SC6A inclusion complex.

Stoichiometry and inclusion constant of the inclusion complex

The addition of a nonfluorescent host (H) results in the formation of 1:1 host-guest inclusion complexes (H-G) which, in turn, enhances the fluores-
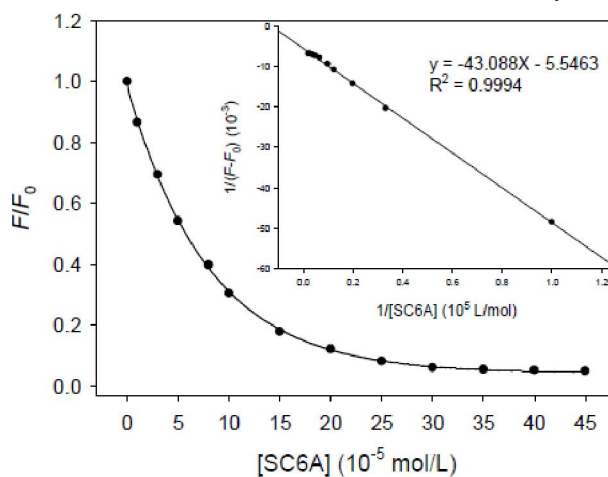
Figure 3 : Fluorescence quenching of PC fluorescence as a function of added SC6A (pH 7.5), the solid line shows the best fit of the data to Eq. (1). The inset shows the linear double reciprocal plot indicating 1:1 complexation

cence of the guest (G). The value of the enhanced fluorescence is dependent on the concentration of the added H,[29, 30] as shown in the following equation:

$$F / F_0 = 1 + \left(F_\infty / F_0 - 1 \right) \frac{[H] K}{1 + [H] K} \qquad (1)$$

where F_0 is the fluorescence intensity of G in the absence of H, F is the observed fluorescence intensity at each H concentration tested, F_∞ is the enhancement when 100% of G is complexed, and K is the equilibrium association constant for the 1:1 complexation.

$$H + G \Leftrightarrow H\text{-}G \qquad (2)$$

$$K = \frac{[H\text{-}G]}{[H][G]} \qquad (3)$$

The 1:1 complexation (and, hence, the applicability of Eq. 1) can be confirmed from the double reciprocal plot of $1/(F - F_0)$ *vs.* $1/[H]$. The plot will be linear if only 1:1 complexation occurs and will be nonlinear if higher-order complexes also form.

In the interaction between SC6A and PC, the equilibrium reaction is as follows:

$$SC6A + PC \Leftrightarrow SC6A\text{-}PC \qquad (4)$$

The equilibrium association constant is defined as follows:

$$K_{SC6A-PC} = \frac{[SC6A\text{-}PC]}{[SC6A][PC]} \qquad (5)$$

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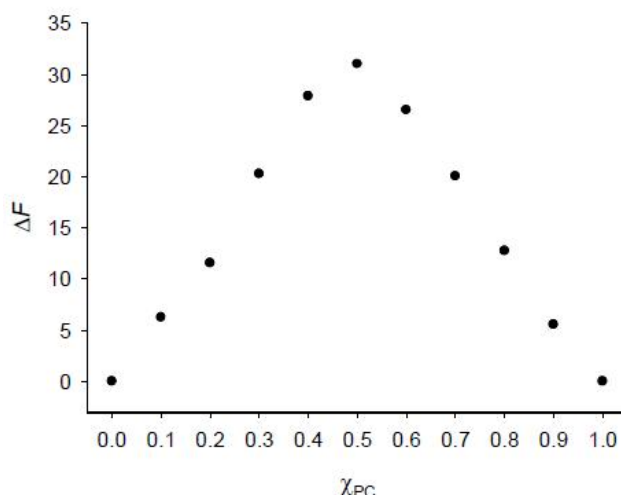**Figure 4 : Job's plot for PC-SC6A inclusion complex in Britton-Robinson buffer solution (pH 7.5)**

Figure 5 : Dependence of fluorescence intensity of PC-SC6A inclusion complex on pH: C_{PC} = 1.0×10^{-5} mol/L, C_{SC6A} = 3.0×10^{-4} mol/L

As shown in Figure 3, the quenching of PC fluorescence as a function of added SC6A. The solid line shows the best fit of the data to Eq. (1) using the nonlinear least squares method. The inset shows the linear double reciprocal plot (R^2 = 0.9994), confirming the 1:1 stoichiometry of the complex. The inclusion constant (pH 7.5) can be calculated as 1.29×10^4 L/mol. This value was determined by dividing the intercept by the slope of the corresponding line. The large inclusion constant demonstrated the strong interaction of PC with SC6A.

To further evaluate the stoichiometry for PC-SC6A inclusion complex, Job2 s plot was performed. A series of solution, in which the total concentration is 1.0×10^{-5} mol/L, were prepared and the mole ratio of PC changed from 0 to 1. The fluorescence intensity in absence (F_0) and presence of SC6A (F) were determined, respectively. The plot of ΔF vs. χ_{PC} was shown in Figure 4. A maximum value of 0.5 for χ_{PC} was observed, meaning that the stoichiometry of PC-SC6A inclusion complex is 1:1. This is in agree-
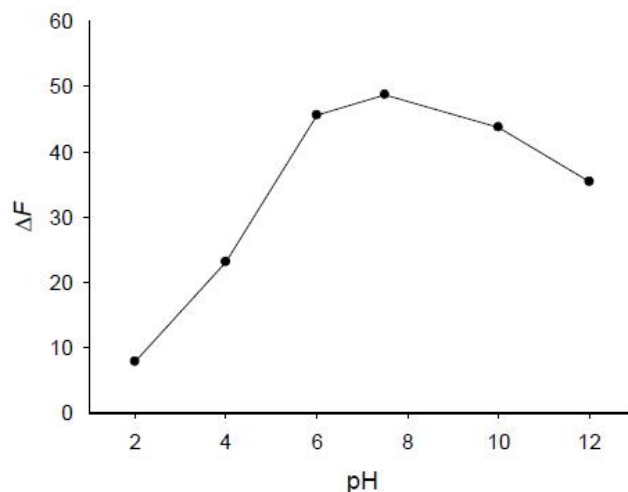
ment with the double reciprocal plot.

Effect of pH

Figure 5 showed the effect of pH on the fluorescence intensities of PC in the presence of SC6A. The fluorescence intensities variation (ΔF) of PC in the presence of SC6A increased with the increase of pH and reached the highest at pH 7.5, however, it decreased with further increase of pH. It suggested that the inclusion interaction of PC with SC6A was occurred suitably at pH 7.5. Using the nonlinear curve fitting method, the inclusion constants of the inclusion complexes between PC and SC6A in different pH solutions were calculated and listed in TABLE 1. It is noted that inclusion constants at pH = 4–7.5 were slightly bigger than at pH<4 or pH>10, implying that the inclusion interactions of PC with SC6A were not basically impacted by pH values. Only take into consideration the optimal inclusion condition of SC6A (6.0–8.5)[31] and physiological environment of drug action, the buffer of pH 7.5 was chosen in

TABLE 1 : Inclusion contants (K) for 1:1 inclusion complexes of PC with SC6A in the absence and presence of NaCl at different pH

	pH	2	4	7.5	10	12
		m = 1:1	*m* = 1:1	*m* = 1:1	*m* = 1:1	*m* = 1:1
Without NaCl	K (L/mol)	7.32×10^3	1.10×10^4	1.29×10^4	1.08×10^4	5.96×10^3
	R^2	0.9998	0.9984	0.9994	0.9991	0.9967
With NaCl	K (L/mol)	3.01×10^3		1.31×10^4	1.07×10^4	
	R^2	0.9916		0.9994	0.9979	

the following study.

Effect of ionic strength and SC6A concentration

The effects of ionic strength on the fluorescence quenching intensity of SC6A-PC system at pH 2.0, 7.5 and 10.0 were examined and the corresponding inclusion constants of SC6A-PC in the presence of NaCl were calculated and listed in TABLE 1, respectively. It could be seen that NaCl ionic strength has obvious effect on the inclusion process at pH 2.0, which implies that the electrostatic interaction maybe has some contribution for the formation of inclusion complex between PC and SC6A. However, compared with pH 2.0, it was noted that the variations of inclusion constant at pH 7.5 and 10.0 were unconspicuous in the absence and presence of 0.25 mol/L NaCl. This demonstrated that the electrostatic interaction should not be the main driving force for the inclusion of PC by SC6A at pH 7.5 and 10.0.

In addition, the effect of SC6A concentration on the fluorescence intensity of PC was also investigated. PC concentration was held constant at 1×10^{-5} mol/L, while the concentration of SC6A varied from 0 to 5.0×10^{-4} mol/L. The results demonstrated that the remarkable fluorescence quenching effect occurred with the addition of $0–3.0\times10^{-4}$ mol/L SC6A, however, when the concentration of SC6A was larger than 3.0×10^{-4} mol/L, the variation of the fluorescence intensity gradually leveled off. This means that the inclusion process has got to the equilibrium condition.

Effect of temperature

Effect of temperature was examined in detail. The inclusion constants (K) of the inclusion com-

TABLE 2: Effect of temperature on inclusion constants (K) of PC with SC6A in Britton-Robinson buffer solution

pH	T (K)	K (L/mol)	R^2
7.5	293	1.28×10^4	0.9996
	298	1.29×10^4	0.9995
	303	1.17×10^4	0.9998
	308	1.23×10^4	0.9993
	313	1.04×10^4	0.9989

plex between PC and SC6A were determined at various temperatures ranging from 293 to 313 K and listed in TABLE 2. It was obvious that the inclusion constants were little sensitive to temperature, suggesting that the supramolecular system PC-SC6A was relatively stable in the temperature range of 293 to 313 K. This is favorable for the delivery of drug in the body.

Inclusion mechanism

To investigate the possible inclusion model between SC6A and PC, molecular modeling calculations were optimized at the B3LYP/6-31G(d)[32] level of density functional theory[33,34] with the Gaussian 03 program[35]. The results indicated the partial inclusion of PC in the SC6A hydrophobic core (Figure 6). In the energy-minimized structure, the partial penetration of the aliphatic chain of PC into the SCX6 cavity and the location of the lipophilic aromatic in the vicinity of negatively charged sulfonyl groups can be verified.

In complex PC–SC6A, the aromatic plane of the guest molecule was slanted outside the SC6A cavity with unconventional hydrogen bonds. Two ethyl arms of the aliphatic moiety pointed inside the SC6A, which were stabilized by C–H···π interactions. The remaining interaction between SC6A and PC was a

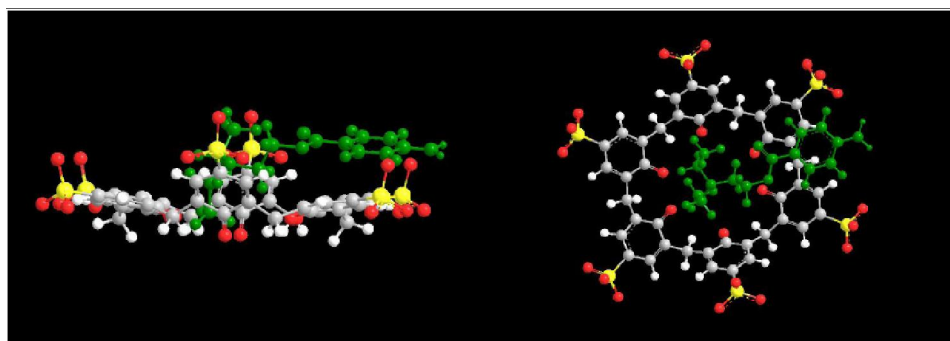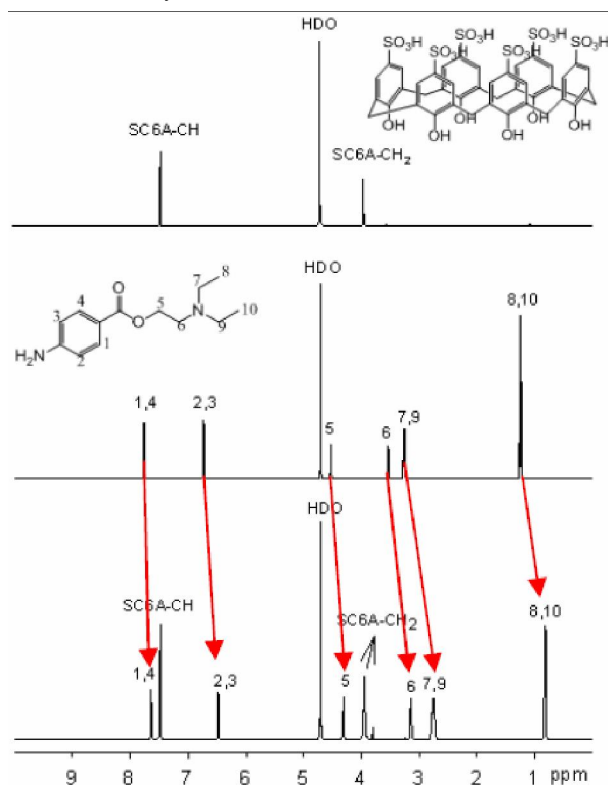**Figure 6 : Two views on the optimized structure of the inclusion complex of PC with SC6A**

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Figure 7 : ¹H NMR (600 MHz) spectrum of SC6A (upper), PC (middle) and SC6A-PC complex (lower) in D_2O

hydrogen bond, which is the more flexible cone-shaped conformation of the SC6A cavity. This structural distinction is ascribed to the wider SC6A cavity. Thus, SC6A can be considered as a shallow-dish shape.

To verify the inclusion model between SC6A and PC, ¹H NMR experiments were carried out in D_2O at room temperature. Figure 7 shows the ¹H NMR spectra of a 1:1 host–guest complex between PC and SC6A. Compared with the proton resonances of the free PC molecules, the chemical shifts of PC protons changed after complexation with SC6A. This result suggests that PC may penetrate into the SC6A cavity to form the inclusion complexes, which led to the shielding of the PC protons. The ¹H NMR spectrum displayed that the presence of SC6A caused significant upfield shifts for H_{10}, H_8, H_7, H_9, H_6, and H_5 of PC. This observation is characteristic of the protons of the aliphatic chain of PC included into the SC6A cavity. The signals of H_1, H_4, H_2, and H_3 protons of the PC slightly experienced upfield chemical shifts, which is characteristic of the protons of the lipophilic aromatic moiety located in a less

acclivitous orientation in the negatively charged sulfonyl groups of SC6A. These deduced complex structures were verified by previous studies on PC–SC6A complexes.

Based on the aforementioned structure analyses and ¹H NMR investigations, C–H···π interactions between the ammonium groups in PC guests and the aromatic cavities of calixarenes, charge interactions of the PC guest's cationic moiety with sulfonate groups, and hydrogen bonds between aniline and sulfonate groups were the dominant driving forces that led to exothermic enthalpy changes in the host–guest complexation.

CONCLUSIONS

The inclusion interaction between PC and SC6A was investigated and characterized via fluorescence spectroscopy and ¹H NMR. The inclusion stoichiometric ratio of 1:1 was verified, and the inclusion constant was estimated. Density functional theory calculations were carried out to propose the possible molecular inclusion model of PC with SC6A. Apparent chemical shift variations of H_{10}, H_8, H_7, H_9, H_6, and H_5 of PC validated that the aliphatic chain was penetrated into the hydrophobic cavity of SC6A with a tilt inconformation to form the host–guest complex. ¹H NMR results are in agreement with those obtained from density functional theory calculations. This study provides useful information for the analytical application of PC and stimulates further investigation to exploit the interactions between PC and other calixarenes.

ACKNOWLEDGEMENTS

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