J Incl Phenom Macrocycl Chem (2012) 72:389–395
DOI 10.1007/s10847-011-9994-6

ORIGINAL ARTICLE

Spectroscopic studies on the inclusion interaction of *p*-sulfonatocalix[6]arene with vitamin B_6

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Received: 20 January 2011 / Accepted: 26 May 2011 / Published online: 18 June 2011

Abstract The formation of the inclusion complex of *p*-sulfonatocalix[6]arene (SCX6) with different forms of vitamin B_6 (VB_6) was studied by using fluorescence spectroscopy. VB_6 can exist in one of three forms (the acidic form, neutral zwitterionic form and basic form) depending on pH. The fluorescence intensities of acidic and basic forms of VB_6 remarkably decreased in presence of SCX6. SCX6 preferred to form 1:1 inclusion complexes with acidic and basic forms of VB_6 but hardly form inclusion complex with neutral zwitterionic form. According to the nonlinear curve fitting method, the inclusion constant (K) for the formation of inclusion complexes of acidic and basic forms of VB_6 with SCX6 were evaluated to be 1.4×10^4 and 9×10^3 L/mol, respectively. The binding affinity of SCX6 towards acidic form is attributed to hydrogen bonds and hydrophobic interaction, furthermore, additional electrostatic interaction also plays a crucial role. The possible inclusion mode was given by ^{1}H NMR technique.

Keywords *p*-Sulfonatocalix[6]arene · Vitamin B_6 · Spectrofluorometric titration · Inclusion

Introduction

Extensive interest on the inclusion complexation between the host molecules and drug guest molecules and their applications have been attracted in chemical and medicinal fields toward drugs with great efficiency and selectivity [1–6]. With classes of macrocyclic compounds used as host receptors are focused on cyclodextrin [7, 8] and calix[*n*]arenes [9, 10]. Initially, natural and substitutional α, β, γ-cyclodextrins were served to encapsulate the drugs into their hydrophobic cavities, hoping to improve the biological utility of the drugs. Indeed, studies have also verified that cyclodextrins as good carriers could deliver drugs to the target and mildly release the active ingredient, leading the enhancement of the drug efficacy in vivo [4, 8].

More recently, compared to cyclodextrins, calix[*n*]arenes have been introduced as drug receptors in biomedical field due to their unique properties including excellent biocompatibility [11, 12] and amenable functionalization of the macrocyclic skeleton [13–15]. In order to further improve the solubility of calix[*n*]arenes in aqueous solution, great efforts have been made to functionalize the platform of calix[*n*]arene at the lower rim and upper rim to conquer the poor solubility of calixarenes [16, 17]. As a result, modified calixarenes have been exploited as molecular hosts for modification of numerous drug molecules [18, 19]. Among these calixarenes, especially, *p*-sulfonated calixarenes with low biological toxicity and good water solubility exhibit potential pharmaceutical and biomedical application [9, 20, 21]. For instance, *p*-sulfonated calixarenes have been reported to increase the solubility of some insoluble drug such as niclosamide, furosemide and nifedipine [12, 22, 23]. Calix[4]arene-tetrasulfonate improved the catalytic reaction on hydrolysis of adenosine triphosphate (ATP) in aqueous solution through the formation of supramolecular complex between calix[4]arene-tetrasulfonate and ATP [24]. Furthermore, it has also been reported that sulfonated calixarene could inhibit chloride channel activity associated with expression of P64-protein in Hela cells in vitro [25].

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Vitamin B_6 (VB_6, Fig. 1a) plays a vital role as the cofactor of a large number of essential enzymes in the human body. It is effective in treatment of pregnancy sickness, symptoms of the pre-menstrual syndrome, muscular weakness, recurrent oxalate urolithiasis and hyperkinetic syndromes in children [26, 27]. VB_6 is mainly involved in the metabolism of amino acids, carbohydrates, fats and the formation of haemoglobin. However, it will be decomposed when suffering from heat, sunlight, acid and alkali during manufacture and storage process. In addition, low bioavailability and weak assimilate are also thorny issues. Several researches were involved the inclusion interaction between vitamin and calixarene. Li [28] employed *p-tert*-butyl-calix[8]arenes-bonded silica gel as the stationary phase for the separation of six vitamins. Ijeri [29] introduced electrocatalytic method for the determination of vitamin C based on carbon paste electrode modified with *p-tert*-butyl-calix[n]arenas. *p*-sulfonated calixarenes and *p*-(*p*-sulfonated benzeneazo) calix[6]arene have been used to the inclusion of vitamin K_3 [30, 31].

In this paper, we aim to investigate inclusion interaction of *p*-sulfonatocalix[6]arene (SCX6, Fig. 1b) to different VB_6 form in aqueous solution based on fluorescence spectroscopy. The inclusion interactions between SCX6 and VB_6 will be evaluated. Moreover, main interaction forces affecting the molecular recognition are discussed.

Experimental

Apparatus

All the fluorescence measurements were performed with a Cary Eclipse fluorescence spectrophotometer (Varian) using a conventional 1 cm × 1 cm quartz cell. Excitation and emission bandwidths were both set at 5 nm. A model pHs-2 meter (the 2nd Instrument Factory, Shanghai, China) was used for accurate adjustment of pH. Absorption spectra were recorded on a Puxi TU-1901 double-beam spectrophotometer (Beijing, China). The measurement of ^{1}H NMR was performed on DKX-300MHZ (Bruker, Switzerland). All experiments were carried out at room temperature.

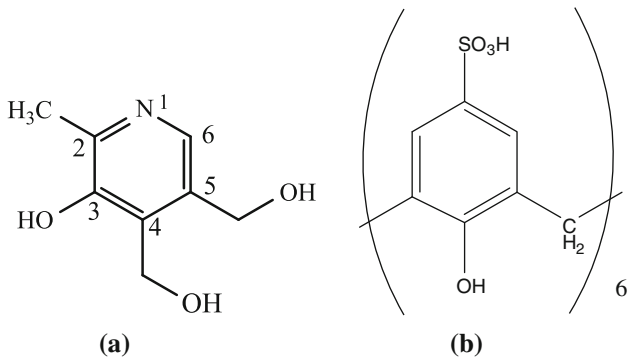

Fig. 1 Molecular structures of (**a**) VB_6 and (**b**) the 4-sulfonated calix[6]arene

Reagents

VB_6 (Biochemical reagent) was purchased from Shanghai Reagent Factory. SCX6 hydrate was obtained from Acros Organics. The stock solution of 0.25 mM VB_6 and 10 mM SCX6 were prepared by directly dissolving their powder in doubly distilled water, respectively. 0.5 M Na_2HPO_4–NaH_2PO_4 buffer solution was used to control the pH-value. All other reagents were analytical-reagent without purification. Doubly distilled water was used throughout.

Procedure

A 1-mL aliquot of the stock solution (0.25 mM) of VB_6 was transferred into a 10 mL volumetric flask, and an appropriate amount of 10 mM SCX6 was added. The pH was controlled by 0.5 M phosphate buffer solution. The mixed solution was diluted to the final volume with distilled water and shaken thoroughly, then equilibrated for 30 min at room temperature. The fluorescence spectra or absorption spectra were measured by using 1 cm quartz cell.

Results and discussion

Inclusion complexation of VB_6 with SCX6

VB_6 itself could emit strong fluorescence (pH 4.0) with maximum emission wavelength at 397 nm (corresponds to the maximum excitation wavelength of 290 nm). With addition of SCX6, it was observed that fluorescence intensity of VB_6 was noticeable decreased accompanying with a red shift of maximum excitation wavelength from 290 to 298 nm (Fig. 2). These remarkable changes of the fluorescence spectra were attributed to the interaction between VB_6 and SCX6, implying the formation of VB_6–SCX6 inclusion complex.

Effect of pH

Figure 3 showed the effect of pH on the fluorescence spectra of VB_6 in the absence and presence of SCX6. The fluorescence emission spectra were very sensitive to pH. With addition of SCX6, a noticeable decrease of fluorescence intensity of VB_6 was observed at pH 4.0 or 10.0, however, at pH 7.5, the fluorescence intensity remained unchanged. In addition, it was also observed that the

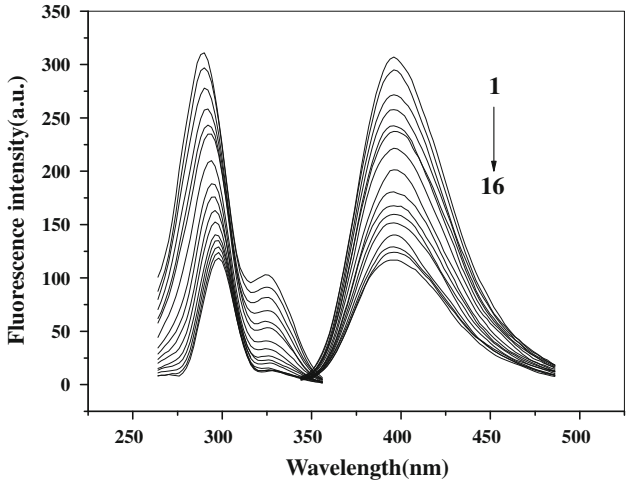

Fig. 2 Fluorescence spectra of 25 μM VB_6 in the absence and presence of SCX6 at pH 4.0. The concentration of SCX6 (μM): (*1*) 0, (*2*) 0.667, (*3*) 13.3, (*4*) 23.3, (*5*) 33.3, (*6*) 43.3, (*7*) 60, (*8*) 86.7, (*9*) 110, (*10*) 133, (*11*) 160, (*12*) 193, (*13*) 227, (*14*) 260, (*15*) 293, (*16*) 327

Table 1 Fluorescence properties of 2.5×10^{-5} mol/L VB_6 at different pH media

pH	4.0	7.5	10.0
λ_{ex} (nm)	290	324	310
λ_{em} (nm)	397	397	380

maximum emission wavelength of VB_6 was shifted from 380 to 392 nm at pH 10.0. The detailed fluorescence excitation and emission wavelength of VB_6 in different media was listed in Table 1. These phenomena verified that VB_6 could be included into the cavity of SCX6 in the acidic or alkaline media, but in the neutral media, the inclusion complex between VB_6 and SCX6 hardly be formed.

It was noted that the fluorescence intensity variations of VB_6 with addition of SCX6 were very sensitive to pH. One of the major factors affecting the inclusion interaction is the hydrophobicity of the guest, which is related to the molecular form of VB_6. In addition, hydrogen bonding and electrostatic interaction between SCX6 and VB_6 may also influence the inclusion interaction.

As shown in Fig. 4, VB_6 participates in the following equilibria in different pH media (Fig. 4). Due to the p*K*a values of VB_6, 4.9 and 8.91 [32, 33], it is in the acidic form and basic form in acidic ($pH \leq 4.9$) and alkaline ($pH \geq 8.91$) media, respectively, however, the neutral zwitterionic form is predominant for $4.9 < pH < 8.91$. With regard to the neutral zwitterionic form, in presence of SCX6, unmeasurable fluorescence variation revealed that

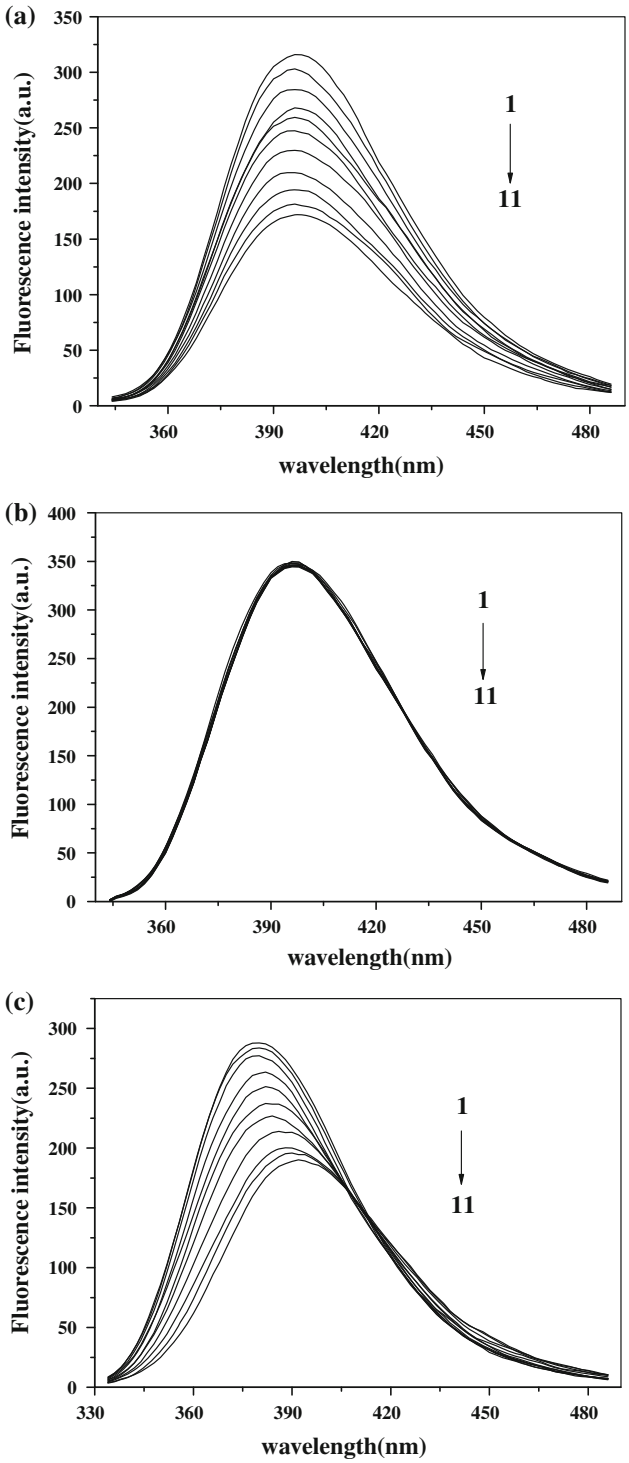

Fig. 3 Fluorescence emission spectra of 25 μM VB_6 in the absence and presence of SCX6 at (**a**) pH = 4.0, (**b**) pH = 7.5, (**c**) pH = 10.0. The concentration of SCX6 (μM): (*1*) 0, (*2*) 0.667, (*3*) 13.3, (*4*) 23.3, (*5*) 33.3, (*6*) 43.3, (*7*) 60, (*8*) 86.7, (*9*) 110, (*10*) 133, (*11*) 160

the inclusion interaction of SCX6 with VB_6 was very weak or even do not occur. It was attributed to relatively strong hydrophilicity in comparison with other forms.

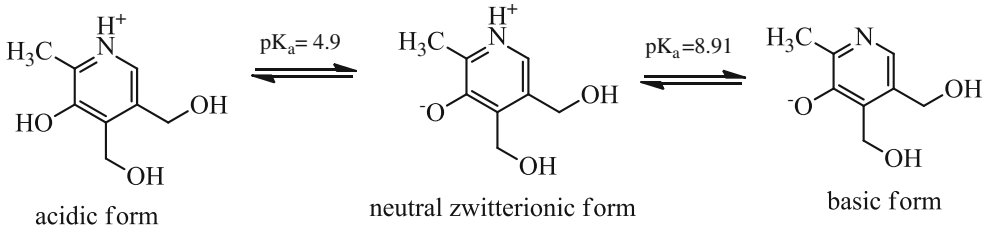

Fig. 4 The reversible equilibria of three forms of VB_6 in the water solution

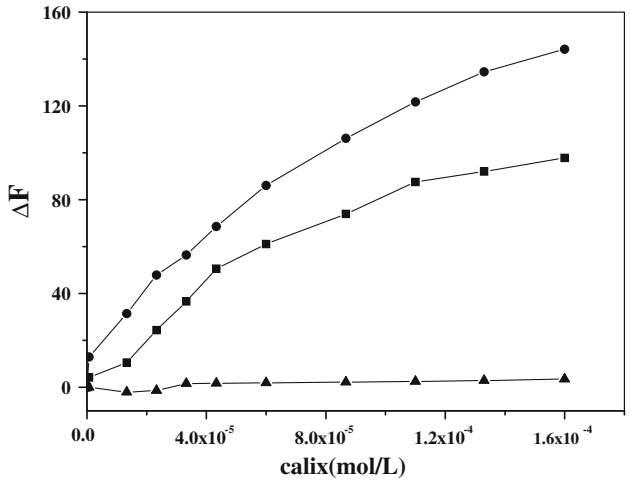

Fig. 5 Dependence of fluorescence intensities of VB_6 on SCX6 concentrations: (*filled circles*) pH = 4.0, (*filled triangles*) pH = 7.5, (*filled squares*) pH = 10.0

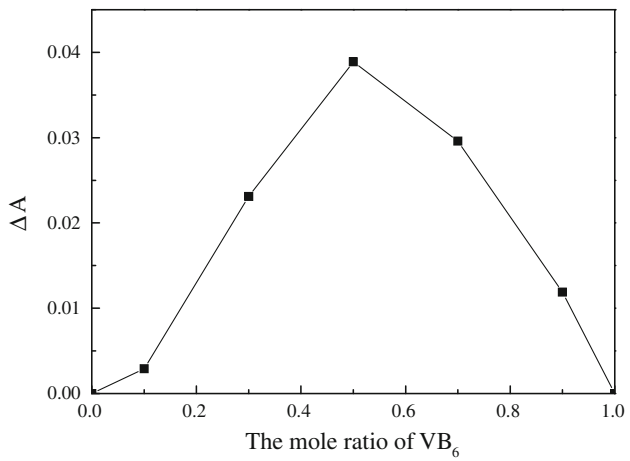

Fig. 6 Continuous variation plot (Job Plot)

In case of pH 4.0, N atom of VB_6 can be firstly protonated [34], accompanying with the formation of intramolecular hydrogen bond ($O_{3'}\cdots H\cdots O_{4'}$). On the other hand, phenolic –OH of SCX6 are not dissociated, so the hydrogen bond between –OH (C_5) of VB_6 and phenolic –OH of SCX6 can be formed. Moreover, there exists additional electrostatic attraction between the positively charged VB_6 ($-NH^+$) and the negatively charged substituent groups ($-SO_3^-$) in the flexible calixarene ring.

But at higher pH (pH = 10.0), the proton is fully removed from on –OH (C_3) of VB_6, leading to the negative charge of VB_6. Except the hydrogen bonding interaction between the –OH (C_4 and/or C_5) of VB_6 and phenolic –OH of SCX6, additional electrostatic repulsion between the negatively charged VB_6 molecule (O^-) and the negatively charged SCX6 ($-SO_3^-$) cumbered the formation of inclusion complex. Herein, compared with the basic form, acidic form of VB_6 is more ease of inclusion by SCX6.

Effect of SCX6 concentration

The effect of the SCX6 concentration on the fluorescence intensity of VB_6 was investigated. The concentration of VB_6 was held constant at 25 μM while that of the SCX6 was varied from 0 to 160 μM. Figure 5 showed that the fluorescence intensity of VB_6 was gradually decreased with increasing SCX6 concentration until the stable inclusion complex was formed. Especially, it was noted that the acidic form of VB_6 resulted in a more effective inclusion interaction with SCX6 which was ascribed to the synergy results of hydrogen bonding interaction, electrostatic interaction and hydrophobic interaction.

Stoichiometry and inclusion constant

The determination of stoichiometry of the inclusion complex was carried out using equimolar variation method. A series of solution, in which the total concentration is 100 μM, were prepared and the mole ratio of VB_6 changed from 0 to 1. The absorbance in absence (A_0) and presence of SCX6 (A) were determined, respectively. A plot of ΔA ($A - A_0$) versus the mole fraction of VB_6 was given in Fig. 6. It showed a maximum at $x_A = 0.5$, implying that the inclusion complexes of VB_6–SCX6 with 1:1 stoichiometry were formed.

The inclusion constant (K) is a measure of the molecular recognition interaction, which reflects the inclusion ability of the host to the guest. In our research, in the case of 1:1 complexation, K was evaluated by the nonlinear curve fitting function that was described in the literature [35].

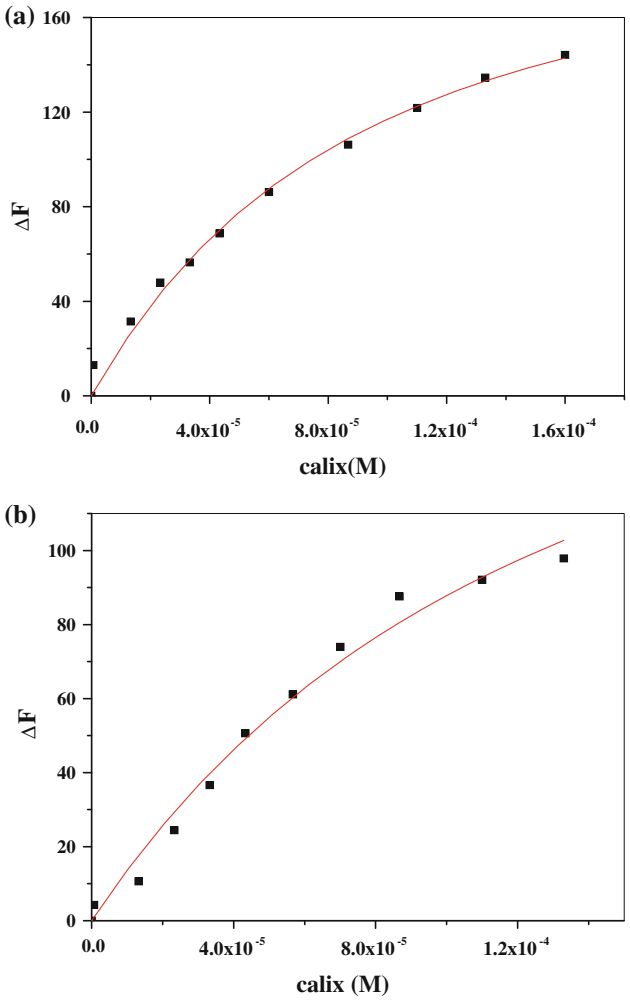

Fig. 7 The plot of the nonlinear curve-fitting for constants in different buffer solution: (**a**) pH 4.0, (**b**) pH 10.0

$$\Delta F = \frac{1}{2}\left\{\alpha\left([\mathrm{H}]_0 + [\mathrm{G}]_0 + \frac{1}{K}\right) - \sqrt{\alpha^2\left([\mathrm{H}]_0 + [\mathrm{G}]_0 + \frac{1}{K}\right)^2 - 4\alpha^2[\mathrm{H}]_0[\mathrm{G}]_0}\right\} \quad (1)$$

where $[H]_0$, $[G]_0$ are the initial concentration of host SCX6 and guest VB_6, respectively. ΔF signifies the change of the fluorescence intensity of VB_6 with the addition of SCX6. α is the proportionality coefficient, which may be taken as a sensitivity factor for the fluorescence variation. K is the inclusion constant. The nonlinear curve-fitting analysis at pH 4.0 and 10.0 were shown in Fig. 7. Good curve-fitting plots ($R > 0.99$) exhibited the formation of inclusion complex between SCX6 and VB_6 with a stoichiometry of 1:1. The relative data were summarized in Table 2. The inclusion constant was very sensitive to the pH values. The SCX6 exhibited different affinity for the three species of VB_6. Generally speaking, this molecular recognition ability of VB_6 by SCX6 followed the order: $K_{\mathrm{pH\ 4.0}} > K_{\mathrm{pH\ 10.0}} > K_{\mathrm{pH\ 7.5}}$. That further suggested, except for hydrogen bonds and electrostatic interaction, the hydrophobicity also is an important role affecting the formation of inclusion complex between host and guest.

Table 2 Inclusion constants K (L/mol) for VB_6–SCX6 complexes at different pH values

pH	4.0	7.5	10.0
n	1:1	–	1:1
K (10^4 L/mol)	1.4	–	0.9
R	0.9959	–	0.9927

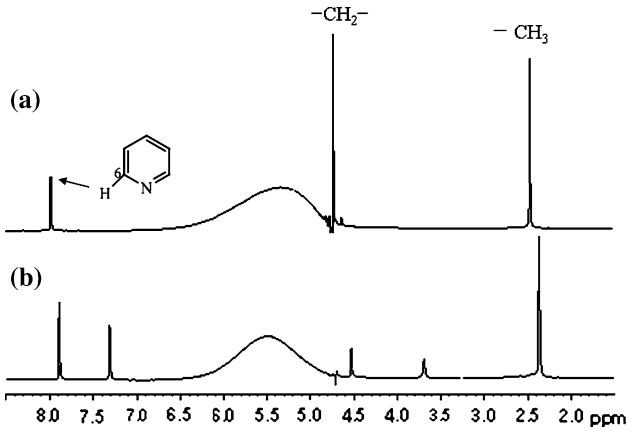

Fig. 8 ^{1}H NMR spectra of VB_6 (*a*) and VB_6–SCX6 complex (*b*) in pD 4.0 solution at 25 °C. The concentrations of VB_6 and SCX6 are 1 mM, respectively

^{1}H NMR studies

To explore the possible inclusion mode between SCX6 and VB_6, ^{1}H NMR spectra were recorded in a pD 4.0 buffer solution (Fig. 8). As can be seen, the δ values of VB_6 protons shift to higher fields after complexation with SCX6 as compared with the free guest. This suggested that VB_6 is encapsulated into the cavity of SCX6 to form the inclusion complex, resulting in an efficient shield toward guest protons. A close comparison of the $\Delta\delta$ values of VB_6 protons after complexation with SCX6 showed that the presence of SCX6 caused significant upfield shifts for the methylene proton ($\Delta\delta = -0.189$), which indicated that the VB_6 may penetrate into the cavity of SCX6 from the 5-position of the guest molecule. The possible inclusion mode was illustrated in Fig. 9. According to this inclusion mode, the hydrogen bond between –OH (C_5) of VB_6 and phenolic –OH of SCX6 can be formed, on the other hand, the protonated N atom of VB_6 is just closed to the anionic sulfonate tails of SCX6, giving additional electrostatic interactions between SCX6 and VB_6.

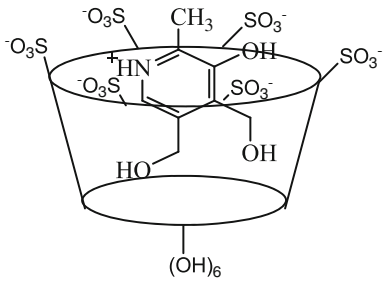

Fig. 9 The possible geometry of inclusion complex of VB_6 with SCX6

Conclusion

The inclusion behavior between SCX6 and VB_6 was studied by spectrometry and ^1H NMR technique. The inclusion constants of SCX6 with VB_6 were evaluated. VB_6 exists in three molecular forms depending on pH and SCX6 is most suitable for inclusion of the acidic form of VB_6. Hydrophobic interaction and hydrogen bonding interaction play important roles in the formation of VB_6–SCX6 inclusion complex. The additional electrostatic effect also contributes the inclusion interaction. This finding will stimulate further investigations to exploit the interactions between other vitamin and *p*-sulfonated calixarenes.

Acknowledgments The work was supported by the National Natural Science Foundation of China (no. 20875059) and the Natural Science Foundation of Shanxi Province of China (no. 2009011012-1).

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