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Dedicated to the memory of
Professor Victor-Emanuel Sahini (1927–2017)

EXPERIMENTAL AND THEORETICAL CHARACTERIZATION OF THE DANOFLOXACIN-γ-CYCLODEXTRIN INCLUSION COMPLEXES

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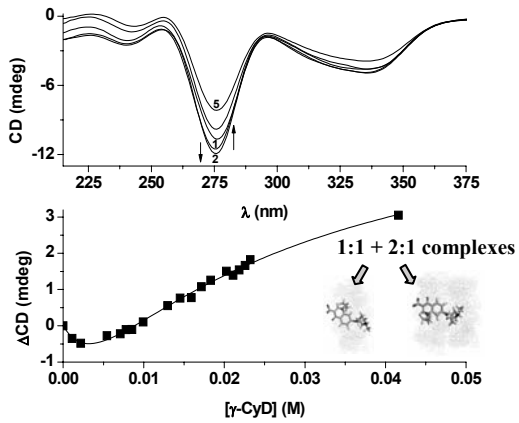
The interest on the drugs encapsulation in the cyclodextrin cavity is due to a series of benefits like the increase in the drug's solubility, bioavailability and the controlled release. In the followings, the interaction of danofloxacin, an antibiotic from the fluoroquinolone class, and γ-cyclodextrin (γ-CyD) will be studied by means of fluorescence, circular dichroism and molecular modeling. Both stoichiometry and association constants were obtained by fitting the dependences of the fluorescence intensity and ellipticity vs. γ-CyD concentration to a non-linear regression model. The signs of the induced circular dichroism spectra were correlated, according to the Harata-Kodaka rules, with the orientation of the guest transition moments (obtained by TDDFT/B3LYP/631++G(d,p)) to the cavity axis. Starting with this information on the ligand orientation in the cavity, the structures of the inclusion complexes were optimized using molecular mechanics.



INTRODUCTION

The interest on the drugs encapsulation in the cyclodextrin cavity and the characterization of the inclusion complexes was continuously increased owing mainly on two aspects. Firstly, from the medical and pharmaceutical point of view, due to a series of benefits like the increase in the drug's solubility, bioavailability and the controlled release.[1,2] Secondly, the application of new experimental spectroscopic and/or calorimetric methods in both solution and solid state open new perspectives in the accumulation of data allowing to obtain a more reliable image of structural aspects of the inclusion complexes. It was found that the analysis of the induced circular dichroism (ICD) spectrum recorded upon complexation of achiral drugs with cyclodextrins (CyD) or the changes in the dichroic signals of chiral drugs allows not only for the possibility to estimate the binding constants but brings about information on the inclusion mode of the guest in the host cavity. The correlation of the experimental signs of the induced dichroic bands with high level TDDFT calculations on the polarisation of the electronic transitions via the semiempirical Harata–Kodaka rules[3,4] contributes to obtain a starting structure of the supramolecular host:gost system.

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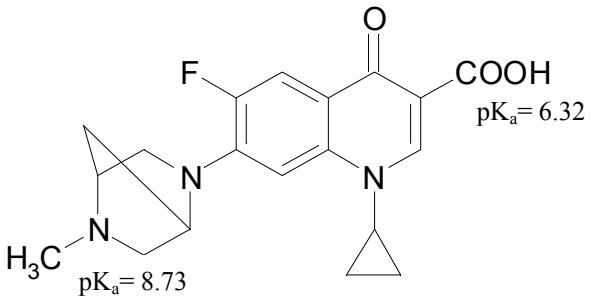

Fig. 1 – Structural formula of danofloxacin. The pKa values reported in the literature[12] are labeled.

In the followings, we continue our previous studies on the spectral and theoretical characterization of the cyclodextrin inclusion complexes of several guests/drugs.[5-9] In the present case, the guest is danofloxacin (Fig. 1), a synthetic chiral antibiotic from the fluoroquinolone class used in veterinary medicine against respiratory diseases[10,11] and the guest is γ-cyclodextrin (γ-CyD), a cyclodextrin preferred due to its larger cavity capable to encapsulate compounds with a complex structure.

Given the presence of carboxylate (pKa_1= 6.32) and amino (pKa_2=8.56) groups[12], danofloxacin could exist in three forms: anion, cation and zwitterion. At pH 7.4, which is approximately equal to the semi-sum of the pKa_1 and pKa_2 values, the zwitterion is the predominant species. We have chosen to work at pH=7.4 in order to have results that will be further compared to those obtained for the interaction of the same guest with albumins.

The methodology used contains three steps: i) the experimental estimation of the stoichiometry and association constant of the guest/host system by two different spectral methods, the steady-state fluorescence and the circular dichroism spectroscopy; ii) the high level DFT/TDDFT characterization of the electronic transitions of the guest, and iii) the modeling of the supramolecular system by molecular mechanics (MM) method in vacuo and water for estimating the main factors contributing to the interaction energy. Our main purpose is to explore the capability of the circular dichroism spectroscopy in correlation with theoretical calculations to well describe the structure of the system.

RESULTS AND DISCUSSION

Fluorescence measurements

Given its high sensitivity and good selectivity, the fluorescence spectroscopy is a powerful tool for determining the association constants of complexes. In this case, the use of this technique is possible because danofloxacin is a fluorescent molecule and, conveniently, γ-CyD is not. Thereby, in water at pH 7.4, the danofloxacin fluorescence spectrum presents a maximum located at 423 nm (Fig. 2A). Gradual addition of γ-CyD causes a moderate decrease of the fluorescence intensity (Fig. 2A), which attests the complexation process. The lack of any isoemissive point is an indication for the existence of multiple equilibria in solution. These equilibria may involve either different types of 1:1 complexes or complexes with different stoichiometry.[13]

A quantitative analysis of the experimental data was made fitting the dependence of the monitored properties (P) on the γ-CyD concentrations to the equations of most common host:guest complexes types, i.e. 1:1; 2:1 and the simultaneous presence of both complexes, respectively:

$$P = \frac{1+P_{11}K_{11}[\gamma-CyD]}{1+K_{11}[\gamma-CyD]} \quad (1)$$

$$P = \frac{1+P_{21}K_{21}[\gamma-CyD]^2}{1+K_{21}[\gamma-CyD]^2} \quad (2)$$

$$P = \frac{1+P_{11}K_{11}[\gamma-CyD]+P'_{21}K_{11}K'_{21}[\gamma-CyD]^2}{1+K_{11}[\gamma-CyD]+K_{11}K'_{21}[\gamma-CyD]^2} \quad (3)$$

where P_0 is the guest property in the absence of the host and P is the guest property at different γ-CyD total concentrations. The indices 11 and 21 refer to the 1:1 and 2:1 host:guest complexes, respectively.

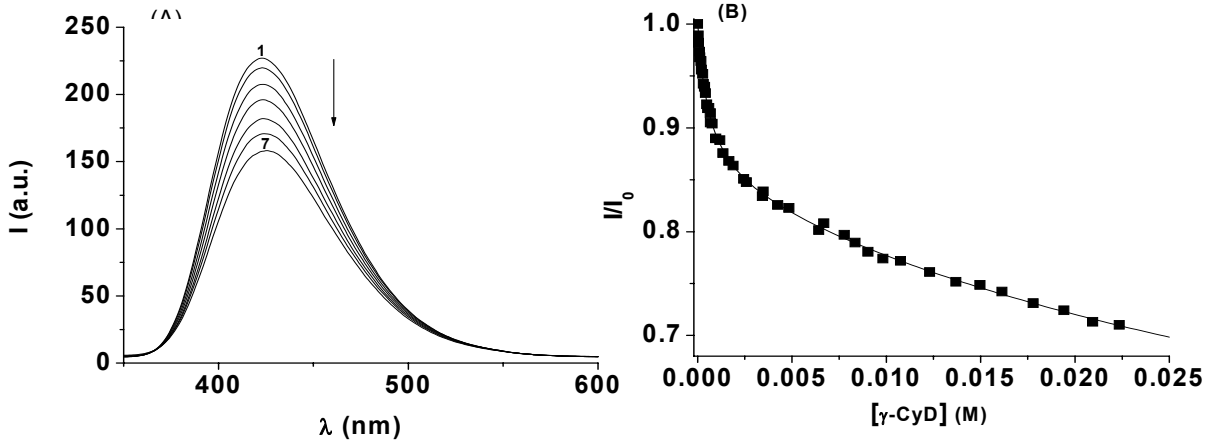

Fig. 2 – (A) Fluorescence spectra of danofloxacin (6.57×10^{-6} M) in water at pH 7.4 (1) and in the presence of different γ-CyD concentrations: (2) 4.58×10^{-4} M; (3) 6.78×10^{-4} M; (4) 1.88×10^{-3} M; (5) 6.42×10^{-3} M; (6) 1.37×10^{-2} M; (7) 2.36×10^{-2} M. (B) Best fit of the normalized intensity vs. γ-CyD concentration was obtained using equation (3) and the parameters are given in Table 1.

Table 1

Values of the association constants of the danofloxacin–γ-CyD complexes obtained by fluorescence and circular dichroism measurements

Method	K_{11} (M^{-1})	K'_{21} (M^{-1})	F-stat *	r^{2} **
Fluorescence	1722 ± 108	20 ± 6	12189	0.998
Circular dichroism	176 ± 64	37 ± 10	1086	0.995

* F-stat is the statistical Fisher parameter; ** r^2 is the correlation coefficient

Equations (1) - (3) were all tested and the best fit was obtained for equation (3), attesting that both 1:1 and 2:1 complexes are formed (Fig. 2B). The association constants are listed in Table 1. It can be seen that the binding constant for the 1:1 complex is much larger compared to the 2:1 complex. A survey of the literature revealed that, at least in the CyD concentration domain used, fluoroquinolones such as ofloxacin,[14] norfloxacin,[15,16] ciprofloxacin,[17] sparfloxacin[18,19] and enrofloxacin[20] give mostly 1:1 complexes. The values reported for their association constants extend in a wide range (17 M^{-1} to 14250 M^{-1}), depending on the CyD type and pH conditions. However, the formation of a 2:1 complex was deliberately obtained in solid state for norfloxacin with β-CyD and 2-hydroxypropyl-β-CyD.[16] Here, using high host concentrations we have shown that, for danofloxacin, a 2:1 complex is also formed.

Circular dichroism measurements

As danofloxacin is a chiral compound, the circular dichroism measurements start with an analysis of the CD and absorption spectra of the free guest in the same conditions as will be used for the inclusion process. In water, at pH 7.4, the absorption spectrum of danofloxacin shows three bands with different intensities (inset of Fig. 3A): at 231 nm (ε = 10430 $M^{-1}cm^{-1}$), at 274 nm (ε = 36470 $M^{-1}cm^{-1}$) and a broad one at 332 nm (ε = 13400 $M^{-1}cm^{-1}$). The values of the molar absorption coefficients indicate that the transitions responsible for all these bands are $\pi\rightarrow\pi^*$. The CD spectrum exhibits two negative bands (274 nm, 332 nm) and a positive one (231 nm) situated at the corresponding wavelengths from the absorption spectrum (Fig. 3A).

Upon addition of γ-CyD up to 2.19×10^{-3} M the bands become more negative (Fig. 3A, 1-2). Further increase in the γ-CyD concentration generates a reverse effect, showing the fact that two types of complexes are present in the solution. After subtracting the CD spectrum of the free danofloxacin, the dependence of the ellipticity readings at 274nm on the γ-CyD concentrations was fitted to the Eqs (1)-(3). Similar to the analysis of the fluorescence data, the best fit was obtained using Eq. (3) in which P was replaced by ellipticity (Fig. 3B). The resulted association constants are also listed in Table 1. Thus, both experimental methods have yielded the same stoichiometry, but different association constants. One explication given for this fact was correlated to the different nature of the processes involved, *i.e.* a ground state for circular dichroism *vs.* an excited state process for fluorescence.[21]

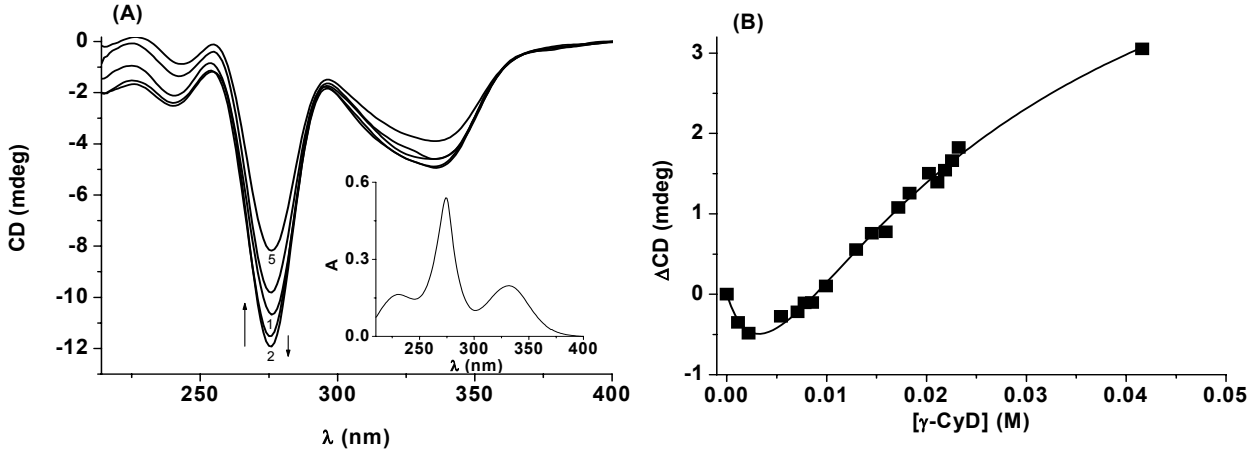

Fig. 3 (A) – Circular dichroism spectra of danofloxacin (4.71×10^{-5} M) in water at pH 7.4 (1) and at different γ-CyD concentrations: (2) 2.19×10^{-3} M; (3) 1.30×10^{-2} M; (4) 4.16×10^{-2} M. Inset: absorption spectra of danofloxacin in water at pH 7.4. (B) Best fit for the danofloxacin ellipticies vs. γ-CyD concentrations dependency, using eq. (3) and the parameters are given in Table 1. The CD data were read at 274 nm, where the spectral changes are more pronounced.

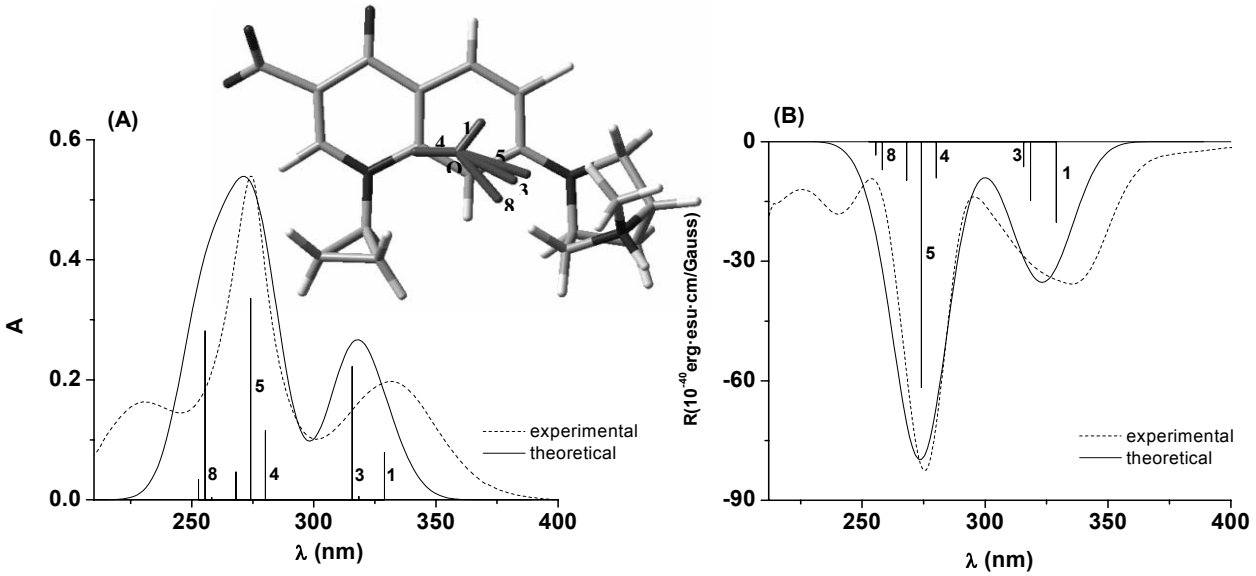

Fig. 4 – (A) Normalized theoretical absorption spectra of danofloxacin obtained by TDDFT calculation on the fully optimized conformation vs. experimental spectrum. Inset: directions of the main electronic transitions (1) 329 nm; (3) 315 nm; (4) 280 nm; (5) 274 nm; (8) 256 nm. (B) ECD spectrum vs. experimental circular dichroism spectrum of danofloxacin.

As the γ-CyD causes at first a decrease and then an increase of the intensity of the danofloxacin CD, it means that the ICD signal is negative for the 1:1 complex and positive for the 2:1 complex. These signs can provide information about the structure of the inclusion complexes, as will be seen below.

Free guest and inclusion complexes calculations

The characterization of the geometry of the inclusion complexes was made in two steps. Firstly, we have obtained information about the guest orientation inside the γ-CyD cavity by corroborating the ICD sign found experimentally with quantum calculations on the free guest. Then, starting with this information, different complexes were optimized by molecular mechanics. The results obtained will be presented in the followings.

A positive/negative ICD signal is, according to Harata-Kodaka rules, an indication that the electronic transition moment of the guest is parallel/perpendicular to the CyD symmetry axis. In order to determine the directions of the electronic transition moments, TDDFT calculations were performed on the fully DFT optimized geometry of danofloxacin, presented in the inset of Fig. 4A, and considering the first nine electronic transitions. The main geometrical parameters are the torsion angles of the carboxylate, cyclopropyl and piperazinyl groups

with respect of the fluoroquinolone ring which were found to be –50°, –41° and –150°, respectively.

The directions of the transition moments characterized by an oscillator strength (f) greater than 0.07 are also displayed in the inset of Fig. 4A. These transitions are located at 329 nm (f=0.0790), 315 nm (f=0.2223), 280 nm (f=0.1153), 274 nm (f=0.3358) and 255 nm (f=0.2816). Figures 4 A,B show the experimental versus calculated absorption and circular dichroism spectra of free guest. As it can be seen the calculated values for the band positions, the relative intensities and the signs in the case of dichroic bands are quite well predicted, excepting for the band located at 231 nm. Despite of the large number of excited states considered, the position for this band was overestimated and the sign is not well predicted.

Since the transitions with larger value of the oscillator strength are polarized along the long axis of the molecule and the ICD signs for the 1:1 and 2:1 complexes are negative and positive, based on empirical Harata-Kodaka rules, we expect an orthogonal and axial inclusion, respectively.

Because fluorescence and circular dichroism measurements have showed two types of danofloxacin–γ-CyD complexes, the structures of both were investigated. First, we have generated a large number of 1:1 structures having in mind the orientation predicted by the ICD data and TDDFT calculations. The structures were obtained considering that the guest can penetrate the cavity from the wide and narrow rim, respectively. Likewise, we have ensured that different moieties of the guest gradually penetrate the cavity. Each structure was optimised and the interaction energy was calculated according to eq. (4). Among them, the structure with the smallest interaction energy (Table 2) was chosen as the most stable and thus the most probable (Fig. 5A). This optimised structure was tested by checking the perpendicular orientation of the guest electronic transitions with respect to the main CyD axis. As it can be seen from Fig. 5A, the danofloxacin molecule is included in a quasi-perpendicular position to the main CyD axis, an angle between the electronic transitions moments and CyD axis greater than 54.7° being accepted for such an assignment.

Table 2

Calculated interaction energies in vacuo and in water, together with the relative contribution of the electrostatic and van der Waals interactions in both complexes

Complex	In vacuo			In water		
	E_{int} (kcal/mol)	% el	% vdw	E_{int} (kcal/mol)	% el	% vdw
1:1	–35.77	8	92	–34.80	8	92
2:1	–45.61	3	97	–42.39	3	97

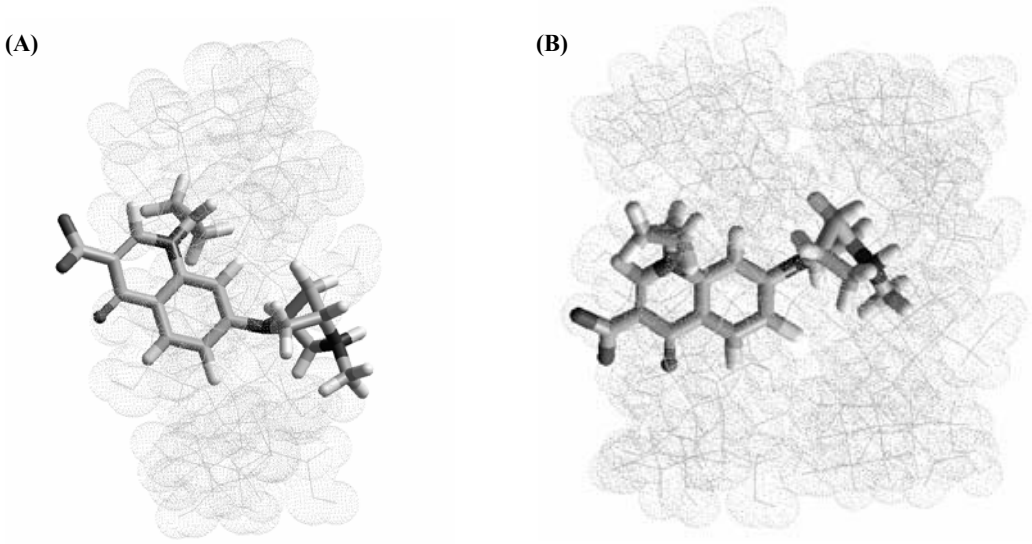

Fig. 5 – Optimised structures of 1:1 (A) and 2:1 (B) γ-CyD:danofloxacin complexes in water obtained from molecular mechanics calculations.

Starting from the 1:1 structure, the 2:1 complex was found by approaching a second host molecule. We have generated complexes considering that the included fragment is either the piperazine moiety or the carboxylate fragment. The most stable proved to be the 2:1 complex with the piperazine moiety included in the cavity (Fig. 5B). In this case, the transition moments proved to be parallel to the CyD axis as ICD data indicated. The smaller extent of the fragment included may explain the smaller association constant of 2:1 complex compared with 1:1 complex.

In molecular mechanics, each energy term in the interaction energy is given as a sum of the van der Waals and electrostatic contributions. Inspection of the values from the Table 2 shows that the driving forces for the formation of danofloxacin–γ-CyD complexes are the van der Waals interactions.

EXPERIMENTAL DATA AND COMPUTATIONAL DETAILS

High purity danofloxacin (Fluka) and γ-CyD (Sigma) were used. Fluorescence and circular dichroism measurements were carried out with a Jasco spectrofluorometer (FP-6300 model) and spectrometer (J-815 model), respectively, in a 1 cm path length cuvette in phosphate buffered solutions with pH 7.4. The circular dichroism (CD) spectra were acquired in the wavelength range of 200–450 nm with a time response of 8 s, scanning rate of 50 nm/min and 1 nm bandwidth.

The inclusion process was analysed by adding aliquots of γ-CyD over a solution of danofloxacin, maintaining a constant drug concentration while varying the γ-CyD concentration.

Density functional theory (DFT) and time dependent-DFT (TDDFT) calculations for the free guest were performed with the Gaussian03 software[22] using the functional B3LYP[23] and the basis set 631++G(d,p). The solvent effect (water) was introduced based on the continuum polarizable model (PCM).[24] For the TDDFT calculations nine transitions were considered. The absorption and electronic circular dichroism (ECD) spectra were plotted with the aid of the Gabedit software.[25]

The structures of the complexes were optimized using molecular mechanics. The procedure applied for in vacuum and in water calculations is described elsewhere.[5] The inclusion process was characterized in terms of the interaction energy (E_{int}) defined as:

$$E_{int} = E_{complex} - (E_{guest} + E_{\gamma\text{-CyD}}) \quad (4)$$

where $E_{complex}$ is the energy of the complex, E_{guest} and $E_{\gamma\text{-CyD}}$ are the guest and host energies that correspond to the geometries frozen in the complex.

CONCLUSIONS

A combined experimental and theoretical study of the inclusion complexes of danofloxacin in γ-CyD allows for the following conclusions. Both experimental techniques used, fluorescence and circular dichroism spectroscopies evidence the presence of two inclusion complexes with 1:1 and 2:1 host:guest stoichiometry dependent on the range of the host concentration. The binding constants of the 1:1 complex estimated by the fluorescence and circular dichroism methods are different. A possible explanation consists in the different nature of the implied state, the ground state in dichroism and the excited states in fluorescence. The changes in the signs of the dichroic bands upon the inclusion process in correlation with the direction of the transition moments let us assume an orthogonal inclusion in the cavity for the 1:1 complex and an axial one for the second complex. Starting with these assumed geometries, a molecular modeling of the structures of the complexes in vacuo and water show that the interaction is mainly van der Waals with a very small percentage of an electrostatic contribution. Another conclusion consists in the important role of the circular dichroism measurements, which combined with high level theoretical methods give an insight on the molecular structure of the complexes.

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