INCLUSION COMPLEXES
OF 3-CARBOXY- AND 7-DIETHYLAMINO-3-CARBOXY-COUMARIN WITH
α-CYCLODEXTRIN: SPECTRAL STUDY AND MOLECULAR MODELING

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The inclusion complexes of the dissociated 3-carboxy-coumarin (3CCA) and 7-diethylamino-3-carboxy-coumarin (DEAC) with α-cyclodextrin (α-Cyd) were investigated using absorption, fluorescence and circular dichroism measurements. Although the changes in the absorption spectra reflect the inclusion process, only the fluorescence data were found suitable for the quantitative characterization of the complexes, the determination of the stoichiometry and the association constants. We have found the formation of a 1:1 complex in the case of 3CCA and of both 1:1 and 1:2 complexes for DEAC. The positive sign of the induced circular dichroism (ICD) spectrum, observed only for 3CCA, was explained, according to the to Harata-Kodaka rule, with the axial inclusion of the ligand in the cavity. The experimental data were supported by theoretical calculations performed on the isolated ligands (DFT-TDDFT) and on the supramolecular ligand-cyclodextrin system (MM, PM3 and TDDFT–ZINDO). The calculated ICD spectrum of the complex was in good agreement with the experimental one, allowing for obtaining a reliable structure of the complex.

INTRODUCTION

The inclusion process of several small organic ligands and/or drugs in cyclodextrins was extensively investigated as a starting point in the further study of their interactions with proteins, especially with serum albumins. There was found that the main features of the inclusion complexes, the stoichiometry and the association constant are dependent on the cavity dimension[1-6] fact that can be correlated with the albumin binding sites.

In the present paper, continuing our previous studies on the coumarin derivatives,[7-9] we wish to report a spectral study on the behaviour of two coumarin derivatives, 3-carboxy-coumarin (3CCA) and 7-diethylamino-3-carboxy-coumarin (DEAC)[10] in the presence of the smallest cyclodextrin, α-CyD.

The main difference between the two guests consists in the presence of the bulky diethyl-amino substituent that can influence the coumarin electronic molecular system and its capacity to interact with cyclodextrins and albumins. The two carboxylic acids, 3CCA and DEAC are characterised by pKa values of 3.28 and 3.46, respectively. Therefore the experiments were conducted at pH>7 to ensure the presence in the system of a single species, the dissociated ions (Fig. 1).

Experimental measurements were performed by absorption, fluorescence and circular dichroism spectroscopy in order to obtain information on the thermodynamic parameters of the inclusion process (stoichiometry and association constants) and on the structure of the complex. Considering this aspect, the ICD spectrum provides valuable information on the inclusion mode of the guest in

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the host cavity. According to the Harata-Kodaka semiempirical rules,[11-16] the sign of the dichroic band shows that the molecule is included in the cavity with the transition moment correspondent to the band aligned along (positive sign) or orthogonal (negative sign) to the cavity axis.
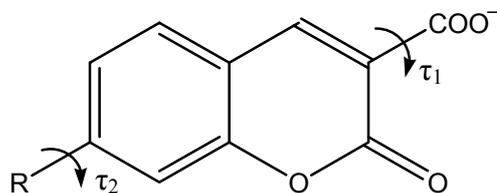

Fig. 1 – Molecular structure of the dissociated forms of 3-carboxycoumarin acid (R=H) and 7-diethylcoumarin-3-carboxylic acid (R=N(C$_2$H$_5$)$_2$); τ_1 and τ_2 denote the torsion angles of the two substituents in respect to the coumarin ring.

The experimental observations on the ICD spectra are discussed in correlation with the results of DFT-TDDFT calculations on the isolated ligands. The molecular modelling of the supramolecular system ligand: cyclodextrin was performed by Molecular Mechanics and semiempirical methods in order to obtain information on the interaction energies and the relative contributions of the van der Waals and electrostatic terms. Starting with the geometries found for the supramolecular system, TDDFT (ZINDO) calculations were carried out to obtain the ICD spectrum. The comparison of the experimental and theoretical data of the ICD spectra allows for a better estimation of the inclusion complex structure.

EXPERIMENTAL AND THEORETICAL METHODS

3CCA was purchased from Aldrich, DEAC and α-CyD from Sigma and all were used without further purification.

The absorption, circular dichroism and emission spectra were recorded on Jasco V-560 UV-VIS spectrophotometer, Jasco J-815 CD spectrometer and a Jasco FP-6300 spectrofluorometer, respectively. All the measurements were made in 1 cm path length cell and in buffered solutions (pH>7). Each CD spectrum is the average of ten scans at a speed of 50 nm/min with a time response of 8 s. According to the absorption spectra, the excitation wavelength was set at 330 nm for 3CCA and 410 nm for DEAC. The quantum yields were estimated against quinine sulfate solution in 0.1 N H$_2$SO$_4$ (Φ=0.55).

The inclusion complexes were prepared by adding to the guest solutions aliquots of stock solutions of CyD maintaining at each determination a constant guest concentration. Due to its low solubility in water, 3CCA was firstly dissolved in methanol and then a 1:9 (v/v) methanol:water dilution was made.

The DFT and TDDFT calculations were performed with the Gaussian03 program[17] using the B3LYP[18] and PBE[19]

functionals, and the 6-311++G(d,p)[20] basis set. The calculations were performed considering water as solvent in the frame of the Polarisable Continuum Model, PCM.[21]

The α-CyD structure was taken from literature.[22] All the necessary computational steps to find the most stable complexes in vacuo and in water were detailed in a previous paper.[23] The guest-host interaction energy in vacuo was calculated as the difference between the energy of the complex and the guest, host energies, corresponding to the geometries frozen in the complex:

$$E_{int} = E_{complex} - E_{guest} - E_{CyD} \qquad (1)$$

The same equation was applied for the interaction energy in water, but only after the removal of water molecules.[24]

RESULTS AND DISCUSSION

Photophysical properties of the guest

The electronic absorption and fluorescence spectra of the two dissociated species are presented in Fig. 2, the main features being listed in Table 1.

The spectrum of 3CCA in the dissociated form is characterized by an intense maximum at 290 nm and a shoulder with a smaller intensity located at 329 nm. By substitution of the hydrogen atom from the position 7 of coumarin with diethyl amino, an electron donating group, the whole spectrum changes. Hence, for DEAC the bands are more intense and the main band is red shifted to 410 nm. Although the values of the extinction coefficients attest that for both coumarins the transitions involved are π–π*, the differences between the electronic spectra of the two coumarin derivatives show that these transitions might involve energy levels correlated with different molecular fragments, as will be discussed below.
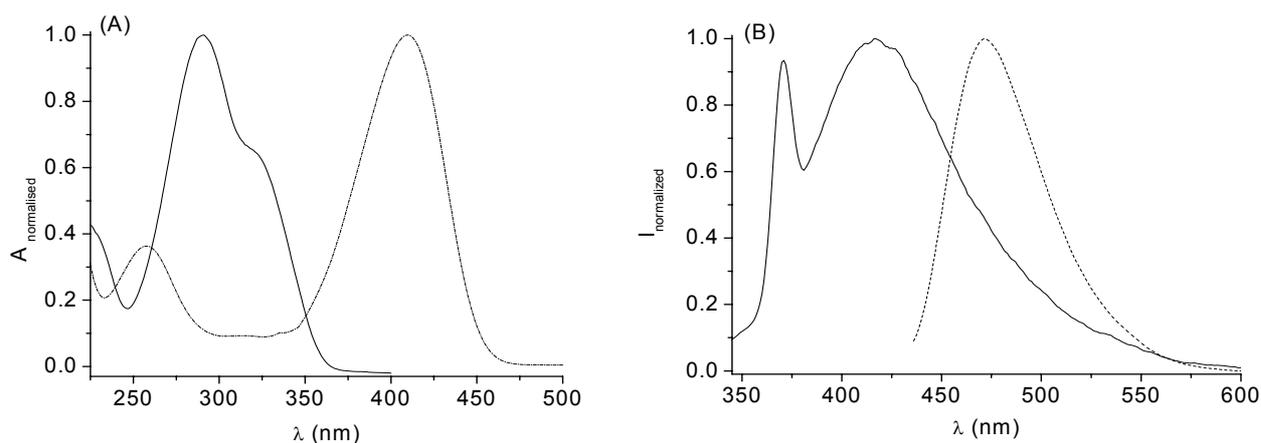
Fig. 2 – Normalized absorption (A) and fluorescence (B) spectra of the dissociated 3CCA (solid curve) and DEAC (dotted curve).

Table 1

Photophysical properties of the dissociated forms of 3CCA and DEAC

Coumarin	λ_{abs} (nm)	ε (M^{-1}cm^{-1})	λ_f (nm)	ϕ
3CCA	290/329	12115/5600	418	0.0002
DEAC	258/410	14502/40283	472	0.0200

The same significant bathochromic shift of the band is also observed in the fluorescence spectra. It can be seen that the fluorescence quantum yield of 3CCA is very low, in agreement with literature data for the undissociated acid.[25-26] The fluorescence spectrum presents a large width indicating a possible existence in the system of several conformers that would be differently influenced by the inclusion process.

It is known that the first excited state of the undissocited DEAC molecule has an ICT (intramolecular charge transfer) character.[27] The drastically decrease of its quantum yield and lifetime in polar solvents has been explained by an additional fast deexcitation pathway attributed to a nonfluorescent TICT (twisted intramolecular charge transfer) state.[27] Regarding the dissociated form of DEAC, its ICT state has the same behavior in water, *i.e.* a very low quantum yield, and therefore it is expected to be very sensitive to the polarity changes imposed by the inclusion in the CyD cavity.

Guest-Host interaction

Absorption measurements. Increasing the concentration of α-CyD, the absorption spectra of both coumarin derivatives are modified, reflecting the inclusion process (Fig. 3).

The 3CCA absorption bands gradually shifts to lower wavelengths, fact that can be attributed to a less polar microenvironment inside the CyD cavity. The band shift is made through a well-defined isosbestic points at 330 nm and 290 nm. A new band located at 365 nm can also be observed.

In the presence of α-CyD, the main absorption band of DEAC initially decreases and then, at higher CyD concentration, a new increase was noted. A hypsochromic shift of the main absorption band was also observed as for 3CCA, but much less pronounced. Given the absorbance behaviors, we infer that for DEAC two types of complexes are formed (*vide infra*).

Although the changes in the absorption spectra attest the inclusion process, the variation of the absorbances were not large enough to allow us a precise determination of the association constants.

Fluorescence spectroscopy. The changes generated by α-CyD on the fluorescence spectra are depicted in Figs. 4 and 5.

The addition of α-CyD suppresses the 3CCA fluorescence intensity and more unexpected, determines the appearance of a new band at 500 nm (Fig. 4). Up to about 8.74×10^{-4} M α-CyD, the family of spectra is characterised by an isoemissive point located at 461 nm. After this value the isoemissive point disappears suggesting a more complex process. This new band can be due to

several causes such as excimer formation,[28-30] a stabilization of a possible existent conformer by favorable interactions within the cavity. The hypothesis of an excited state dimer formation was ruled out since the dilution had no the expected result *i.e.* it did not influence the monomer-dimer

equilibrium by increasing the intensity of the monomer band on the expence of this new one. Therefore, the new band could reflect the modification of the conformer populations in the excited state upon the inclusion process.
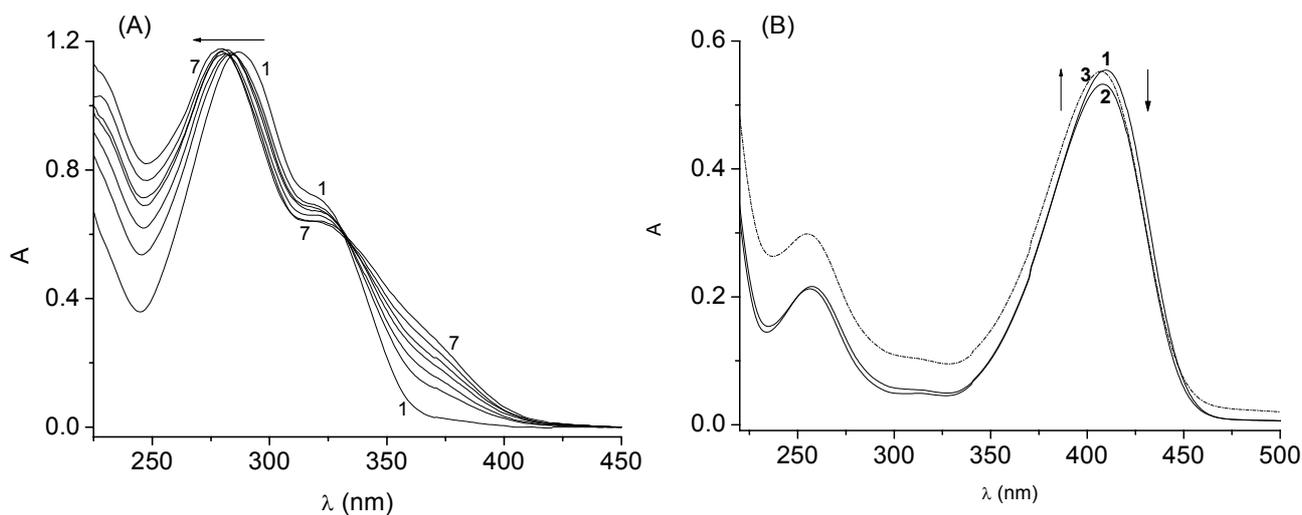

Fig. 3 – Absorption spectra of the dissociated species of 3CCA (A) and DEAC (B) in the presence of different concentrations of α-CyD. [3CCA] = 10^{-4} M and [DEAC] = 1.36×10^{-5} M.
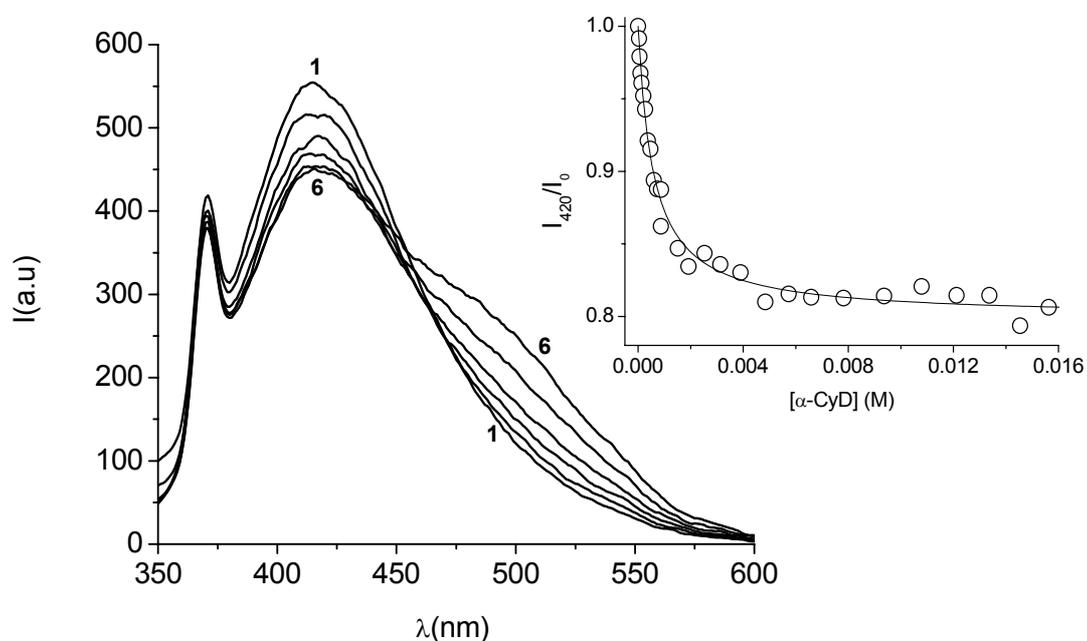

Fig. 4 – Fluorescence spectra of 3CCA (10^{-5} M) in the presence of increasing α-CyD concentrations. Inset: Variation of the fluorescence intensity at 420 nm versus α-CyD concentration. The solid line represents the best fit according to eq. (2).

Fig. 5 – Fluorescence spectra of DEAC (2.22×10^{-6} M) in the presence of increasing α-CyD concentrations. Inset: Variation of the fluorescence intensity at 420 nm versus α-CyD concentration. The solid line represents the best fit according to eq. (4).
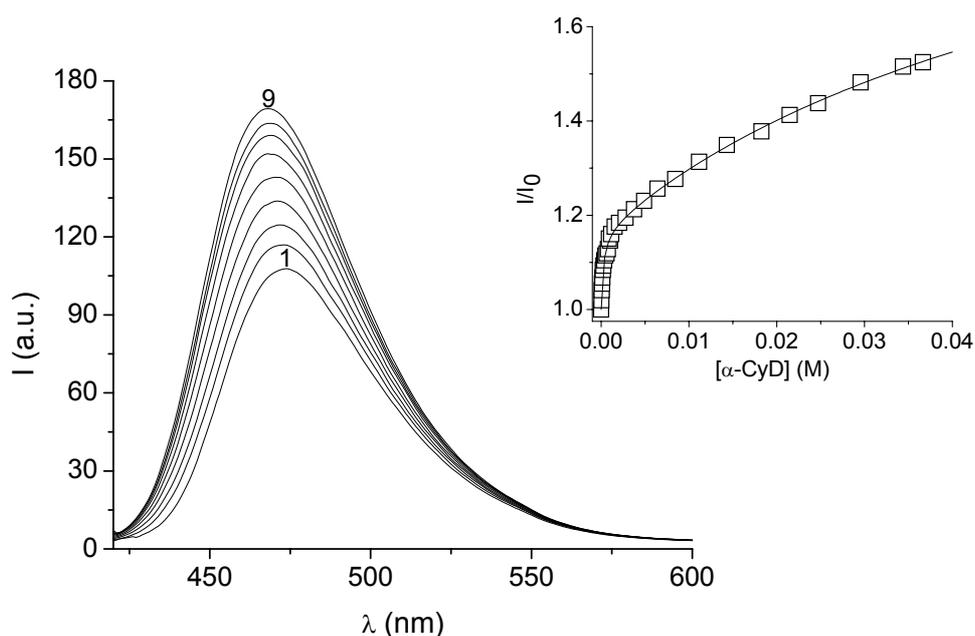
Regarding the DEAC behavior, the addition of α-CD induces an increase of the fluorescence intensity together with a hypsochromic shift of 6 nm (Fig. 5). The enhancement of the fluorescence can be attributed to the guest passage from the bulk solution in the CyD cavity where it experiences a less polar microenvironment determining an increase in its quantum yield.[31] The same explanation, *i.e.* the guest encapsulation, applies for the hypsochromic shift. As no isoemissive point was encountered, it means that the system may involve different kinds of 1:1 complexes and/or of complexes with different stoichiometry.[31]

In order to find the association constants (K) and the stoichiometry of the complexes we have monitored the variation of the fluorescence intensity with the total CyD concentration, [CyD].

Three equations corresponding to different guest:host stoichiometries (*i.e.* 1:1, 1:2 and a mixture of the two) were tested:

$$I = \frac{I_0 + I_{11}K_{11}[CyD]}{1 + K_{11}[CyD]} \qquad (2)$$

$$I = \frac{I_0 + I_{12}K_{12}[CyD]^2}{1 + K_{12}[CyD]^2} \qquad (3)$$

$$I = \frac{I_0 + I_{11}K_{11}[CyD] + I'_{12}K_{11}K'_{12}[CyD]^2}{1 + K_{11}[CyD] + K_{11}K'_{12}[CyD]^2} \qquad (4)$$

where I_0 and I stands for the guest fluorescence intensity in the absence and the presence of the CyD.[32] The fluorescence intensities were read at 420 and 472 nm for 3CCA and DEAC respectively. The results of the best fits are displayed in the insets of Figs. 4 and 5 and are summarized in Table 2. It can be seen that for 3CCA, only the 1:1 complex was evidenced, while for DEAC, a mixture of 1:1 and 1:2 guest: host complexes were found, even as the second constant is very low. Previously, in the case of a related coumarin carboxylate derivative, the 3-carboxy-5,6-benzo-coumarin we have also found the formation of both 1:1 and 1:2 complexes in the presence of α-CD with constants in the same range of values.

Circular dichroism spectroscopy. In the presence of cyclodextrins, even the achiral compounds can present an induced dichroic signal due to the asymmetric interaction within the cavity. The ICD bands are located in the range of the electronic spectrum of the ligand and are characterised by intensity and sign, depending on the position of the ligand inside the cyclodextrin, and, consequently, on the effect exerted by the cavity asymmetry.

Table 2

Association constants of the inclusion coumarin - α-CyD complexes determined by fluorescence spectroscopy.
r^2 and F are the correlation coefficient and the Fisher factor, respectively

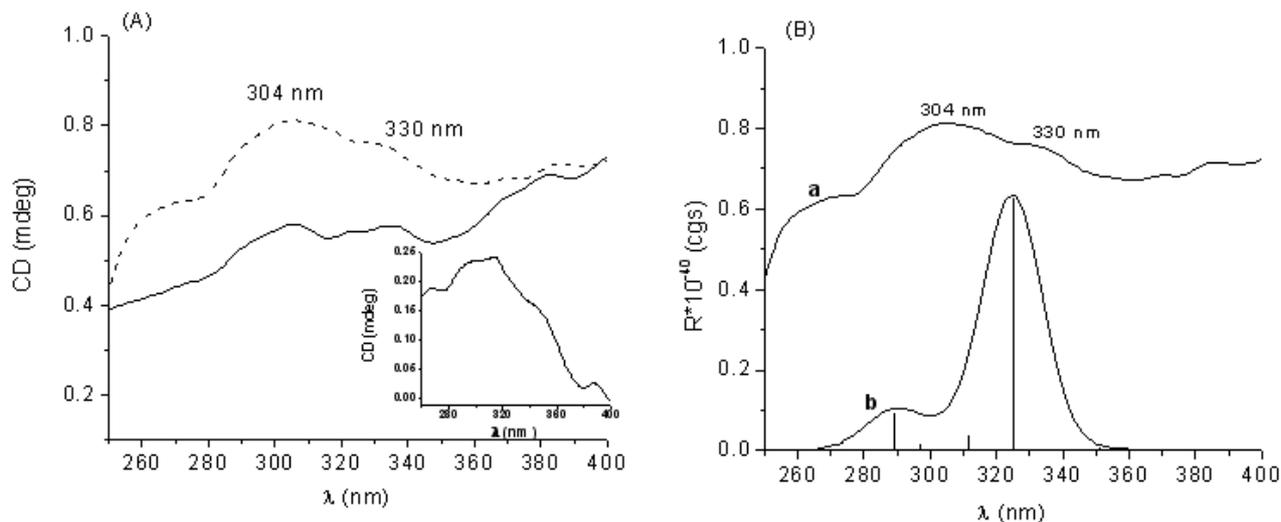| Coumarin	K_{11} (M^{-1})	K'_{12} (M^{-1})	I_{11}/I_0	I'_{12}/I_0	r^2	F
3CCA	1730 ± 76	-	0.80 ± 0.01	-	0.990	3827
DEAC	4239 ± 339	16 ± 2	1.17 ± 0.01	2.14 ± 0.08	0.999	8685

Fig. 6 – A) The ICD spectra of 3CCA 7×10^{-5} M in the absence (solid) and in the presence of α-CyD 6×10^{-2} M (dotted); inset: the difference spectrum. B) The experimental (a) and TDDFT simulated (b) ICD spectra of the supramolecular system 3CCA - α-CyD.

Although 3CCA is achiral, in the absence of cyclodextrin, a very weak ICD signal (around 0.5 mdeg) without contoured bands, like a background was obtained, likely due to the possible conformers differing by the position of the COO$^-$ group. Adding α-CyD, a rather weak but clear dichroic signal was obtained, characterized by two positive bands located at 304 and 330 nm, at the same positions with those observed in the absorption spectra. The ICD spectrum obtained from 3CCA is displayed in Fig. 6A. A slight positive band at 386 nm, at a position similar with that evidenced in the absorption spectrum could also be observed in the difference spectrum displayed in the inset of Fig. 6A, but its intensity is too weak to be certain.

In the case of the second derivative, DEAC, a noticeable ICD spectrum could not be obtained, attesting that the chromophore correlated with the main electronic transition was less perturbed by the inclusion process. As we have obtained an intense positive ICD signal in the presence of β- and 2-hydroxy-β-CyD, the lack of the signal in this case could be explained by the lower dimension of the cavity.

Correlating the experimental ICD data for 3CCA with the Harata-Kodaka rules, it is expected that the ligand is included in the cavity with the transition moments of the two bands oriented along the cavity axis. In order to check this assumption, the polarisation of the transition moments was calculated by TDDFT calculations.

Previous DFT calculations performed on 3CCA for several values of the torsion angle of the COO$^-$ group in respect to the coumarin ring (τ_1) showed that the minimum energy conformation corresponds to $\tau_1 = 90^o$, but the barrier to rotation of the group was estimated as 1.54 kcal mol^{-1} and 1.30 kcal mol^{-1}, using the B3LYP and PBE functionals respectively. These theoretical data point to a free rotation of the carboxylate group meaning that in solution, in the absence of any constraint, all the conformers can be found. The inclusion process can change the population of conformers, both in the ground and excited states, some conformations being more affected by the CyD cavity as was seen by the changes in the absorption and fluorescence spectra. Considering for the discussion of the ICD band only two limiting conformations, the total planar ($\tau_1 = 0^o$) and the orthogonal one ($\tau_1 = 90^o$), the calculated directions of the transition moments for the electronic transitions with a significant value of the oscillator strength, f, are displayed in Fig. 7.
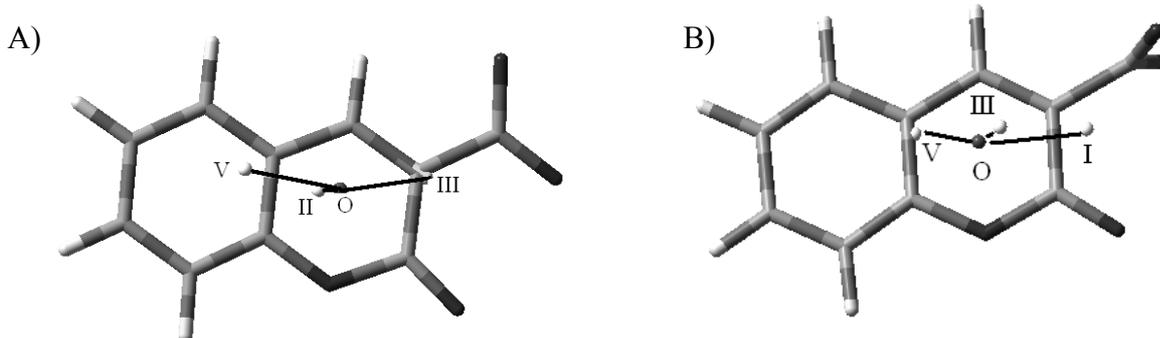
A)   B)

Fig. 7 – Directions of the calculated transition moments (B3LYP/ 6-311++G(d,p)) of the most intense electronic transitions
for two conformers of 3CCA: A) $\tau = 0^o$; B) $\tau = 90^o$ (O represents the center of mass of the molecule).

It can be seen that although the implicated transitions are influenced by the COO⁻ position, *i.e.* transitions II, III, V for $\tau_1 = 0^o$ and I, III, V for $\tau_1 = 90^o$, the polarisations of the transition moments are parallel to the long molecular axis, explaining the experimental results.

As concerns the second compound, given the presence of two flexible fragments –COO⁻ and –N(C₂H₅)₂, we have considered several possible conformers depending on the position of both fragments in respect to the coumarin ring. Each conformer was described by the corresponding torsion angles τ_1 and τ_2, shown in Fig. 1. For the minimum energy conformers the frontiers molecular orbitals are depicted in Fig. 8 A. It can be seen that both *homo* and *lumo* orbitals have a π character and are mainly localized on the coumarin ring and the -N(C₂H₅)₂ fragment with a reduced contribution from the COO⁻ group. When –N(C₂H₅)₂ is twisted at $\tau_2=90^o$, as a consequence of the interrupted conjugation, the localization of the frontier orbitals changes dramatically. Thereby, *homo* remains localized on the nitrogen atom of the –N(C₂H₅)₂, while *lumo* only on the coumarin ring

(Fig. 8 B). A general conclusion of the MO calculations is the major influence of the ethyl amino group on the overall π electronic system, supporting the differences already discussed on the spectral properties of 3CCA and DEAC.

Molecular modeling of the host-guest supramolecular system. The optimisation of the host-guest complexes was made in the framework of MM and PM3 methods. The structures of the most stable 1:1 complexes are given in Fig. 9 and the interaction energies, the van der Waals and electrostatic contributions are listed in Table 3. The values show that DEAC forms with α-CyD a more stable complex than 3CCA, with a smaller electrostatic contribution.

For the 3CCA- α-CyD complex we have calculated the ICD spectrum and the result is presented together with the experimental one in Fig. 6B. It can be seen that excepting the relative intensities of the bands the theoretical spectrum is in good agreement with the experimental one predicting well the main features of the ICD spectrum, the sign and the positions of the bands.
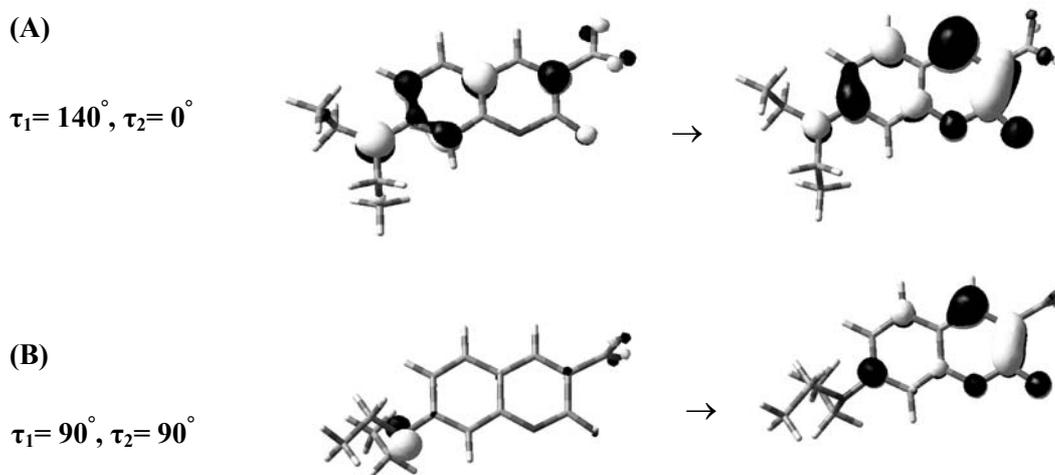
(A)

$\tau_1= 140^o, \tau_2= 0^o$

(B)

$\tau_1= 90^o, \tau_2= 90^o$

Fig. 8 – Isodensity plots of the molecular orbitals involved in the *homo → lumo* transitions for different DEAC conformers.
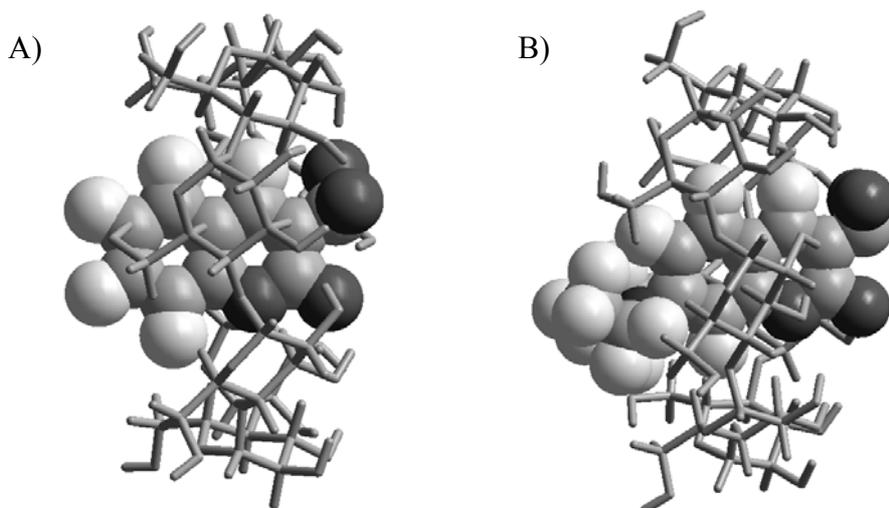
Fig. 9 – Structures of the most stable 1:1 complexes found for 3CCA (A) and DEAC (B).

Table 3

Interaction energy with the van der Waals and electrostatic contribution

Coumarin	vacuo			water		
	E_{int}(kcal/mol)	% vdW	% el	E_{int} (kcal/mol)	% vdW	% el
3CCA	-47.34	35	65	-22.43	70	29
DEAC	-52.02	49	51	-31.45	63	37

CONCLUSIONS

The characterization of the inclusion complexes of the dissociated species of 3-carboxycoumarin and 7-diethylamino-3-carboxycoumarin with α-CyD using three spectral methods, absorption, fluorescence and circular dichroism spectroscopy bring about complementary information on the overall inclusion process. Although no used for quantitative determinations, the modifications recorded in the absorption spectra in the presence of α-CyD, attest the inclusion process and outline the differences between the electronic system of the two derivatives brought about by the donor character of the diethylamino group in DEAC. The fluorescence spectra allow for the estimation of the stoichiometry and association constants. We have found a single 1:1 complex in the case of 3CCA and a mixture of 1:1 and 1:2 guest: host complexes for DEAC; it must be remarked that the presence of two complexes with similar constants was previously found for a related carboxycoumarin, the 5,6-benzo-3-carboxy coumarin.[7] Differing from DEAC, the fluorescence spectra for the 3CCA-α-CyD system reflect also a change in the conformer population upon inclusion. The circular dichroism spectroscopy led to a useful result only for 3CCA; for this derivative, a weak but clear positive ICD signal was recorded, attesting, according to the Harata-Kodaka rules, the axial inclusion of the ligand in the cavity. In what concern the other derivative the very uncertain ICD signal obtained reflects that no asymmetry was induced by the ligand-CyD interaction, or that the inclusion process did not perturb the chromophore responsible for the bands in the observed range.

The DFT and TDDFT calculations support all the experimental observations and explain the differences between the two compounds. Starting with experimental ICD data on the inclusion way of the 3CCA in the cavity, we have performed a molecular modeling of the supramolecular system 3CCA- α-CyD. The main result of the theoretical TDDFT calculations was the simulation of the ICD spectrum in good agreement with the experimental one, attesting the reliability of the complex structure.

One of the main conclusions of our work is that the correlation of several different kinds of experimental data is necessary in order to have a more consistent image on the inclusion complexes.

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