Inclusion Complex of Fluorescent 4-Hydroxycoumarin Derivatives with Native β-Cyclodextrin: Enhanced Stabilization Induced by the Appended Substituent

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This work studies the interaction of substituted coumarins with β-cyclodextrin (β-CD). The role of the substituent in the 3 position was investigated. The guest molecules were two original 4-hydroxycoumarin derivatives, bearing an ethyl furoate (**1**) or a benzodioxanyl (**2**) substituent in the 3 position. Comparison was made with a nonsubstituted analogue (**3**). It must be noted that molecules **1** and **2** potentially offer very different complexation sites. Formation of the inclusion complex was checked by UV/vis absorption and fluorescence spectroscopy. A strong revival of the fluorescence intensity of **1** and **2** was observed in the presence of β-CD and partly attributed to the fact that the heterocyclic substituent was prevented from rotating freely. The 1:1 stoichiometry of the complex was established, and the values of the binding constants (7×10^2 and 3.4×10^2 M^{-1} for **1** and **2**, respectively) were extracted from the spectrophotometric data. A much lower (8.1×10^1 M^{-1}) binding constant was found for unsubstituted compound **3**. The ^1H NMR study confirmed that the coumaryl ring system was engulfed in the cyclodextrin cavity. The proximal moiety of the heterocyclic substituent also interacted with the β-CD, whereas the rest protruded outside the cavity. This explains that the substituent controls the structure of the inclusion complex and contributes to its stabilization.

1. Introduction

Cyclodextrins (CDs) are cyclic polysaccharides usually composed of six (α-CD), seven (β-CD), or eight (γ-CD) glucose units. They are hollow, toroidally shaped structures and have long been known for their ability to accommodate conveniently sized molecules,[1−5] for example, dyes.

Regarding coumarins, which are the topic of this study, inclusion in CDs has been investigated for a variety of purposes. Coumarins are widely used as dyes[6] and drugs.[7] It has been shown that CDs orientate their chemical[8] and photochemical[9,10] reactivity. From a photophysical viewpoint, one important objective was to increase with CDs the solubility and stability of coumarins in water, in an attempt to use them as laser dyes.[10−14] CDs also proved to be useful in the spectrophotometric analysis of chiral coumarin-based pesticides.[15,16] Finally, the coumarin/CD system allowed the solvation dynamics in a restricted environment to be studied, mimicking the biological process that occurs within the cavity of proteins and lipid membranes.[17]

In the above-cited cases, there have been a number of observations that the spectroscopic properties of the dyes were modified in the presence of CDs.[11−16,18] The effects observed varied strongly with the nature of the coumarin derivative used. Remarkable differences were also encountered for the binding constants. For example, it is surprising to note that fairly high binding constants were reported for 4-hydroxycoumarin derivatives substituted in the 3 position, although the presence of the hydroxyl group is known to disadvantage inclusion.

However, there is obviously a lack of information concerning the importance of coumarin substitution with respect to inclusion in CDs. The present work is an attempt to clarify this point. In this aim, original compounds were employed, namely, 4-hydroxycoumarin derivatives (HCD) **1** and **2** (Scheme 1). These compounds differ by the nature of the substituent borne in the 3 position. Their spectroscopic and photophysical properties in organic solvents have been the subject of a previous paper.[19] In these solvents, molecule **1** displayed excellent fluorescence properties (a high quantum yield and a lifetime of approximately 2 ns) very weakly altered by variations in temperature or viscosity of the medium. In contrast, **2** emitted with low quantum yield and short lifetime, but it was extremely sensitive to factors affecting the rotation of the benzodioxanyl substituent. In this respect, **2** can be considered as a microviscosity or "constraint" probe. In the present study, the behavior of these probes was investigated in water and in the absence and in the presence of cyclodextrins. For comparison, hydroxycoumarin derivative **3**, deprived of a substituent in the 3 position, was also studied. So, the three compounds are good candidates to highlight the role of the substituent in the 3 position. It must also be noted that in compounds **1** and **2**, either the coumaryl

SCHEME 1: Chemical Structures of 4-Hydroxycoumarin Derivatives 1−3

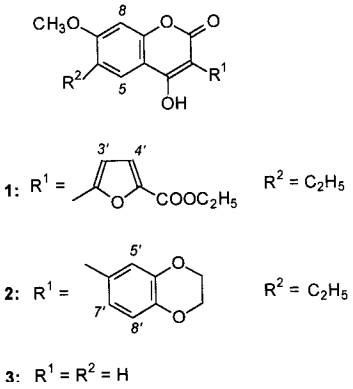

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TABLE 1: Spectrophotometric Characteristics of Compounds 1−3 in Ethanol and in Water in the Presence and Absence of β-CD and α-CD: Maximum Absorption Wavelength λ_{abs}, Molar Absorption Coefficient ϵ, Maximum Emission Wavelength λ_{em}, Fluorescence Quantum Yield Φ, and Fluorescence Lifetime τ

	ethanol		water[a] pH 1.9	water[a] pH 9.4	water[a] pH 5.4							
HCD	λ_{abs}/nm	ϵ/M^{-1} cm^{-1}	λ_{em}/nm	λ_{abs}/nm	λ_{abs}/nm	λ_{abs}/nm	ϵ/M^{-1} cm^{-1}	λ_{em}/nm	Φ	τ/ns	k_r/10^8 s^{-1}	k_{nr}/10^8 s^{-1}
1	364	24 000	432	348	318, 338	318, *338*	25 000[b]	440	3.6×10^{-2}	<0.5[c]	>0.72	>19.3
2	324	21 000	430	324	316	316	24 400	446	2.2×10^{-3}	<0.5[c]	>0.04	>19.9
3	282, *308*	11 400[b]	344			286, *302*	10 500[b]	349	7.3×10^{-3}	<0.5[c]	>0.14	>19.8

	water[a] pH 5.4 + β-CD (10^{-2} M)							water[a] pH 5.4 + α-CD (10^{-2} M)	
HCD	λ_{abs}/nm	ϵ/M^{-1} cm^{-1}	λ_{em}/nm	Φ	τ/ns[b]	k_r/10^8 s^{-1}	k_{nr}/10^8 s^{-1}	λ_{abs}/nm	λ_{em}/nm
1	318, *344*	22 700[b]	428	2.3×10^{-1}	1.3	1.77	5.92	318, 342	440
2	316	22 000	434	2.2×10^{-2}	1.1	0.2	8.89	316	439
3	286, *302*	10 500[b]	349	1.7×10^{-2}	<0.5[c]	>0.34	>19.7	286, 304	352

[a] Water contains 1.3% ethanol. [b] ϵ at the wavelength in italics. [c] Detection limit of our apparatus.

moiety or the heterocycle in the 3 position may be able to enter the CD cavity. This work therefore allowed some information to be gained about the inclusion of substituted dyes, which potentially offer very different complexation sites.

2. Experimental Section

2.1. Materials. Absolute ethanol was from Carlo Erba Reagenti. Cyclodextrin hydrate was purchased from Aldrich and used as received. Coumarins **1** and **2**, prepared as previously described,[20,21] were a gift from Pr. V. P. Khilya. Coumarin **3** was synthesized according to the literature.[22]

2.2. Apparatus and Methods. The pK_a measurements were carried out using a Bioblock Scientific WTW pH330 pH-meter, equipped with a SenTix 97T electrode. Spectrophotometric measurements were conducted in a thermostated cell at 25 °C. Absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. Steady-state fluorescence work was performed on a Photon Technology International (PTI) Quanta Master 1 spectrofluorometer. All fluorescence spectra were corrected. The fluorescence quantum yields were determined using coumarin-6 in ethanol ($\Phi_f = 0.78$) as standard.[23] Fluorescence decay was measured with the stroboscopic technique using a Strobe Master fluorescence lifetime spectrometer from PTI. The excitation source was a flash lamp filled with a mixture of nitrogen and helium (30/70). Data were collected over 200 channels with a time base of 0.1 ns per channel. Each measurement was repeated four times. Fluorescence decay was analyzed using the software from PTI. The goodness of the fit was established by using the χ^2, Runs test Z, and Durbin−Watson parameters. The estimated experimental error was 2 nm for the band position, 15% for the molar extinction coefficient, 10% for the fluorescence quantum yield and 10% for the fluorescence lifetimes. NMR spectra were recorded on an ARX 400 MHz Bruker spectrometer, with presaturation of the water peak. Chemical shifts were measured with respect to TMS as an external reference. Simulations and iterations were carried out using Win-Daisy 4.0 software (Bruker). Molecular modeling was performed using the Discover program within the Insight II version 98 interface (Biosym/MSI). The force field was CVFF. The Conjugate Gradient algorithm was used for energy minimization calculations. For molecular dynamics, the temperature chosen was 298 K, which corresponds to our experimental conditions. The structure of a β-CD hydrate clathrate, used to build a model of β-CD, was taken from the Cambridge Database.

2.3. Data Analysis. For the determination of the binding constants, the fluorescence data were processed on a HP 9000 series 710 workstation. Equation 3 (see below) was numerically solved by an iterative method. The concentrations of bound [CD−HCD] and free [HCD] species, and the total fluorescence intensity $F = \Phi_{meas}[HCD_0] = \Phi_0[HCD] + \Phi[CD-HCD]$ were successively calculated (Φ_{meas}, Φ_0, and Φ being the measured quantum yield and the quantum yield of the free and bound species, respectively). The error was given by $E = \sum_{i=1}^{n}(F_{calc} - F_{meas})^2/n$, where n is the number of experimental points. The sum of the squares of the differences between the experimental values and those of the numerical calculation was minimized by a Powell nonlinear optimization algorithm.

2.4. Preparation of the Solutions. In method 1, used for spectrophotometric studies, solutions of HCD (7.6×10^{-4} M) in absolute ethanol were prepared and sonicated for 5 mn. Then, 40 μL of this solution were added to 3 mL of deionized water containing up to 10^{-2} M β-CD. The hydroxycoumarin derivative concentration (10^{-5} M) was thus kept constant, whereas the concentration of β-CD was allowed to vary. The solutions were stirred for 5 min prior to measurement. Solutions of HCD with α-CD (1×10^{-2} M) were also prepared according to this method.

In method 2, the hydroxycoumarin derivative (9.6×10^{-6} mol) was dissolved in 10 mL of acetone and mixed with an equimolecular amount of β-CD in 1.5 mL of deionized water. The solvent was evaporated off and the mixture dried under vacuum (15 mmHg) for 6 h. The resulting powder was dissolved in 1 mL of D_2O for ^{1}H NMR measurements. For spectrophotometric measurements, 10^{-6} mol of complex was dissolved in 100 mL of a 10^{-2} M^{-1} β-CD solution. Final concentrations were 10^{-5} M HCD and around 10^{-2} M β-CD.

3. Results and Discussion

3.1. Behavior of the Probes in Water. Before undertaking the study of the hydroxycoumarin derivatives in the presence of cyclodextrin, it was necessary to clarify the behavior of these probes alone in water. Of particular interest was the determination of the ionization state of the three dyes and that of the degree of aggregation, which was carried out with compound **2**.

UV/vis Absorption Properties. The HCD studied in this work were only slightly soluble in water. Consequently, these compounds were first dissolved in absolute ethanol, and then a tiny volume of this concentrated solution was diluted in deionized water. The final concentrations were 1×10^{-5} M for the HCD and 1.3% v/v for ethanol in water. In deionized water at pH = 5.4, compound **1** displayed two UV/vis absorption maxima at 318 and 338 nm, whereas **2** exhibited a narrow band, peaking at 316 nm (Table 1).

When a solution of NaOH was added to the hydroxycoumarin derivative solution, no significant variation in the shape of the

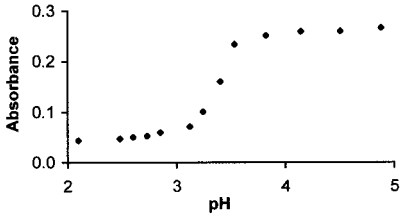


Figure 1. Variation of absorbance at 316 nm vs pH for **2** (1×10^{-5} M) in deionized water (1.3% ethanol).

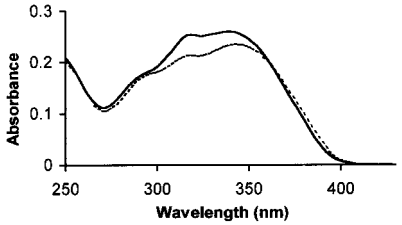


Figure 2. Absorption spectrum of **1** (1×10^{-5} M) in deionized water (1.3% ethanol) in the absence (full line) and in the presence (dotted line) of 10^{-2} M β-CD.

UV/vis absorption spectrum was observed. This suggests that in water at pH 5.4 both HCD were mainly in their ionized form. In contrast, addition of hydrochloric acid led to a strong shift toward the red, with a decrease in absorbance and a marked variation in the shape of the spectrum, peaking at 348 and 324 nm, at pH = 1.9 for **1** and **2**, respectively.

Concerning the position and shape of the spectra, it was shown previously that in organic solvents the UV/vis absorption characteristics of the three probes showed a very low sensitivity to the solvent nature.[19] In the present work, it was observed for **2** (1×10^{-5} M) that the absorption maximum of the neutral species in water (1.3% ethanol v/v) at pH 1.9 was identical to the maximum in ethanol, a solvent in which the dye is neutral (Table 1). The shape of the spectra was similar too. In contrast, for compound **1** (1×10^{-5} M), the UV/vis absorption spectrum of the neutral form in water (1.3% ethanol v/v) at pH 1.9 was shifted by 16 nm toward lower wavelengths, compared with that in ethanol. However, it was shown by calculation for **1** in a vacuum that an intramolecular hydrogen bond was formed between the hydroxyl group in the 4 position and the oxygen atom of the furoate cycle.[19] This hydrogen bond contributes in making the molecule planar, which leads to a good conjugation of the π-electron system. It is most likely that this bond exists in most of organic solvents but is disrupted in water. Therefore, the substituent cycle may be twisted with respect to the coumaryl cycle, as is the case for **2**.[19] This can explain that a blue shift was observed on the UV/vis absorption spectrum of **1** in acidic water, compared with ethanol.

Determination of the pK_a Values. The variations of the UV/vis absorption spectrum were analyzed versus the pH of the solution, measured with a pH meter (Figure 1). The pK_a values were found to be about 3.3 for compounds **1** and **2**. For compound **3**, the pK_a value was found to be close to 4, but it must be underlined that the spectroscopic variations were very weak, leading to some inaccuracy in the measurement. Such values are not surprising, because among all hydroxycoumarins, that bearing the hydroxyl group in the 4 position is known to be the most acidic.[24] However, it is interesting to note that the pK_a values found for our compounds were even lower than that of unsubstituted 4-hydroxycoumarin, which is equal to 5.1 according to the literature.[24] The difference can be attributed to the presence of the substituents.

Search for Aggregation. Owing to the low solubility of the HCD in water, aggregation was likely to occur. The occurrence of this phenomenon was searched for in the case of **2**. As described above, no evidence was found for aggregation in the shape of the UV/vis absorption spectra. Then, the absorbance was regarded with respect to the probe concentration, because deviations from the Beer−Lambert law are generally found when aggregation takes place. The concentration of **2** was allowed to vary from 5×10^{-7} to 1×10^{-5} M in water (1.3% ethanol v/v) pH 5.4. The molar absorption coefficient was found to be invariant. Consequently, no aggregation was detected at the concentrations used in this study.

Fluorescence Properties. When passing from ethanol to water, the emission spectrum of the three dyes was shifted to the red. The fluorescence quantum yield and lifetime of **1** were drastically reduced in water compared with organic solvents.[19] This behavior can be related to the disruption of the hydrogen bond and loss of planeity of compound **1** in water.

3.2. Spectrophotometric Study in the Presence of Cyclodextrin. *UV/vis Absorption Properties.* Two methods were used to bring the hydroxycoumarin derivatives and β-cyclodextrin together. In method 1, a concentrated ethanol solution of the 4-hydroxycoumarin derivatives was added to a solution of β-cyclodextrin in water. In the presence of β-CD, weak variations of the UV/vis absorption spectrum of the probes were then observed. For 10^{-2} M β-CD, the UV/vis spectrum of **1** exhibited a 6 nm bathochromic effect, together with a weak decrease of the absorbance, the molar absorption coefficient ϵ passing from 25 000 to 22 700 M^{-1} cm^{-1} at the maximum wavelength (Figure 2). For **2** and **3**, no shift was detected, but the absorbance was also slightly decreased (Table 1) in the case of **2**.

Samples were then prepared according to method 2. An aliquot of β-CD, dissolved in water, was mixed with an equimolecular amount of hydroxycoumarin derivative, dissolved in an organic solvent. The mixture was dried, and the resulting powder was redissolved in a 10^{-2} M cyclodextrin solution, so that the concentrations were comparable to those used in method 1. Spectroscopic variations strictly identical to those obtained using method 1 were observed, suggesting that the same phenomenon was observed in both cases.

Fluorescence Properties. The spectral characteristics are gathered in Table 1. In deionized water, all three hydroxycoumarin derivatives emitted weak fluorescence, which was red-shifted compared with that observed in organic solvents, for example, ethanol. Upon addition of 10^{-2} M β-CD, a 12 nm hypsochromic shift of the fluorescence spectrum was observed for **1** and **2**. The same variation was noted whatever the method used to prepare the samples. This variation, together with the shift observed in the UV/vis absorption spectrum of **1**, suggests that the HCD are here experiencing a new medium, the properties of which are close to those of organic solvents. This medium could very well be the β-CD cavity, which is well established to be much less polar than water.[25] No wavelength shift was detected for **3**.

All three compounds exhibited a drastic increase of the fluorescence intensity upon addition of β-CD (Figure 3). The fluorescence quantum yield was multiplied by 6.4, 10, and 2.3 for **1**, **2**, and **3**, respectively, when the β-CD concentration reached 10^{-2} M. In parallel, the lifetimes of compounds **1** and **2**, which were very short (below 0.5 ns) in water, were increased to 1.3 and 1.1 ns, respectively. These data are reminiscent of the behavior of HCD in organic solvents.[19] From the values obtained for the fluorescence quantum yield Φ and the radiative lifetime τ, the radiative ($k_r = \Phi/\tau$) and nonradiative ($k_{nr} =$

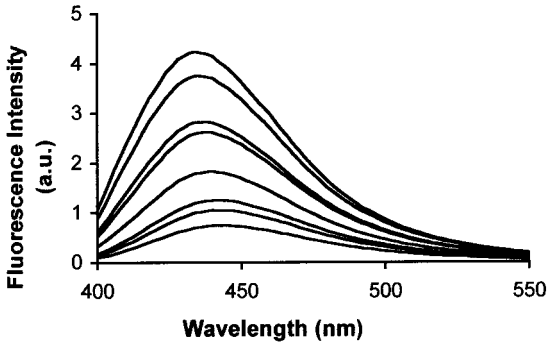


Figure 3. Fluorescence spectrum of **1** (1×10^{-5} M) in deionized water (1.3% ethanol) in the presence and absence of β-CD. From bottom to top: [β-CD] = 0, 1×10^{-4}, 2×10^{-4}, 1×10^{-3}, 2×10^{-3}, 4×10^{-3}, 7×10^{-3}, and 1×10^{-2} M.

$(1 - \Phi)/\tau$) deactivation constants were calculated (Table 1). The variations of the k_r values in the absence and in the presence of CD are difficult to discuss, because of the unreliable measurement of the lifetime in water. However, it is clear that the k_{nr} value was markedly decreased for **1** and **2** in the presence of β-CD in comparison with water, meaning that thermal deactivation has been reduced. The whole data support the hypothesis of the inclusion of HCD within β-CD. The variations observed here may be partly attributed to the protecting effect of the CD cage with respect to the surrounding solvent. However, it is worth noting that the strongest spectroscopic effects were encountered with the substituted compounds, especially **2**, which is particularly sensitive to hindrance of the rotation of the substituent in the 3 position.

The excitation spectrum remained unchanged whatever the emission wavelength. It was similar to the absorption spectrum, suggesting that the emitting species is the one that absorbs. Conversely, no variation in the shape or position of the emission spectrum was observed when varying the excitation wavelength, for any of the three compounds in the presence of 10^{-2} M β-CD. Although there is an equilibrium between bound and free HCD, only one emitting species was detected. For **2** and **3**, this can be explained by the fact that the absorption spectra of the bound and free species are similar. For **1**, a weak shift was expected. However, at this concentration of β-CD, most of the HCD was complexed (see below), and the quantum yield of the free species was much lower than that of bound HCD. For example, it can be calculated for **1** that 87.5% HCD was bound to β-CD (10^{-2} M) and exhibited a quantum yield of 0.23. The remaining 12.5% free species is only responsible of 2% of the fluorescence signal. This is probably the reason the fluorescence decays were correctly fitted with only one exponential.

Interaction Model and Calculation of the Binding Constant. The binding constants were calculated from the emission data obtained using method 1. The fluorescence quantum yield was measured for **1**, **2**, and **3** and analyzed versus the β-CD concentration. Among the different models which were investigated, the simplest, assuming a 1:1 stoichiometry, gave a satisfactory fit, which was not improved by taking further equilibria into account (Figure 4). The equilibrium can be written

$$\mathrm{CD} + \mathrm{HCD} \leftrightarrows \mathrm{CD{-}HCD} \qquad \text{(I)}$$

with the binding constant $K = [\mathrm{CD{-}HCD}]/[\mathrm{CD}][\mathrm{HCD}]$.

According to the mass conservation equations

$$[\mathrm{HCD}_0] = [\mathrm{HCD}] + [\mathrm{CD{-}HCD}] \qquad (1)$$

$$[\mathrm{CD}_0] = [\mathrm{CD}] + [\mathrm{CD\text{-}HCD}] \qquad (2)$$

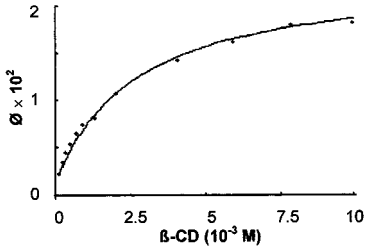
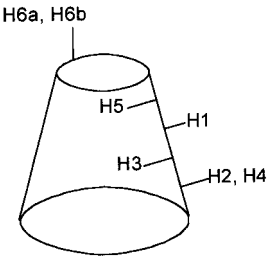


Figure 4. Fitted spectrofluorimetric data for compound **2** vs β-CD concentration. $\lambda_{ex} = 344$ nm. The points are experimental, and the curve is calculated.

SCHEME 2: Schematic Representation of the β-CD Protons



in which the subscript 0 refers to initial concentrations. Therefore, the equilibrium equation is the following:

$$K[\mathrm{CD{-}HCD}]^2 - (1 + K[\mathrm{HCD}_0] + [\mathrm{CD}_0]) \times [\mathrm{CD{-}HCD}] + K[\mathrm{HCD}_0][\mathrm{CD}_0] = 0 \qquad (3)$$

It was solved by an iterative method. The binding constants were found to be 7×10^2 M^{-1} for **1**, 3.4×10^2 M^{-1} for **2**, and only 8.1×10^1 M^{-1} for compound **3**. The error was estimated to be about 10%. The first two values compared well with the mean value of binding constants collected by Connors in the literature for 1:1 inclusion complexes of β-CD.[26] In contrast, the third value was much lower. This suggests that the substituent in the 3 position plays a major role in the stabilization of the inclusion complex.

Spectroscopic Properties in the Presence of α*-CD.* α-CD (cavity diameter ~ 5.2 Å) was used in the same conditions as β-CD (6.6 Å), following method 1. Variations much weaker than those observed with β-CD were noted for substituted compounds **1** and **2** (Table 1). This is probably due to the fact that the cavity diameters of α-CD did not suit the volume of these probes, which rather interact with the outer surface of α-CD. By comparison, this observation supports the hypothesis of the formation of an inclusion complex with β-CD.

3.3. ^{1}H NMR Study. As recently reviewed,[27] ^{1}H NMR spectrometry has been widely used to give evidence for inclusion complexes of CD and is an excellent tool to do so.[28,29] Cyclodextrins possess outer-surface (H2 and H4) and inner-surface (H3 and H5) protons (Scheme 2). The H6 protons are situated at the smaller opening of the CD torus. The spectral characteristics of these protons have long been known. In the presence of a guest molecule, the reasoning is that upon formation of an inclusion complex, the "inside" protons are more strongly affected than the others. Alternatively, if the interaction takes place outside the torus, the shift mainly concerns the "outside" protons.[30−33]

The ^{1}H NMR spectra were recorded in D_2O and analyzed using a program system designed for simulation and iteration. The fit of the experimental spectra with the calculated spectra are given in the Supporting Information. For the sake of

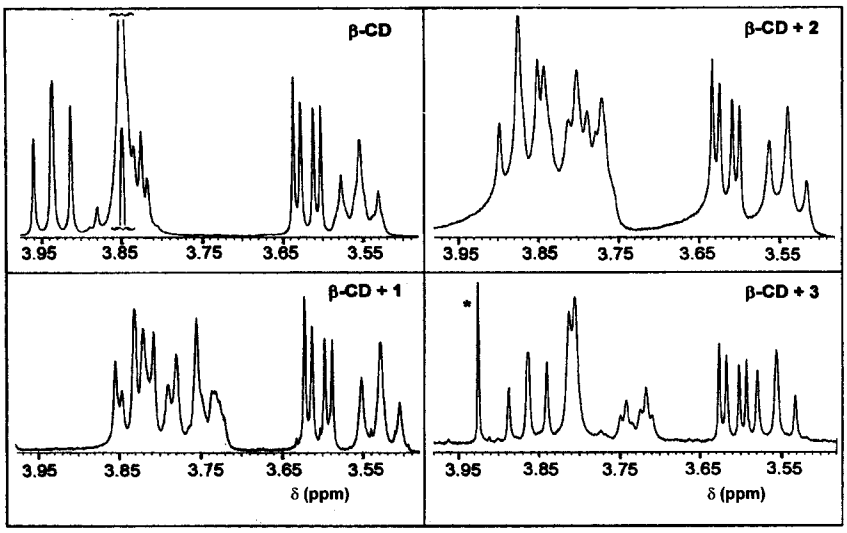


Figure 5. ^{1}H NMR spectra of β-CD (9.6×10^{-3} M) in the absence and in the presence of equimolecular amounts of 4-hydroxycoumarin derivatives in D_2O. The star indicates a coumarin proton.

TABLE 2: ^{1}H NMR Multiplicity (*m*), Shifts (δ), and Coupling Constants (*J*) for the β-CD Protons, in the Absence and Presence of the 4-Hydroxycoumarin Derivatives[a]

		β-CD	β-CD + **1**		β-CD + **2**		β-CD + **3**	
	m	δ	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$
H1	d	5.04	5.01	−0.03	5.03	−0.01	5.03	−0.01
H2	dd	3.62	3.61	−0.01	3.62	0.00	3.61	−0.01
H3	dd (t_{app})	3.94	3.83	−0.11	3.87	−0.06	3.86	−0.07
H4	dd (t_{app})	3.55	3.53	−0.02	3.54	−0.01	3.56	−0.00
H5	m	3.84	3.74	−0.10	3.78	−0.06	3.73	−0.11
H6a	dd	3.84	3.77	−0.06	3.80	−0.04	3.81	−0.03
H6b	dd	3.86	3.83	−0.03	3.85	−0.01	3.81	−0.05
J_{1-2}		3.6	3.6		3.7		3.8	
J_{2-3}		9.9	9.9		9.9		9.8	
J_{3-4}		9.0	9.1		8.8		9.2	
J_{4-5}		10.4	9.9		10.5		10.1	
J_{5-6a}		4.6	4.3		5.2		4.3	
J_{5-6b}		1.6	1.8		2.2		2.7	
J_{6a-6b}		−12.1	−12.3		−12.1		−12.6	

[a] The $\Delta\delta$ values were calculated from the exact δ values with 4 decimals.

TABLE 3: ^{1}H NMR Shifts (δ), Multiplicity (*m*), and Coupling Constants (*J*) for the 4-Hydroxycoumarin Derivatives Protons, in the Absence and Presence of β-CDs

	m	δ	δ	$\Delta\delta$
		1	**1** + β-CD	
H5	s	7.72	7.75	0.03
CH_2CH_3 (6)	t	1.17	1.25	0.08
CH_2CH_3 (6)	q	2.63	2.75	0.12
CH_3O (7)	s	3.89	3.99	0.10
H8	s	6.91	6.83	−0.08
H3′	d	6.74	6.69	−0.05
H4′	d	7.40	7.40	0.00
CH_2CH_3 (5′)	t	1.34	1.35	0.01
CH_2CH_3 (5′)	q	4.35	4.35	0.00
$J_{CH_2CH_3}$ (6)		7.5	7.7	
$J_{3'-4'}$		3.6	3.6	
$J_{CH_2CH_3}$ (5′)		7.1	7.1	
		2	**2** + β-CD	
H5	s	7.70	7.73	0.03
CH_2CH_3 (6)	t	1.18	1.26	0.08
CH_2CH_3 (6)	q	2.65	2.74	0.09
CH_3O (7)	s	3.91	4.00	0.09
H8	s	6.95	6.84	−0.11
O−CH_2−CH_2−O	m	4.32	4.32	0.00
H5′	d	6.84	6.79	−0.05
H7′	dd	6.81	6.77	−0.04
H8′	d	6.92	6.93	0.01
$J_{CH_2CH_3}$ (6)		7.5	7.6	
$J_{5'-7'}$		1.9	1.9	
$J_{7'-8'}$		8.2	8.0	
		3	**3** + β-CD	
H5	d	7.78	7.82	0.04
H6	dd	6.94	7.01	0.08
OCH_3 (7)	s	3.88	3.92	0.04
H8	d	6.93	6.90	−0.03
J_{5-6}		9.1	8.8	
J_{6-8}		2.5	2.4	

comparison, the spectrum of β-CD alone was recorded and found to be in good accordance with the literature data.[34] The spectrum of the hydroxycoumarin derivatives alone was also recorded. Then, the ^{1}H NMR spectrum of β-CD (9.6×10^{-3} M) was recorded in the presence of stoichiometric amounts of hydroxycoumarin derivatives **1**−**3**. The spectral characteristics of β-CD are reported in Table 2 and those of the coumarin derivatives in Table 3.

In the presence of the hydroxycoumarin derivatives, a negligible effect was observed on the CD protons H1, H2, and H4. In contrast, the spectral region δ 4.00−3.70 ppm, which corresponds to protons H3, H5, and H6, exhibited substantial changes (Figure 5). It appeared that proton H3 underwent strong shielding, which reached 0.11 ppm in the presence of **1**. Strong shielding was also observed for proton H5. The H6a and H6b protons showed a small upfield shift in most cases. The magnitude of the shifts of protons located within or near the CD cavity (H3, H5, and H6) is comparable with that reported for inclusion complexes.[27,30−33] It is therefore very likely that the hydroxycoumarin derivatives are positioned within the β-CD cavity.

Very weak variations were observed in the coupling constants. It must be noted that, because of the insufficient resolution of the NMR spectrum (400 MHz), the J_{5-6a} and J_{5-6b} constants were difficult to obtain precisely. These constants are related to the conformation of the glucose units. Pyranosides may exist under three staggered conformations, differing by the rotation about the C5−C6 bond. The magnitude of the coupling constants involving the H5 and the two H6 protons, namely, J_{5-6a} and J_{5-6b}, generally allow the contribution of each rotamer to be estimated. Djedaïni et al. reported that the host could have an influence upon the J_{5-6a} and J_{5-6b} constants and, hence, upon the glucose configuration,[35] although this situation seems to be

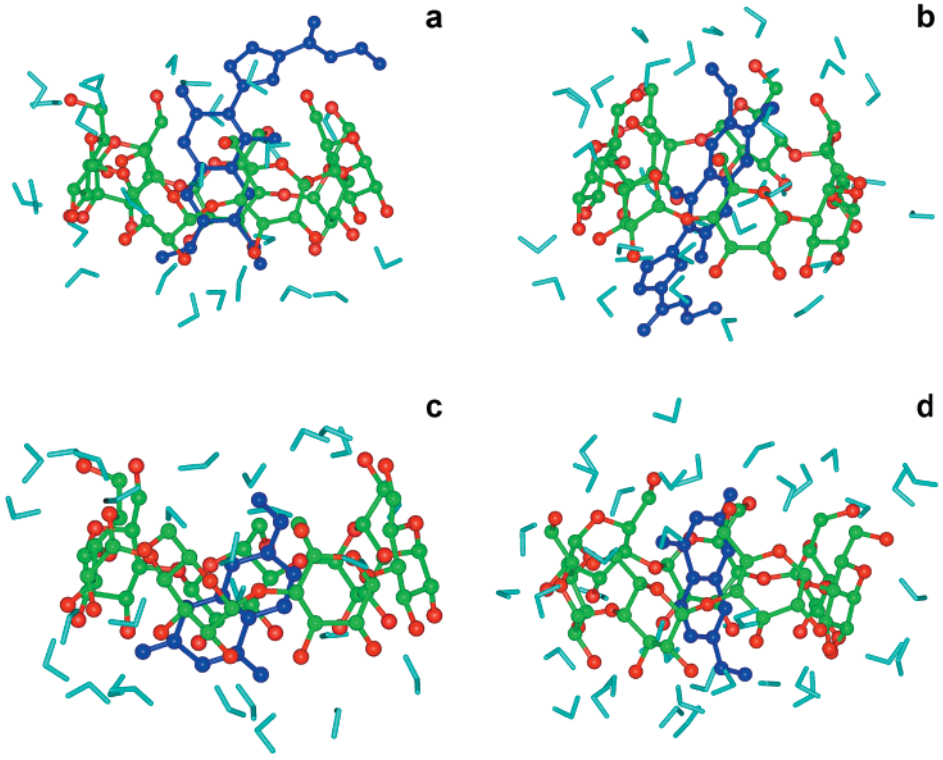


Figure 6. Result of the molecular modeling calculation for molecules **1** and **3** in β-CD. Only the water molecules which solvate the complex were represented. For the sake of simplification, the hydrogen atoms were omitted.

quite rare. In our case, the significant errors obtained on these constants do not allow them to be discussed.

Let us now turn our attention toward the ^{1}H resonance of the probes. Substantial differences were also found in the presence of β-CD. For all three hydroxycoumarins, a deshielding of the protons borne by the phenyl ring in the 5, 6, and 7 positions was observed, as well as a shielding of the H8 proton. This indicates that for all three compounds the coumaryl moiety interacts with β-CD, in which it is most likely included. However, for **3**, the $\Delta\delta$ values concerning H8 and the protons of the methoxy group in the 7 position were much lower than those found for **1** and **2**. It is thus possible that the location of the coumarin derivative in the β-CD cavity differs in the two cases.

Concerning the substituent moieties borne on the 3-position of analogues **1** and **2**, the H3′ proton of **1** was shifted upfield, whereas the other protons of the furoate group were not affected. For **2**, both the H5′ and H7′ protons, which are close to the coumaryl rings, experienced shielding. The signals assigned to the dioxanyl group were unchanged. This implies that the heterocycle in the 3 position lies outside the β-CD cavity, but its protons situated near the coumaryl cycle are nevertheless involved in the interaction with β-CD.

No signal corresponding to the free molecules was detected on the NMR spectra of β-CD in the presence of the hydroxycoumarin derivatives. This indicates that the association−dissociation process is rapid with respect to the ^{1}H NMR time scale and probably takes place in the microsecond to millisecond range. Moreover, the seven glucose units display identical signals: the complex possessing an apparent symmetry. According to Wood et al.,[30] this symmetry could be due to the guest rotating within the cavity, which could provide an identical shielding contribution to each glucose unit.

3.4. Molecular Modeling. The use of computational chemistry in the area of cyclodextrins has been the topic of recent reviews which underlined well its difficulties and limitations.[36−38] The present molecular modeling mainly aims at giving an idea of the complex structure in accordance with the NMR data. To build a model of β-CD, the structure of a β-CD hydrate clathrate, determined by X-rays analysis, was taken from the literature.[39] This molecule was solvated by 12 molecules of water. The starting geometry of the hydroxycoumarin derivatives was subjected to minimization, first in a vacuum, then after solvation by a 5 Å thick water layer. The host and guest molecules were then dehydrated, and their spatial arrangement was manually generated in two different ways. For **1** and **2**, either the coumaryl ring system or the substituent in the 3 position was introduced through the β-CD wide opening. For **3**, either the lactone-bearing cycle or the benzene ring was introduced. The complex was solvated by a 5 Å thick water layer and subjected to energy minimization calculation, to molecular dynamics, then to another energy minimization.

For **1**, it appeared that the hydrogen bond between the oxygen atom of the furoate cycle and the hydrogen atom of the hydroxyl group was disrupted in the presence of water. This is in total agreement with the results obtained by spectrophotometry. Whatever the mode of introduction, the coumaryl ring system was engulfed within the β-CD cavity. However, when the substituent was introduced through the β-CD wide opening, no interaction could be seen between the ethyl furoate group and the β-CD (Figure 6a). In contrast, when the coumaryl ring system was introduced through the wide β-CD rim, the proximal moiety of the ethyl furoate subtituent was found to be close to the secondary hydroxyl groups of the β-CD (Figure 6b). This model is on line with the NMR study, which indicates that proton H3′ was affected by the inclusion. According to this calculation, it also seems that the angle between the coumarin residue and the ethyl furoate substituent was increased when passing from water (49°) to the complex (65°). This suggests the adjustment of the guest molecule within its host.

For **2**, according to molecular modeling, both the incorporation of the coumaryl ring system and that of the benzodioxanyl

group were likely to occur. This confirms that the β-CD may accommodate molecules of the size of two benzene rings.[1] However, only the incorporation of the coumaryl moiety is in line with the experimental data.

For **3**, each mode of incorporation leads to a possible complex (Figure 6 parts c and d), but no experimental evidence allows one to choose between those structures.

4. Conclusions

In the present work, the spectrophotometric study gave strong indications that an interaction takes place between the HCD and β-CD. The spectral properties of HCD in the presence of β-CD were strongly reminiscent of those obtained in organic medium, suggesting the formation of an inclusion complex. However, despite their sensitivity, optical methods do not allow a clear distinction to be made between the interactions involved, and ^{1}H NMR spectroscopy was necessary to allow a thorough understanding of the nature of the inclusion complex.

Similar results were obtained using two different methods to prepare the dye/CD mixture. Checking this was essential for the homogeneity of this work, so the data obtained by spectrophotometric measurement (methods 1 or 2) were fully comparable with the NMR spectroscopy data (method 2). Another important consequence is that our inclusion complexes do not seem to be sensitive to the presence of tiny amounts of cosolvent, as reported by Bergmark et al. for other coumarin derivatives.[13]

Hydroxycoumarin derivative **3** led to complexes of low stability, although its size was appropriate for good insertion within the CD cavity. For the sake of comparison, it can be noted that the stability constant of coumarin with β-CD, determined spectrophotometrically, was reported to be around 1.4×10^3 M^{-1}.[8] This corresponds to a fairly stable complex. Consequently, the hydroxyl group in the 4 position, which is ionized at the pH considered, has a negative influence on complex formation. This can be connected with the report that other CDs (α-CD) showed no affinity for phenols or phenates.[40] In contrast, grafting a substituent onto the 3 position of the 4-hydroxycoumarin led to a marked increase of the binding constants for **1** and **2**. This was observed too in the literature for other HCDs substituted in the 3 position. Binding constants in the $4 \times 10^2 - 1.2 \times 10^3$ M^{-1} range have been reported for warfarin, coumachlor, and phenprocoumon in the presence of β-CD.[15,16] It is interesting to note that in **1** and **2** the 3 substituent was part of the conjugated system and displayed a weak electron-withdrawing effect.[19] However, in the structures reported in the literature, the 3 subtituent did not extend the coumarin π-electron system, probably having a weak inductive effect. So, the stabilization of the complex cannot solely be explained by the electronic influence of the 3 substituent on the coumaryl ring system. It was shown here that the substituent itself establishes interactions with the β-CD. Then, it appears that the presence of the substituent in the 3 position counterbalances the unfavorable effect of the hydroxyl group.

Concerning the inclusion complex structure, it can be underlined that no 1:2 complex, i.e., one HCD molecule sandwiched between two β-CD, was detected. The size of the substituent in the 3 position allowed this structure to be considered for **1** and **2**. That such a complex does not form is probably due to the high entropy associated with this system. Moreover, only one type of 1:1 complex was detected, at least for **1** and **2**, although several different complexes were expected, because of the dissymmetry of the HCD and β-CD molecules. This probably results from the presence of the substituent in the 3 position, which governs the structure of the inclusion complex formed. Our data are in line with the literature, which indicates that the coumarin derivatives are positioned within the CD cavity with the lactone group close to the CD wide opening.[8,41] It must be noted that in these conditions a hydrogen bond could be established between the coumarin lactone group and the CD secondary hydroxyl groups.

The present study shows that despite the considerable number of β-CD inclusion complexes reported up to now the multiplicity of the possible interactions is far from being fully described. Fluorescent probes, which allow the powerful technique of fluorescence spectroscopy to be used, prove to be an excellent tool for this type of investigation.

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Supporting Information Available: Fit of the ^{1}H NMR spectra with the calculated spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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