Effects of Natural Cyclodextrins on the Photophysical Properties of Dibenzofuran-2-carboxylic Acid

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The absorption and fluorescence spectral characteristics of dibenzofuran-2-carboxylic acid (DBFCA) have been studied in aqueous solutions and in several organic solvents. The fluorescence emission is structured both at basic pH and in nonaqueous media, whereas at acidic pH it is broad and largely Stokes-shifted. The red-shifted emission emerges solely as a consequence of an intramolecular charge-transfer (ICT) state, stabilized by hydrogen bonding with the solvent, and is not due to changes in the molecular structure upon excitation, to excimer emission, or to any other multimolecular processes. The p*K*'s in the ground and first electronic excited states are pK = 4.19 and pK^* = 8.5, as calculated by the Förster cycle from the absorptometric and fluorimetric data. DBFCA forms inclusion complexes with α-, β-, and γ-cyclodextrins (CDs), with the spectral behavior being very dependent on the type of CD and pH. At alkaline pH, only the β- and γ-CDs form complexes, with a 1:1 stoichiometry. ^{1}H NMR spectra point to the most likely structure of the complex in solution, with the carboxylate group located at the narrower end of the CD. The observed fluorescence quenching is static in nature, as deduced from the temperature dependence of the emission and from the absorption spectra. The decrease in fluorescence has been used to assess the formation constants by nonlinear regression analysis, and from their dependence on the temperature, the thermodynamic parameters of the binding, enthalpy, and entropy have been deduced. At acidic pH, fluorescence enhancement occurs with α- and β-CDs, and DBFCA forms complexes more stable than those at alkaline conditions with all the CDs. γ-CD induces the formation of an excimer of 2:1 stoichiometry, DBFCA$_2$:γ-CD. The effect of the −COOH group on the stability is analyzed by comparing the DBFCA complexes with those formed with the nonsubstituted dibenzofuran (DBF).

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides built up from α-D-glucopyranose residues linked by glycosidic bonds α-1,4, with the most common being those formed by six, seven, or eight glucose units (α-, β-, and γ-CD, respectively). Due to the lack of free rotation about the glycosidic bonds, CDs display a torus-like or hollow truncated cone shape, with a hydrophobic cavity and two hydrophilic rims in which the primary and secondary OH groups are inserted. The main feature that makes CDs of interest is their ability to form inclusion complexes with a variety of guest molecules, in solution or in solid phase. This quality offers many interesting applications, which have been described extensively in the literature.[1] The first condition required for a molecule to form an inclusion complex with CD is that it fits in the cavity, either totally or partially. In addition to this, a favorable energetic balance is required, which depends on the nature of the guest, the inner diameter of the CD, and its substitution degree.[2]

Fluorescent compounds are appealing molecules to form complexes with CDs. Since the inclusion can affect the ground and/or excited states of the fluorophore, modifications of the spectral properties of the substrate upon inclusion are frequently observed. Fluorescence enhancement is the most common situation, which has found a number of interesting analytical uses. This emission enhancement has been ascribed to factors such as the protection against collisional quenching, changes in the polarity of the microenvironment or an increase in the rigidity of the guest, among others.[3] The opposite effect (quenching of fluorescence) is, however, less common. In the presence of natural and modified CDs, certain heterocycles, such as acridine[4] and phenazine[5] display predominantly a static quenching, as well as some naphthalene derivatives, such as 2-acetylnaphthalene.[6,7] Pyrene exhibits both enhancement and quenching of fluorescence, together with complexes of different stoichiometries, depending on the type of host or the concentration range.[8,9]

In addition to the changes in the photophysical processes of the guest upon the inclusion, the photochemistry can also be modified.[10] Thus, the CD behaves as a protective shell for the fluorophore against photobleaching or side reactions or, on the contrary, can act as a catalyst, inducing excimer formation.[11,12] The reactivity in the excited state, for example, proton transfer, can also be altered in the presence of CD, due to interactions of a protonable group with the hydrophilic borders of the cavity.[13] The CDs have also proven to restrict the twisting of functional groups in molecules that display twisted intramolecular charge transfer (TICT), for example, 4-*N*,*N*-dimethylaminocinnamic acid[14] or 2-(4′-*N*,*N*-dimethylaminophenyl)benzimidazole.[15] This space restriction caused by the inclusion has been used advantageously to probe the dual fluorescence of

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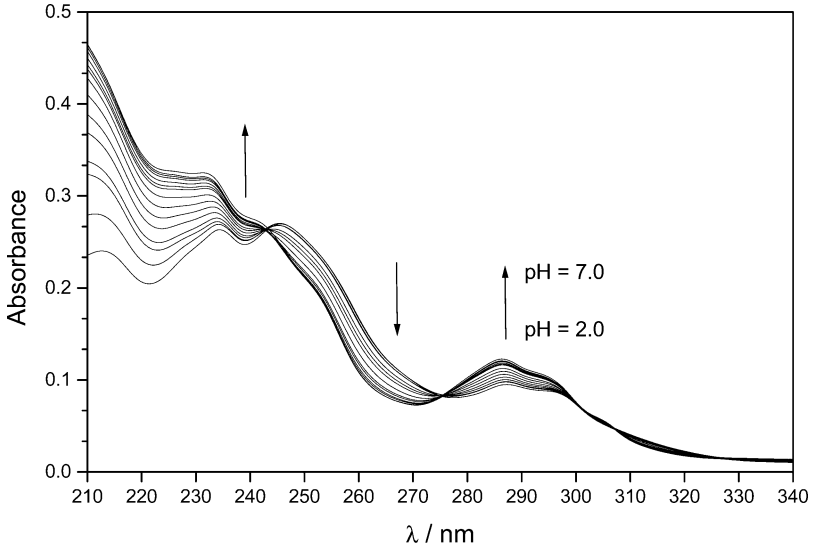

Figure 1. Absorption spectra of DBFCA as a function of pH (arrows indicate the changes in *A* with increasing pH).

9-anthroic acid, ending the controversy about the nature of such emission.[16,17]

In a previous paper, we investigated the quenching processes produced in dibenzofuran (DBF), upon the inclusion within natural CDs.[18] Some important differences with respect to heterocycles containing N in the stoichiometries and binding constants were found, especially with α-CD, and γ-CD. The latter CD was also found to induce excimer emission by complexes of 2:2 stoichiometry at high γ-CD/DBF ratios.

In this framework, we have carried out the study of a DBF derivative, dibenzofuran-2-carboxylic acid (DBFCA), a molecule capable of giving proton transfer in the excited state. The aim of this paper has been, on one hand, to explore the spectral features (absorption, steady-state fluorescence, mainly, and also ^{1}H NMR) of DBFCA in water and other solvents and, on the other hand, to investigate the complexes formed between DBFCA and natural CDs in aqueous solution. The effect of the −COOH group on the stability has been analyzed by comparing the DBFCA complexes with those formed with the parent compound, DBF.

Experimental Section

β-CD was manufactured by Roquette, and α-CD and γ-CD were manufactured by Wacker. The water contents were 13.89%, 8.29%, and 7.91%, respectively, as determined by thermal analysis. Dibenzofuran-2-carboxylic acid (DBFCA) was obtained from Aldrich (Rare Chemicals Library). All the reactants were used as received. Cyclohexane (Panreac), ethanol (Merck), and acetonitrile (Scharlau) were of spectrophotometric grade. Water solutions were prepared with deionized water using Wasserlab type I reagent grade water equipment.

Steady-state fluorescence measurements were performed using a Perkin-Elmer LS-50B spectrofluorimeter. The excitation wavelength in each case corresponded to the λ_{max} in the excitation spectrum at the longest wavelength to minimize possible photobleaching. The current scan rate was 300 nm/min, and the excitation and emission slits were fixed at 2.5 and 3.0 nm, respectively. The temperature of the cell (1.000 cm path length quartz cuvettes) was controlled with a heated circulating bath at 15, 25, 35, and 45 °C (±0.1 °C).

UV spectra were acquired with a HP 8452A diode array spectrophotometer, with an integration time of 1 s (10 spectra averaged per record) using quartz cells of 1.000 cm path length. The temperature was kept constant within ±0.1 °C with an external thermostat.

In the experiments with CDs, the DBFCA concentration was fixed at 1.5×10^{-5} M and 2.3×10^{-5} M (pH 2 and 10, respectively) for UV measurements; for fluorescence, it was 4.6×10^{-6} M at both pH's. The ratio CD/DBFCA was varied by direct titration in the cell with a stock solution containing DBFCA + CD. This procedure permits us to minimize the errors due to the manipulation of the cuvette or to slight changes in its position with reference to the radiation source.

^{1}H NMR spectra were recorded with a Bruker Avance DPX-300 spectrometer operating at 300 MHz, at 300 K, by averaging 64 scans. The solutions were prepared in D_2O (Aldrich Chemical Co., 99.9% minimum in D), with the necessary amount of NaOD (Merck, 40% solution of NaOD in D_2O, 99.5% in D) to reach alkaline pH ≈ 10. The HDO signal was used as the reference.

Results

Solvent Dependence of DBFCA Spectral Properties. The absorption spectrum of DBFCA in water at pH = 10 is characterized by two partially structured bands: a long wavelength band (LW), with an absolute maximum at 286 nm, and a short wavelength one (SW), at 228 nm (Figure 1). Acidic pH does not dramatically affect the appearance of the spectrum, except for a bathochromic shift of the LW band (2 nm) and the resolution into two components of the SW one. This slight effect of the pH on the LW band of the absorption spectrum indicates that the carboxyl group, either ionized or neutral, has little influence on the energy of the electronic states responsible for the absorption.

The spectra do not suffer important changes in their shape when using less polar solvents, such as cyclohexane, ethanol, and acetonitrile (Figure 2). The only significant alterations occur in cyclohexane, namely, a slightly better resolution of the vibronic structure and a red shift of the LW band with respect to the cases of other solvents (ca. 2 nm). In all cases, the spectrum resembles that of DBF in water, the main differences being the red shift of the LW band of DBF, at 280 (ca. 8 nm), and the broadening of the sharp transition at 250 nm (Figure 2). This similarity points again to the little conjugation between the functional group and the dibenzofuran nucleus.

As far as the fluorescence is concerned, two remarkable facts are found: the lack of mirror similarity between absorption and emission spectra, and the effect of the solvent polarity and pH.

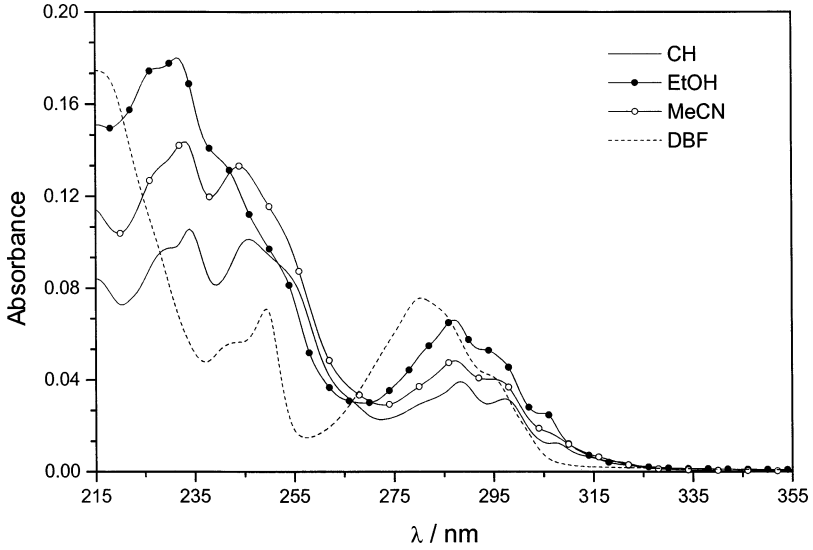

Figure 2. Absorption spectra of DBFCA in several solvents (CH, cyclohexane; EtOH, ethanol; MeCN, acetonitrile) and DBF in water.

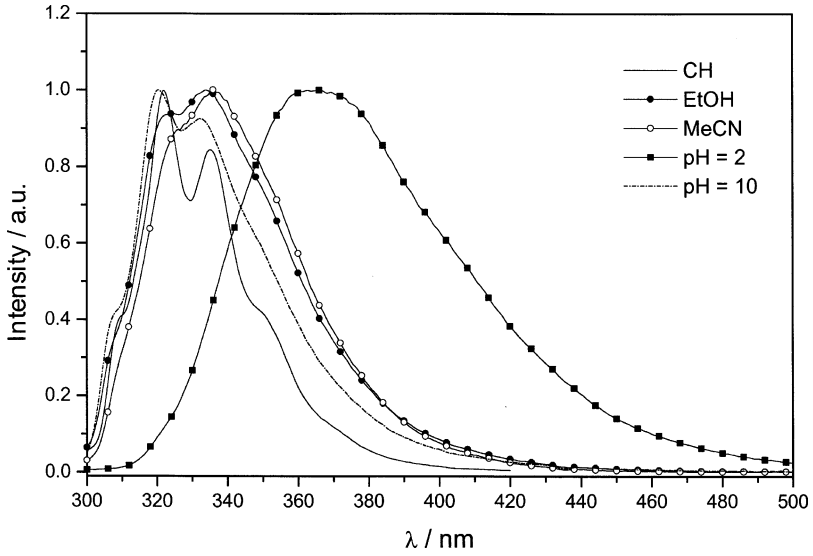

Figure 3. Normalized fluorescence emission of DBFCA in several solvents (same as Figure 2).

The emission of DBFCA is well structured in cyclohexane (Figure 3), but there is no correspondence to the UV spectrum as concerns the relative intensity of the vibronic bands. There are two possible explanations for this fact: (i) the molecule changes in geometry upon excitation; (ii) the LW band is composed of several overlapping transitions whereas emission occurs only from one excited state, according to Kasha's rule.

As for the first possibility, it is known that DBF is a planar molecule in both ground and excited states (a class I molecule according to Berlman's classification[19]). The only possibility of a geometrical distortion that the addition of a carboxyl group to DBF can induce is twisting around the −COOH bond. This is known to occur in 9-anthroic acid.[16,20−22] In this compound, the carboxyl group is oriented at an angle of 55° with respect to the aromatic ring in the ground state, and upon excitation, the COOH twists toward a more coplanar conformation, increasing the conjugation with the aromatic moiety.

To verify the possibility of a geometry change caused by photoexcitation, we have carried out semiempirical calculations, based on the PM3-CI (6−6) Hamiltonian, with Hyperchem software.[23] The procedure consisted of obtaining the energies of the ground and first excited singlet states as a function of the twist angle, introducing the torsion as a restraint into the minimization at each angle, prior to the calculation of the energy. In Figure 4 these energies are plotted as a function of the twist angle of the −COOH group. The plot suggests that the coplanar conformation is the most stable in both states, showing a small rotational barrier. No change in the geometry upon excitation is then expected to happen.

The spectrum of DBFCA should parallel that of the parent compound, DBF, whose interpretation has been a subject of research for many years. The most recent one, by using quantum chemical calculations and linear dichroism, has settled some remaining questions about the current assignments.[24] According to this investigation, the UV spectrum involves five electronic transitions, with the $^1A_1$ (302 nm) and $^1B_2$ (282 nm) being those that configure the LW band, that is, the vertical transitions from the ground state to the first singlets, S_1 and S_2. The presence of more than one transition contributing to the LW band is the most plausible explanation for the nonmirror similarity between absorbance and fluorescence in DBFCA. This evidence has been also observed in 2-anthroic acid and explained as the result of the contribution of two overlapping bands, by comparison with the L_a and L_b states of anthracene.[21] The observed differences between absorption and excitation spectra when using different λ_{em} values (not shown) seem to reinforce this conclusion.

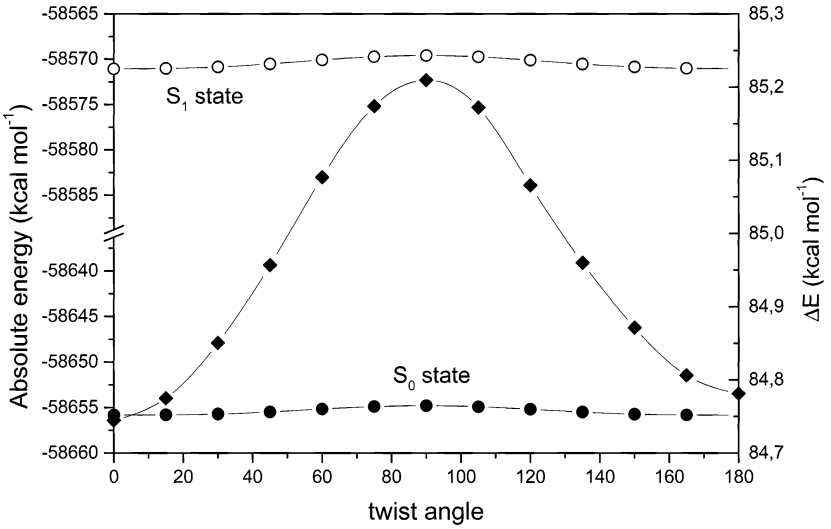

Figure 4. Left axis: energies of the ground (●) and first (○) singlet electronic states versus the twist angle DBF−COOH. Right axis: $E(S_1) - E(S_0)$ (◆).

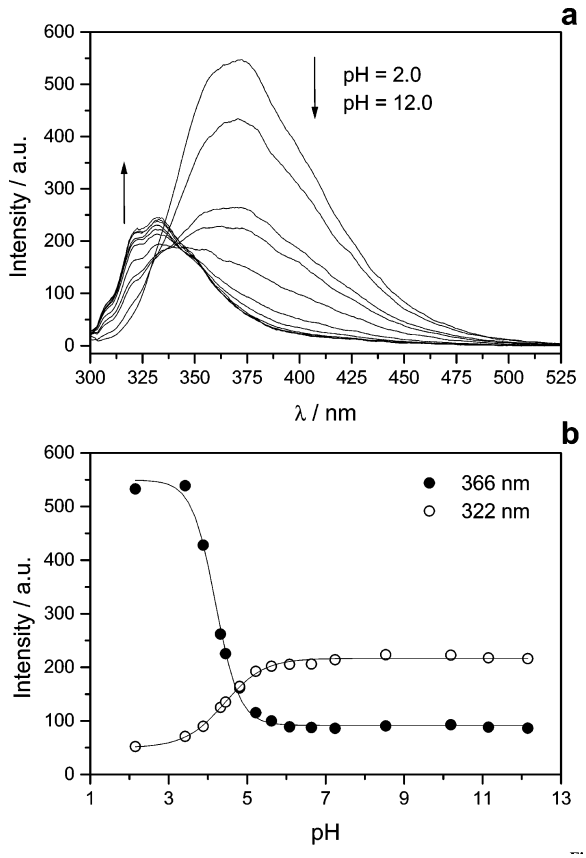

Figure 5. (a) Fluorescence emission of DBFCA as a function of pH. (b) Fluorimetric titration of DBFCA at different emission wavelengths.

Another remarkable aspect of the fluorescence emission of DBFCA is the effect of the solvent polarity. In the presence of solvents more polar than cyclohexane, the emission spectrum widens, losing gradually its structure, in the sequence cyclohexane < water (basic) < ethanol ≈ acetonitrile < water (acid). The emission in water is strongly pH dependent (Figure 5a), with the Stokes shift reaching its maximum value in acidic aqueous solutions (6760 cm^{-1}). Regarding the quantum yields, they decrease in the order acetonitrile ≈ cyclohexane > water (acid) > water (basic) > ethanol (0.205, 0.218, 0.140, 0.073, and 0.044, respectively).

The broad and Stokes-shifted emission at acidic pH is common in molecules having an electron withdrawing group, such as −COOH, attached to an aromatic nucleus. However, the nature of such an emission is not always easy to ascertain, since it can be the result of a variety of causes, including dimer formation in the ground state (or other kinds of aggregates), excimer emission, changes in the conformation upon excitation, or charge-transfer processes. An example of this is 9-anthroic acid, which has been the subject of a number of studies in the past, providing different interpretations of its fluorescence emission.[16,17]

In the case of DBFCA, the fact that the broad band does not appear at alkaline pH could suggest the possibility of DBFCA forming dimers in the ground state (linked by hydrogen bonding), which give rise to excimer emission upon excitation.[25] These dimers cannot form if the carboxylate group is ionized, and accordingly, the emission would be structured, as observed. However, the low concentrations currently used, as much as 5×10^{-6} M, rule out this possibility. Even at concentrations as low as 2×10^{-7} M, the broad emission can be detected.

Excimer emission could also result from some kind of packing of the DBFCA molecules, even if hydrogen bonding is not the driving force. This is a planar molecule, a feature that is known to facilitate this type of emission, as in DBF,[18,26] naphthalene,[27] or anthracene.[28] However, this mechanism must be rejected, as will be explained in the following section when discussing the influence of CDs on the DBFCA fluorescence.

The most likely reason is not a multimolecular process, as those described, but a unimolecular one: the emission of a state with a high intramolecular charge-transfer character (ICT), stabilized by hydrogen bonding with the solvent. In such a state, the donor group is the aromatic moiety of the molecule, and the acceptor group is the −COOH. In an acidic medium, the carboxyl group becomes more conjugated with the aromatic π system, in a situation in which there is a marked charge separation within the molecule (resonant structure I).

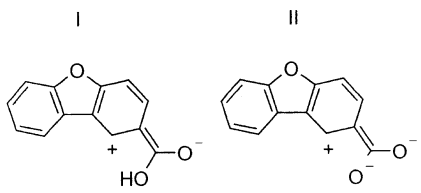

TABLE 1: Spectral Characteristics of DBFCA with Natural CDs at 298 K

	absorption	emission[a]	λ_F (nm)	Stokes shift (cm^{-1})	spectral width compared to that of DBFCA
		(a) Acidic pH; $\lambda_{ex} = 296$ nm			
α-CD	overall lowering of A	I. pt. 384 nm	353	5460	narrower (+)
β-CD	overall lowering of A	I. pt. 378 nm	354	5540	narrower
γ-CD	isosbestic points that shift with [γ-CD]	I. pt. 370 nm	374	7050	broader (+)
		(at high [γ-CD])	356	5690	broader
		(b) Basic pH; $\lambda_{ex} = 286$ nm			
α-CD	no effect	no effect			
β-CD	defined isosbestic points	quenching	320	3720	narrower
γ-CD	overall lowering of A	quenching	320	3720	identical

[a] I. pt.: isoemissive point.

In nonpolar solvents, the state that lies lower in energy and is responsible for the structured emission is the locally excited (LE) state. The energies of these states, LE and charge transfer (CT), are not equally affected by changes in the solvent polarity. Hydrogen bonding between the carboxyl and the solvent molecules lowers the energy of the CT form relative to that of the locally excited one, to the point where crossover has taken place and the CT state lies lower in energy than the LE state. Semiempirical calculations on model compounds in which CT is expected, such as *p*-dimethylaminobenzonitrile (*p*-DMABN), by simulating different solvents, prove that this crossing over happens.[29]

The Stokes shift and the resolved spectra in cyclohexane, ethanol, and acetonitrile would indicate a primarily dipolar interaction between the solute and the solvent molecules, which is in contrast to the broad and largely red-shifted spectrum in acidic aqueous solution. This means that hydrogen bonding is added to the dipolar contribution in acidic media, with the subsequent stabilization of the CT state. The carboxylate anion, on the other hand, already containing a negative charge (resonant structure II), will not readily accept a second negative charge necessary for the resonance interaction, and consequently, the emission arises from the LE state, that is, a structured spectrum, as in cyclohexane.

The position of the substituent in the ring is key for the absorption and fluorescence behavior, because of the different electronic densities of the HOMO on each atom. Thus, substitution at position 1 would stabilize the CT state better, since the electronic density is higher at this point, as the chemical shifts in the ^{1}H NMR spectrum of DBF show.[30] It is to be expected that 1-DBFCA would yield spectra even more diffuse and unstructured than those of 2-DBFCA, as has been observed for 1-anthroic and 2-anthroic acid.[21]

Ground- and Excited-State p*K*'s of DBFCA. Despite the low solubility and the limited pH dependence of the absorption spectrum, the p*K* could be calculated from the changes in the absorbance by a nonlinear least-squares fit to the Henderson−Hasselbach equation. At $\lambda_{max} = 286$ nm, the p*K* resulted in 4.19 ± 0.03, a value similar to that of other aromatic carboxylic acids, such as benzoic, 2-naphthoic, or 2-anthroic.[31]

Fluorescence and absorption data allow the calculation of the acidity constant in the excited state, p*K**, by means of the Förster cycle[32] according to

$$pK - pK^* = \frac{E_{HX} - E_X}{2.303RT} \qquad (1)$$

where E_{HX} and E_X are the energies of the electronic transitions for the acid and base pair, determined as the 0−0 transition energies. These can be estimated by averaging the energies corresponding to the absorption and fluorescence maxima of both acid and base forms, that is,

$$E_{HX} = \frac{Nhc}{2}(\bar{\nu}^a_{HX} + \bar{\nu}^f_{HX}); \quad E_X = \frac{Nhc}{2}(\bar{\nu}^a_X + \bar{\nu}^f_X) \qquad (2)$$

where the terms in parentheses refer to the wavenumbers for the maxima of absorption (a superscript) and fluorescence (f superscript). An alternative procedure for estimating the 0−0 transition is to determine the wavenumber corresponding to the intersection point of the normalized absorption and emission spectra (intensity vs energy). The p*K**s obtained by both approaches are, respectively, 8.92 and 8.07. Regardless of the method used, the acid is weaker in the excited state, a result which is consistent with the ICT state expressed by the resonance form I. The p*K* shift ($\Delta pK = pK^* - pK$) falls within the same range as that reported for other carboxylic acids of polycyclic aromatic hydrocarbons.[31,33]

Fluorescence titrations can tell us something about the time scale in which the excited state proton exchange and fluorescence decay occur. The fluorescence intensity at 322 and 366 nm has been plotted versus the pH in Figure 5b. In these representations, only one inflection point is detected for both wavelengths, at a mean value of 4.30, that matches almost perfectly the p*K* of DBFCA in its ground state, and no inflection point appears in the zone of higher pH. This indicates that the emission occurs from the excited species unperturbed by excited-state proton-transfer reactions; that is, both the rate of pseudo-first-order protonation of the excited DBFCA and the rate of dissociation of the excited conjugate acid are small in comparison to the inverse of the fluorescence lifetimes. Therefore, the variations of the intensity of fluorescence of the acid or base will depend on the thermodynamics of the ground-state reaction, and the plots parallel those of the absorptometric titrations.[28] Thus, only one inflection point is observed, which corresponds to the p*K* in the ground state. 9-Anthroic acid[17] is a well-known example of this same behavior, and so is quinine sulfate.[34] Time-resolved fluorescence experiments, together with kinetic analysis methods, would help to support these results.

Studies of DBFCA + CD in Water. *Absorption Spectroscopy.* The main effects that the addition of the three natural CDs has on the UV spectrum of DBFCA, both at acidic and alkaline pH's, have been collected in Table 1.

At pH = 2 the changes in the spectra upon the addition of CD are noteworthy. With α- or β-CD the absorbance falls, and no isosbestic points are detected. In contrast, with the wider macrocycle, γ-CD, some isosbestic points arise, whose positions shift with the CD concentration (Figure 6).

At basic pH, on the contrary, the changes are not so significant. α-CD does not modify the DBFCA spectrum, whereas γ-CD produces an overall lowering of the absorbance

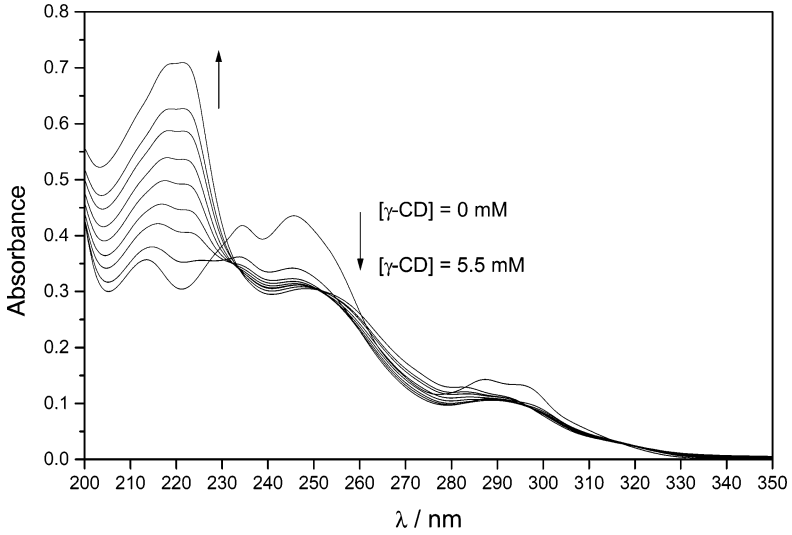

Figure 6. UV spectra of DBFCA (1.48×10^{-5} M, pH = 2) in the presence of variable amounts of γ-CD (arrows indicate the changes in intensity with increasing [γ-CD]).

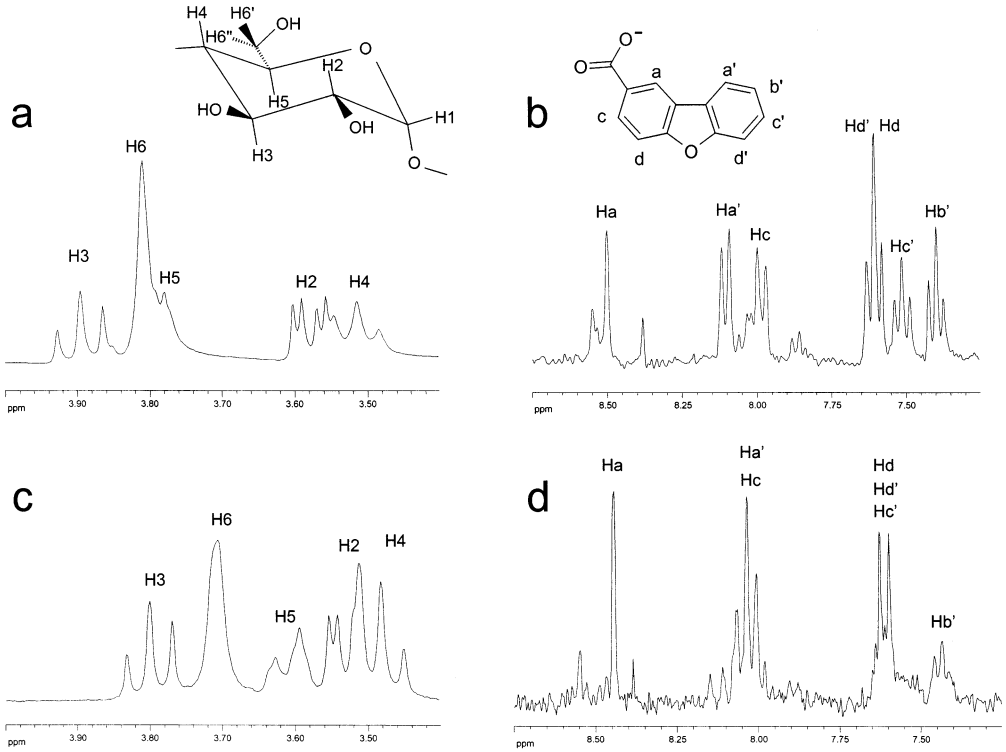

Figure 7. ^{1}H NMR spectra in basic D_2O of (a) 2×10^{-3} M β-CD; (b) 2×10^{-3} M DBFCA; and (c and d) 2×10^{-3} M DBFCA + 2×10^{-3} M β-CD.

and β-CD yields well-defined isosbestic points at 296 and 302 nm, together with a red shift of 2 nm for the transition at 286 nm.

The exact interpretation of all these data is not easy, since the LW, as has been explained in the previous section, is composed of two overlapping bands, which can be modified diversely upon the addition of CD. In any case, the absorption data point to some kind of interaction in the ground electronic state between DBFCA and the three CDs, with the exception of α-CD at pH = 10.

^{1}H NMR. The poor solubility of this substance in acidic conditions precluded any qualitative or quantitative studies by NMR. Henceforth, the discussion will refer to DBFCA at basic pH. The ^{1}H NMR spectrum of DBFCA and its proton assignation is displayed in Figure 7, along with the elucidation of the signals accomplished with the COSY spectrum and the simulation of spin subsystems with MestRe-C software.[35]

The addition of β-CD to a DBFCA solution (1:1 mole ratio) produces considerable shifts in all the protons of the CD, especially the inner H5 and H3, as well as in DBFCA ones (Figure 7). γ-CD gives less remarkable variations of the chemical shifts, whereas with α-CD no changes in any of the resonances can be detected. These data agree qualitatively with UV experiments and with those of fluorescence, which we will discuss in the following section. The shifts of the resonances of the CD cavity undoubtedly indicate that the complex is of inclusion type and not a simple association between both molecules.

All the protons of β-CD shift upfield, with the inner ones being the most remarkable, H3 (−0.08 ppm) and H5 (−0.16

ppm). In addition to this, the H6 protons, which belong to the primary rim of the cavity, suffer a magnetic shielding (−0.09 ppm) that is even higher than that of H3, which seems to indicate the participation of the narrower edge of the CD in the complex structure. Such a shielding can be caused by the nearby presence of the $-COO^-$, which would be located at the narrower end of the CD. The external protons H1, H2, and H4 scarcely change (−0.03, −0.03, −0.02 ppm, respectively), most probably due to distortions of the structure of the macrocyclic ring than to any kind of physical contact.

Regarding the proton shifts induced by the β-CD on the guest, all the resonances of DBFCA experience changes with the exception of those for the d (meta with respect to $-COO^-$) and d′ (ortho with respect to the heteroatom) protons. However, b′, c, and c′ shift downfield, whereas a and a′ shift upfield. The cause of the different directions of the shifting is not straightforward. In principle, it would reflect the steric interactions of the guest with the inner part of the CD, which are primarily given by the shielding tensor of the CD. However, upon the inclusion, the electronic distribution of DBFCA will be presumably modified. The oxygen heteroatom, which is hydrogen bonded in water, is shielded within the CD, and this effect is transmitted throughout the aromatic structure, changing the charge distribution on each atom. Something similar can occur with the $-COO^-$ group, as the relatively high $\Delta\delta$ in H6 protons seems to indicate. Therefore, little can be said about the abnormal shifting, except that the aromatic moiety must be lodged within the CD cavity.

According to geometric considerations, the stoichiometry is 1:1. The β-CD cavity is 7.9 Å in height, with a mean inner diameter of 6.2 Å.[36] The aromatic nucleus of DBFCA is about 9 Å in length and 5 Å in width, as estimated by using the standard bond lengths for C−H, C−O and C−C bonds in an aromatic ring. On this basis, it is clear that the guest can be lodged completely into the β-CD cavity along its axis. These considerations, together with the NMR data, depict a structure in which the DBFCA lays axially inside the CD, with the aromatic moiety completely buried and with the carboxylate group located at the primary rim.

Steady-State Fluorescence. In contrast to the absorption, the fluorescence behavior of DBFCA is quite different depending on the CD considered and the pH. For the sake of clarity, the main facts have been brought together in Table 1 and we will discuss each CD separately.

(a) β-Cyclodextrin. Figure 8b shows the fluorescence spectra of DBFCA in acidic aqueous solutions upon the addition of β-CD. The intensity of the broad emission increases, and a blue shift of ∼16 nm is observed, with an isoemissive point appearing at 378 nm, indicative of an equilibrium between emitting species. This evidence rules out the hypothesis of DBFCA being in the ground state in dimer form. If this were the case, that is, the broad band were due to the emission of an excited dimer, as has been suggested by some authors for 9-anthroic acid,[25] a decrease in the intensity would be expected, together with the appearance of a structured fluorescence near 320 nm, as a consequence of the disruption of such species and subsequent complexation, since the β-CD cavity cannot lodge more than one DBCA molecule. Instead, an emission enhancement is observed. As a consequence, we must conclude that the fluorescence displayed by DBFCA at acidic pH is due solely to the ICT state and that it does not come from multimolecular processes.

The quantum yield increase together with the diminution in the Stokes shift is in accordance with the nonpolar character of the cavity and a less effective solvation, which reduce the nonradiative pathways of deactivation. This great dependence of the fluorescence on the solvent polarity is common in molecules having ICT bands, for example, 9-methylester anthroate.[37]

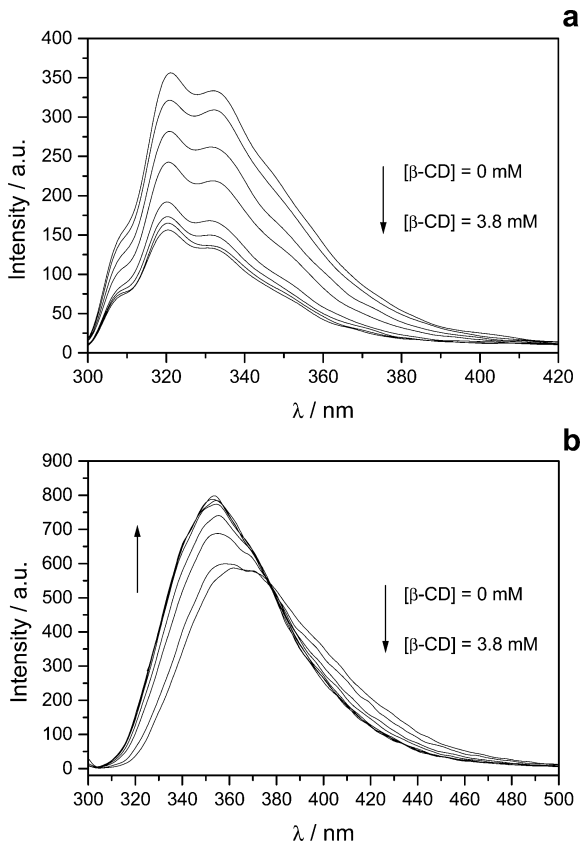

Figure 8. Fluorescence spectra of DBFCA in the presence of variable amounts of β-CD at 25 °C: (a) basic pH; (b) acidic pH (arrows indicate the changes in intensity with increasing [β-CD]).

At alkaline conditions (pH = 10), where only the structured fluorescence is present, β-CD produces the opposite effect, that is, fluorescence quenching (Figure 8a). Neither isoemissive points nor band shifting is perceived. However, the complex is not dark, and the fluorescence reduces, approaching a constant level.

The type of quenching, whether static or collisional, can be deduced by temperature dependence studies or by time-resolved fluorescence spectroscopy. The aforementioned investigation on the quenching of fluorescence in DBF points to a mainly static mechanism; that is, the attenuation in the fluorescence is a consequence of the formation of a complex between the fluorophore and the quencher which is, in this case, less emissive than the fluorophore itself.[38] Provided the complex formation is exothermic (the most common situation in complexes with CDs), a temperature increase will reduce the stability of the complex, raising the concentration of free fluorophore, which will result in a lower slope in a F_0/F versus [CD] plot.

The ratios F_0/F versus the concentration of β-CD at four temperatures are plotted in Figure 9a. A reduction in the quenching upon increasing the temperature from 15 to 45 °C is observed, in accordance with a static quenching process, as explained above. The result is similar to that observed in DBF, although in that case the quenching was not so intense. Besides, the changes observed in the absorption spectrum of DBFCA upon the addition of β-CD constitute an additional proof of the type of process, since the complex formation involves the

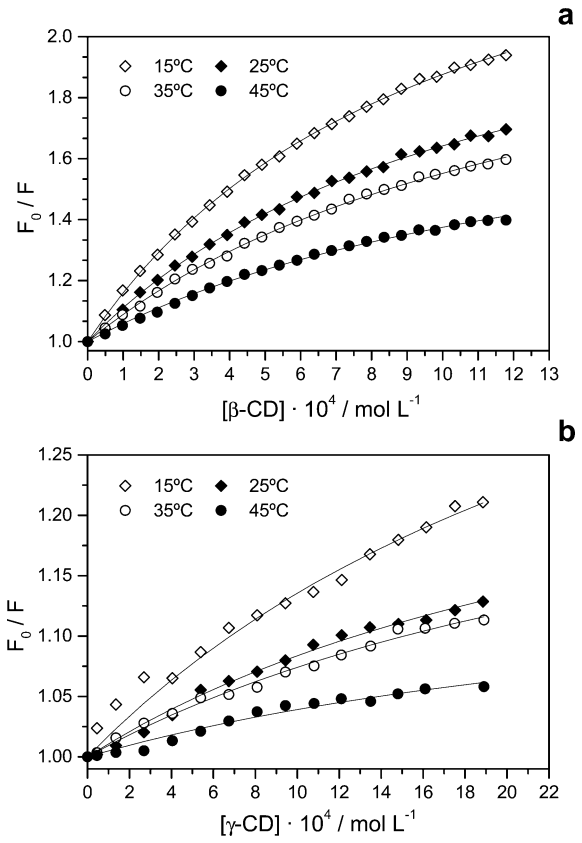

Figure 9. F_0/F plots at different temperatures for (a) DBFCA + β-CD, pH = 10; and (b) DBFCA + γ-CD, pH = 10 (solid lines are the fits to eq 3).

ground state of the fluorophore and, as a result, it gives rise to alterations in the absorption spectrum, whereas collisional quenching would only affect the excited state of the fluorophore.

In the general case of a two-state system, in which the complex is fluorescent, the following relationship stands[39]

$$\frac{F_0}{F} = \frac{1 + K[\mathrm{CD}]}{1 + aK[\mathrm{CD}]} \tag{3}$$

where F_0 is the fluorescence of DBFCA in the absence of CD, F is the measured fluorescence at each point, [CD] is the concentration of nonbonded CD, K is the binding constant, and a is related to the quotient between the products of the quantum yields, ϕ, and the molar absorptivities, ϵ, of DBFCA in its complexed and free forms through $a = \epsilon_{cx}\phi_{cx}/\epsilon_{DBFCA}\phi_{DBFCA}$. This equation can be linealized to give

$$\frac{1}{F_0/F - 1} = \frac{a}{1 - a} + \frac{1}{(1 - a)K[\mathrm{CD}]} \tag{4}$$

Both equations reduce to the classical Stern−Volmer equation for a dark complex (i.e., $a = 0$). Assuming that the total CD concentration added, $[CD]_0$, is much higher than that of DBFCA, the amount of free cyclodextrin, [CD], can be set approximately to $[CD]_0$, and by plotting a double reciprocal plot, $1/(F_0/F - 1)$ versus $1/[CD]_0$, a straight line must be obtained if the complex is 1:1. Conversely, if a 1:2 stoichiometry is expected (DBFCA: CD_2), in which $K_2 \gg K_1$, then $1/(F_0/F - 1)$ should be linear[9] when plotting it against $1/[CD]_0^2$. For DBFCA + β-CD at 25 °C and both pH's, the plot (not shown) is linear with $1/[CD]_0$, whereas, by assuming a 1:2 binding, the graph displays an upward curvature, which confirms the 1:1 stoichiometry of this complex.

The association constant can be obtained by fitting the experimental data by a nonlinear least-squares regression analysis to eq 3. The K values calculated at each temperature are given in Table 2.

It is possible to obtain the thermodynamic parameters of the binding, that is, the enthalpy and the entropy, from the dependence of K on the temperature through the van't Hoff equation, provided these magnitudes remain constant within the considered temperature range (Figure 10a):

$$R \ln K = \Delta S - \frac{\Delta H}{T} \tag{5}$$

The entropy and enthalpy have been calculated by a weighed least-squares method, where the statistical weight of each point is given by its error in the corresponding binding constant (Table 2). ΔH is negative, as usual in complexes with CDs, and the entropy is practically zero. It is worthy of mention that the binding enthalpy is practically the same as that for DBF with this CD (-22 ± 3 kJ mol^{-1}). Considering that the only difference is the extra $-COO^-$ group, the result suggests that the carboxylate would be preferentially hydrated by the solvent, having only a little effect on the binding. Regarding the entropy, its interpretation is not staightforward since this parameter is usually known with less precision than the enthalpy, and its value results from the sum of several contributions of different signs and of difficult evaluation. On one hand, there is an entropy loss associated with the binding of the fluorophore; on the other hand, there is a release of water molecules that are inside the cavity and around the guest, which results in a positive contribution to the entropy. When comparing DBF and DBFCA, the entropy is only slightly higher for DBFCA, within the experimental uncertainty, which seems to reinforce what has been said about the arrangement of the guest in the complex.

The binding constants for protonated DBFCA are considerably higher than those of DBF or DBFCA in its ionized form. It is well-known that substituents of aromatic rings capable of hydrogen bonding can bind the OH groups of the CD edges.[40] The energy involved in such H-bond interactions is responsible for the higher binding constants found, when compared to those of the unsubstituted molecule. This pattern in the binding has been detected in the complexes between benzene derivatives and β-CD. Thus, benzene[41] has a formation constant of 107 M^{-1}, whereas that for benzoic acid[42,43] ranges between 126 and 501 M^{-1}. This seems to be the case for DBFCA: the acid will be "anchored" to the CD by the −COOH group, a factor that confers stability to the complex.

Fluorescence measurements cannot give us detailed information about the orientation of DBFCA inside the cavity. Unfortunately, the low solubility of DBFCA in acid pH has avoided any qualitative or quantitative NMR studies, which would be the most appropriate to elucidate the inclusion mode. Liu et al. have proposed an empirical model for predicting the binding energies and orientation of mono- and disubstituted benzene derivatives.[44] An interesting conclusion is that, from the two possible orientations of benzoic acid along the cavity, the preferred one is that with the carboxylate located near the primary rim of the CD. According to the comparatively high values of the H6 shifts for ionized DBFCA, the results suggest that the carboxylate group is at the narrower edge of the CD. This can also be the case with the acid form of the guest.

(b) γ-Cyclodextrin. In contrast to β-CD, DBFCA undergoes fluorescence quenching, both at high and low pH's, on increasing the ratio γ-CD/DBFCA, although the trends in the F_0/F plots are different in both cases (Figure 11).

TABLE 2: Binding Constants, $K \times 10^{-3}$ (L mol^{-1}), and Thermodynamic Parameters for DBFCA Complexes with CDs

	pH = 2	pH = 10					
	25 °C	15 °C	25 °C	35 °C	45 °C	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
α-CD	1.10 ± 0.08						
β-CD	9 ± 1	2.81 ± 0.04	2.12 ± 0.05	1.71 ± 0.04	1.33 ± 0.05	−19.1 ± 0.4	0 ± 1
γ-CD	12 ± 1[a]	0.50 ± 0.06	0.45 ± 0.06	0.40 ± 0.06	0.3 ± 0.1	−9.0 ± 0.6	21 ± 2

[a] Apparent constant assuming a 1:1 complex (see text).

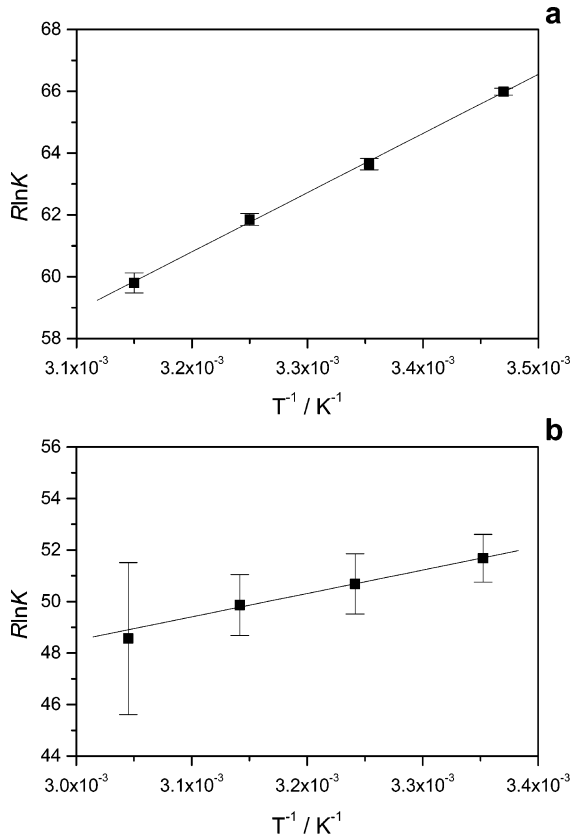

Figure 10. Van't Hoff plots for (a) DBFCA + β-CD, pH = 10; and (b) DBFCA + γ-CD, pH = 10.

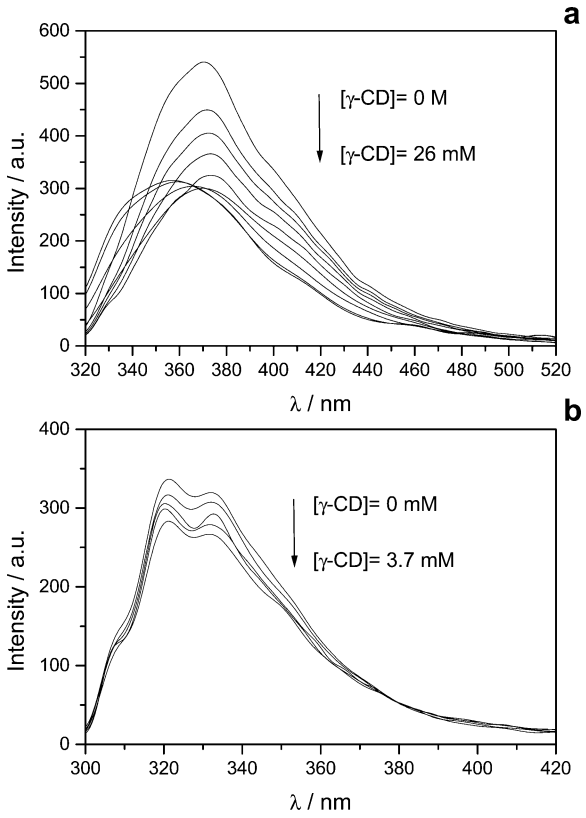

Figure 11. Fluorescence spectra of DBFCA in the presence of variable amounts of γ-CD at 25 °C: (a) acidic pH; (b) basic pH (arrows indicate the changes in intensity with increasing [γ-CD]).

At basic pH, the quenching (much less pronounced than that of β-CD) follows the trend described by eq 3. By assuming a 1:1 stoichiometry, the binding constant at basic pH and 25 °C is 450 M^{-1}, a relatively low value compared to that of β-CD but reasonable according to the wider cavity size of γ-CD, in which DBFCA fits loosely. The slope of the F_0/F plots reduces when raising the temperature (Figure 9b), in accordance with a static quenching mechanism, which permits the calculation of the thermodynamic parameters of the binding through eq 5 (Table 2).

The enthalpy value is smaller than that for β-CD, in accordance with the poorer fit of DBFCA into γ-CD, but the entropy is positive and nearly the same as that of DBF with this CD (14 ± 3 J mol^{-1} K^{-1}). An important contribution to the overall entropy change is the structured water of the cavity that converts into bulk water upon inclusion. β-CD has a cavity volume of 0.262 nm^3, whereas γ-CD has one of 0.427 nm^3.[36] Since only one molecule of DBFCA can be housed in the CD, there will be more conversion of highly structured water for the γ-CD. In addition to this, the poorer fit of DBFCA in the wider macrocycle would allow a rotational mobility around the longitudinal axis of the CD, a possibility which is absent for β-CD. The "rattling" of the guest inside the cavity represents a situation more favorable entropically than the "locked" one with β-CD, giving a positive contribution to ΔS upon binding.

At a low pH, however, appreciable changes with γ-CD concentration are observed (Figure 11a). The quenching is accompanied by a red shift of ∼4 nm and a broadening of the emission. In principle, one would expect that DBFCA in a neutral state would also form a less stable complex with this CD than with β-CD. The narrower cavity of β-CD fits better the DBFCA molecule, which, in addition, can be stabilized by hydrogen bonding with the OH groups of the edges. Surprisingly, the binding constant calculated according to eq 3 (1:1 model, fluorescence at 352 nm) results in 1.2 × 10^4 M^{-1}, that is, the highest value found with any of the CDs at any of the pH's.

An explanation for this fact, and for the abnormal quenching displayed with this CD when we compare it to the enhancement of fluorescence with β-CD, is the possibility of having higher stoichiometries along with the 1:1 one, in which DBFCA forms a dimer within the cavity of the γ-CD. It has been proven by theoretical studies[26] that, for DBF, the oscillator strength for the emission of the excimer is small and that interplanar distances of ∼3.5 Å agree with the experimental fluorescence wavelengths. This range of distances can be attained when packing two DBF molecules within the γ-CD, and the same holds for DBFCA. Indeed, this is the case with DBF, which forms a 2:2 complex for high concentrations of γ-CD.[18] Other condensed aromatic derivatives, such as pyrene[45] or 2-acetyl-

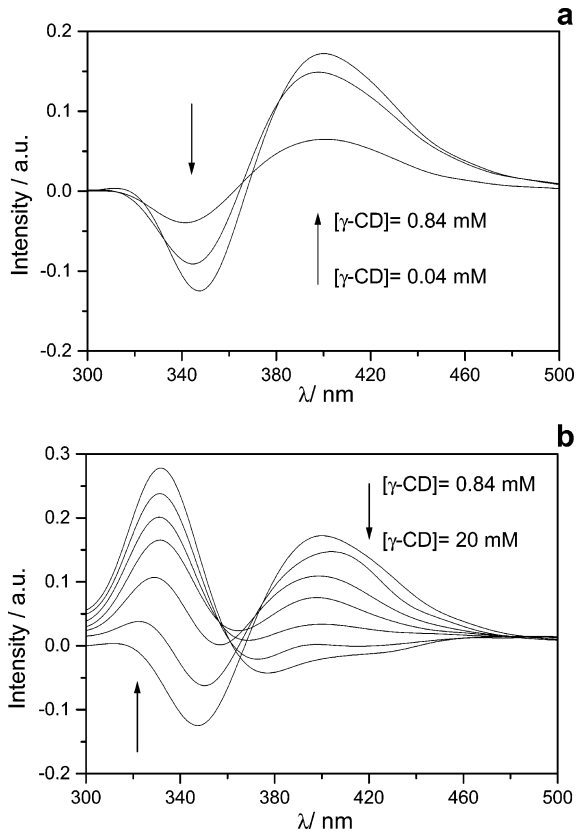

Figure 12. Subtraction normalized fluorescence spectra of DBFCA in the presence of variable amounts of γ-CD: (a) low γ-CD concentrations; (b) higher γ-CD concentrations.

naphthalene[46] also have these stoichiometries with γ-CD and yield excimer emission. This emission, although weak, was used to prove the existence of the DBF complex in solution and its stoichiometry. In our case, the existence of stoichiometries different from 1:1 is compatible with the absorption data, that is, the mismatch of the isosbestic points when [γ-CD] increases.[47]

The actual stoichiometry, whether 2:2 or 2:1, is not easy to ascertain, since both complexes could equally give excimer emission. If the excimer appears far to the red from the λ_{max} of the guest, it is possible to discriminate between both types of complexes. In such a case, an isoemissive point is usually observed, the fluorescence due to each species can be split, and with an adequate mathematical manipulation, the correct stoichiometry can be established. This can be done in the case of DBF, since the maxima are at least 60 nm apart. However, the red-shifted and broad band due to the ICT in DBFCA overlaps to a great extent the emission of the excimer.

A careful study of the effect of the concentration of γ-CD on the emission spectra can help us to establish the correct stoichiometry of the excimer (Figure 11a). In a first stage, at moderately low CD concentrations, the quenching is associated with a red shift of the band. Upon increasing the amount of γ-CD, it undergoes a hypsochromic shift of 14 nm and the intensity increases. In addition to this, an isoemissive point arises at 370 nm.

By normalizing the spectra and subtracting the spectrum of DBFCA, a band centered at 400 nm appears, whose intensity increases up to a value of [γ-CD] that matches that of the largest Stokes shift (Figure 12a), and can be ascribed to the excimer. However, the excimer does not exist along the entire γ-CD concentration range. Thus, when [γ-CD] increases, the excimer dissociates, diminishing its contribution to the overall intensity,

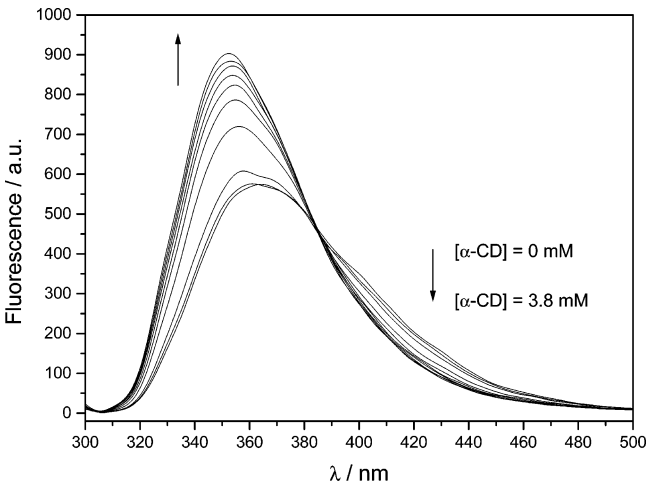

Figure 13. Fluorescence spectra of DBFCA in the presence of variable amounts of α-CD at acid pH (arrows indicate the changes in intensity with increasing [α-CD]).

and another new band, centered at 330 nm, comes into view. Hence, there are three species in solution, namely, free DBFCA, DBFCA:γ-CD, and the excimer. The rising of the excimer band up to a maximum and the subsequent decrease as CD concentration increases indicates undoubtedly that the complex is 2:1. In this way, for high [γ-CD], the excimer band decreases, given that DBFCA is "spread out" among the increasingly available CD molecules, and the 1:1 complex raises at the expense of the 2:1 complex. On the contrary, for the 2:2 model, the complex $DBFCA_2$:γ-CD_2 monotonically increases its concentration along the entire [γ-CD] interval, and the excimer band should not vanish. This can be easily verified by simulating the concentrations of the different species, according to the models 2:1 or 2:2, giving different values for $K_{1:1}$, $K_{2:1}$, and $K_{2:2}$, within a plausible range for complexes with CDs (between 10 and 10^4 L mol^{-1}, for instance).

This stoichiometry makes sense, considering that a 2:2 complex involves a second CD to share two axially stacked molecules of DBFCA and, necessarily, it must trap the carboxyl group. This seems improbable in view of the polarity of the −COOH and considering the hydrophobic character of the cavity. On the other hand, no excimer emission has been detected at basic pH, which seems reasonable considering the low likelihood of two negatively charged molecules joining within the γ-CD.

At this point, one possible strategy could be to perform a NLSF analysis of the curves at a certain wavelength to estimate the binding constants and quantum yields of the complexes. We have tried this approach with unsuccessful results, due to the high number of parameters that must be estimated, namely, two binding constants (one for each equilibrium) and two quotients between the quantum yields for the complexes. Although the 2:1 model seems to fit the data well, the errors estimated in the parameters are too high to make sense, even when imposing some restraints.

(c) α-*Cyclodextrin.* As was expected, the narrower α-CD gives the least stable complexes with DBFCA at any pH's. In fact, with the ionized DBFCA, either the complex does not form or the constant formation is very low: no alterations are observed in the fluorescence and absorption spectra, and the chemical shifts of the inner protons of the CD, H3 and H5, do not undergo any changes.

At low pH, however, an enhancement of the emission is observed, similarly to that of β-CD, together with an isoemissive

point at 384 nm, indicative of the equilibrium between the free DBFCA and the complex (Figure 13). According to the length of this heterocycle and the cavity size of α-CD, too narrow to fit the DBFCA along the longitudinal axis, higher stoichiometries than 1:1 could be expected (i.e, binding of more than one CD per guest). However, DBF does not form 1:2 complexes with α-CD, and neither do other molecules structurally like it (e.g. acridine, studied by fluorescence,[4] or anthracene, by HPLC[48]). The inclusion of such molecules is only shallow and gives complexes of rather low stability. However, this stability can be enhanced if a group capable of establishing H-bond interactions, such as −COOH, is incorporated in the molecule. The high value of $(1.10 \pm 0.08) \times 10^3$ obtained for DBFCA according to eq 3, when compared to that of DBF, seems to reinforce this conclusion.

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