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ORIGINAL ARTICLE

Changes in the reactivity of the fluorescent reagents carbazole-9-carbonyl chloride and 9-carbazolylacetic acid in the presence of cyclodextrins

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Abstract Carbazole-9-carbonyl chloride (C9CC) and 9-carbazolylacetic acid (9CAA) were selected as model fluorescent reagents. The effect of different chemically modified cyclodextrins (CDs) added to the aqueous solutions of these reagents was studied in water and in buffered aqueous solutions at pH 4.5 and 8.8. The CDs employed were 2-hydroxypropyl-β-cyclodextrin (HP-βCD), 2,3-di-*O*-methyl-β-cyclodextrin (DM-βCD) and 2,3,6-tri-*O*-methyl-β-cyclodextrin (TM-βCD). The inclusion of these reagents inside the cavities of the CDs was verified and this process can affect the derivatization reaction because CDs can modify the reactivity of the guest molecules. The basic conditions necessary for the derivatization reaction between C9CC and amines lead to the formation of carbazole anion through hydrolysis followed by decarboxylation. In the presence of CDs, the hydrolysis-decarboxylation of carbazole-9-carbonyl chloride is faster than in buffered aqueous homogeneous solutions. The behaviour observed for these reagents in aqueous solutions of CDs was compared to the one observed in basic ethanolic solutions. These changes are particularly noticeable in the case of 2,3-di-*O*-methyl-β-CD and 2-hydroxypropyl-β-CD. The characteristics of the fluorescent reagents are compared to carbazole and 9-methylcarbazole as model compounds.

Keywords Fluorescent derivatization reagents · Carbazole derivatives · Cyclodextrins catalysis · Inclusion complexes

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Introduction

Derivatization reactions are frequently employed in HPLC analysis of a great variety of compounds [1, 2]. New fluorescent derivatization reagents are being introduced at an increasing pace, and some differences can be found between them and the more traditional fluorogenic reagents. Thus, for the capillary techniques, pre-column derivatization is preferred to post-column derivatization for technical reasons. Carbazole-related derivatization reagents exhibit native luminescence and have the advantage over their better-known fluorene analogues of their higher solubilities in chromatographic mobile phases containing polar solvents like methanol, leading to more appropriate conditions of elution. A carbazole reagent for amino derivatives related to FMOC (9-fluorenylmethyl chloroformate), namely carbazole-9-carbonyl chloride has the advantage of leading to urea derivatives that are much more stable than those arising from the FMOC reagent [3]. Several other carbazole derivatization reagents have been employed for the determination of amines [4], including amino acids [5], biogenic amines [6] and aromatic amines [7].

Many organic reactions such as hydrolysis [8, 9], substitution, reduction, oxidation [10] and photochemical reactions [11] among others [12] can be catalyzed by CDs [13]. The catalytic activity is related to the presence of a hydrophobic pocket and several hydroxy groups that are activated by intramolecular hydrogen bonding. CDs have been employed as chiral selectors in HPLC and capillary electrophoresis for the enantiomeric separations of amino acids and peptides [14]. 2-Hydroxypropyl-β-CD is adequate for the resolution of amino acid derivatives of AQC (6-aminoquinolyl-*N*- hydroxysuccinimidyl carbamate) [15]. Chiral separations of proteins and amino acids derivatized with FMOC [16] and AEOC (2- (9-anthryl) ethyl chloroformate) [17] have been successfully achieved using β-CD and SDS in capillary electrophoresis. The increase in resolution is related to the above-mentioned characteristics of the CDs and also to their capability to accommodate the derivatization products in their cavities. CDs have been employed to enhance the sensitivity and the selectivity (chiral or non chiral compounds) of different analytes after derivatization reactions, but the influence of the presence of CDs on derivatization reactions has not been previously studied. In this paper we describe the degradation reactions of C9CC and 9CAA in the presence of different β-cyclodextrins and consider the consequences derived from the modifications in the reactivity of these fluorogenic reagents.

Experimental

Apparatus and reagents

Uncorrected excitation and emission spectra were obtained with a Perkin–Elmer MPF 2A spectrofluorimeter (Xenon lamp 150 W) using quartz cells with 1 cm of path length. Ultraviolet-visible absorption spectra were obtained with an automatic double beam Kontron Uvikon 810 spectrophotometer. A pH-meter Crison micro-pH 2001 was used to monitor the adjustment of the final pH values of the different buffered solutions prepared. A SBS thermostated multi-magnetic stirrer was employed for preparing the cyclodextrin inclusion complexes and maintaining the temperature in the 22 ± 2 °C range.

All reagents and solvents were analytical or spectroscopic grade and they were used without further purification. Water was doubly distilled and deionised prior to its use. Carbazole was obtained from Merck, and carbazole-9-carbonyl chloride and 9-carbazolylacetic acid were purchased from Fluka. The reference compound 9-methylcarbazole (9-MeCZ) was synthesized from carbazole (CZ) using a modification of a literature procedure for the *N*-alkylation of pyrroles and indoles as described in reference [18]. 2,6-Di-*O*-methyl-β-cyclodextrin (DMβ-CD) and 2,3,6-tri-*O*-methyl-β-cyclodextrin (TMβ-CD) were purchased from Sigma, and 2-hydroxypropyl-β-cyclodextrin (HPβ-CD) was a generous gift from Rhône-Poulenc (France).

Procedures

Inclusion complexes formation

Stock solutions of the carbazole derivatives were prepared in concentration 5.0×10^{-3} M using acetonitrile as solvent. In the preliminary experiments the stock solutions were prepared in ethanol. Aliquots of these stock solutions were placed in a round-bottomed flask and the solvent was evaporated in vacuo while the solutions were mechanically stirred at room temperature. By application of this method, a thin film of the carbazole reagent was left on the wall of the flask. To this film 10 ml of the buffered aqueous solutions were added. This procedure was used to ensure that the same concentration of the carbazole derivatives is present in all solvents or buffered aqueous solutions for each batch of assay.

Measurements at specific pH values involve the use of buffered solutions. Buffered aqueous solution at pH 4.5 was prepared with 0.5 M glacial acetic acid to which the corresponding volume of a solution 4.0 M of NaOH was added to obtain the desired pH value. A buffered aqueous solution at pH 8.8 was prepared with 0.2 M H_3BO_3, adding the corresponding volume of a solution 4.0 M of NaOH to obtain the desired pH value.

The final concentration of the carbazole derivatives was 1.0×10^{-6} M. After 30 min under magnetic stirring the fluorescence excitation and emission spectra were recorded, and an exactly weighed amount of the suitable CD was added to the aqueous solution of carbazole derivatives producing a concentration of 1.0×10^{-2} M. The mixture was magnetically stirred in a thermostated (22 ± 2°C) water bath for 90 min. An adequate aliquot was taken and the fluorescence excitation and emission spectra were recorded again. Then the suitable CD was added in increasing amounts to produce three dissolutions of concentrations 2.0×10^{-2} M, 3.0×10^{-2} M and 4.0×10^{-2} M. These solutions were magnetically stirred for 90 min after each CD addition and the fluorescence spectra were also obtained. For each experiment a

blank solution containing the carbazole derivative in the aqueous solution without CDs was prepared and measured. The sample and the blank solutions were measured again after being kept for 24 h.

Additions of NaOH to the inclusion complexes with CDs

Considering that alkaline conditions are required to the derivatization reaction with C9CC, additions of small amounts of NaOH to the solutions containing the inclusion complexes of C9CC and 9CAA at pH 4.5 and 8.8 were carried out. The inclusion complexes were prepared as described in the previous paragraph. The inclusion complexes solutions were magnetically stirred during 24 h. Then the fluorescence spectra were obtained and small volumes of NaOH solution were added. The final concentrations of NaOH in the inclusion complexes solutions were 0.04 M, 0.2 M and 0.4 M. The concentrations of carbazole derivative (C9CC and 9CAA) and the CDs were respectively 1.0×10^{-6} M and 4.0×10^{-2} M. After each addition the fluorescence emission spectra were recorded.

Results and discussion

Carbazoles exhibit peculiar photophysical and photochemical properties, specially the dependence of their luminescence behaviour on the environment. Recently, we have described the influence of solvent polarity and pH on the fluorescence of the carbazole derivatization reagents C9CC and 9CAA, whose emission maxima are clearly affected by the nature of the substituent on the pyrrolic nitrogen [18]. The formation of inclusion complexes between the carbazole reagents and the CDs under study was evaluated on the basis of the enhancement in the fluorescent emission intensity. A notable increase in the fluorescence intensity was observed for the carbazole reagents C9CC and 9CAA and also for the model compounds carbazole (CZ) and 9-methylcarbazole (9-MeCZ) for all CDs studied (HPβ-CD, DMβ-CD and TMβ-CD). These results are presented in Table 1 for the different pH conditions studied. Important differences in the enhancement of the fluorescence intensity were detected and can be explained on the basis of the chemical structures and hydrophobicities of the guest molecules. Thus the fluorescence intensity increase in the case of 9-MeCZ for all CDs studied can be explained considering its lipophilic behaviour and the small size of its substituent on the pyrrolic nitrogen. In the case of CZ the presence of the ionizable pyrrolic nitrogen increases the hydrophilic character and therefore the fluorescence enhancement is smaller. 9CAA presents a substituent with a notable steric hindrance on the pyrrolic nitrogen and consequently a small increase in the fluorescence intensity is observed for HPβ-CD and DMβ-CD and a decrease in the fluorescence intensity for TMβ-CD complexes. This behaviour can be explained considering that the interaction of 9CAA and TMβ-CD was hampered by the presence of methyl groups on OH-2 and OH-3 of the glucose units. For C9CC a notable increase in the fluorescence intensity is observed for all CDs studied. The highest increase is observed for C9CC/TMβ-CD complexes; C9CC can be

Table 1 Fluorescence excitation and emission maxima and fluorescence enhancement of the carbazole reagents studied in the presence of different CDs at concentration 4.0×10^{-2} M. The concentration of carbazole reagents in the media was 1.0×10^{-6} M

Cyclodextrin	Conditions	Enhancement of the fluorescence intensity (%)			
		C9CC (λ_{ex} = 287 nm, λ_{em} = 320, 335 nm)	9CAA (λ_{ex} = 292 nm, λ_{em} = 356, 370 nm)	9-MeCZ (λ_{ex} = 293 nm, λ_{em} = 357, 372 nm)	CZ (λ_{ex} = 291 nm, λ_{em} = 350, 363 nm)
HPβ-CD	Water	54.8	13.3	183.3	13.2
	Buffered aqueous solution pH 4.5	40.0	1.3	107.5	26.0
	Buffered aqueous solution pH 8.8	66.7	6.1	175.0	13.7
DMβ-CD	Water	60.0	8.9	154.3	18.5
	Buffered aqueous solution pH 4.5	54.8	18.7	100.0	37.8
	Buffered aqueous solution pH 8.8	50.0	–11.1	128.9	13.6
TMβ-CD	Water	100.0	–1.9	157.1	11.6
	Buffered aqueous solution pH 4.5	91.2	–1.7	79.6	22.2
	Buffered aqueous solution pH 8.8	118.6	–22.2	175.0	23.5

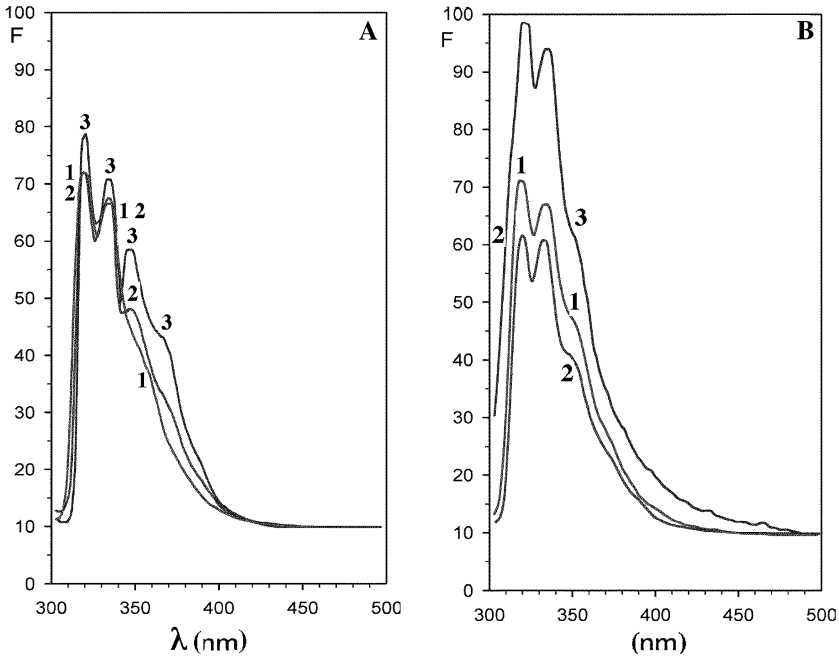


Fig. 1 Emission spectra of the carbazole reagent C9CC inclusion complexes with different CDs at concentration 4.0×10^{-4} M. (A) Complexes in buffered aqueous solutions at pH 4.5 and (B) Complexes in buffered aqueous solutions at pH 8.8. (1) HPβ-CD, (2) DMβ-CD and (3) TMβ-CD

accommodated on the rim of TMβ-CD producing stable complexes similar to those described for the complexes of amino acids and TMα-CD which are more stable than the those with DMα-CD [19].

Figure 1 shows the emission spectra of the inclusion complexes of C9CC with the CDs studied. At pH 4.5 the spectral shape shows the characteristic maxima at λ_{em} 320 and 335 nm close to those observed in organic solvents (cyclohexane or ethanol) together with a peak at 347 nm and a shoulder at 367 nm. These peaks are not present in the inclusion complexes solutions (2 h under magnetic stirring) but they appear after 24 h of stirring. In the solutions at pH 8.8 these peaks do not appear and a band broadening is observed in the region of 400–430 nm corresponding to the emission of carbazole anion, which is generated by hydrolysis of the acyl chloride function followed by elimination of the *N*-substituent through decarboxylation in the presence of CDs. Then small amounts of concentrated NaOH were added to the inclusion complexes of C9CC. The observed behaviour is presented in Fig. 2 and can be explained as follows: for DMβ-CD and TMβ-CD in buffered aqueous solution at pH 4.5 the emission intensity of the characteristic maxima and the peaks at 347 and 367 nm decreased when the NaOH concentration was increased. No emission was detected in the region of 400–430 nm. For the C9CC/ HPβ-CD inclusion complexes at pH 4.5, the fluorescence intensity of the characteristic maxima at 320 and 335 nm decreased when NaOH concentration was increased together with the appearance of an isoemissive point at 390 nm and the emission band at 430 nm corresponding to the carbazole anion. This band was increased with increasing NaOH concentration. This special behaviour observed in the presence of HPβ-CD suggests that the hydroxypropyl chain plays an important role in the catalytic effect of CD on the hydrolysis-decarboxylation reaction. At pH 8.8 and for all CDs studied the peak and shoulder at 347 and 367 nm are not observed and the increase in the NaOH concentration causes the emission of the anion of carbazole. With the aim to verify that emission at 347 and 367 nm correspond to the intermediate product in the hydrolysis-decarboxylation reaction different experiments were carried out. Additions of NaOH to the aqueous and buffered aqueous solutions of C9CC cause the emission of the carbazole anion to appear. Upon addition of HCl acid the equilibrium is shifted to produce neutral carbazole emission as can be seen in Fig. 3. Similar experiments were developed in ethanolic solution. Thus additions of NaOH or sodium ethoxide increase notably the emission corresponding to the carbazole anion with a decrease in the intensity of the maxima at 320 and 335 nm. The additions of bases also originate a weak emission peak at 347 nm. This peak is more defined and intense in ethanolic solution. The appearance of this peak can be attributed to carbazole-9-carboxylic acid or to neutral carbazole according to the Scheme 1. Thus, when the spectra obtained in homogeneous (ethanolic and aqueous

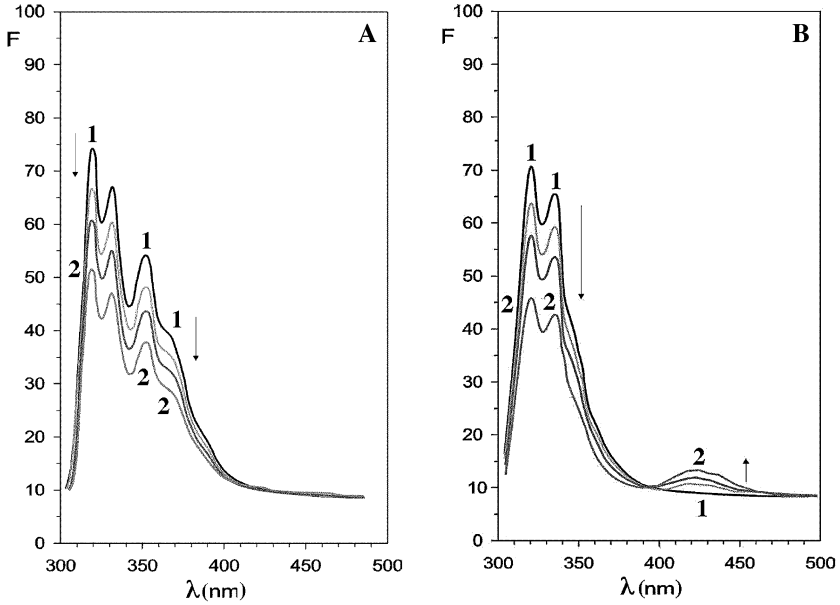


Fig. 2 Effect of NaOH additions on the emission spectra of C9CC inclusion complexes obtained in buffered aqueous solutions at pH 4.5. (A) Complexes of DMβ-CD and (B) Complexes of HPβ-CD. (1) Starting solutions without NaOH, (2) After NaOH additions up to concentration 0.4 M

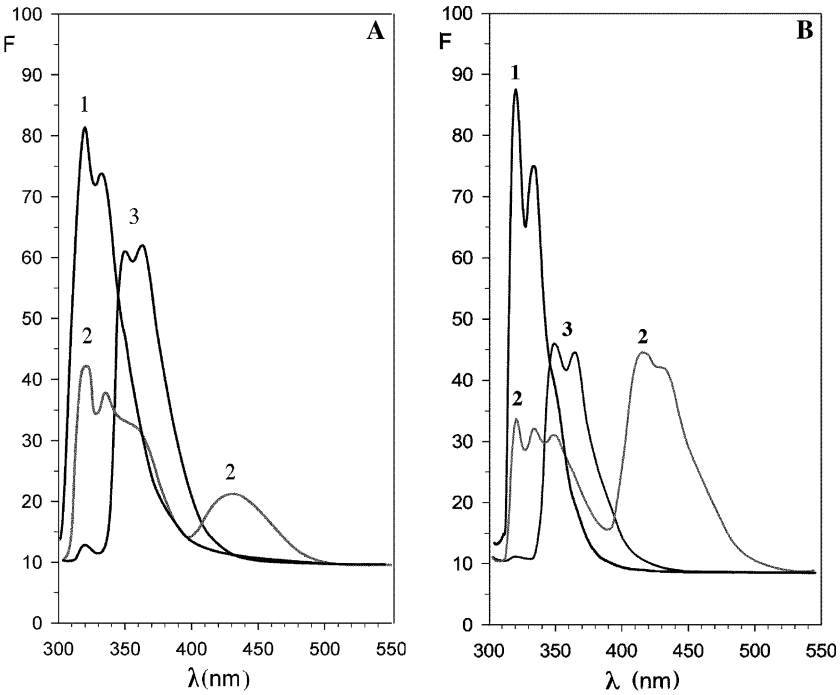
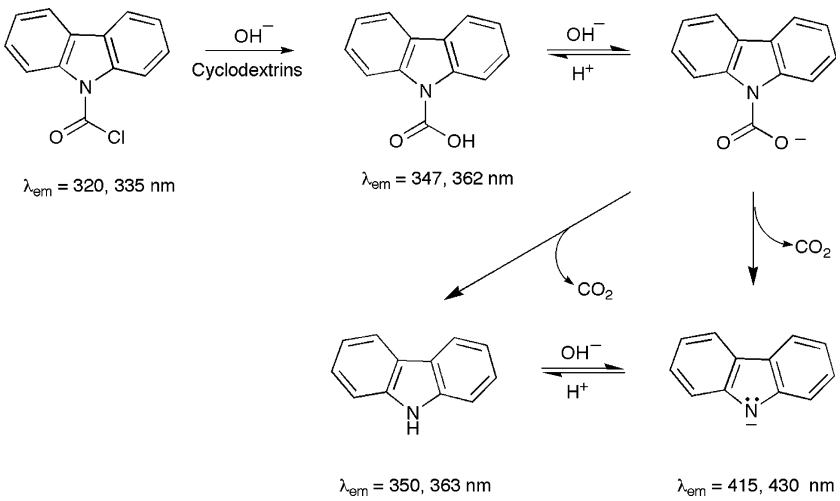


Fig. 3 Effect of NaOH additions on the emission spectra of C9CC in homogeneous solution. (A) Buffered aqueous solution at pH 8.8 and (B) ethanolic solution. (1) Starting solutions without NaOH, (2) After NaOH additions up to concentration 0.4 M and (3) Addition of HCl up to obtain neutral pH

solutions) and CDs solutions are compared it can be concluded that bases (OH^- or EtO^-) cause the hydrolysis-decarboxylation reactions and then formation of the carbazole anion. In the presence of CDs the peak at 347 nm appears without addition of NaOH (for DMβ-CD and TMβ-CD) suggesting that the CDs environment catalyzes this reaction. The anionic carbazole is generated in the presence of CDs but the intensity of this band depends on the nature of the CDs (the highest intensity is observed in the presence of HPβ-CD), and therefore it can be concluded that the molecular recognition by different CDs leads to a specific catalytic effect on the reaction of C9CC with important analytical consequences on the derivatization reactions using this fluorescent reagent.

With regard to the other fluorescent reagent studied, 9-carbazolylacetic acid (9CAA), the formation of the inclusion complexes with CDs do not affect the spectral shape and the position of the emission maxima but the fluorescence intensity is changed depending on the

Scheme 1 Proposed reactions of hydrolysis-decarboxylation of C9CC in the presence of CDs



nature of the CDs as has been described in Table 1. The addition of NaOH to the aqueous or ethanolic solutions of 9CAA does not change the spectra and only a decrease ($\approx$ 20%) in the fluorescence intensity at the maxima of 356 and 370 nm was observed. However, in the presence of CDs, the additions of NaOH produce a decrease in the fluorescence intensity of these two peaks together with the broadening of the band in the region of 400–430 nm, depending on the experimental conditions. In the case of HPβ-CD the emission band at 430 nm corresponding to the anionic carbazole is observed with a weak intensity (Fig. 4). These results suggest the loss of the acetic acid side chain in 9CAA in the presence of HPβ-CD, a process that can be explained according to the mechanism in Scheme 2, where the acetic acid chain is transferred to the hydroxypropyl chain in the CD, showing again that this chain exerts an important influence on the stability and reactivity of this kind of fluorescent reagents. In the case of the model compound 9-MeCZ, the methyl group on the pyrrolic nitrogen was not lost upon additions of bases in homogeneous media or in the presence of CDs. For carbazole, the addition of bases (OH^- or EtO^-) produces the emission of carbazole anion as a consequence of the proton transfer process in excited state as has been described previously [20].

In conclusion the presence of CDs in the derivatization reaction media can modify the derivatization

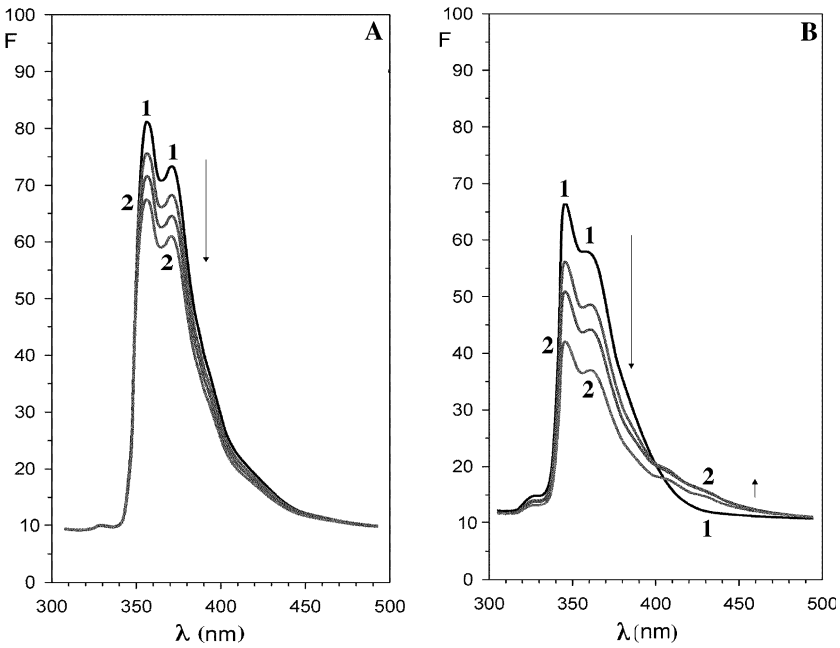
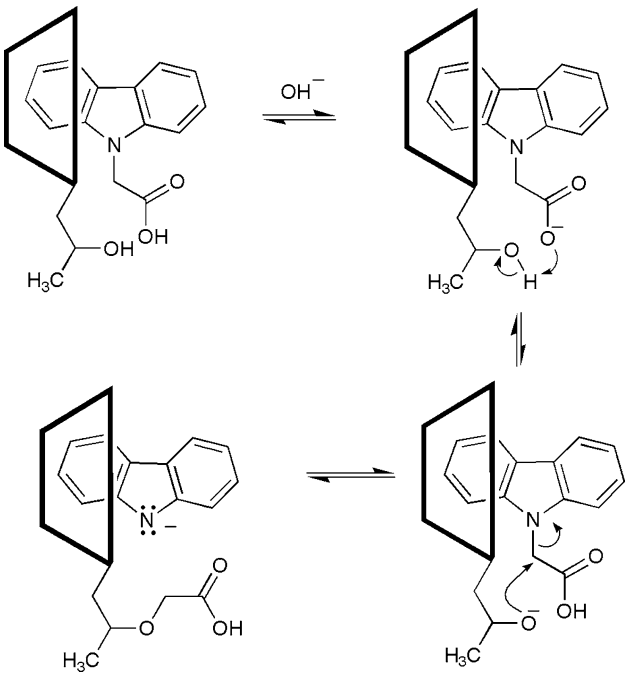


Fig. 4 Effect of NaOH additions on the emission spectra of 9CAA. (A) Aqueous solution and (B) Complexes of HPβ-CD at pH 8.8. (1) Starting solutions without NaOH, (2) After NaOH additions up to concentration 0.4 M



Scheme 2 Proposed mechanism explaining the loss of the acetic acid chain from 9CAA in the presence of HPβ-CD

reactions. The reactions observed introduce a note of caution in the use of the derivatization reagents C9CC and 9CAA. Finally, the hydrolysis-decarboxylation reaction can serve as a model for other CD catalyzed hydrolytic processes.

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