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# The Impact of Dihydrogen Phosphate Anions on the Excited-State Proton Transfer of Harmane. Effect of β-Cyclodextrin on These Photoreactions

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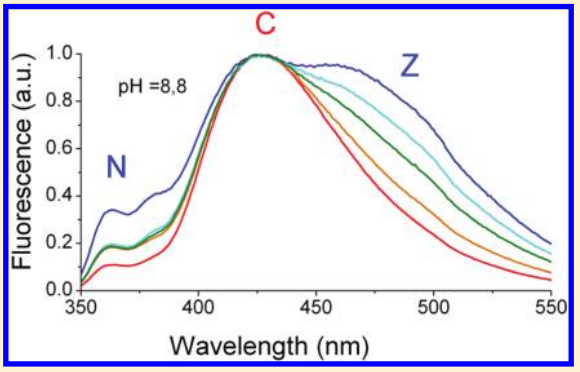
**ABSTRACT:** Photoinduced proton transfer reactions of harmane (1-methyl-9*H*-pyrido[3,4-*b*]indole) (HAR) in the presence of a proton donor/acceptor such as dihydrogen phosphate anions in aqueous solution have been studied by stationary and time-resolved fluorescence spectroscopy. The presence of high amounts of dihydrogen phosphate ions modifies the acid/base properties of this alkaloid. Thus, by keeping the pH constant at pH 8.8 and by increasing the amount of $NaH_2PO_4$ in the solution, it is possible to reproduce the same spectral profiles as those obtained in high alkaline solutions (pH >12) in the absence of $NaH_2PO_4$. Under these conditions, a new fluorescence profile appears at around 520 nm. This result could be related to the results of a recent investigation which suggests that a high intake of phosphates may promote skin tumorigenesis. The presence of β-cyclodextrin (β-CD) avoids the proton transfer reactions in this alkaloid by means the formation of an inclusion complex between β-CD and HAR. The formation of this complex originates a remarkable enhancement of the emission intensity from the neutral form in contrast to the cationic and zwitterionic forms. A new lifetime was obtained at 360 nm (2.5 ns), which was associated with the emission of this inclusion complex. At this wavelength, the fluorescence intensity decay of HAR can be described by a linear combination of two exponentials. From the ratio between the pre-exponential factors, we have obtained a value of $K$ = 501 M for the equilibrium of formation of this complex.




## INTRODUCTION

Proton transfer is one of the most important processes involved in the chemical reactions of biological systems; for example, hydrogen bonds in DNA base pairs. Nevertheless, although a large number of studies have been undertaken on hydrogen bonding and proton transfer processes,[1−10] the role and the mechanism of hydrogen transfer in DNA base pairs have not been completely clarified. On the other hand, phosphate is the major anion in living systems. This anion can act as a donor and acceptor of protons, and it is found in nucleotides and phospholipid molecules. Its concentration in extracellular and intracellular fluids is in the 0.5−2 mM range. Recent research showed that a high intake of phosphates can promote skin tumorigenesis.[11] We think that the development of these tumors could be caused by an alteration in the mechanism of hydrogen transfer in DNA base pairs by phosphate ions presence .

The rates of protonation for reactions in biological fluids are further enhanced by the presence of proton donating and accepting groups. A special class, within systems presenting proton transfer, is represented by azaaromatic molecules containing both hydrogen-bonding donor and hydrogen-bonding acceptor groups in their structure. The prototype of these molecules is exemplified by 7-azaindole, which is an N-heteroaromatic compound ubiquitous in nature that takes an active part in most of the macromolecules of biological interest. In this compound, the presence of proton donating and accepting groups leads to a double hydrogen atom transfer, producing two imino−enol tautomeric forms that could play a role in mutagenesis.[12−21]

A β-carboline derivative (*H*-pyrido[3,4-*b*]indole derivatives), harmane (HAR), also belongs to a compound type with two proton donor/acceptor groups, both with nitrogen atoms in their structure. These molecules make up a group of drug-binding alkaloids that are widely distributed throughout nature, with interesting biological and photophysical properties.[22−28] The lone electron pair of the pyridinic nitrogen atom has excellent hydrogen bonding properties and enables β-carboline to interact with a variety of hydrogen bonding donors. On the other hand, the pyrrolic N−H group enables the interaction of these derivatives with hydrogen bonding acceptors. The presence of these two groups may give rise to a double proton transfer when

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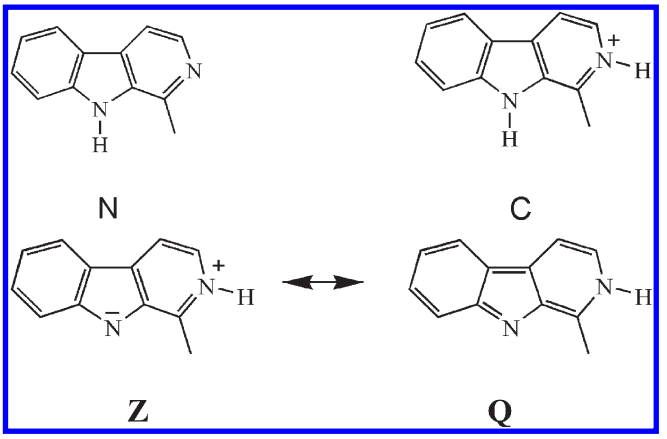
**Scheme 1. Structure of the Different Forms of HAR Found in Aqueous Solution**




hydrogen bonding donors and acceptors are present in the surrounding environment. This double transfer may also induce a phototautomerization process. Furthermore, when these compounds are excited to the $S_1$ state, photoinduced changes in their electronic distribution occur, modifying the acidic and basic properties of the two nitrogen atoms at the $\beta$-carboline ring, leading to a variation in their reactivity. This electronic distribution produces a much more basic pyridinic nitrogen and much more acidic pyrrolic NH group in the first excited electronic state than in the ground state. In this way, the surrounding environment modifies strongly the fluorescence of HAR, leading to a complicated photophysicochemistry in aqueous solution, with different equilibria in ground and excited states, involving neutral (N), cationic (C) and zwitterionic (Z) forms (Scheme 1). Nowadays, the main controversy[29−35] involves how to establish the structure, kinetics, and formation mechanism of the species with an emission band around 520 nm (Z). Initially, this emission was observed in aqueous solutions of norharmane (another, $\beta$-carboline derivative) at pH values greater than 12. A zwitterionic structure (Z) was associated with this emission, in which the pyridinic and pyrrolic nitrogen atoms are protonated and deprotonated, respectively. Carmona et al.[36,37] hypothesized that the zwitterionic structure (Z) might also present a quinoid structure (Q). Recenly, we have proposed[38] an oscillating system between two tautomers to explain this mechanism of Z/Q formation.

On the other hand, it is known[39−48] some $\beta$-carboline derivatives form inclusion complexes with the $\beta$-cyclodextrin ($\beta$-CD). The formation of inclusion complexes between HAR and $\beta$-CD reduces the number of species in aqueous solution, and this could help us understand the formation mechanisms of all of these species.

The main goal of this work was to study how, independently of pH, the presence of phosphate proton donor/acceptor groups in the surrounding environment of an azaaromatic molecule such as HAR modifies its reactivity in the $S_1$ state. This behavior could be extrapolated to the nitrogenous bases that are part of DNA. The interactions between these bases and phosphate ions could hinder the correct DNA replication.

## MATERIALS AND METHODS

**Chemicals.** 1-Methyl-9*H*-pyrido[3,4-*b*]indole, $\beta$-cyclodextrin, sodium dihydrogen phosphate, and $Na_2B_4O_7 \cdot 10H_2O$ (borax) were purchased from Aldrich and used as received without further purifications. Milli-Q quality water was used as the solvent.

Absorption spectra were obtained using a Hewlett−Packard UV−vis spectrophotometer HP 8453. The steady-state fluorescence measurements were made using a Shimadzu spectrofluorometer RF-5301 PC with a 3 nm bandwidth in excitation and a 1.5 nm bandwidth in emission. The fluorescence lifetime measurements were carried out using a time-correlated single photon counting fluorimeter from Edinburgh Analytical Instruments.

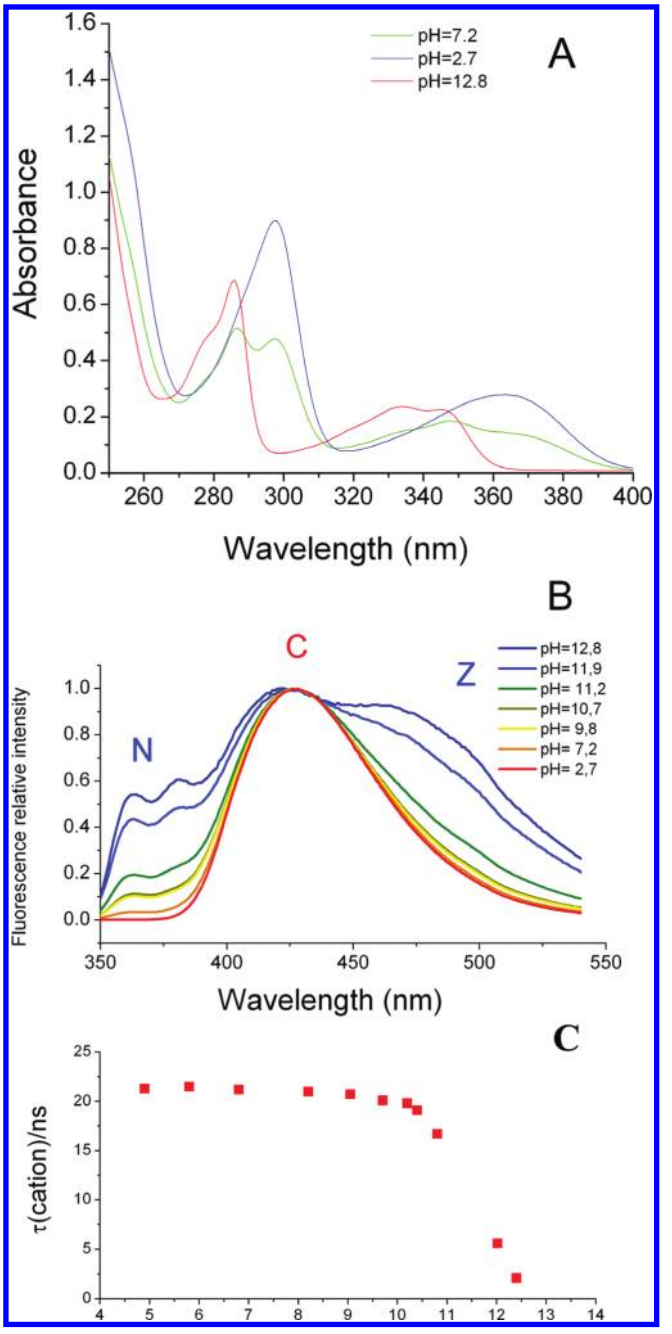



**Figure 1.** HAR in aqueous solution at 298 K. (A) Absorption spectra at different pH values (pH = 2.7, cationic form absorption; pH = 7.2, neutral and cation forms absorption; pH = 12.8, neutral form absorption). (B) Fluorescence spectra at different pH values, $\lambda_{ex}$ = 320 nm. (C) Cation lifetime versus pH ($\lambda_{em}$ = 445 nm).


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**Table 1. Fluorescence Lifetimes of HAR($\lambda_{exc}$ = 320 nm) in Aqueous Solution at Different pH Values (Adding NaOH)[a]**

| pH | $\tau(\lambda_{em}$ = 445 nm)/ns | $\tau(\lambda_{em}$ = 520 nm)/ns |
|---|---|---|
| 2.7−8.2 | 21.2 | |
| 9.1 | 20.7 (0.088) | |
| | 0.6 (−0.074) | |
| 9.8 | 20.1 (0.090) | |
| | 0.6 (−0.043) | |
| 10.2 | 19.8 | |
| 10.4 | 19.1 | |
| 10.7 | 16.7 | |
| 11.9 | 5.6 | 4.6 (−0.143) |
| | | 8.0 (0.205) |
| 12.4 | 2.1 | 2.1 (−0.075) |
| | | 8.1 (0.127) |
| 12.8 | 1.5 | 1.5 (−0.071) |
| | | 8.1 (0.122) |

[a] Recording emission wavelengths at 445 and 520 nm. The numbers in parentheses are the pre-exponential factors of the decay time components.

The excitation source was a hydrogen nanosecond flash lamp: the repetition rate was 40 kHz and the excitation pulse width was less than 1 ns. Fluorescence decay profiles and the quality of the fits was judged by the reduced chi-square ($\chi^2$) values and the autocorrelation function of the residuals. All experiments were carried out at 25 °C and analyzed by nonlinear least-squares iterative deconvolution.

**$^1$H NMR Spectra.** Three aqueous solutions were prepared in a borax buffer (pH 9.2). Solution 1: saturated in $\beta$-CD. Solution 2: saturated in HAR. Solution 3: (1) + HAR (saturated). All of the mixtures were sonicated and transferred to NMR tubes (final volume 0.5 mL).

The proton spectra were recorded at 298 K in a Bruker Avance AV-500 spectrometer (11.7 T) by averaging 32 scans with a digital resolution of 0.30 Hz. The HDO signal was used as the reference signal.[49] The signal assignment of the HAR was established by conventional NMR methods.

**ROESY Experiments.** A Bruker Avance DPX-300 spectrometer (7.05 T) was used by applying the pulse sequence defined in the literature.[50] Different spin-lock mixing times (ranging between 200 and 800 ms) were used to ensure the validity of the linear approximation for the ROE cross-peaks and to obtain the best signal-to-noise ratio, which was achieved with 600 ms. Thirty-two scans were collected in each spectrum. Before the subsequent Fourier transformation and 2D phase tuning, linear prediction in F1 and cosine square apodization in both dimensions were applied to the FIDs. The temperature was kept constant in these experiments at 298 K

## RESULTS AND DISCUSSION

**Spectral Changes Induced by the pH.** The absorption and fluorescence spectra at different pH (pH 2.7−12.8), as well as the results of the time-resolved measurements for these solutions, are shown in Figure 1 and Table 1. For the pH 9.2−12.1 range, only the absorption of N is observed. At pH 7.2, both C and N absorbances are shown, whereas at pH 2.7, only the absorption of C is observed. These results agree with the p$K_{a1}$ value of around 7 for the equilibrium between cation/neutral forms.[51] Thus, two different species could be found in the ground state: C (in acid solution) and N (in alkaline solution), because the transformation from neutral to anion occurs in highly alkaline solutions with a p$K_{a2}$ value of around 14.[52,53]

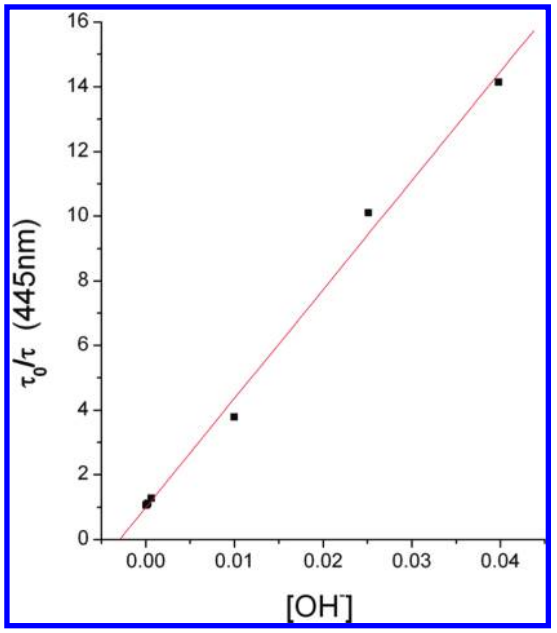


**Figure 2.** Plot of $\tau_0/\tau$ versus [OH$^-$] for HAR in aqueous solution (Stern−Volmer equation); $\lambda_{ex}$ = 320 nm, $\lambda_{em}$= 445 nm.

According to the fluorescence measurements, the sequence of p$K_a$ values was reversed for the lowest excited singlet state $S_1$;[51] thus p$K^*_{a1}$ ∼ 13 and p$K^*_{a2}$ ∼ 8, and therefore, three different fluorescence emissions were observed (pH 1−13): C, N, and Z. It is only possible to observe the fluorescence of anions in very basic solutions (pH >14), outside the pH range of the present study. In our case, the fluorescence spectra were excited at 320 nm (N absorption) from pH 2.7 to pH 9.0, and only showed C emission ($\lambda_{max}$ = 430 nm). From pH 9.0 and upward, a small band with a vibrational structure arose (with maximum fluorescence around 360−380 nm), corresponding to N emission. Above pH 11.0, a new shoulder appeared at 520 nm, which was attributed to Z emission. Recording of the time-resolved fluorescence measurements at 445 nm and from pH 2.9 to 8.2 found that the fluorescence decay was a single-exponential decay, with a lifetime of around 21 ns (lifetime of C), in agreement with the results of other studies (Figure 1, Table 1). At pH 9.0, a short component with a lifetime of about 0.6 ns (lifetime of N) appeared with a negative pre-exponential factor. We associated this short component with the formation of C from N in the $S_1$ state. Between pH 10.2 and pH 12.6, the decay time recorded at 445 nm continuously decreased with increasing pH level. Above pH 12.0, a double-exponential decay was recorded at 520 nm with a short component (with a negative pre-exponential factor) that decreased with the increase in pH, and a pH-independent component with a constant lifetime of around 8.0 ns (lifetime of Z). From these results, we associated the changes in the lifetime of C (from 21 to ∼1 ns) at pH 9.0−12.6 with the formation of Z from C in the $S_1$ state. This assumption was supported by the negative pre-exponential factor recorded at 520 nm with a lifetime similar to that for C (Table 1).


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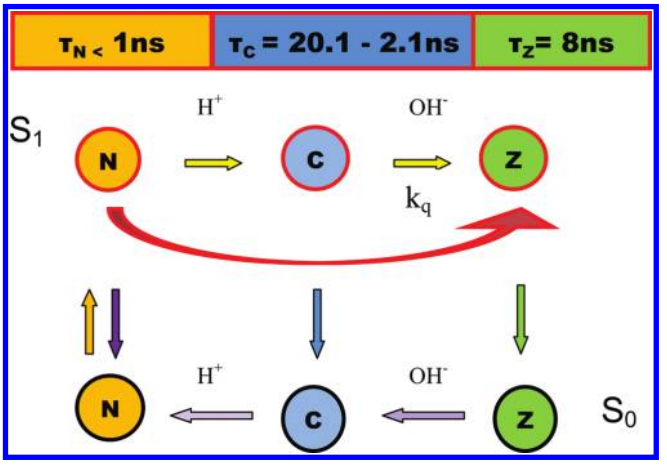
**Scheme 2. Photophysical Parameters of HAR in Aqueous Solution within the pH Interval of 10.0−12.5 (Adding NaOH) or pH at 8.8 Fixed with Sodium Dihydrogen Phosphate Buffer (0.2 M)**




These changes in the fluorescence lifetime of C with the increasing of $[OH^-]$, follow the Stern−Volmer equation:

$$\frac{\tau_0}{\tau} = 1 + K_D[OH^-] \quad (1)$$

where $\tau_0$ is the fluorescence lifetime of C at pH ≤ 9 and $\tau$ is the lifetime in the range of pH 10.0−12.5, $K_D$ is the dynamic quenching constant in the $S_1$ state, which competes with the intrinsic de-excitation of C, and $K_D$ = 340 ± 10 $M^{-1}$ was determined from the slope in the plot of $\tau_0/\tau$ vs $[OH^-]$ (Figure 2).

The above results can be explained according to the kinetics proposed in Scheme 2, where $K_D = k_q\tau_0$, with $k_q$ being the kinetic constant of the photophysical intermolecular deactivation process of C transformed into Z above pH 10.0. These results constitute another argument that justifies the connection between C and Z in the $S_1$ state.

It is well-known that in absence of proton donors the characteristic lifetime of N is around 3.0 ns (2.9 ns for HAR in benzene and dioxane and 3.1 ns for HAR in acetonitrile).[31] Thus, low lifetime values for the neutral species in aqueous solution (<1 ns) were justified by the connection between N and C in the $S_1$ state, as shown in Scheme 2. According to these photophysical kinetics, a biexponential decay would be obtained for C with a negative amplitude that corresponded to neutral species decay. However, this decay time was so low that, in most of the cases, it was out of the spectrofluorimeter's sensitivity range (100 ps), whereas good parameters were obtained for the monoexponential fits. On the other hand, according to Scheme 2, Z would present a triexponential decay with two negative amplitudes that corresponded to neutral and cation species. Also, in this case, the fact that the lifetime was below the spectrofluorometer's sensitivity range again justifies a biexponential fit for neutral species.

**Spectral Changes Induced by $NaH_2PO_4$.** *Low Concentrations of $NaH_2PO_4$.* When low concentrations of $NaH_2PO_4$ (<4 × $10^{-3}$ M) were added to the aqueous HAR solution, the absorption and fluorescence profiles were slightly modified in the pH range pH 7.7−12.1.The only relevant difference was shown by the lifetime of C, which decreased from 21.0 ns, obtained in the absence of $NaH_2PO$, to 15.4 ns, obtained in the presence of small amounts of this compound.

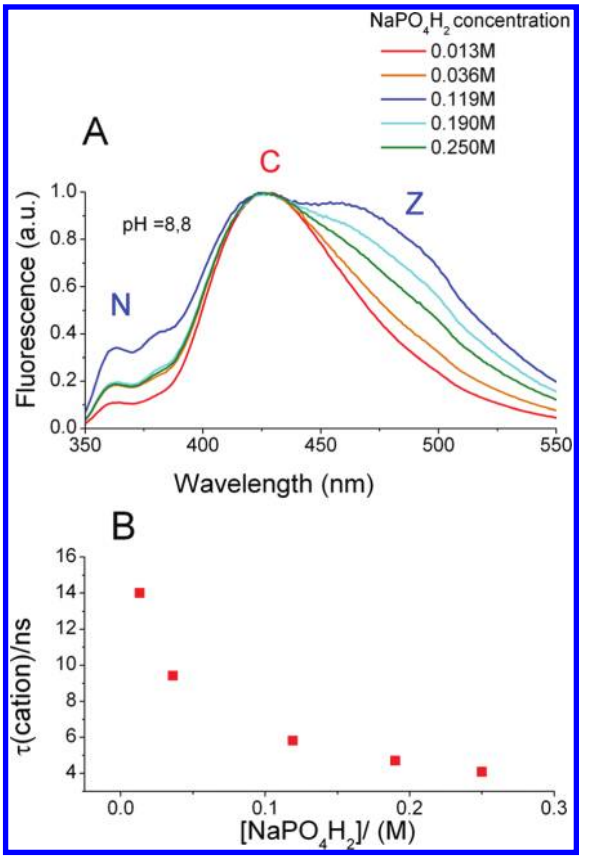



**Figure 3.** (A) Fluorescence profiles of HAR at different $NaH_2PO_4$ concentrations ($\lambda_{ex}$ = 320 nm). (B) Cation lifetime versus $NaH_2PO_4$ concentration ($\lambda_{em}$ = 445 nm).

*High Concentrations of $NaH_2PO_4$.* On the other hand, working with a buffer concentration of $NaH_2PO_4$ (0.2 M) and fixing the pH at pH 8.8, the fluorescence profiles were similar to those obtained in the absence of $NaH_2PO_4$ and pH >12 (Figure 3). That is, the same photophysical behavior was observed at high concentrations of phosphates and pH 8.8, and without phosphates and pH >12 (an environment that is four pH units more basic). In this case, the lifetime of the cation (wavelength emission at 430 nm) strongly decreased (4.1 ns), a double exponential decay was observed at 520 nm with a new decay time of 8.1 ns (Z), and a negative pre-exponential factor was associated with the lifetime of C (Table 2). When the emission at 370 nm (neutral species) was recorded, a single-exponential decay was observed with a lifetime of 0.5 ns. Undoubtedly, Scheme 2 could also explain this behavior by replacing the pH increase with an increase in $NaH_2PO_4$ concentration.

For concentrations of $NaH_2PO_4$ within the range of 0.03−0.25 M and pH 8.8, this system also follows the Stern−Volmer equation, confirming the kinetics mechanism presented in Scheme 2. In this case, the dynamic quenching constant for $NaH_2PO_4$ was $K_D$ = 9.7 ± 0.7 $M^{-1}$, taking $\tau_0$ = 21 ns.

Using $Na_2B_4O_7.10H_2O$ (borax) as buffer and fixing the pH at pH 8.8, the lifetime of C was around 21.0 ns, no shoulder was observed at 520 nm and upon recording the emission at 370 nm a single-exponential decay was obtained with a lifetime of 0.7 ns

**Table 2. Fluorescence Lifetimes of HAR ($\lambda_{exc}$ = 320 nm) in Buffer Solutions (Sodium Dihydrogen Phosphate or Borax) versus the $\beta$-CD Concentration (pH 8.8)[a]**

| | borax buffer | | phosphate buffer (0.2M) | | |
|---|---|---|---|---|---|
| [$\beta$-CD] | $\tau(\lambda_{em}$(370 nm))/ns | $\tau(\lambda_{em}$(440 nm))/ns | $\tau(\lambda_{em}$(370 nm))/ns | $\tau(\lambda_{em}$(440 nm))/ns | $\tau(\lambda_{em}$(520 m))/ns |
| 0.00 | 0.7 | 21.0 | 0.5 | 4.1 | 4.0 (−0.057) |
| | | | | | 8.1 (0.085) |
| 0.48 | 0.7 (72.7%) | 21.7 | 0.5 (81.4%) | 4.2 | 4.0 (−0.049) |
| | 3.8 (27.3%) | | 2.5 (18.6%) | | 8.5 (0.075) |
| 2.00 | 0.7 (66.5%) | 21.4 | 0.5 (44.0%) | 4.4 | 3.8 (−0.048) |
| | 3.8 (33.5%) | | 2.5 (56.0%) | | 9.3 (0.069) |
| 3.97 | 0.7 (53.8%) | 21.8 | 0.5 (30.2%) | 4.7 | 3.6 (−0.040) |
| | 3.8 (46.2%) | | 2.5 (69.8%) | | 9.8 (0.022) |
| 6.17 | 0.7 (49.4%) | 21.7 | 0.5 (22.5%) | 4.9 | 3.7 (−0.040) |
| | 3.8 (50.6%) | | 2.5 (77.5%) | | 10.6 (0.061) |

[a] Two-exponential global fit was realized for the emission recorded at 370 nm. The numbers in parentheses are the pre-exponential factors of the decay time components.

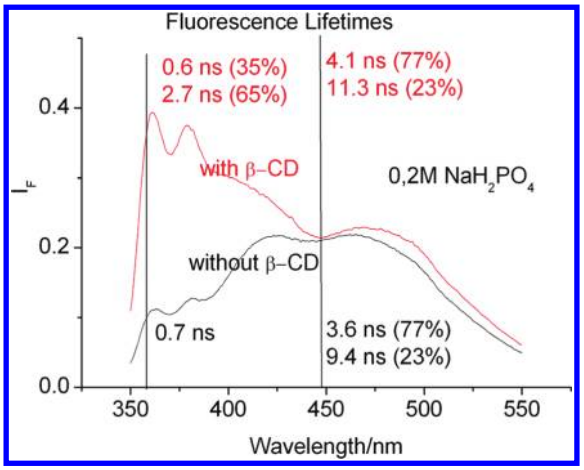


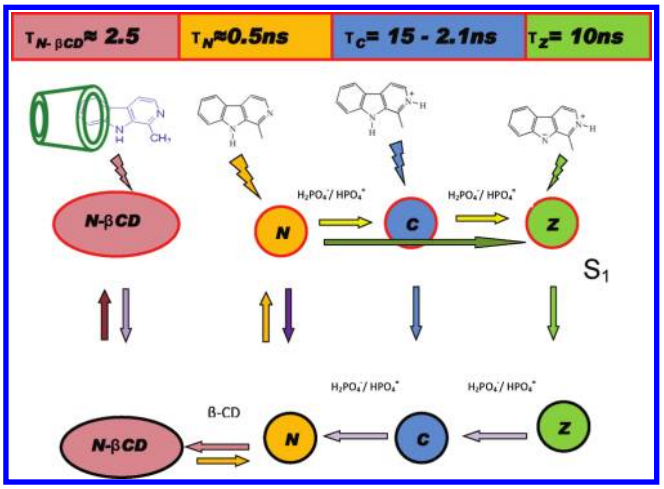

**Figure 4.** Fluorescence profiles and lifetimes of HAR in the absence and presence of $\beta$-CD. Lifetimes obtained at $\lambda_{ex}$ = 320 nm and $\lambda_{em}$ = 370 and 445 nm.

(Table 2). Therefore, in this case, only N and C were connected in the excited $S_1$ state.

The same photophysical behavior as in the phosphate buffer solution at pH 8.8 was observed for HAR in aprotic solvents such as dioxane, dichloromethane, and chloroform in the presence of a donor/acceptor such as acetic acid.[31−34] However, in these aprotic solvents, another fluorescence band was observed around 400 nm, which was related to the interactions between HAR and the hydrogen donor/acceptor molecules. We think that this species was not stable in aqueous solution because water is a highly dissociative solvent. Therefore, no fluorescence was observed for HAR at 400 nm in aqueous media.

**Spectral Changes Induced by $\beta$-Cyclodextrin.** To clarify the complicated photophysicochemistry of HAR, we added $\beta$-CD to the aqueous solution. The optical properties of HAR were also measured in the presence of both buffer solutions (borax and sodium dihydrogen phosphate) with different concentrations of $\beta$-CD. Figure 4 shows the fluorescence spectra of HAR in 0.2 M $NaH_2PO_4$. The emission was observed in both the absence and presence of $\beta$-CD. The relative intensities observed under these conditions changed with the amount of $\beta$-CD in solution.

**Scheme 3. Photophysical Parameters of HAR in Aqueous Solution at pH 8.8 in Sodium Dihydrogen Phosphate Buffer and in the Presence of $\beta$-CD**




Independently of phosphate, a remarkable enhancement took place in the emission intensity of N in contrast with C and Z in presence of $\beta$-CD.

Regarding the time-resolved fluorescence measurement of both types of buffer solution (borax/sodium dihydrogen phosphate) and recording the emission at 370 nm (pH 8.8), the goodness of fits unambiguously show the necessity of the two-exponential. For the borax buffer solution two lifetimes were recorded (0.7 and 3.8 ns). A global fit realized at different concentrations of buffer at 370 nm shows the shorter lifetime (0.7 ns) with a pre-exponential factor that decreased with the concentration of $\beta$-CD. In contrast, the contribution of the longer decay (3.8 ns) increased with the concentration of $\beta$-CD (Table 2). When borax was replaced by sodium dihydrogen phosphate, a similar behavior was observed with lifetimes equal to 0.5 and 2.5 ns.

When the emission at 440 nm was recorded, lifetimes of C of around 21 and 4 ns were obtained for the borax and sodium dihydrogen phosphate buffer solutions, respectively. In the presence of $NaH_2PO_4$ buffer solution, the Z lifetime (520 nm)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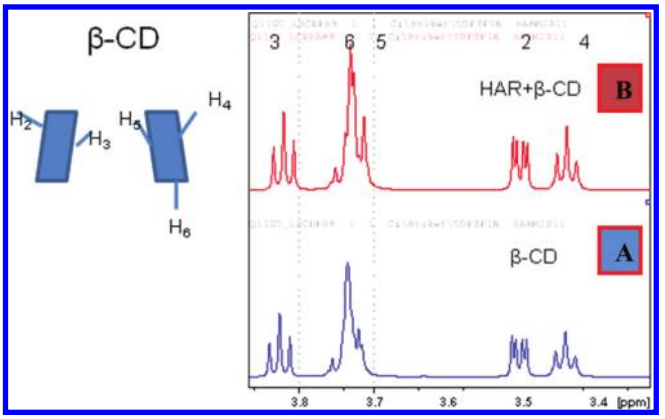


**Figure 5.** $^1$H NMR spectra in aqueous buffer solutions (pH 9.2) containing $\beta$-CD in the absence (A) and presence (B) of HAR.

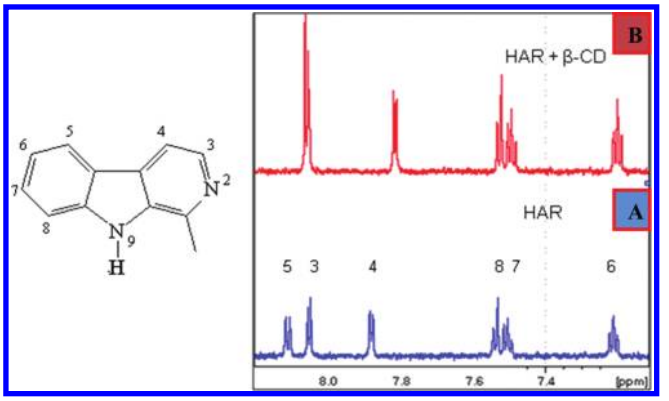


**Figure 6.** $^1$H NMR spectra in aqueous buffer solutions (pH 9.2) containing HAR in the absence (A) and presence (B) of $\beta$-CD.

slightly increased from 8 to 10 ns with the concentration of $\beta$-CD. There was no emission at this wavelength in the borax solutions.

These results proved that the addition of $\beta$-CD notably alters the proton transfer of HAR in aqueous alkaline solutions. These changes are due to the formation of an inclusion complex between the N and $\beta$-CD. The shorter decay times (0.5 and 0.7 ns) were related to the uncomplexed neutral species. The longer decay times (3.8 and 2.5 ns) corresponded to the N$-\beta$-CD complex. The other proton transfer processes (Scheme 3) in HAR were not affected by the presence of $\beta$-CD. No inclusion complex formation seemed to occur with the other HAR species, although the lifetime of Z, around 10 ns, longer than that obtained without $\beta$-CD (around 8 ns), could have been associated with a (Z$-\beta$-CD)* complex. The alcoholic groups inside the CD's cavity can favor the formation of a (Z$-\beta$-CD)* species from (N$-\beta$-CD), as was obtained in alcoholic media.[54] In acid solutions, where only C exists in the ground state, no changes were observed in the spectra profiles, or in the lifetimes. No interaction takes place between C and $\beta$-CD.

From these results, we propose a system formed by a mixture of two independently emitting species in the ground state: the N (uncomplexed) and N$-\beta$-CD complex. In this way, the fluorescence intensity decay $I_0$ of HAR can be described by a linear combination of two exponentials: $I_0 = \alpha_{13}e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}$, where the ratio between their amplitudes $\alpha_i$ is proportional to the ratio between the concentration of species in equilibrium in the ground state ($S_0$):

$$\frac{[N-CD_n]}{[N]} \propto \frac{\alpha_1}{\alpha_2} \qquad (2)$$

Furthermore, the equilibrium constant, $K_{CD}$, for a reaction involving $n$ $\beta$-CD molecules

$$N + n\beta\text{-}CD \rightleftarrows N-\beta\text{-}CD_n$$

may be expressed as

$$K_{CD} = \frac{[N-CD_n]}{[N][CD]^n} \qquad (3)$$

From eqs 2 and 3

$$\frac{\alpha_1}{\alpha_2} = K[CD]^n$$

where $K = K_{CD}(\varepsilon_1 k_1^F/\varepsilon_2 k_2^F)$ ($\varepsilon$ represents the molar absorption coefficient at the wavelength of excitation and $k^F$ is the rate constant of fluorescence). Therefore, plotting ln $\alpha_1/\alpha_2$ versus ln [CD] can yield the number of $\beta$-CD molecules in a complex. In both buffers, a 1:1 stoichiometry was obtained with the same value of $K$ = 501 M. The formation of this complex slightly modifies the absorption and fluorescence profiles of N as well as their intensities, so it possible to consider $\varepsilon_1 k_1^F \approx \varepsilon_2 k_2^F$ and, consequently, $K_{CD} \approx K$.

Inclusion complexes of some $\beta$-carboline alkaloids with $\beta$-CD have been already described in the literature.[39−48] In these studies, the $\beta$-CDs exerted a significant influence on the proton transfer of some $\beta$-carboline derivatives. In the presence of $\beta$-CD, access of the protons to the pyridinic nitrogen was not possible due to the lower diffusion rate of protons inside these cavities with a lower polarity than water, decreasing the basicity of these organic bases. Some of these studies revealed the formation of two types of inclusion complexes (1:1 and 1:2) between $\beta$-carboline and $\beta$-CD, respectively. Some of these works were realized at pH 8.0 using $NaH_2PO_4$ as the buffer. In all cases, no interactions between phosphates and the alkaloid were recorded. We think that some of the interactions shown in these works were due to interactions with the buffer and not with $\beta$-CD, as was proposed.[44]

The $^1$H NMR spectra and 2D ROESY experiments in aqueous buffer solutions (pH 9.2) containing HAR in the absence and presence of $\beta$-CD, as well as pure $\beta$-CD in the buffer solution, were also used to determine the complexation between HAR and $\beta$-CD. Two sets of signals were distinguished in the $^1$H NMR spectra of the $\beta$-CD: the internal hydrogens termed $H_3$ and $H_5$, the external hydrogens $H_2$ and $H_4$, and $H_6$, an edge hydrogen (Figure 5). Due to the low solubility of HAR in water at pH 9.2, significantly lower than that of $\beta$-CD, the chemical shifts of these hydrogens are small with the presence of HAR. However, the internal hydrogens, particularly $H_5$, suffered greater displacement (Figure 5). On the other hand, the chemical shifts experienced by the HAR hydrogens were greater than those shown for $\beta$-CD due to the higher solubility of $\beta$-CD in the medium studied. The behavior observed was similar to that shown by HAR in the presence of a hydrogen donor:[29,38] $H_3$, $H_4$, and $H_5$ (assigned by COSY) were the HAR hydrogens that suffered a greater upfield shift due to the interaction between the pyridine nitrogen and the hydrogen donor (Figure 6). The formation of the inclusion complex HAR$-\beta$-CD could also


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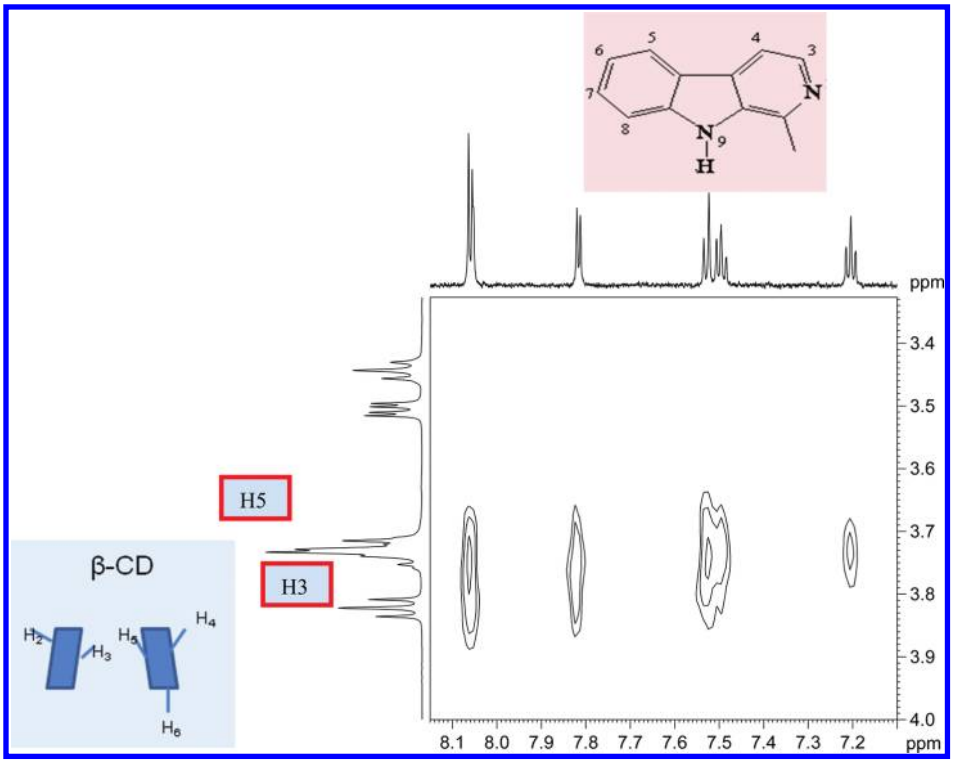


**Figure 7.** 2D ROESY experiment of HAR versus $\beta$-CD. This figure shows that only the internal hydrogens of the $\beta$-CD cavity were coupled with all of the HAR hydrogens.

explain this behavior by means of an interaction between pyridine nitrogen and the internal hydrogens of $\beta$-CD.

The ROESY experiments confirmed the formation of this inclusion complex (Figure 7). This figure shows that only the internal hydrogens of the $\beta$-CD cavity were coupled with all of the HAR hydrogens.

## CONCLUSIONS

The concentration of phosphate in extracellular and intracellular fluids is within the 0.5−2.0 mM range. In this range, no changes in the photophysical parameters behavior of HAR due to the presence of phosphates were observed. In $NaH_2PO4$ >36 mM, the presence of phosphates produced dramatic changes in the photophysicochemistry of this compound, which have to be taken into account. Analyzing the ground and excited-state effects in HAR induced by a high $NaH_2PO_4$ concentration (0.2 M) in aqueous solution, we demonstrated that, independently of the pH value, the presence of this donor/acceptor molecule in a high concentration medium can modify the acid/base properties of this alkaloid and consequently its ability to form hydrogen-bonded complexes. If we extrapolate this behavior to all of the nitrogenous bases that are part of DNA (N-heteroaromatic compounds similar to the $\beta$-carboline derivative), we can relate the development of tumors, as a consequence of the high intake of phosphates, to the interactions between these bases and phosphate ions, hindering correct DNA replication.

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