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Excited state tautomerization of camptothecin in aqueous solution

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Abstract

The effect of pH on the absorption and fluorescence spectral characteristics of 20(S)-camptothecin was studied in aqueous solution. In alkaline solution, the lactone ring of camptothecin hydrolyzes to produce camptothecin carboxylate. The equilibrium constants of the lactone ring hydrolysis of camptothecin at various pH values were determined using steady state fluorescence measurements. The ^{1}H nuclear magnetic resonance (NMR) and circular dichroism spectra were measured to characterize the structure of camptothecin in strongly alkaline medium. The fluorescence intensity of camptothecin is quenched by OH^- ions. A mechanism of fluorescence quenching of the carboxylate form by OH^- ions, which involves tautomerization on electronic excitation, is proposed. The ground and excited state pK_a values of the quinolinium ion are reported.

Keywords: Acidity constants; Camptothecin; Circular dichroism; Fluorescence; Tautomerization

1. Introduction

Camptothecin (CAM) (**I**) (Scheme 1), a plant alkaloid, was first isolated and characterized by Wall et al. [1] in 1966 from a Chinese tree *Camptotheca acuminata* (family Nyssaceae). This pentacyclic alkaloid contains a quinoline ring system (rings A and B), a pyridone ring (ring D) and a terminal α-hydroxy-lactone ring (ring E). It has a chiral center (C-20) within the lactone ring. The lactone ring is labile and will easily hydrolyze to form sodium camptothecin (CAM-Na) (**II**) in basic medium [1].

Following its discovery and chemical identification, CAM was found to be active in tests against L1210 leukemia [2] and Walker 256 carcinosarcoma [1,3,4]. It was also found that CAM inhibits both DNA and RNA synthesis in mammalian cells. This attracted immediate interest in CAM as a potential cancer chemotherapeutic agent. Its high antitumor activity against a wide range of experimental tumors has been confirmed [5]. However, because of its poor aqueous solubility, CAM was clinically evaluated as its water-soluble sodium salt **II**, and was found to be one-tenth as active as the parent drug [6]. In fact, CAM-Na is biologically inactive. The low activity of CAM-Na was thought to be due to its partial conversion to CAM in plasma. Thus the intact α-hydroxy-lactone ring is a structural requirement for its anticancer effects [7–11]. The configuration of the C-20 chiral center is also important. 20(R)-Camptothecin, which does not exist in nature, was found to be inactive in both in vitro and in vivo tests [11–13]. The patients under clinical phase I trials, mostly with cancer of gastrointestinal origin, encountered severe dose-dependent toxicities, such as vomiting, diarrhea, hemorrhagic enterocolitis, leukopenia and thrombocytopenia [14]. As a result, in spite of the apparent success in gastrointestinal cancer and neck tumor, the interest in CAM decreased. The results of preclinical and clinical developments of CAM and its analogs have been reviewed in two recent papers [7,15].

One of the important effects of CAM that has been reported is the rapid and reversible fragmentation of cellular DNA in cultured mammalian cells [16]. However, CAM by itself does not cleave purified DNA. The mechanism of DNA breaking was unknown until Hsiang et al. [17] in 1985 reported that the only cellular target of CAM is DNA which, in the presence of an enzyme (topoisomerase I (Top-I)) forms a ternary complex with CAM and is nicked subsequently. The detailed mechanism of DNA single-strand scission by CAM has been discussed in two recent reports

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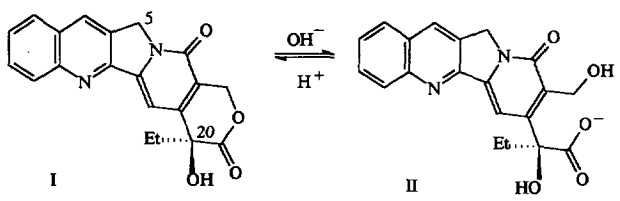

I II

Scheme 1.

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[18,19]. This discovery resulted in renewed clinical interest in CAM. Assuming that the undesirable and unpredictable toxicities of CAM are due partly to its poor solubility in water, several water-soluble CAM analogs have been synthesized [20] and clinically evaluated [21]. However, only 9-, 10- and 11-substituted derivatives have shown anticancer activity in a number of in vitro and in vivo studies [22–29]. Of these, the topotecan analog (10-hydroxy-9-dimethylaminomethyl-camptothecin) has been under phase I and phase II clinical trials for the past few years, while 9-aminocamptothecin (9-AC) and irinotecan (7-ethyl-10[4-(1-piperidino)-1-piperidino]-carboxycamptothecin (CPT-11)) are presently undergoing phase I clinical trials in the USA.

Recently, Burke and coworkers, in a series of publications, have reported the binding properties of CAM and its amino and hydroxy analogs with liposomes [30,31] and human serum albumins [32–36] by exploiting the brilliant fluorescence of these compounds. More recently, we have studied in detail the electronic absorption and fluorescence spectral properties of CAM in organic and aqueous solutions, and have reported the fluorescence quenching of CAM as well as CAM-Na by I^- ions [37]. Although much research has been performed on the structure–activity relationships of CAM and its synthetic analogs, the mechanism of DNA cleavage by CAM in the presence of Top-I is not yet completely understood. Hsiang et al. [17] mentioned the possible involvement of a tautomeric species of CAM in the process of DNA cleavage. Therefore we have undertaken a detailed investigation of the structure of the molecule in acidic and basic aqueous solutions. In this paper, we discuss the effects of pH on the absorption and fluorescence spectral properties of camptothecin and the structure of camptothecin in strongly alkaline solution. The equilibrium constants of lactone ring hydrolysis of camptothecin at various pH values are measured. We also report the ground (S_0) and excited (S_1) state pK_a values of the molecule.

2. Experimental details

2.1. Materials

20(S)-Camptothecin was procured from Aldrich and was used without further purification. Analytical grade perchloric acid, sodium hydroxide, sodium perchlorate, sodium acetate, sodium bicarbonate, sodium carbonate and monosodium and disodium hydrogen phosphate were used directly from the bottle. Deionized water was used for the preparation of aqueous solutions.

2.2. Fluorescence quenching measurements

For the quenching studies of CAM-Na, a 0.5 ml aliquot of the stock solution of CAM-Na was diluted to 10 ml in a volumetric flask by an NaOH solution of appropriate concentration (in the range, 0–0.2 M). The ionic strength was maintained constant at 0.2. The pH of the final solution was measured by a pH meter using glass electrodes. The same solutions were used for lifetime measurements. A modified form of the Stern–Volmer equation [38] was used to analyze the static and dynamic processes

$$F_0/F = 1 + (K_D + K_S)[Q] + K_D K_S [Q]^2 \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher Q and K_S and K_D are the static and dynamic quenching constants respectively. The K_D value was obtained from the Stern–Volmer plot of the lifetime data

$$\tau_0/\tau = 1 + K_D[Q] \quad (2)$$

where τ_0 and τ are the fluorescence lifetimes in the absence and presence of quencher respectively. The collisional quenching rate constant k_q was calculated from the relation $K_D = \tau_0 k_q$. Both steady state and dynamic quenching measurements were performed at 25 °C.

2.3. Determination of the pK_a values

The pK_a value of the quinoline functional group in CAM was determined using a spectrophotometric method from the absorbance at 370 and 415 nm as a function of pH/H_0 in acetate buffer and perchloric acid. The ground state pK_a value was obtained from the Henderson–Hasselbalch plot as well as from the absorbance titration curves. The excited state (S_1) pK_a value was obtained from fluorescence titration curves [39] constructed from the relative fluorescence intensities at 430 nm and 510 nm. For fluorometric titration, the solutions were excited at the isosbestic point (380 nm) of the absorption spectrum in the acidity range. The pK_a value thus obtained was corrected for the fluorescence lifetimes of both the quinolinium ion (CAMH) and CAM using the following equation [39]

$$pH = pK(S_1) - \log(\tau_0/\tau'_0) \quad (3)$$

where pH is the acidity at which the titration curves show an inflexion and τ_0 and τ'_0 are the fluorescence lifetimes of CAMH and CAM respectively.

2.4. Absorption and fluorescence measurements

The absorption spectra were measured on a double-beam Shimadzu UV-3101PC spectrophotometer equipped with a constant-temperature circulator. All measurements were carried out at 25 °C using 1 cm^2 quartz cuvettes. Absorption spectra were recorded with reference to the solvent.

The fluorescence spectra were measured on a Spex-Fluorolog model F2T21I spectrofluorometer equipped with a cell compartment thermostatically controlled with a VWR model 1160 constant-temperature circulator. All fluorescence measurements were conducted at 25 °C. The slit widths of both excitation and emission monochromators were 5 nm. The fluorescence intensity was measured at a right-angled configuration.

The circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter using a quartz cuvette of 1 cm path length under an N_2 atmosphere. For each spectrum, three to five scans were accumulated. The respective solvent spectrum was subtracted from the sample spectrum. The ^1H nuclear magnetic resonance (NMR) spectra were measured using a Brucker 400 MHz instrument. TSP (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt) was used as external reference.

2.5. Fluorescence lifetime measurements

Fluorescence lifetimes were measured on a PTI Inc. LS-100 luminescence spectrometer. An N_2 and He gas mixture was used in the discharge tube for excitation. A dilute solution of colloidal starch was used as a scatterer to determine the excitation lamp flash profile. The 337 nm emission of N_2 was used for sample excitation. The decay curves were obtained using the time-correlated single-photon counting (TC-SPC) method. To obtain fluorescence decay curves, $(2–5)\times10^4$ counts were collected in the peak channel. Each data set was collected in 256 channels. The data were analyzed using a multiexponential decay analysis program. The goodness of fit between the experimental and computed decay curves was evaluated by the randomness of the weighted residuals, the autocorrelation function of the residuals and the reduced χ^2 (0.9–1.2), Durbin–Watson (greater than 1.7) and run time ($Z > -1.96$) parameters. All measurements were repeated more than once to obtain the best data set.

3. Results and discussion

The absorption and fluorescence spectra of CAM were measured in the pH/H_0 range from 13 to −2. The spectral changes are depicted in Fig. 1 and the spectral properties are listed in Table 1. At pH > 5, the absorption and emission spectra show a red shift. The shift in the absorption spectrum is small compared with that in the emission spectrum. As noted above, the lactone ring of CAM hydrolyzes in neutral to basic solution to produce the sodium salt **II**. Therefore the above spectral changes can be associated with the formation of camptothecin carboxylate (CAM-Na). The data in Table 1 show that the fluorescence quantum yield of CAM-Na is slightly lower than that of CAM, whereas the lifetime of CAM-Na is higher than that of CAM. This may be due to the partial loss of rigidity of the molecule, which increases the dipole moment and hence increases the solute–solvent interaction, thereby increasing the non-radiative decay rate. The high fluorescence quantum yield suggests that the emitting state of CAM-Na is also of π,π^* character.

Table 1
Absorption (λ_{max}(abs)) and fluorescence (λ_{max}(flu)) maxima, fluorescence quantum yields (Φ_f) and lifetimes (τ_f) of CAM, CAM-Na and CAMH

	CAM (pH 5.1)	CAM-Na (pH 9.1)	CAMH (4 M $HClO_4$)
λ_{max}(abs) (nm)	370	373	405
	355(s)	360(s)	300
	252	254	250
λ_{max}(flu) (nm)	428	448	510
Φ_f	0.64	0.62	0.29
τ_f (ns)	3.99	4.48	3.86

(s), shoulder.

The rate of lactone hydrolysis is slow at low pH values, but increases with increasing pH. The details of the kinetics and mechanism of the reaction have been reported elsewhere [30,40,41]. The fluorescence intensity measured after 24 h of equilibration shows a gradual decrease with increasing pH reaching a minimum at pH 8. The relative intensity changes for both CAM and CAM-Na are plotted in Fig. 2 as a function of pH. The relative fractions of each species present at different pH values in the range pH 5–8 are listed in Table 2. The plot in Fig. 2 indicates that, at pH > 8, CAM is mainly present in the carboxylate form and, at pH < 5, in the lactone form. The equilibrium constant calculated from the relative fractions of each species at pH 7 is 2.7, which is in good agreement with the reported value (2.7 at pH 7.13) obtained by high performance liquid chromatography (HPLC) measurements [40–42].

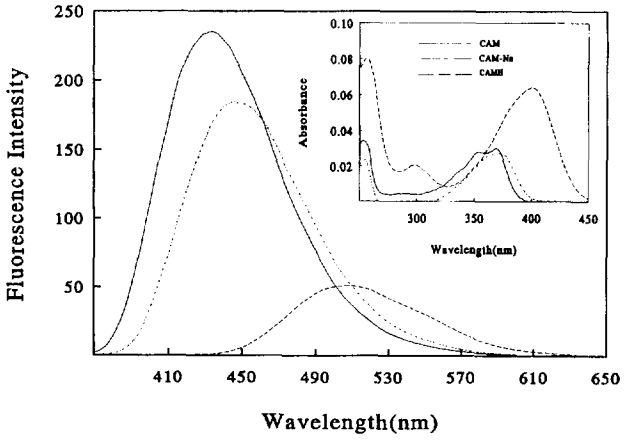


Fig. 1. Absorption (inset) and fluorescence spectra of CAM, CAM-Na and CAMH.

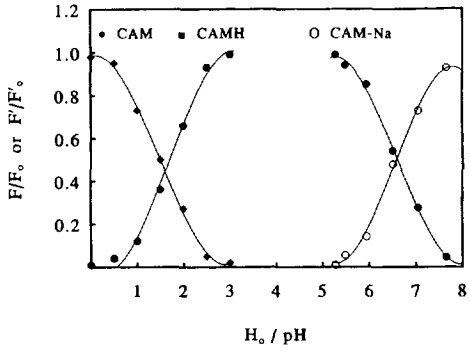


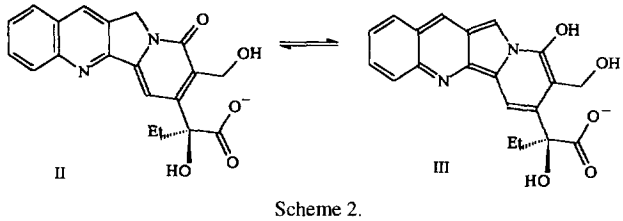
Fig. 2. Plot of the relative fluorescence intensities of CAM, CAM-Na and CAMH vs. pH.

Table 2
Relative fractions of CAM and CAM-Na and equilibrium constants (K_{obs}) at various pH values

pH	Relative fraction of CAM	Relative fraction of CAM-Na	K_{obs}
5.2	0.99	0.01	0.01
5.48	0.94	0.06	0.06
5.94	0.85	0.14	0.16
6.50	0.54	0.47	0.87
7.05	0.27	0.73	2.70
7.65	0.05	0.93	18.6
8.00	0.01	0.99	99.0

Above pH 9, the absorption spectrum of CAM-Na remains unchanged even at a high OH^- ion concentration, whereas the fluorescence intensity decreases as the concentration of OH^- ion is increased. Lown et al. [43], on the basis of NMR spectral data, proposed the tautomerization of CAM in basic solution to yield the tautomer **III** (Scheme 2) to explain the mechanism of DNA strand scission by CAM in the presence of Top-I.

The tautomerization involves an electronic reorganization of the molecule and should be manifested in the electronic spectrum. However, no change in the spectrum was observed at higher pH values. Therefore the formation of the tautomer at room temperature in the ground electronic state is unlikely. To substantiate this conclusion further, we measured the ^1H NMR spectrum of CAM in 2 M NaOD solution in D_2O. The spectrum is depicted in Fig. 3(a). For comparison, the ^1H NMR spectrum of CAM was also measured in 4 M DCl solution (Fig. 3(b)) in which the molecule is mainly present in the protonated lactone form (CAMH) as discussed later in this section. In acidic solution, the $ArCH_2{<}N$ protons appear as a singlet at 5.4 ppm. However, in basic solution, no peak corresponding to these methylene protons was observed. Also, no change in the NMR spectrum was observed even after 1 week. If tautomerization does occur, we would expect this peak to appear as a singlet corresponding to a single proton further downfield in the aromatic region (7–8 ppm). The absence of the $ArCH_2{<}N$ peak may be due to deuterium exchange with the solvent molecules. It is interesting to note that the $ArCH_2O{<}$ protons give an AB-type quartet (5.4 ppm) in both acidic and basic solutions. In addition, the methylene protons of the ethyl group (CH_3CH_2C-20), which appear as a quartet (2 ppm) in acidic solution, are split into two distinct sets of quartets, each integrating to one proton, in basic solution. This is contrary to expectation. If the molecule is present as the carboxylate form, the



Scheme 2.

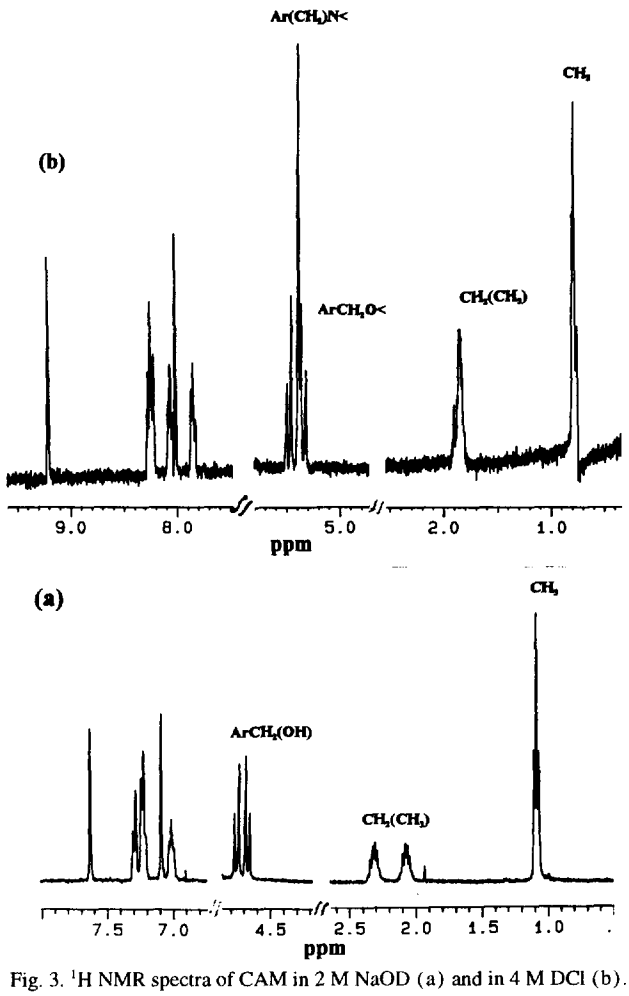


Fig. 3. ^1H NMR spectra of CAM in 2 M NaOD (a) and in 4 M DCl (b).

$ArCH_2O{<}$ protons should appear as a singlet and the $(CH_3)CH_2C$-20 protons should still give a quartet due to the free rotation of the groups around the respective Ar–C bond.

It has been proposed that CAM, on photolysis at 360 nm in basic solution, produces a racemic hemiacetal [44]. However, it is difficult to understand the formation of such a hemiacetal under the experimental conditions employed in this study. To substantiate this conclusion further, we measured the circular dichroism (CD) spectra of CAM in 1 M NaOH solution, methanol and acetate (pH 5.1) and bicarbonate (pH 9.1) buffers. The spectra are shown in Fig. 4. The CD spectra in methanol and acetate buffer, in which CAM is present in the lactone form, are similar and show a negative CD band in the long-wavelength region and a positive band in the short-wavelength region. The CD spectra in bicarbonate buffer and 1 M NaOH are similar to each other, but different from those in methanol and acetate buffer. This suggests that the species present at pH 5.1 and in 1 M NaOH have opposite configurations at the C-20 chiral center or the electronic transitions involved are different. However, as already mentioned above, there is no plausible mechanism which can explain the inversion of configuration. We have already demonstrated that the electronic transitions involved in the long-wavelength absorption of the lactone and carbox-

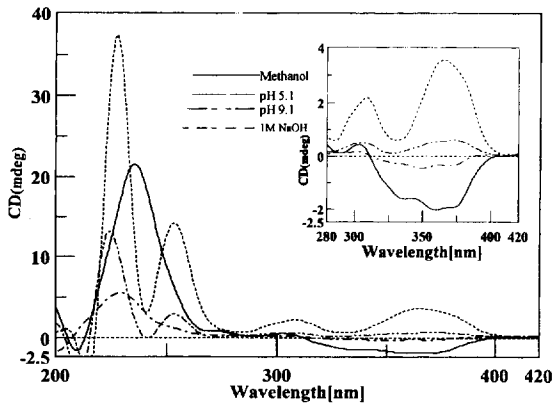


Fig. 4. Circular dichroism spectra of CAM in methanol, buffer (pH 5.1), buffer (pH 9.1) and 1 M NaOH.

ylate form are of π,π^* type. However, it may be possible that they are polarized in directions perpendicular and parallel to the long axis of the molecule. That the species at pH 9.2 and in 1 M NaOH are the same carboxylate form is further substantiated by the fact that acidification of both solutions gives CD and absorption spectra similar to that of the lactone form. This also rules out the possibility of formation of the proposed hemiacetal. Therefore the above NMR spectrum in 1 M NaOH is characteristic of the carboxylate form.

As mentioned above, the fluorescence intensity of CAM-Na decreases with increasing OH^- ion concentration. The Stern–Volmer plot of the ratio of the steady state fluorescence intensities in the absence and presence of quencher against the quencher concentration in the range 0–0.2 M is linear (Fig. 5). The fluorescence lifetime was also measured in solutions of different OH^- ion concentration. The lifetime decreases as the concentration of OH^- increases, suggesting that the fluorescence quenching is due to a dynamic process. The Stern–Volmer plot of τ_0/τ vs. OH^- concentration gives a good linear fit with the quenching constant $K_D = 16.4$. The quenching constant obtained from the slope of the steady state Stern–Volmer plot is 14.3. The quenching rate constant calculated using the lifetime data at pH 9.2 is $k_q = 3.7 \times 10^9$ M^{-1} s^{-1} which is identical within experimental error with

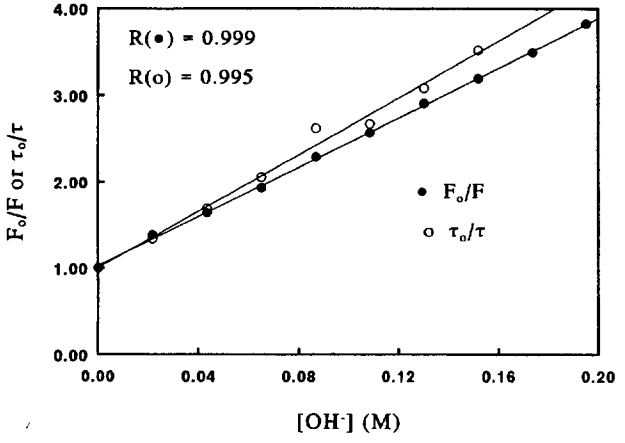
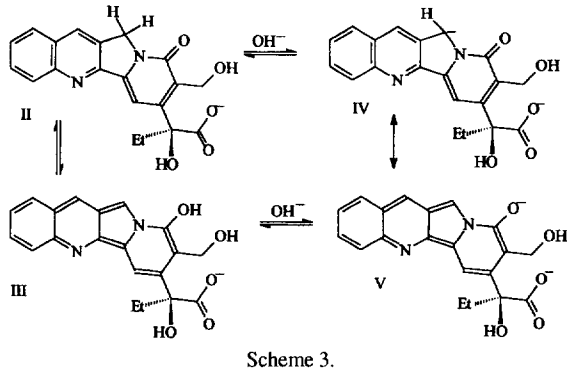


Fig. 5. Stern–Volmer plots of the fluorescence quenching of CAM-Na by OH^- ion.

that ($k_q = 3.2 \times 10^9$ M^{-1} s^{-1}) calculated from steady state fluorescence quenching.

It appears from the above discussion that the fluorescence quenching is due to an excited state proton transfer reaction involving the $ArCH_2N <$ protons. The ^1H NMR spectrum of CAM in basic solution suggests that, in the ground electronic state, these protons are in exchange with solvent molecules. It seems that the deuterium exchange is catalyzed only by OH^- ions, since no deuterium exchange is observed in acidic solution. It is quite reasonable that these protons become photolabile on electronic excitation to the S_1 state and dissociate to give carbanions. The formation of carbanions via ionization of carbon acids is well known in the ground electronic state [45]. In the S_0 state, the pK_a value of carbon acids is usually in the range 30–38 [46]. However, Forster cycle [39,47] calculations of a number of hydrocarbons and their conjugate bases have shown that the benzylic protons of these compounds become very acidic in the S_1 state by as much as 30 orders of magnitude. Recently, the formation of carbanions from the excited singlet state of suberene [48] and other analogs [49] has been reported in the literature. These workers have also reported that the fluorescence of suberene is quenched in acetonitrile by H_2O.

The rates of deprotonation from C–H acids in the S_0 state are typically slow [50], because of a lack of hydrogen bonding to the solvent and the substantial geometric and solvation changes generally required on deprotonation of carbon acids. If this were true for electronically excited states as well, such intrinsically slow rates for the deprotonation of C–H bonds would not compete favorably with the fast rates of decay available to the S_1 state. It seems clear from the above observations that OH^- deactivates the S_1 state of CAM-Na by acting as a base for protons at the C-5 carbon atom, resulting in the generation of a transient carbanion intermediate **IV**. Thus k_q may be taken as a measure of the observed rate at which protons from C-5 are transferred to solvent water in the S_1 state, if not the rate of deprotonation itself. The driving force for deprotonation is the electronic and geometric rearrangement in the molecule, as shown in Scheme 3, which results in the formation of the tautomer. The tautomer **III** thus produced in the S_1 state rapidly equilibrates with the corresponding phenoxide ion **V** in basic solution. It seems that the phenoxide ion is non-fluorescent in aqueous solution.



Scheme 3.

In the acidity region of pH 4 to $H_0 - 2$, the absorption and fluorescence spectra of CAM shift to longer wavelengths. The shift in the emission spectrum is larger than that in the absorption spectrum. This can be attributed to the protonation of the quinoline ring nitrogen giving rise to the formation of the quinolinium ion CAMH. The red shift in the absorption and emission spectra of CAMH suggests that the electronic transition is of π,π^* type. Unlike quinoline and acridine, the fluorescence quantum yield of CAMH is less than that of the neutral species. Since protonation is the extreme case of hydrogen bonding, the quantum yield of CAM should increase on protonation. However, in the present case, the transition is π,π^* type and therefore stabilized due to protonation. This reduces the S_1–S_0 and S_1–T_1 energy gaps. As a result, the rates of radiationless decay increase and thus the fluorescence quantum yield is reduced.

The ground state pK_a value (0.7) of the conjugate acid CAMH is lower than that reported (1.18) in the literature [40]. In addition, the pK_a value is less than that of quinoline (4.87) [51], which suggests that the molecule is a weak base in the S_0 state. The low basicity, compared with that of quinoline in the S_0 state, is because of the large resonance stabilization of the lone pair electrons of the quinoline nitrogen due to the extended conjugation in the ring system. However, the basicity increases on electronic excitation to the S_1 state as indicated by the pK_a^* value (1.65) obtained from the fluorometric titration curves (Fig. 2). The pK_a^* value was corrected for the fluorescence lifetimes of the species involved in the excited state equilibrium to obtain the pK_a (S_1) value (1.64). As can be seen from Table 1, the lifetimes of CAM and CAMH are very similar, which indicates that prototropic equilibrium is established during the lifetime of the excited species. This is reflected in the small correction factor (-0.01).

4. Conclusions

Above pH 8, camptothecin is present predominantly in the carboxylate form as indicated by the equilibrium constants. Both ^{1}H NMR and circular dichroism spectra suggest that camptothecin is present as the carboxylate form even in strongly basic solution. No hemiacetal or tautomer as reported by other workers was observed in the ground electronic state. However, camptothecin tautomerizes in basic solution on electronic excitation to the S_1 state. This suggests that the mechanism of photolytic DNA strand breaking in the presence of Top-I, as reported in the literature, involves the phototautomer of camptothecin. The ground and excited state pK_a values indicate that the quinoline nitrogen becomes more basic in the S_1 state.

Acknowledgements

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