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Ground and excited-state ionization behavior of 9-aminocamptothecin: An absorption and fluorescence spectral study

Ashis Biswas & Joykrishna Dey*
Indian Institute of Technology, Kharagpur 721 302, India

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The absorption and fluorescence spectra of 9-amino-20(S)-camptothecin (ACAM) have been measured as a function of *p*H. The photophysical properties, for example, fluorescence quantum yields and lifetimes, and radiative and nonradiative decay rates of different prototropic species of ACAM in aqueous solution have been estimated. The neutral lactone form as well as the ring-opened carboxylate form of the drug are found to be nonfluorescent in aqueous medium. The ground and excited-state acidities of the prototropic forms of the molecule have been determined. The semi-empirical AM1 calculations have been performed to calculate the ground and excited-state charge densities at the basic centers of the molecule. The results have been compared with that for the parent drug, 20(S)-camptothecin.

The states of ionization of drug molecules are extremely important in determining the action of drugs. The site of ionization is also equally important in case of molecules having more than one ionizable group. Therefore, an accurate knowledge of the sites of ionization corresponding to the pK_a values of the drug molecule is necessary to study the structure-activity relationship (SAR). The ionization states of a molecule in aqueous solution can be readily obtained from the studies of its absorption and fluorescence properties.

The 9-amino-20(S)-camptothecin, ACAM (Fig.1) is an anticancer drug. The parent drug 20(S)-camptothecin, CAM (Fig.1) was found to be active in tests against L1210 leukemia[1] and Walker 256 carcinosarcoma.[2-4] It was also found that CAM inhibits both DNA and RNA synthesis in mammalian cells. This attracted a huge interest in CAM as a potential cancer chemtherapeutic agent. Its high antitumor activity against a wide range of experimental tumors has been confirmed.[5] Due to its poor solubility in water, CAM was clinically evaluated as its water-soluble sodium salt. Several water-soluble CAM analogs were also synthesized[6] and clinically evaluated.[7] However, only 9-, 10-, and 11-subsituted derivatives have shown anticancer activity in a number of *in vitro* and *in vivo* studies.[8-15] The details of pre-clinical and clinical developments of CAM and its analogs have been reviewed elsewhere[16,17].

However, in most studies reported so far, attention has been mainly focused on the SAR of CAM and its synthetic analogs. Burke *et al.*, in a series of publications, have reported the binding of the parent drug as well as its analogs with human serum albumin and lipid bilayers.[18-23] The binding was confirmed by exploiting the fluorescence properties of the drug. These authors used the *p*H sensitive fluorescence of ACAM to estimate its binding constant with phospholipid vesicles.[24] They reported that the fluorescence intensity of ACAM increases upon decrease in *p*H of the solution. This was attributed to the protonation at the amine group. However, the spectrum reported in the paper showed an emission band which was red-shifted relative to that of the neutral CAM molecule. In case of protonation at the

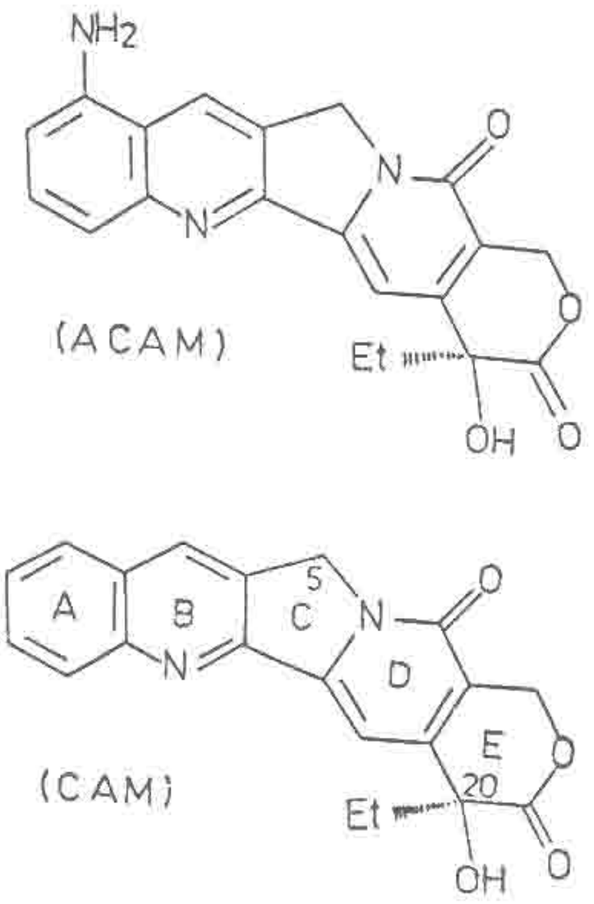

Fig. 1—Molecular structures of ACAM and CAM

spectrum of the monocation species of ACAM shifts to longer wavelength relative to that of CAM upon protonation at the ring nitrogen atom. The protonation at the amine group destroys the CT character of the ^1L_a state thus increasing the fluorescence from ^1L_b state as a result of reduction of the rate of intersystem crossing. That is, a reversal of the emitting state occurs upon protonation at the amine group. However, the fluorescence quantum yield of the monocation species of ACAM is only one half of that of the neutral CAM molecule.[27] This, as discussed above, is due to the stronger solute-solvent interactions in the case of protonated species of ACAM. Indeed, the nonradiative decay rate of the monocation species is much higher than that of the neutral CAM molecule (k_{nr} = 0.9 x 10^8 s^{-1})[26]. The low fluorescence quantum yield of the dication as compared to monocation species could be due to the stabilization of the lowest excited ^1L_b state which enhances the singlet-triplet interaction and hence increases the intersystem crossing rate. Clearly, this is so as indicated by the large nonradiative decay rate (Table 1) of the dication species. The increase in nonradiative decay rate of the dication species as compared to the monocation species may be also due to greater solute-solvent interactions. That is, the strong solute-solvent interaction is partly responsible for the low fluorescence quantum yield of the dication species of ACAM. The red shift of the fluorescence maximum of the dication species relative to that of the monocation species of CAM can be attributed to the difference in solvation energy. The former species, being doubly charged, are expected to be strongly solvated as compared to the latter.

The pK_a^* value of the monocation species obtained from fluorometric titration is equal to the corresponding ground-state value (3.0). This suggests that the basicity of the amine group does not change upon excitation. The data in Table 2 also suggest that the charge density at the amine nitrogen does not change significantly upon electronic excitation to the S_1 state. However, it should be remembered that solvation of the functional groups of the molecules/species was not taken into account in the MO calculations. The other possibility is that the equilibrium is not established during the lifetime of the molecule in the excited state. This seems to be most likely as the nonradiative decay rate of the neutral molecule is very high making it essentially nonfluorescent. In fact, the shift in the position of the emission maximum upon protonation clearly indicates a decrease in the pK_a value of the corresponding ammonium ion upon excitation to the S_1 state. According to Forster-cycle calculations[35] using fluorescence maxima (567 nm for the neutral and 465 nm for the monocation species) of the respective species, the pK_a^* value should be equal to –5.1. However, it should be noted that the nature of the emitting states of the neutral and the monocation species is different. Also, the degree of hydration of the species is different. Therefore, such calculation is not appropriate. Although the calculated value cannot be accepted as the pKa^*, this definitely suggests that the NH_2 group becomes strong electron donor and hence less basic when the molecule is excited to the S_1 state. In the case of protonation at the ring nitrogen atom, the pK_a^* (1.0) value is larger than the corresponding ground state pK_a (0.3) value which indicates the increase in basicity of the quinolinic ring nitrogen atom. Similar increase in pK_a value upon excitation was also observed in the case of CAM.[27]

Conclusions

The introduction of the NH_2 group at the 9-position of CAM dramatically changes the absorption and fluorescence spectral properties of the parent drug. Unlike CAM the neutral form of ACAM is non-fluorescent in aqueous medium. The *p*H effect on the absorption spectra of ACAM in aqueous solutions suggests that the first protonation occurs at the NH_2 group in ground as well as in the S_1 state. The protonation at the amine nitrogen reverses the order of emitting states in the monocation species. That is, the emitting state in the neutral molecule is ^1L_a/CT type and that in the monocation is ^1L_b type. The large red shift of the monocation emission of ACAM as compared to the neutral form of the parent drug is a result of stabilization of the emitting state due to its strong solvation. The low fluorescence quantum yield of the dication species compared to the monocation is largely due to higher nonradiative decay rates. The results suggest that the basicity of the quinolinic nitrogen increases upon excitation.

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amine group, one would expect the resulting fluorescence spectrum to be closely similar to that of the neutral form of CAM in water. Recently, Dey and coworkers have reported the solvent dependence of the fluorescence spectra and photophysical properties of ACAM[25]. In this work, Dey and Warner have also noted a strong red-shifted fluorescence of ACAM in acidic medium. A detailed study on the ground and excited-state proton-transfer properties of the parent drug, CAM, has been reported by these authors[26,27].

We have undertaken this study in order to understand the ionization behaviour of ACAM. The aims of the present work are: (i) to study the *p*H effect on the absorption and fluorescence spectra, and photophysical properties of ACAM in aqueous solution, (ii) to estimate the pK_a values of the ground and excited-state proton-transfer equilibria, and (iii) to perform theoretical calculations to calculate charge densities at various atomic centers of CAM as well as ACAM to substantiate the experimental data.

Materials and Methods

Materials—The 9-amino-20(S)-camptothecin (Lot # 94B10A) was received as a gift from Pharmacia and was used without further purification. Analytical grade NaH_2PO_4, Na_2HPO_4, $HClO_4$ and H_2SO_4 (S.D. Fine) were used as received. The spectroquality grade 1,2-dimethoxyethane was obtained from Aldrich. Millipore (18 MΩ) deionized distilled water was used for making aqueous solutions.

Methods—Because of its poor aqueous solubility, a stock solution (~10^4 *M*) of ACAM was made in 1,2-dimethoxyethane. For *p*H studies, an aliquot (100 µL) of this solution was taken in a 10 ml flask and the volume was made up with appropriate buffer solution. Hammett's H_o acidity function[28] was followed to prepare aqueous solutions below *p*H<1. Fluorescence quantum yields were determined according to the method of Parker and Rees[29] with reference to quinine sulphate in 0.1 *N* H_2SO_4 (ϕ_f = 0.545).[30] The absorbance of the solutions including quinine sulphate at the wavelength of excitation (360 nm) was maintained at <0.05.

The theoretical calculations were performed using the semi-empirical AM1 method. The molecular geometry was preoptimized using Tripo's SYBYL 5.2 force field before AM1 calculations were performed.

Apparatus—The absorption spectra were measured on a double beam Shimadzu UV-1601 spectrophotometer using 1 cm^2 cuvette. A SPEX FL3-11 spectrofluorometer was used to measure the fluorescence spectra. The slit width of the excitation and emission monochromators was respectively set at 2 and 3 nm. Fluorescence lifetimes were measured on a PTI Inc LS-100 luminescence spectrometer. The 358 nm emission of N_2 was used for sample excitation. The decay curves were obtained by use of the time correlated single photon counting technique. The data were analyzed by using a multiexponential decay analysis program provided by the company. All other measurements, except fluorescence lifetimes were made at room temperature (~25 °C). The fluorescence lifetimes were measured at 25 °C.

Results

Absorption and fluorescence spectra—The absorption and fluorescence spectra of ACAM were measured in a series of buffered aqueous solution in the H_o/*p*H range of –2 to 13. Figure 2 shows the absorption spectra of the protonated forms in water. The corresponding fluorescence spectra appear in Fig. 3. The relevant spectral data are listed in Table 1. The absorption spectra above *p*H 7 when measured after equilibration (for 24 hr) showed no change except a small decrease in intensity of the ^1L_a band.[25] A decrease in intensity of the ^1L_a band was also observed below *p*H 7. However, this is accompanied by a blue shift of the shortest wavelength absorption band. The resulting spectrum at *p*H 2 resembles the spectrum of the neutral form of CAM in the same solvent. At *p*H < 2, the spectrum shows a large red

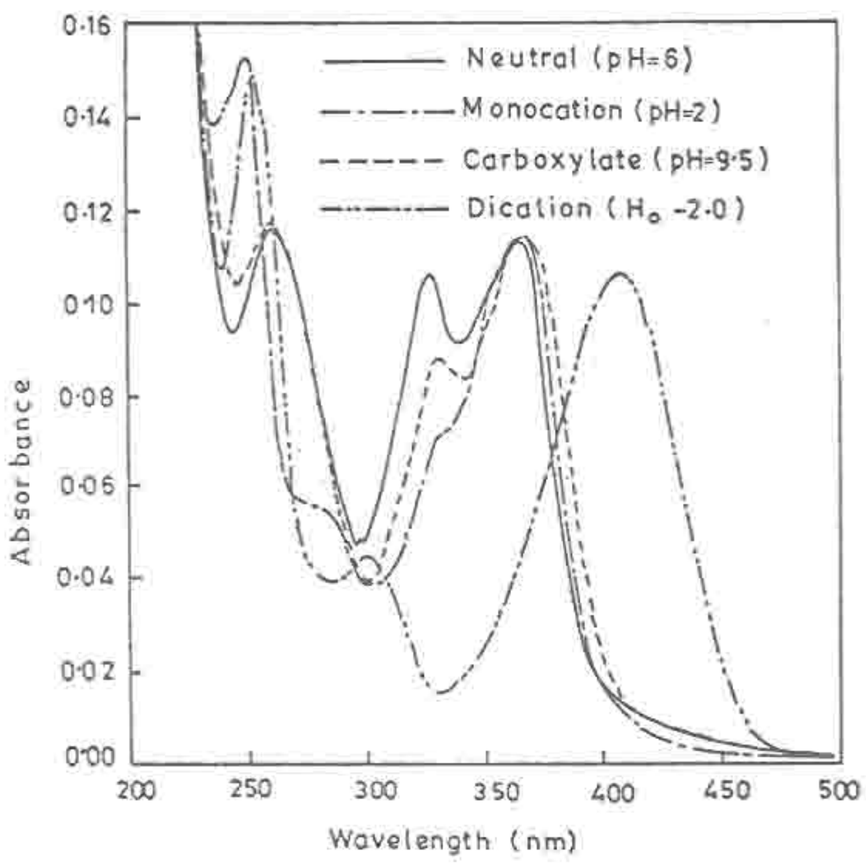

Fig. 2—Absorption spectra of the prototropic species of ACAM in water

shift with the decrease in *p*H. No further change in the absorption spectrum was observed at $H_o < -2$.

The molecule is essentially nonfluorescent in neutral and at higher *p*Hs although a weak fluorescence band can be observed in this *p*H range. The intensity of the weak fluorescence was observed to decrease with an increase in *p*H and finally it disappeared at *p*H 13. At lower *p*Hs (< 6) a strong fluorescence band at ~465 nm rises with a decrease in *p*H. However, at *p*H < 2 the intensity of the band decreased with a further increase in acidity of the medium and a weak but large red-shifted (λ_{max} = 548 nm) fluorescence spectrum appeared at H_o –2 which resembles monocation spectrum of CAM.[27]

The fluorescence quantum yields and lifetimes of the prototropic species of ACAM measured in water are also included in Table 1. The radiative (k_r) and nonradiative (k_{nr}) decay rate constants calculated from the corresponding ϕ_f and τ_f values ($k_r = \phi_f / \tau_f$ and $k_{nr} = (1-\phi_f)/\tau_f$) are also listed in the same table. The neutral form is essentially nonfluorescent in water. However, the ϕ_f of the monocation is much higher as compared to that of dication species. The ϕ_f values of the monocation and dication species of ACAM are respectively less than those of the neutral and monocation forms of CAM.[27] The fluorescence decay of the short-lived component of the monocation decays is similar to the decay time of the dication species and, therefore, is associated with the dication species. The fluorescence lifetime of the monocation species is greater than that of dication. The nonradiative decay rate of the monocation species of ACAM is larger than that of neutral CAM (k_{nr} = 0.9 x 10^8 s^{-1})[26] in the same solvent.

Theoretical calculations—The quantum chemical molecular orbital (MO) calculations were performed for ACAM as well as CAM using the semi-empirical AM1 method[31] to calculate charge densities (CDs) at the heteroatoms of the molecules. The molecular geometry was preoptimized first using MM+ force field[32] and then by AM1 semi-empirical method before MO calculations were performed. A total of nineteen configurations involving three occupied and three unoccupied MOs were used for calculations. The calculated CDs at various atoms are summarized in Table 2. The results indicate that the CD at the quinolinic ring nitrogen of ACAM is slightly greater than that in CAM. The CD at the quinolinic ring nitrogen increases almost equally upon electronic excitation of the molecules. The CD at the amino nitrogen of ACAM, however, remains unchanged upon electronic excitation. No significant change in

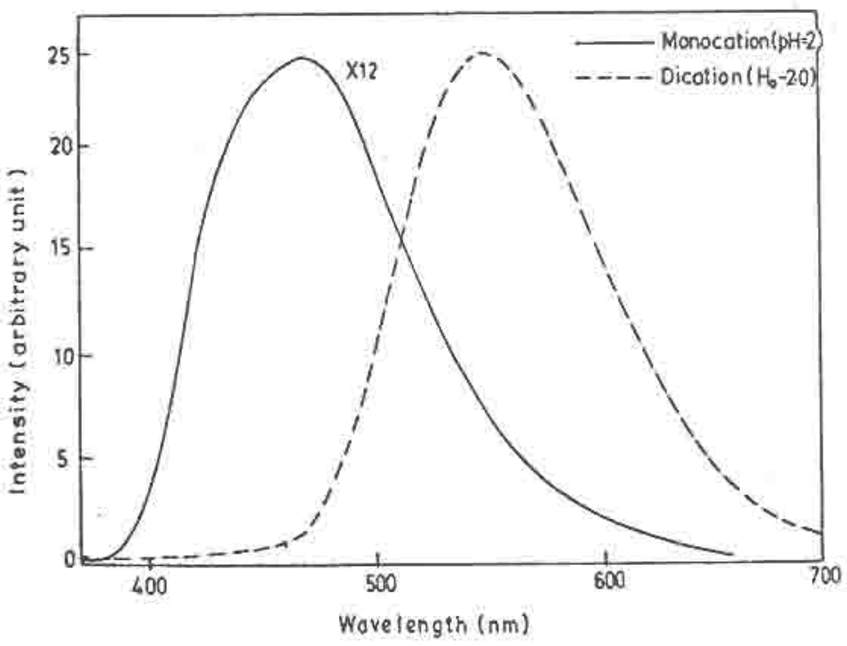

Fig. 3—Fluorescence spectra of the prototropic species of ACAM in water

Table 1—Absorption and fluorescence maxima (λ_{max} (abs) and λ_{max} (flu), respectively), fluorescence quantum yields (ϕ_f), lifetimes (τ_f) and radiative (k_r) and nonradiative (k_{nr}) rate constants of the prototropic species of ACAM.

Species (*p*H/H_o)	λ_{max}(abs) (nm)	λ_{max}(flu) (nm)	ϕ_f (± 0.001)	τ_f [a] (± 0.1 ns)	$k_r \times 10^{-7}$ (sec^{-1})	$k_{nr} \times 10^{-8}$ (sec^{-1})
Neutral	366					
(*p*H 6.0)	337	567[b]	-	-	-	-
	265					
Carboxylate	369					
(*p*H 9.5)	338	-	-	-	-	-
	264					
Mono-cation	368	465	0.227	3.4(0.89)	6.68[c]	2.27[c]
(*p*H 2.0)	251			1.0(0.11)		
Dication	408	548	0.053	1.0	5.3	9.47
(H_o -2.0)	301					

[a]Values within the bracket represent relative amplitudes of the components.
[b]From reference 25; c. calculated using the lifetime (τ_f) of the long-lived component.

the CD values of other heteroatoms of the molecule was observed.

Discussion

As reported by Dey *et al*[25] the long-wavelength absorption band of the neutral molecule consists mainly of ^1L_a and ^1L_b transitions at 335 and 365 nm respectively. Based on the results reported in the literature the changes in the absorption spectrum at *p*H > 7 can be attributed to hydrolysis of lactone ring to give the carboxylate form. The spectrum at *p*H 2, can be associated with the monocation species. The disappearance of the short-wavelength band at 335 nm upon protonation suggests that the transition involved is CT (or l ---> π^*) type. This in conjunction with the pK_a value (3.0) obtained from the absorptiometric titration clearly suggests that the above spectral change is a result of protonation at the exocyclic amine group giving rise to the formation of monocation species. If the protonation occurred at the quinolinic nitrogen then one would have expected a red shift in the absorption spectrum. However, this is in contrast to 5-aminoquinoline in which the first protonation occurs at the ring nitrogen.[33] The pK_a value of the corresponding ammonium ion is much lower than that of aniline (4.5)[34] which suggests that the CT interaction of the NH_2 group of ACAM with its quinoline ring is much stronger than in aniline but weaker than in 5-aminoquinoline.[33] Thus, in the case of ACAM, the second protonation occurs at the ring nitrogen to produce dication species as indicated by the red shift of the long-wavelength absorption band. This suggests that the ^1L_b band is of π ----> π^* type. Therefore, the red-shifted absorption spectrum at H_o – 2 can be ascribed to the dication species. The pK_a value (0.3) of the dication species which is slightly less than that of the monocation species of CAM (0.7)[27] is, however, much lower than that of 5-aminoquinoline (5.64).[33] The low basicity of the ring nitrogen of the protonated ACAM is obviously due to the extended conjugation and the presence of a positively charged NH_3^+ group which reduces the charge density at the quinolinic ring nitrogen atom.

The weak fluorescence at neutral *p*H as established in a previous report[25] is due to the parent drug, CAM, present as an impurity in the sample. However, the level of impurity seems to be very low. The appearance of the CAM emission is due to its very high fluorescence quantum yield (>0.5)[26]. Thus ACAM is nonfluorescent in neutral solutions. The same was also observed by Dey and Warner[25]. When increasing amounts of water were added to dioxane solvent the fluorescence intensity decreased to almost zero in pure water. The charge-transfer structure of ACAM was thus established. The decrease in fluorescence intensity of the weak band in water with an increase in *p*H above 10 as already reported is a result of phototautomerisation of CAM.[27] The ACAM is also expected to undergo similar photoinduced intermolecular proton transfer in the same *p*H range to give corresponding tautomer. In both the cases, the phototautomer being present in the phenolate form at higher *p*H is nonfluorescent.

According to the results described in previous publication, in the absence of quenching in water the CT fluorescence of ACAM would have appeared around the emission maximum at 567 nm.[25] In accordance with the absorption spectrum, the protonation at the amine group should result in a blue shifted fluorescence spectrum closely similar to that of the neutral CAM molecule. Indeed the monocation fluorescence (λ_f = 465 nm) of ACAM is blue-shifted relative to that of the neutral species (λ_f = 567 nm) However, it is interesting to note that the spectrum of the monocation species is red shifted relative to that of neutral form of the latter molecule (λ_f = 428 nm).[27] The red shift of the spectrum is most likely due to stronger solute-solvent interactions of the positively charged monocation species with the water molecules which stabilizes the ^1L_b state. Also, Dey *et al*[25] have demonstrated that hydrogen-bonding interaction with the pyridinic ring nitrogen causes a red shift in the absorption as well as in the fluorescence spectrum of the neutral ACAM molecule. The fluorescence

Table 2—Net charge desities at different heteroatoms of CAM and ACAM

Atom type	Charge densities			
	CAM		ACAM	
	S_o	S_1	S_o	S_1
$-NH_2$	-	-	-0.330	-0.330
⩾N	-0.085	-0.128	-0.091	-0.130
>N	-0.246	-0.232	-0.245	-0.231
=O (arom)	-0.363	-0.302	-0.364	-0.303
=O (lactone)	-0.306	-0.306	-0.307	-0.307
-O(H)	-0.305	-0.311	-0.306	-0.312
>O (lactone)	-0.230	-0.231	-0.230	-0.231

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