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Triplet excited-state characterization and determination of the photoionization mechanism of the antitumoral drug pazelliptine

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Abstract

The triplet properties of the excited triplet state of pazelliptine (PZE), an antitumoral drug derived from ellipticine, were investigated in dioxane, ethanol and buffer aqueous solutions using the laser flash photolysis technique. The triplet absorption spectra and the kinetic parameters associated with the excited state decay were quite similar in the different solvents. ^{3}PZE reacted with unexcited PZE in deaerated solutions ($k=6\times10^{10}$ M^{-1} s^{-1}) and was quenched by oxygen ($k\approx2\times10^{7}$ s^{-1}). The extinction coefficients of the triplet transition were estimated and used to calculate the singlet–triplet intersystem crossing quantum yields of about 5%.

A biphotonic ionization of PZE in buffer aqueous solution has been demonstrated in a previous work. This process was also observed in ethanol but not in dioxane. Mixed yttrium aluminum garnet laser harmonics (355 nm + 532 nm) and delayed-pulse experiments were carried out in order to determine the intermediate excited state involved in this photoionization process. The results indicate that pazelliptine radical cation and e^-_s are formed via a consecutive two-photon absorption in which the first excited singlet state is the only intermediate.

Keywords: Pazelliptine; Photoionization mechanisms; Triplet state

1. Introduction

Ellipticine and many of its derivatives (9-hydroxyellipticinium, azaellipticine, 9-methoxyellipticine, etc.) are a group of drugs which exhibit significant antitumor properties both in vitro and in vivo and which are used in the chemotherapy of leukemia and different forms of cancer [1]. Synthesis of new molecules is at the present time in intensive development [2]. However, the precise mode of action of these drugs remains unknown. Intercalation into deoxyribonucleic acid (DNA) base pairs of these compounds appeared to be an important but insufficient parameter for antitumor activity [3,4]. In vitro experiments have suggested that metabolic oxido-reduction processes involving electron transfer and the formation of free radicals and/or molecular intermediates could be at the origin of their biological activities [5,6]. Recently, irreversible DNA strand break was observed upon light irradiation of an ellipticine derivative covalently attached to an oligonucleotide which recognizes a specific DNA sequence [7]. These results prompted us to study the photodynamic properties of these antitumoral molecules with respect to their possible use in the antigenic strategy or artificial endonuclease.

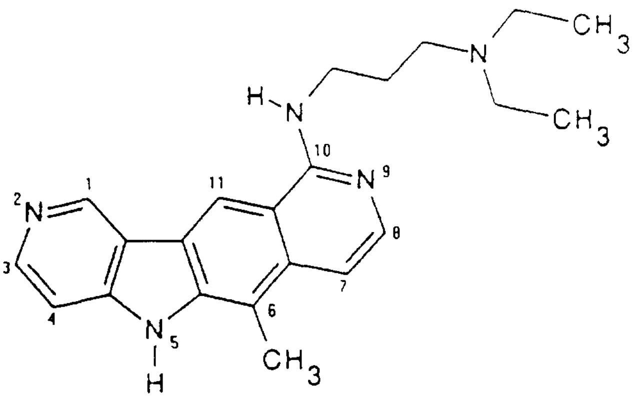

Fig. 1. PZE structure.

Pazelliptine (PZE), a 9-azaellipticine (10-[[3-(diethylamino)propyl]amino]-6-methyl-5H-pyrido-[3′-4′:4.5]pyrrolo[2,3-g]isoquinoline) (Fig. 1), was chosen as a model for this study. In a previous work [8], using high intensity laser pulse excitation at 355 nm, we have reported the obser-

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vation of one electron ejection from PZE in aqueous solution and the formation of the PZE radical cation ($PZE^{\bullet +}$) supposed to be involved in the biological effects of the molecule [5]. It was found that the extent of photoionization increased quadratically with increasing laser fluence and hence it was concluded that this reaction is due to two-photon excitation of PZE molecule. In addition to this photoprocess, triplet-state formation was also detected.

The aim of the present work was to characterize the excited triplet state of PZE and to identify the intermediate excited states (the first excited singlet S_1 and/or the lowest triplet T_1 states) involved in the photoionization mechanism. For this purpose, the triplet-state spectral characteristics, lifetime, molar absorption coefficients and quantum yields were determined in dioxane, ethanol and aqueous solutions. The dependence of the fluorescence quantum yield on the solvent nature was also examined. The mechanism of photoionization was studied using the mixed harmonics ((355+532) nm) of a Nd-doped yttrium aluminum garnet (YAG) laser and delayed-pulse experiments. Complementary results using the heavy-atom effect to enhance the triplet population were performed to confirm the relative importance of both states S_1 and T_1 as intermediates in the photoionization process.

2. Materials and methods

The samples of PZE were a generous gift from Dr. E. Bisagni and Dr. C. Rivalle of the Curie Institute, Orsay, France. The synthesis and characterization of the drug have been described previously [9]. β-carotene was supplied by Aldrich. Dioxane and ethanol were of spectroscopic grade, and potassium iodide (KI) was a Suprapur product, all obtained from Merck. PZE was used in its free basic form for the experiments in dioxane and in its salt form in the other preparations. In aqueous media, PZE was first dissolved in HCl solution (Prolabo, Normapur) and then diluted in phosphate buffer (KH_2PO_4–K_2HPO_4, 5×10^{-2} M; pH 7.0) prepared from twice-distilled deionized water. The samples were deaerated by bubbling argon through the solution prior to the experiments. When necessary, the solvated electron was eliminated by saturation of the solution with N_2O and 0.05 M *tert*-butanol which scavenged the reaction $OH^{\bullet}$ free radicals formed during the electron–N_2O reaction. The oxygenated samples were obtained by maintaining 1 atm O_2 upon the solution. All the gases were from Alphagaz with a purity of 99.99%.

Absorption spectra were recorded using a PC-controlled Varian Cary 210 spectrophotometer. Corrected steady state emission and excitation spectra were measured with an SLM-Aminco 8000 spectrofluorometer (2 nm slits in both excitation and observation monochromators). Determination of the fluorescence quantum yields was carried out using 9,10-diphenylanthracene (DPA) in decalin solvent as a reference ($\lambda_{exc}=355$ nm; $\Phi_f=0.94$) [10]. The reference and samples were matched to have absorbances around 0.1 (optical path length, 1 cm). The fluorescence quantum yields Φ_f were calculated using the following relationship:

$$\Phi_{fx}=\Phi_{fs}\frac{F_sA_x}{F_xA_s}$$

where the subscript x refers to the unknown and the subscript s to the standard, A is the absorbance at the excitation wavelength and F is the integrated emission area across the band. The time-resolved fluorescence measurements were performed using the frequency doubled (532 nm) and the frequency tripled (355 nm) of a *Q*-switched Nd-doped YAG mode-locked laser (BMI; pulse width, 30 ps) [11]. The two beams were directed onto the sample simultaneously or successively and then delayed by about 800 ps with respect to each other. A monochromator, and a streak camera interfaced with a microcomputer were used to measure the laser-induced emission signals at selected wavelengths as a function of time.

Transient absorption measurements were obtained using laser photolysis equipment described previously [12]. Briefly, the excitation source was a Nd-doped YAG laser (Quantel, YG 441) of 3 ns full width at half-maximum with second- and third-harmonic generation (532 nm and 355 nm respectively). In the single-pulse excitation experiments, the 355 nm beam was directed onto one side of the 10 nm square silica cell containing the sample. In the double-pulse excitation experiments, the 355 and 532 nm beams were directed onto the sample either simultaneously or delayed with respect to each other. In the latter case, part of the 532 nm light was extracted before third-harmonic generation and delayed by about 30 ns with respect to the 355 nm beam. The transient transmission variations were monitored at right angles to the excitation in a cross-beam arrangement using a xenon flash lamp, a monochromator, a photomultiplier and a digitized oscilloscope interfaced with a microcomputer. The energy of the laser pulse was varied using glass filters of precisely determined transmission at 355 nm. Variations in the laser output were measured using a joulemeter receiving a small fraction of the laser light extracted before its attenuation by the filters. The fluence of the incident laser pulse in the sample was obtained by calibration of the joulemeter using anthracene in deaerated cyclohexane as a triplet actinometer (6.5×10^{-5} M) [13].

3. Results and discussion

3.1. Absorption spectra

The ground-state absorption spectrum of PZE was measured in different solvents (Fig. 2). The absorbance of PZE solutions followed the Beer–Lambert law in all media, in the concentration range 5×10^{-6}–1.5×10^{-4} M (Fig. 2, inset A); hence we assume that over the concentration range used in this study the molecule in its ground state did not form dimers in solution.

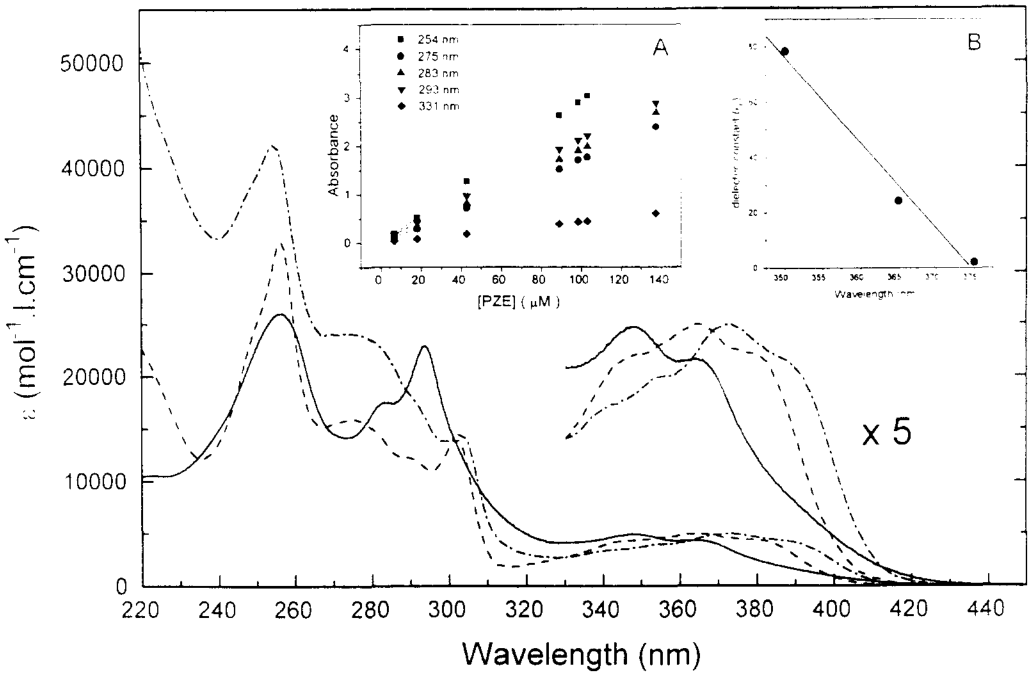

Fig. 2. Ground-state absorption spectra of PZE in dioxane (– · –), ethanol (– – –) and phosphate buffer pH 7 (——). Inset A shows the verification of the Beer–Lambert law in buffer aqueous solution. Inset B shows the absorption maximum of PZE in different solvents as a function of the dielectric constant.

Below 320 nm, the absorption spectra of PZE in dioxane and ethanol have similar shapes but the absorbance was less intense in ethanol than in dioxane. In aqueous solution, we observed an increase and a blue shift of the absorption maximum from 305 to 295 nm. This result must be correlated with different protonation states of the molecule as described elsewhere [14]. Between 340 and 450 nm, the absorption spectra shape as well as the molar extinction coefficient were not affected by the solvent nature ($\epsilon_{max} = 5 \times 10^3$ M^{-1} cm^{-1} [15]). However, the absorption maxima varied with the solvent polarity and had values between 350 and 370 nm. The absorption spectra were blue shifted as the solvent polarity was increased (hypsochromic solvatochromism) and a linear relationship between absorption maxima and dielectric constant was observed as shown in Fig. 2 (inset B). This positive solvatochromism thus established was characteristic of compounds having a weakly polar ground state [16].

3.2. Fluorescence spectra

Corrected fluorescence emission and excitation spectra were obtained in the different solvents (Fig. 3). In all media, there was no difference between fluorescence excitation and absorption spectra. We observed some flattening of the maximum excitation spectra of PZE in dioxane (370 nm) compared with the absorption spectra and owing to the saturation effect of the spectrofluorometer. The fluorescence quantum yield Φ_f was progressively reduced as the solvent polarity increased (Fig. 3, inset). The high values of fluorescence quantum yields obtained for PZE in dioxane and ethanol were consistent with the low triplet quantum yield values (see below).

3.3. Triplet absorption characterization

3.3.1. Dioxane

After the 355 nm pulse photolysis of argon-flushed PZE solutions in dioxane, absorbance changes were measured (Fig. 4(A)). The absorption spectrum recorded at a short time (10 ns) after the laser pulse was characterized by a positive absorbance below 350 nm and from 400 to 700 nm (ΔA_{max} at about 430 nm) and depletion in the region of the ground-state maximum absorption. Our previous experiments in deaerated aqueous solution [8] revealed that the photolysis of PZE led to two photochemical processes, triplet state formation and ionization of the molecule by a biphotonic process, to produce the solvated electron e^-_s and the PZE radical cation $PZE^{•+}$ (absorption between 400 and 700 nm). In dioxane, the strong absorption of e^-_s could not be observed because of its important red shift ($\lambda_{max} = 1700$ nm [17]) but the transient absorption spectrum observed in Fig. 4(A) could include the absorption of $PZE^{•+}$ species. However, we have shown that the e^-_s disappeared on the nanosecond time scale by reaction with the non-excited PZE which resulted in the formation of the $PZE^{•+}$ radical and a build-up of the absorbance between 600 and 700 nm [8]. In deaerated dioxane solution, no spectral evolution was observed. Furthermore, Fig. 4(D) showed that the absorbance variations at 550 nm (absorption maximum of $PZE^{•+}$) increased linearly and not with the square of the increasing laser fluence as for a biphotonic process. These results revealed that no photoionization process occurred in dioxane.

Analysis of the absorption time dependence of the transient represented in Fig. 4(A) at all wavelengths gave a first-order decay rate, which indicated that this spectrum was derived

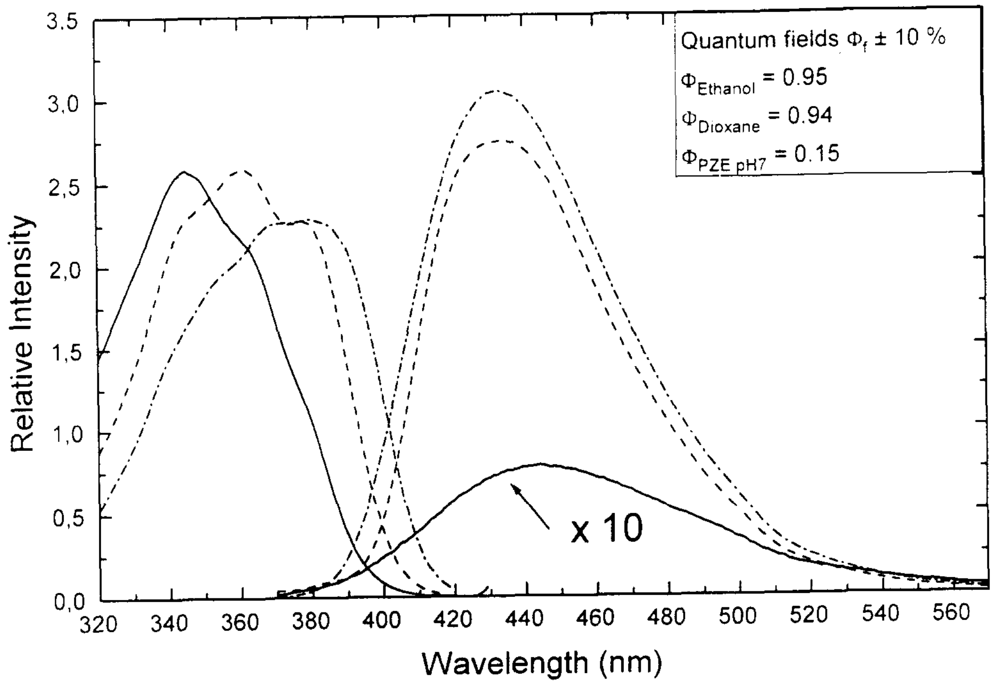

Fig. 3. Fluorescence spectra (excitation wavelength, 355 nm) and excitation spectra (emission wavelength, 440 nm) of 2×10^{-5} M PZE in dioxane (— · —), ethanol (– – –) and phosphate buffer pH 7 (——).

from only one species. In the presence of oxygen, it was quenched with a rate constant of 2.0×10^7 s^{-1}, suggesting that this transient could be attributed to the PZE triplet absorption. The linear dependence of the absorbance variations with the laser fluence (Fig. 4(d)), characteristic of a monophotonic process, agreed with this assignment. In deaerated solutions, the first-order rate constant of ^{3}PZE showed a linear dependence on the PZE ground-state concentration, revealing that ^{3}PZE disappeared by a quenching process with a rate constant $k = (6.0 \pm 0.5) \times 10^{10}$ M^{-1} s^{-1}. The decay of the transient absorbance represented in Fig. 4(A) confirmed this assignment since it showed a further depletion of the ground state below 400 nm.

Sensitization of the β-carotene triplet state [18] was used to confirm that the PZE triplet was generated. Indeed, on addition of 2.8×10^{-5} M β-carotene to 1.4×10^{-4} M PZE in deoxygenated dioxane solution, a build-up in the transient absorption was observed at 520 nm (maximum of absorbance of β-carotene triplet state) compared with PZE alone in the same conditions (Fig. 4(A), inset). The transient absorption decays measured at 520 and 600 nm (a wavelength where the β-carotene triplet state does not absorb), in the absence and presence of β-carotene, led to an energy transfer rate constant of about 7.5×10^{10} M^{-1} s^{-1}. However, the kinetic analysis at 600 nm revealed only a partial transfer between the triplet states.

3.3.2. Ethanol and water

Pulse laser excitation of PZE in deaerated ethanol solution resulted in a transient absorption (Fig. 4(C)) extremely similar to that observed in aqueous solution [8]. In the latter conditions, we have previously established that the broad absorption band with a λ_{max} of about 700 nm corresponded essentially to the e^-_s produced by a biphotonic ionization process. The same behaviour occurred in ethanol solutions. Indeed, in Fig. 4(D), we did not observe any linear dependence of the absorbance changes measured at 700 nm on the laser fluence characteristic of a monophotonic process. Furthermore, the decay rate of the transient absorption band was strongly increased by electron scavengers (N_2O or O_2). The e^-_s and PZE$^{•+}$ yields in ethanol were calculated from the absorption changes at 700 nm at the end of the laser pulse in the presence of e^-_s (deaerated solutions) and in the absence of e^-_s (solution saturated with N_2O) using the extinction coefficient of e^-_s at this wavelength [19]. It was found that 5–10% of the PZE was photoionized in ethanol, a result similar to that obtained in water and consistent with the other two fluorescence and triplet quantum yields.

In N_2O saturated solutions, the shape of the absorption spectrum measured after the solvated electron decay (200 ns) (Fig. 4(C)) was similar to the PZE cation radical spectrum [8]. However, taking into account the molar extinction coefficient [8] and the yield of PZE$^{•+}$, the absorbance was higher than that corresponding to the radical cation alone, revealing that ionization was not the only photochemical process. This other species absorption, quenched by oxygen, could correspond to PZE triplet absorption. Subtraction of the long-lived PZE$^{•+}$ spectrum (Fig. 4(C), spectrum at 40 μs) from the spectrum obtained at the end of the laser pulse (200 ns) led to a spectrum extremely similar to that observed in dioxane or water (Fig. 4(A)) and confirmed the formation of the PZE triplet excited state in ethanol.

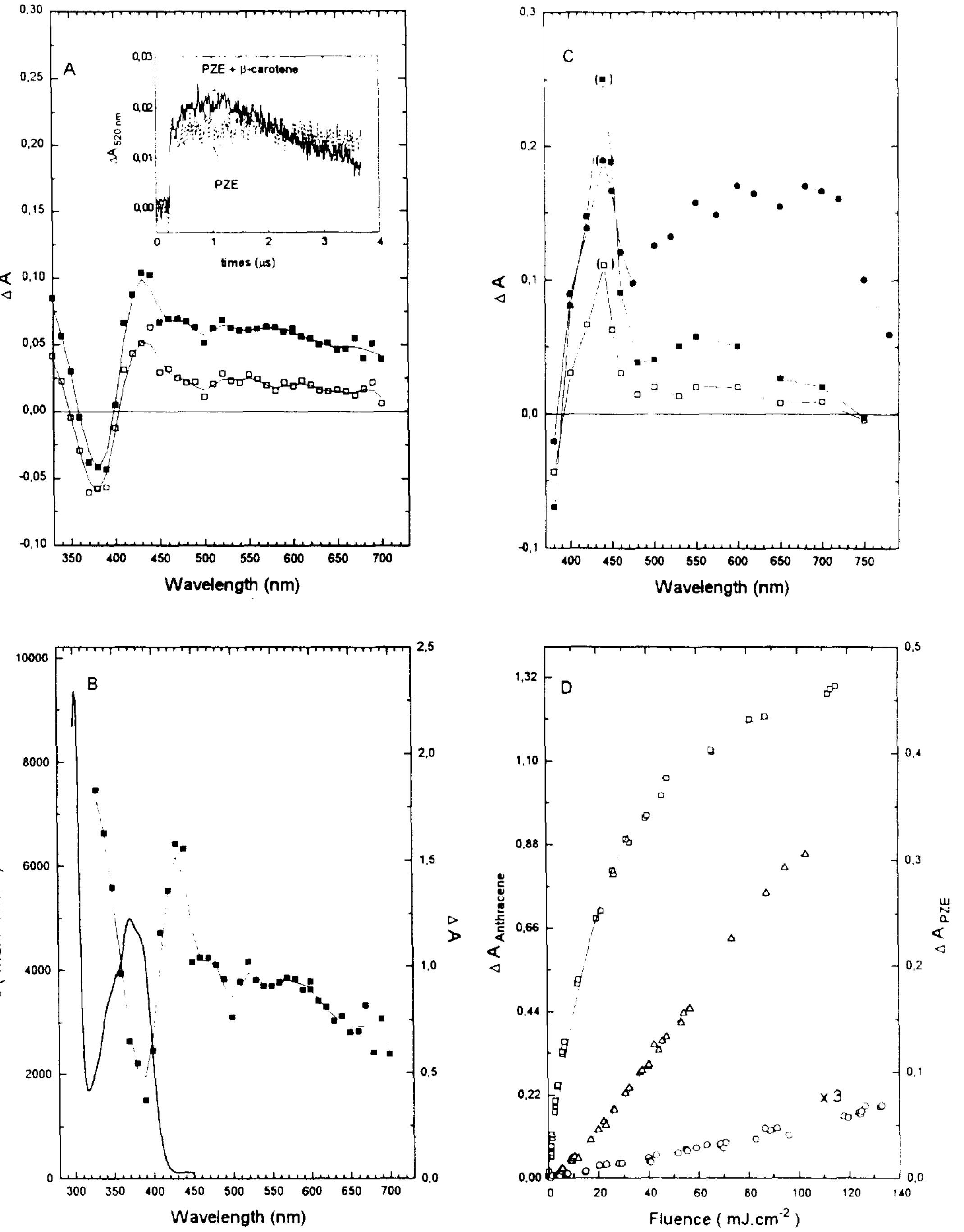

Fig. 4. (A) Transient absorption spectra recorded on 355 nm photolysis of PZE (1.4×10^{-4} M), in deaerated dioxane solution at the end of the laser pulse (■) and at 40 μs after the pulse (□). The inset shows the decay kinetics of triplet state in deaerated dioxane solution at 520 nm in the absence and the presence of β-carotene ($[\text{PZE}]_0 = 1.4\times10^{-4}$ M; $[\beta\text{-carotene}] = 2.8\times10^{-5}$ M). (B) Ground absorption (——) and corrected triplet absorption spectra (■) of PZE in dioxane. (C) Transient absorption spectra recorded on 355 nm photolysis of PZE in deaerated ethanol solution ($[\text{PZE}]_0 = 1.4\times10^{-4}$ M) at the end of the laser pulse (●), in the presence of N_2O at the end of the e^-_s decay (■) and 40 μs after the laser pulse, at the end of the triplet decay (□). Points enclosed in parentheses indicate that the weak fluorescence emission can induce imprecision into the transient absorption measurements at this wavelength. (D) Transient absorption at 422 nm due to the triplet population on laser excitation of 6.5×10^{-5} M anthraceneincyclohexane plotted against the laser fluence (□), transient absorbance at 550 nm due to the triplet population of 1.4×10^{-4} M PZE in dioxane (○) and transient absorbance at 600 nm due to triplet, e^-_s and $PZE^{\cdot+}$ formation on excitation of 1.4×10^{-4} M PZE in ethanol (△).

Kinetic analysis revealed that ^{3}PZE in deoxygenated ethanol solutions loses its energy through quenching by ground state with a rate constant similar to that measured in dioxane. Our previous results [8] obtained in aqueous solution showed that the microsecond absorption evolved with second-order kinetics, a consequence of the high laser energy employed. Additional experiments using a lower laser fluence gave the same kinetics as in dioxane and ethanol. The kinetic evolution of ^{3}PZE in ethanol excluded the possible reaction of the excited state with the abstractable hydrogen atoms of the

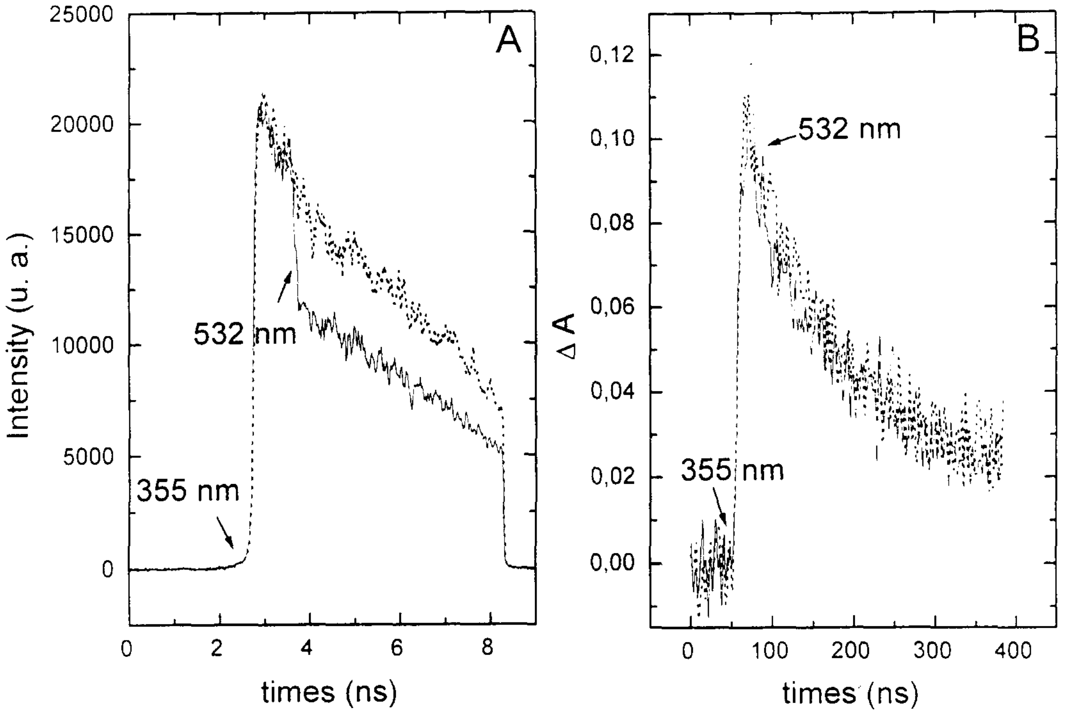

Fig. 5. (A) Fluorescence decay curves at 440 nm with the 30 ps laser pulse: (- - -), 355 nm alone; ——, 355 + 532 nm delayed by 800 ps. (B) Absorption decay curves at 700 nm with the 3 ns laser pulse: - - -, 355 nm alone; ——, 355 + 532 nm delayed by 30 ns.

solvent, to give a semiquinone-type free radical as was observed for other antitumor drugs [20].

3.3.3. Molar triplet absorption coefficients and triplet quantum yield determination

Determination of the molar absorption coefficients ϵ_T of PZE triplet was undertaken in dioxane since no photoionization process occurred in this solvent. As reported above, β-carotene did not quench efficiently the PZE triplet state of PZE, so that the energy transfer method could not be used to determine ϵ_T. As was observed in Fig. 4(D), experimental complete conversion of the PZE ground state to triplet state was not achieved but could be simulated on multiplication of the transient absorption measurements by an appropriate coefficient x. Different values of x were tried and thus the resulting absolute spectrum of the triplet was compared with that of the ground state of the molecule. On the assumption that the triplet spectrum was different from that of the ground state between 350 and 400 nm, acceptable x values were obtained between 12 and 17 (Fig. 4(B)). In these conditions, the ϵ_T precision was about 20%. Since the triplet absorption profiles of PZE were found to be very similar in dioxane, ethanol and water and since the oscillator strength was independent of the medium, it was assumed that the triplet extinction coefficients were of the same order of magnitude in the different solvents.

The quantum yield Φ_T of singlet–triplet intersystem crossing for 355 nm excitation was also measured for PZE in dioxane. The comparative method [18] was employed using anthracene in cyclohexane as the actinometer:

$$\Phi_T(\mathrm{PZE}) = \Phi_T(\mathrm{anthracene})\,\frac{(A_T/E)^{\mathrm{PZE}}}{(A_T/E)^{\mathrm{anthracene}}} \times \frac{\epsilon_T^{\mathrm{anthracene}}}{\epsilon_T^{\mathrm{PZE}}}\,\frac{A_S^{\mathrm{anthracene}}}{A_S^{\mathrm{PZE}}}$$

where $A_T^{\mathrm{anthracene\ and}}\,A_T^{\mathrm{PZE}}$ were the maximum absorbances for anthracene and PZE triplet absorption respectively, E^{PZE} and $E^{\mathrm{anthracene}}$ the excitation fluences, $\epsilon_T^{\mathrm{anthracene}}$ and $\epsilon_T^{\mathrm{PZE}}$ the triplet extinction coefficients, and $A_S^{\mathrm{anthracene\ and}}\,A_S^{\mathrm{PZE}}$ the absorbances of the ground state at the laser excitation wavelength. This method of determining quantum yields is valid if only a small fraction of the molecules are excited, so that the absorbance obtained remains linear with the laser energy; in these measurements, less than 10% of the PZE and anthracene molecules were converted into the triplet state. Furthermore, the anthracene concentration was chosen to give the same absorbance at 355 nm as that of PZE. The triplet concentration of anthracene was monitored at its absorption maximum (422 nm) using a triplet extinction coefficient of $6.47 \times 10^4\ \mathrm{M^{-1}\ cm^{-1}}$ [18]. The PZE triplet concentration was monitored at 550 nm using the triplet extinction coefficient previously determined ($\epsilon_T = (3.6+0.6) \times 10^3\ \mathrm{M^{-1}\ cm^{-1}}$). With $\Phi_T^{\mathrm{anthracene}} = 0.71$ [18], we obtained a value for the PZE triplet quantum yield Φ_T of $(5.2 \pm 1.0) \times 10^{-2}$.

3.4. Photoionization mechanism

3.4.1. Double-pulse experiments

The observation of a photoionization process under excitation of PZE in ethanol or buffer aqueous solutions raised the question of the nature of the intermediates involved in the

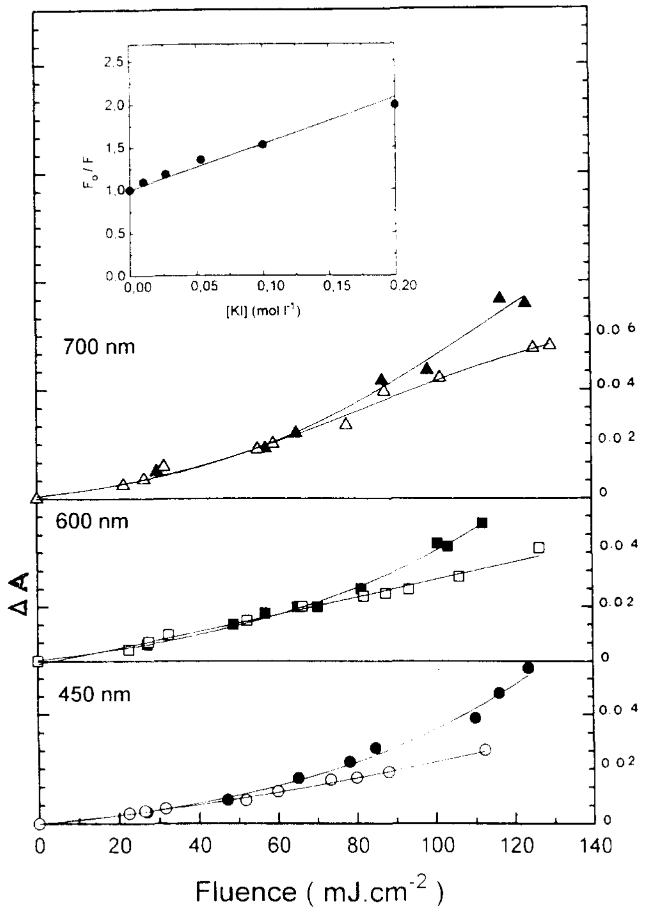

Fig. 6. Effect of 0.1 M KI on the transient absorbance measured at different wavelengths as a function of the laser fluence ([PZE] = 1.4×10^{-5} M in buffer aqueous solution): △, □, ○, without KI; ▲, ■, ●, with KI. The inset shows the Stern–Volmer representation of the PZE fluorescence quenching at 440 nm as a function of KI concentration ([PZE] = 1.8×10^{-5} M).

photoionization mechanism: the lowest triplet state T_1, the first excited state S_1 or both states. At the second-harmonic wavelength (532 nm) of the laser, the fundamental state of the antitumoral molecule does not absorb. Therefore, in the mixed harmonic excitation experiments, only S_1–S_n and/or T_1–T_n transitions may be induced.

Direct evidence of the S_1 state as the intermediate for biphotonic PZE radical formation was given by comparing the fluorescence signals after single-harmonic (355 nm) and mixed-harmonics excitation (355 nm + 532 nm). As shown in Fig. 5(A), an appreciable decrease in fluorescence was observed at the arrival of the 532 nm excitation when the latter was delayed by 800 ps. During the 30 ps mixed-harmonics laser excitation, the triplet population is quite small compared with the singlet-S_1-state population since the intersystem crossing time constant is only about 5 ns (unpublished result). The PZE free radical formation on mixing the 355 nm and 532 nm laser pulses thus obviously proceeded by excitation of the S_1 state at 532 nm.

Excitation of the triplet T_1 with the second harmonic of the laser was not found to lead to an increase in PZE ionization. Indeed, no modification in the transient absorption was observed on excitation by the 532 nm pulse when delayed by about 30 ns with respect to the 355 nm pulse (Fig. 5(B)). 30 ns after the 355 nm excitation, only the excited state T_1 remains present in the solution ($\tau_{S_1} = 5$ ns). This result attested to the fact that the two-photon dissociation of PZE did not proceed via T_1 as the intermediate.

3.4.2. Photoionization efficiency and fluorescence quenching

To verify the relative contribution of S_1 and T_1 to the photoionization mechanism, experiments in which the intersystem crossing was enhanced by the addition of KI were carried out using the 3 ns pulse. KI is known to lower the selection rule barrier for S_1–T_1 intersystem crossing. Furthermore, KI is unreactive towards e^-_s. The acid–base reactions of KI were avoided in the present study by carrying out the experiments at pH 10.1. For higher pH, at the concentrations used in the absorption experiments, PZE precipitates. The concentration effect of KI on the fluorescence decay of PZE was monitored at 440 nm maximum emission wavelength. The quenching rate constant was determined using the Stern–Volmer relation (Fig. 6, inset). The value obtained, $k_q = 8 \times 10^9$ M^{-1} s^{-1} was consistent with a diffusion-controlled reaction. Fig. 6 represents the effect of 0.1 M KI on the transient absorbance measured at the end of the laser pulse compared with PZE alone in deoxygenated aqueous solution. The results showed that, at different wavelengths, the absorption relative to the triplet, e^-_{aq} and PZE$^{\bullet +}$ concentrations increased by a factor about 1.3 while the fluorescence was reduced by about 1.5. A pathway involving only T_1 in the photoionization process would show an increase in the transient absorption depending on the wavelength since the e^-_{aq} extinction coefficient values vary by a factor of about 3 between 450 and 700 nm [19]. In our experiments, the enhancement of the intersystem crossing from S_1 to T_1 led to identical transient absorption variations at all wavelengths, corresponding to an increase in the triplet concentration. This result attested to the fact that T_1 was not implied in PZE photoionization process.

4. Conclusion

This study has revealed that 355 nm photolysis of PZE leads to the triplet formation in the different solvents with a quantum yield of about 5%. Since the fluorescence quantum yield of PZE in aqueous buffer solution is 0.15, it can be concluded that internal conversion from S_1 to S_0 is the most dominant process in the photophysics properties of PZE. However, the relatively low triplet quantum yield does not rule out the involvement of this excited state in the photodynamic properties of the intercalating complex PZE–DNA.

Simultaneously with triplet formation, photoionization of PZE occurred in ethanol and in buffer aqueous solution but not in dioxane. Both methods used in the present work (double excitation and fluorescence quenching) led to the same conclusion, namely laser-induced photoionization of PZE at

355 nm in a two-step process which occurred via the lowest excited singlet state. Since photoionization is observed upon two-photon excitation using both 355 and 532 nm, it is inferred that the ionization potential of PZE in aqueous solution is not higher than about 5.8 eV.

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