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PAPER

Photosensitization of DNA by β-carbolines: Kinetic analysis and photoproduct characterization

M. Micaela Gonzalez,[a] Mariana Vignoni,[b] Magali Pellon-Maison,[c] Matias A. Ales-Gandolfo,[d] Maria R. Gonzalez-Baro,[c] Rosa Erra-Balsells,[a] Bernd Epe*[e] and Franco M. Cabrerizo*[d]

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β-Carbolines (βCs) are a group of alkaloids present in many plants and animals. It has been suggested that these alkaloids participate in a variety of significant photosensitized processes. Despite their well-established natural occurrence, the main biological role of these alkaloids and the mechanisms involved are, to date, poorly understood. In the present work, we examined the capability of three important βCs (norharmane, harmane and harmine) and two of its derivatives (*N*-methyl-norharmane and *N*-methyl-harmane) to induce DNA damage upon UV-A excitation, correlating the type and extent of the damage with the photophysical characteristics and DNA binding properties of the compounds. The results indicate that DNA damage is mostly mediated by a direct type-I photoreaction of the protonated βCs after non-intercalative electrostatic binding. Reactive oxygen species such as singlet oxygen and superoxide are not involved to a major extent, as indicated by the only small influence of D_2O and of superoxide dismutase on damage generation. An analysis with repair enzymes revealed that oxidative purine modifications such as 8-oxo-7,8-dihydroguanine, sites of base loss and single-strand breaks (SSB) are generated by all βCs, while only photoexcited harmine gives rise to the formation of cyclobutane pyrimidine dimers as well.

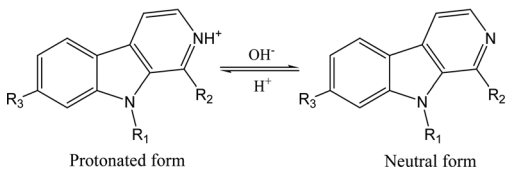
Introduction

β-Carbolines (βCs) are alkaloids belonging to a family of heterocyclic compounds characterized by a 9*H*-pyrido[3,4-*b*]indole ring system. The structures of three βC alkaloids and two of its derivatives are shown in Scheme 1. βC alkaloids are present in a wide range of species. They are found in many plants,[1–4] arthropods[5] and insects.[6] They also occur as minor constituents in foods,[7,8] alcoholic drinks, cigarettes/tobacco smoke, *etc.*[9–11] In mammals, these alkaloids were detected in skin, plasma, platelets and urine (with basal levels ~ 0.1 nmol l^{-1})[8,11,12] After alcohol intake and smoking, basal levels of βCs are considerably increased (*i.e.*, ~ 1 nmol l^{-1}).[12,13]

It has been proposed that some βCs in plants could play a role in the defence response because of their phototoxic effects against insects, webworms, *etc.*[14] Consistent with this assumption, some photoexcited βCs are able to induce chromosome damage in mammalian cells,[15,16] and to inactivate bacteria[17,18] and viruses.[19] Despite their established importance, the main biological role of these alkaloids and the mechanisms involved remain poorly understood.

Recently, the relaxation of supercoiled DNA by norharmane (nHo) under irradiation with UV-A has been demonstrated.[20] The results suggested that the underlying DNA damaging process involves, mainly, electron transfer reactions (*i.e.*, Type I

[a]*CIHIDECAR–CONICET, Universidad de Buenos Aires, Pabellón 2, 3p, Ciudad Universitaria, (1428) Buenos Aires, Argentina*
[b]*INIFTA–CONICET, Universidad Nacional de La Plata, Casilla de Correo 16Sucursal 4, (1900) La Plata, Argentina*
[c]*INIBIOLP–CONICET, Universidad Nacional de La Plata, calle 60 y 120, (1900) La Plata, Argentina*
[d]*IIB–INTECH–CONICET, Universidad Nacional de San Martín, Intendente Marino km 8,2CC 164, (B7130IWA) Chascomús, Buenos Aires, Argentina. E-mail: fcabrerizo@intech.gov.ar*
[e]*Institute of Pharmacy and Biochemistry, University of Mainz, Staudingerweg 5, D-55099 Mainz, Germany. E-mail: epe@uni-mainz.de*



	R_1	R_2	R_3
norHarmane (nHo)	-H	-H	-H
Harmane (Ho)	-H	$-CH_3$	-H
N-Methyl-norharmane (*N*-Me-nHo)	$-CH_3$	-H	-H
N-Methyl-harmane (*N*-Me-Ho)	$-CH_3$	$-CH_3$	-H
Harmine (Ha)	-H	$-CH_3$	$-OCH_3$

Scheme 1 Structures of the five βCs used in this study. The nitrogen of the indolic ring (R_1 = H; nHo, Ho and Ha) has a pK_a value greater than 12.

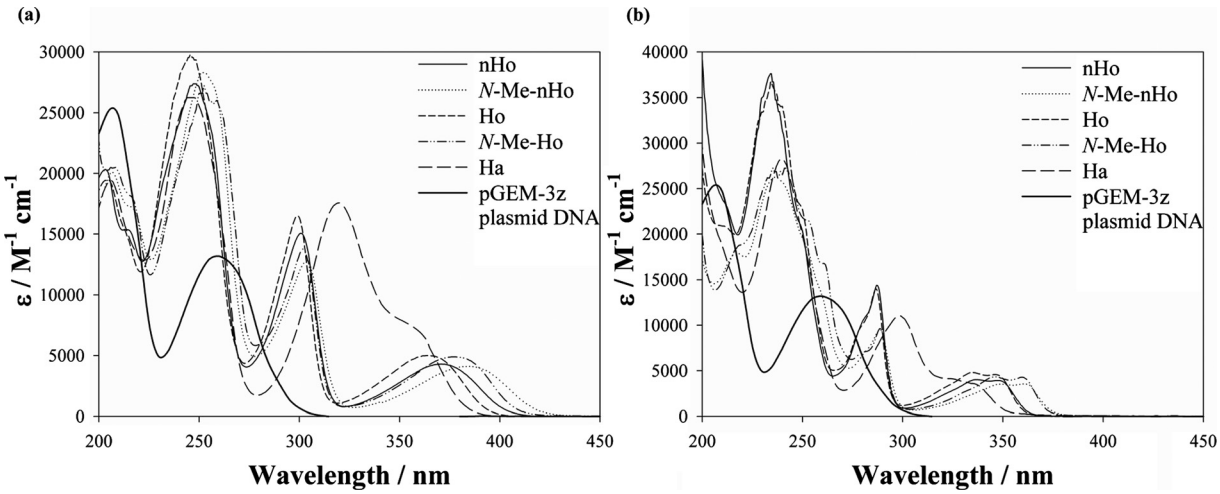


Fig. 1 Absorption spectra of the five βCs studied recorded in air-equilibrated aqueous solutions. **(a)** pH 4.8 and **(b)** pH 10.0.

mechanism). However, no information was obtained about the influence of βC structures on the DNA photodamage. Moreover, the generation of other types of DNA photoproducts formed upon photosensitization was not investigated.

In the present work, we have further analyzed the capability of three βCs [nHo, harmane (Ho) and harmine (Ha)] and two βC derivatives [*N*-methyl-norharmane (*N*-Me-nHo) and *N*-methyl-harmane (*N*-Me-Ho)] to photoinduce DNA damage upon UV-A excitation. Particularly, a comparative analysis was performed to evaluate the type and extent of the DNA damage photoinduced by each βC and to establish the mechanisms involved. In addition, the spectrum of DNA-photoproducts formed upon photosensitization was investigated by experiments using specific DNA repair glycosylases as probes.[21,22]

Experimental

General

β-Carbolines (βCs). Norharmane, harmane and harmine (>98%) from Sigma–Aldrich were used without further purification. The method used to synthesize and purify *N*-methyl-derivatives has been published elsewhere.[23]

DNA material. (*i*) pGEM®-3z plasmid (2,743 bp, Promega) was propagated in *E. coli* JM109 strain and purified from 100 ml culture after 12 h of growth using a commercial kit (Plasmid Maxi kit, Qiagen) following the specifications provided by the company. The purity of the DNA was checked by monitoring the A_{260}/A_{280} ratio. DNA concentrations were determined spectrophotometrically using a molar absorption coefficient of 13 200 M^{-1} cm^{-1} (*i.e.*, an average ε value for a single base pair). (*ii*) DNA from bacteriophage PM2 (PM2 DNA, 10,000 bp) was prepared according to the method of Salditt *et al.*[24]

Enzymes. The restriction endonuclease EcoRI and superoxide dismutase were from Invitrogen. Formamidopyrimidine-DNA glycosylase (Fpg protein)[25] from *E. coli* was kindly provided by S. Boiteux, Fontenay-aux-Roses, France. Endonuclease IV was a gift of B. Demple, Stony Brook, N.Y., U.S.A. T4 endonuclease V was partially purified from an inducible overproducer (*E. coli* strain A 32480 carrying the plasmid ptac-denV) provided by L. Mullenders, Leiden, Netherlands. All repair endonucleases were tested for their incision at reference modifications (*i.e.*, thymine glycols induced by OsO_4, AP sites by low pH and pyrimidine dimers by UV^{254}) under the applied assay conditions (see below) to ensure that the correct substrate modifications are fully recognized and no incision at non-substrate modifications takes place.[26]

pH adjustment. The pH of the βC aqueous solutions was adjusted by adding drops of aqueous NaOH or HCl solutions (concentration ranged from 0.1 M to 2.0 M) with a micropipette. The ionic strength was approximately 10^{-3} M in all the experiments. In experiments using D_2O as solvent, D_2O (> 99.9%; Sigma), DCl (99.5%; Aldrich) in D_2O, and NaOD (Aldrich) in D_2O were used.

DNA damage generation in plasmid pGEM®-3z by photoexcited βCs: Kinetic analysis

Irradiation set-up I. Aqueous solutions containing a mixture of the photosensitizer (βC, Fig. 1) and the target molecule (plasmid DNA at 15.5 μg ml^{-1}) were irradiated, in the presence of air, in a quartz cell (0.4 cm of path length) at room temperature with a Rayonet RPR lamp (Southern N.E. Ultraviolet Co.) emitting at 350 nm (bandwidth ~ 20 nm). Incident photon flux (P_0) at the excitation wavelength was 3.2×10^{-4} Einstein L^{-1} min^{-1}. The method for the determination of P_0 has been described in detail elsewhere.[27,28] To give a crude estimate of the photon flux absorbed by the sensitizer, P_a, in the respective experiments, we used the following expression:

$$P_a = P_0\ (1\ -\ 10^{-A}) \qquad (1)$$

where A is the absorbance of the photosensitizer at 350 nm. For better comparison, all the experiments were performed using solutions of the photosensitizers with the same absorbance at 350 nm (*i.e.*, $A = 0.3$). Thus, the steady-state concentration of the electronic excited states of the βCs ([βCs]*) were the same in the kinetic experiments performed for all the βCs under all pH conditions. It is noteworthy that matching the absorbance at 350 nm does not introduce huge differences in the βCs

Table 1 Rate of DNA relaxation in air-equilibrated aqueous solution, upon UV-A excitation, at different pH

Entry	Reaction conditions	[a]d[Sc]/dt (NI min^{-1}) pH 4.7	[a]d[Sc]/dt (NI min^{-1}) pH 10.2
1	Ho	$-8.1\ (\pm 0.8) \times 10^{-3}$	$-2.4\ (\pm 0.8) \times 10^{-3}$
2	nHo	$-8.2\ (\pm 0.9) \times 10^{-3}$	$-2.3\ (\pm 0.5) \times 10^{-3}$
3	*N*-Me-Ho	$-14\ (\pm 1) \times 10^{-3}$	$-8.6\ (\pm 0.7) \times 10^{-3}$
4	*N*-Me-nHo	$-15\ (\pm 1) \times 10^{-3}$	$-14\ (\pm 1) \times 10^{-3}$
5	Ha	$-35\ (\pm 4) \times 10^{-3}$	—[b]

[a] Averaged d[Sc]/dt value obtained from three independent sets of experiments. [b] Because of the very low ε at 350 nm and the extremely low solubility of Ha in alkaline water, the experiment with this βC could not be done under this pH condition.

concentrations. *i.e.*, βC concentrations ranged from ~80 to ~130 μM. Due to the quite high difference in its absorption coefficient (ε) harmine (Ha) represents an exception, *i. e.*, in acidic and alkaline solution Ha concentration would be of 38 and 366 μM, respectively. However, no experiments with Ha in alkaline solution were performed (see comment in Table 1). On the other hand, the low Ha concentration used under acidic conditions did not affect the data analysis and conclusions.

Electrophoretic analysis of the photosensitized cleavage of pGEM®-3z plasmid. After exposure to light at variable time intervals, samples of 10 μl were taken and 2 μl of loading buffer (Tris 0.04 M, acetate 0.02 M, EDTA 1 mM, 0.25% bromophenol blue, 30% glycerol, pH 7.2) were added to the reaction mixture. The samples were analyzed by 0.8% agarose gel electrophoresis in TBE buffer (Tris 0.05 M; Boric acid 0.05 M; EDTA 1 mM). DNA was visualized with ethidium bromide (Sigma, 0.5 μg ml^{-1}) under UV illumination.

After electrophoresis, all gels were stained and photographed on a UV transilluminator (Hoefer MacroVue UV-20) using a digital camera KD 120 (Kodak). The intensities of the bands were scanned and integrated with the Kodak Digital Science 1D software. In order to compare different lanes of the same or different gels, a normalization procedure was adopted: the intensity of each band was divided by the sum of the intensities of all bands in the same lane yielding the normalized intensity (NI).[29] The lesser intercalation of ethidium into supercoiled (Sc) DNA was taken into account by dividing the corresponding intensity by 0.8.[30] The initial relaxation rate of Sc DNA (d[Sc]/dt) was calculated from the slope of the plot of the NI of Sc form *vs.* irradiation time.

Thermal reactions between the βC and DNA were excluded by control experiments in which solutions containing the mixture were incubated in the dark under different experimental conditions (concentration, pH and time).

Analysis of the contribution of ROS in the photocleavage of plasmid DNA

To test for the participation of ROS, d[Sc]/dt values were determined in the absence and presence of selective scavengers as follows: (*a*) For *hydroxyl radical* (*HO*$^{\cdot}$) *scavenging*, solutions containing a βC and plasmid DNA were irradiated in the presence of 50 mM propan-2-ol.[31] (*b*) For eliminating *superoxide* ($O_2^{\cdot -}$), solutions containing a βC and plasmid DNA, at a given pH (*i.e.*, 4.7 and 10.2) were irradiated in the presence of 100 U mL^{-1} of superoxide dismutase (SOD). (*c*) To test for an involvement of *singlet oxygen* ($^1O_2$), solutions containing a βC and plasmid DNA were prepared in H_2O and D_2O, and the extent of DNA damage generated upon irradiation was compared for the two solvents. In another type of assay, photolysis was carried out in the presences of 1 mM furfuryl alcohol (Riedel-de Haën) as a scavenger for $^1O_2$.[32]

DNA binding studies

UV-vis spectrophotometric analysis. The interaction of βCs with pGEM®-3z was studied by UV-vis spectrophotometric titration. The spectra were recorded on a Perkin-Elmer Lambda25 UV/VIS spectrometer. Measurements were made in quartz (QS) cells of 10 × 2 mm light path and 100 μL of volume (Hellma, type 105.250-QS), at room temperature. The βC acidic (pH 4.4) solutions were prepared in acetic acid-sodium acetate buffer, and the alkaline solutions (pH 10.9) were prepared in K_2HPO_4–KOH buffer. Experimental difference (ED) spectra were obtained by subtracting the spectrum at 0 μM of DNA from the subsequent spectra recorded at different DNA concentration.

The cuvette initially containing 100 μL of a ~100 μM βC buffered solution was progressively titrated by increasing amounts of plasmid pGEM®-3z solution (*i.e.*, from 0 to 200 μM in bp). The absorption spectra obtained were corrected by the corresponding dilution factor. Assuming a 1 : 1 stoichiometry for complexes, the association constant (K_G) can be estimated by using the Benesi–Hildebrand equation (eqn (2)):

$$\frac{1}{\Delta A} = \frac{1}{(\varepsilon_{\beta C \bullet B} - \varepsilon_{\beta C})} \bullet \frac{1}{[\beta C]_o} + \frac{1}{K_G \bullet (\varepsilon_{\beta C \bullet B} - \varepsilon_{\beta C}) \bullet [\beta C]_o} \bullet \frac{1}{[B]} \qquad (2)$$

where $\varepsilon_{\beta C \bullet B}$ and $\varepsilon_{\beta C}$ are the molar absorption coefficients of the βC-DNA complex (βC•B) and βC, respectively, at the titration wavelength. ΔA is the change of absorbance relative to the completely free βC at this wavelength.

Fluorescence measurements. (*i*) Steady-state fluorescence measurements were performed using a Perkin–Elmer LS 50B spectrofluorometer. The fluorescence intensity (I) was obtained by integration of the corrected fluorescence spectra over the entire emission profile. (*ii*) Time-resolved fluorescence measurements were performed using a single-photon-counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon). A NanoLED source (maxima at 341 nm) was used for excitation. The emitted photons, after passing through the iHR320 monochromator, were detected by a TBX-04 detector connected to a TBX-PS power supply and counted by a FluoroHub-B module, controlled using

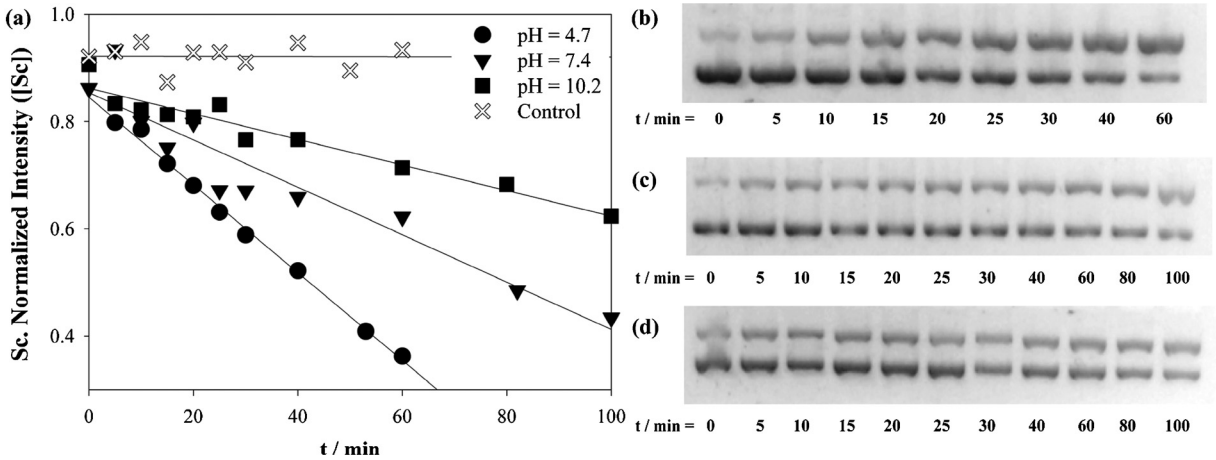


Fig. 2 **(a)** Representative data showing the pH dependence of the normalized intensity of the pGEM-3z Sc form as a function of the irradiation time, in the presence of harmane. For each pH-condition, the absorbance at 350 nm was matched at 0.3. Crosses represent data obtained from DNA solutions irradiated in the absence of Ho (*i.e.*, control experiments). **(b)**, **(c)** and **(d)** show representative electrophoretic runs of the experiments performed at pH 4.7, 7.4 and 10.2, respectively.

the DataStation measurement control software application. The selected counting time window for the measurement reported in this study was 0–200 ns. Emission decays were monitored at 460 nm. Lifetimes were obtained from the monoexponential decays observed after deconvolution from the lamp background signal. (*iii*) Fluorescence measurements were recorded in quartz (QS) cells of 10 × 2 mm light path and 100 µL of volume (Hellma-type 105.250-QS), at room temperature. For determining the quenching of fluorescence of each βC by plasmid DNA, emission spectra and/or fluorescence lifetime of each alkaloid solution (20 µM) was recorded in the absence and in the presence of pGEM®-3z plasmid (*i.e.*, from 0 to 200 µM in bp). The experiments were performed under both pH conditions (*i.e.*, at pH 4.7 and 10.9) in aqueous buffered solutions using the same buffers as those for UV-vis analysis. Data analysis was performed as it was previously described.[33,34]

Characterization of the spectrum of DNA modifications induced by photoexcited βCs

Irradiation set-up II. Buffered aqueous solutions (10 mM KH_2PO_4, 50 mM NaCl, pH 7.4) of a βC and PM2 DNA (at 10 µg ml^{-1}) were irradiated for 20 min on ice in a 96 well-plate with a Philips HPW 125 W lamp emitting at 365 nm (bandwidth ~20 nm), placed at a distance of 10 cm. After treatment, the DNA was precipitated by ethanol/sodium acetate and re-dissolved in BE1 buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) for damage analysis.

Quantification of endonuclease-sensitive modifications in PM2 plasmid. The DNA relaxation assay used to quantify endonuclease-sensitive modifications (ESS) and single-strand breaks (SSB) in PM2 DNA has been described earlier[35] and we have recently reviewed its application to characterize the DNA damage induced by photosensitization.[36] It makes use of the fact that supercoiled PM2 DNA is converted by either a SSB or the incision of a repair endonuclease into a relaxed (nicked) form, which migrates separately from the Sc form in agarose gel electrophoresis.

An aliquot of 0.2 µg of the modified DNA in 20 µL BE1 buffer was incubated for 30 min at 37 °C with 10 µL of BE1 buffer (for the determination of directly produced SSB) or of one of the following repair endonuclease preparations: (*i*) Fpg protein, 3 µg ml^{-1} in BE1 buffer, (*ii*) endonuclease IV, 0.3 µg ml^{-1} in BE1 buffer and (*iii*) T4 endonuclease V, 3 µg ml^{-1} in BE15 buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 15 mM EDTA). The reactions were ended by addition of 10 µl of stop buffer with 2% sodium dodecyl sulfate and the DNA applied to an agarose electrophoresis gel. Fluorescence scanning of the Rel and Sc forms of the DNA after staining with ethidium bromide allowed calculating the number of SSB or, if an incubation with a repair endonuclease preceded the gel electrophoresis, the number of SSB plus ESS. To obtain damage profiles, the data were corrected for the number of modifications in unmodified DNA, and the number of ESS were obtained by subtraction of the number of SSB.

Results and discussion

Kinetic analysis of the photosensitized cleavage of pGEM®-3z DNA by βCs

In aqueous solutions, βCs behave as weak acids, showing one dominant acid–base equilibrium, over the pH-range 4–11, with a characteristic pK_a value ~7 (Scheme 1).[37,38] In order to establish the role of protonation on the generation of photodamage, air-equilibrated aqueous solutions of a βC and supercoiled (Sc) DNA (plasmid pGEM®-3z) were irradiated with UV-A (350 nm) (irradiation set-up I) for various periods of time, under different pH conditions.[39] DNA absorption was negligible at the excitation wavelength (Fig. 1). The generation of the single- and double-strand breaks (conversion of the supercoiled form of the plasmid into the relaxed (Rel) and linear form, respectively) was monitored by agarose gel electrophoresis. For comparative reasons, all the experiments were performed using βCs solutions with the same absorbance at the excitation wavelength (see the Experimental section).

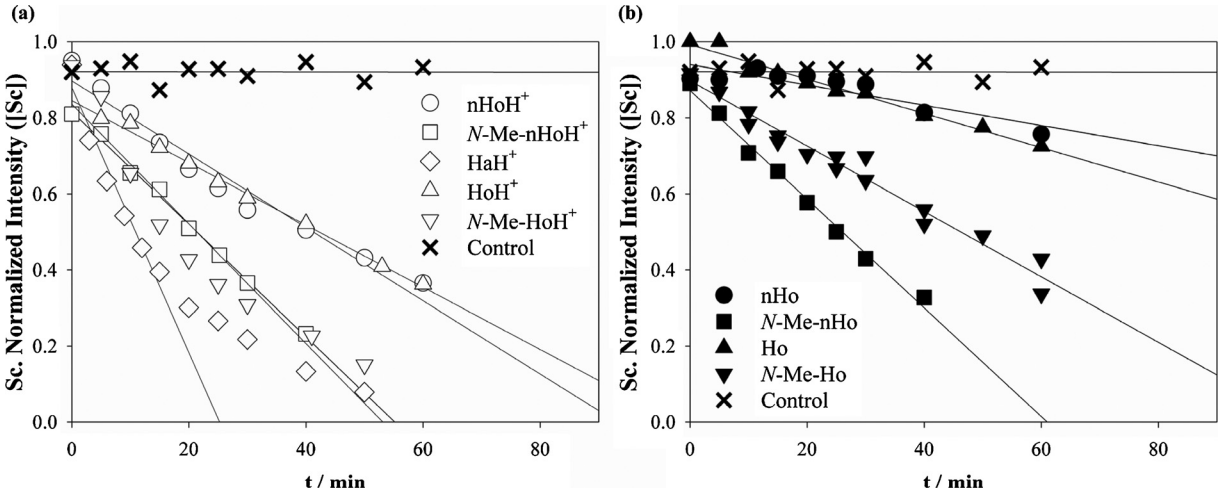


Fig. 3 Representative data showing the evolution of the normalized intensity of pGEM-3z Sc form as a function of the irradiation time, using the five βCs listed in Scheme 1 as photosensitizers. Experiments performed at **(a)** pH 4.7 and **(b)** pH 10.2.

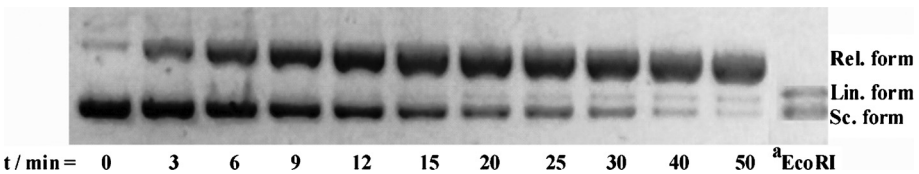


Fig. 4 Agarose gel analysis of pGEM-3z after irradiation with UV-A ($P_0 = 3.2 \times 10^{-4}$ einstein L^{-1} min^{-1}) in the presence of HaH^+ (95 μM) as photosensitizer for the indicated times. Representative experiments performed in air-equilibrated aqueous solution, at pH 4.7. [a] pGEM-3z linearized (Lin) with EcoRI.

Fig. 2 depicts the photocleavage of supercoiled pGEM®-3z plasmid by photoexcited harmane at three different pH values. A decrease of the Sc form as a function of irradiation time was observed in all cases. Clearly, the Sc form of the plasmid is converted exclusively into the Rel form, *i.e.*, double strand breaks are not generated. At pH 4.7, during the first 100 min of irradiation, the rate of Sc form disappearance (d[Sc]/d*t*) was calculated to be $-8.1 \pm 0.8 \times 10^{-3}$ NI min^{-1}. This value is the same, within the experimental error, to that obtained for Rel form appearance (d[Rel]/d*t* = 8.1 (± 0.8) × 10^{-3} NI min^{-1}). At higher pH values, lower DNA damage was observed (d[Sc]/d*t* = $-4.5 (\pm 0.5) \times 10^{-3}$ NI min^{-1} at pH 7.4 and $-2.4 (\pm 0.8) \times 10^{-3}$ NI min^{-1} at pH 10.2, respectively). In all cases, the increase in the relaxed (Rel) form concentration correlated very well with the decrease in the [Sc]. The same tendency was observed for the other βCs investigated (results not shown). Under all pH conditions evaluated, d[Sc]/d*t* matched with (d[Rel]/d*t*) following the SSB model described above. This kinetic pattern has already been described previously for the case of norharmane and its *N*-methyl derivative.[20] In brief, the data confirm that photocleavage by βCs follows the SSB model suggested by Kishikawa *et al.*,[40] which assumes that photocleavage occurs essentially by producing randomly distributed SSB.

The strong pH dependence of the photocleavage can be explained by taking into account the very distinctive behavior of the electronic excited state of βCs in aqueous solution.[20,41–43] In brief, upon excitation, the basic character of the βC pyridine nitrogen is greatly enhanced. Thus, in the whole pH range investigated, excited βC species are readily protonated.[44] Consequently, all the photochemical, photophysical and photosensitized processes of βCs occur mainly from the excited states of their protonated forms ($[\beta CH^+]^*$).[41–43] Note that in alkaline aqueous solutions only a fraction of the excited singlet state (S_1) of the neutral form of each βC becomes protonated.[45] Therefore, under those pH conditions where the neutral form is dominating in its electronic ground state (*i.e.*, at pH 7.4 and 10.2 where neutral βCs are present in ~50% and ~100%, respectively) the yields of the photochemical processes, including photoproduct formation, are lower than in acidic media.[46] In addition, a pH-dependence on the interaction between βCs and DNA would explain the trend observed above (see below).

To further evaluate the dependence of the DNA damage on the chemical nature of the βC, a new set of comparative experiments using five βCs (Scheme 1) as photosensitizers were carried out. βC solutions, with the same absorbance at 350 nm at a given pH, were irradiated in the presence of DNA. Under both, acidic and alkaline solution, a clear dependence of the DNA photocleavage on the chemical structure of the βCs was observed (Fig. 3). Looking at the corresponding d[Sc]/d*t* values (Table 1, entries 1 to 5), the following trend is observed: nHo ~ Ho < *N*-Me-nHo ~ *N*-Me-Ho ≪ Ha.

The highest extent of the DNA damage produced by Ha is also evidenced in Fig. 4, where the appearance of a third band corresponding to the linear form of pGEM®-3z is observed at longer irradiation times. This fact would explain the non-linear kinetic observed in Fig. 3. On the other hand, this result also

Table 2 Rate of DNA relaxation in air-equilibrated aqueous solution, upon UV-A excitation at different pH conditions and in the presence of a given ROS scavenger

Entry	Reaction conditions	d[Sc]/dt^a (NI min^{-1}) pH 4.7	d[Sc]/dt^a (NI min^{-1}) pH 10.2
1	Ho (in H_2O)	$-8.2 (\pm 0.8) \times 10^{-3}$	$-2.4 (\pm 0.8) \times 10^{-3}$
2	Ho + SOD (in H_2O)	$-9.0 (\pm 0.6) \times 10^{-3}$	$-2.2 (\pm 0.5) \times 10^{-3}$
3	Ho + propan-2-ol (in H_2O)	$-5.4 (\pm 0.8) \times 10^{-3}$	$-1.0 (\pm 0.9) \times 10^{-3}$
4	Ho + furfuryl alcohol (in H_2O)	$-8.7 (\pm 0.8) \times 10^{-3}$	$-2.6 (\pm 0.5) \times 10^{-3}$
5	Ho (in D_2O)	$-10.2 (\pm 0.9) \times 10^{-3}$	$-1.1 (\pm 0.9) \times 10^{-3}$

[a] Averaged d[Sc]/dt value obtained from three independent sets of experiments.

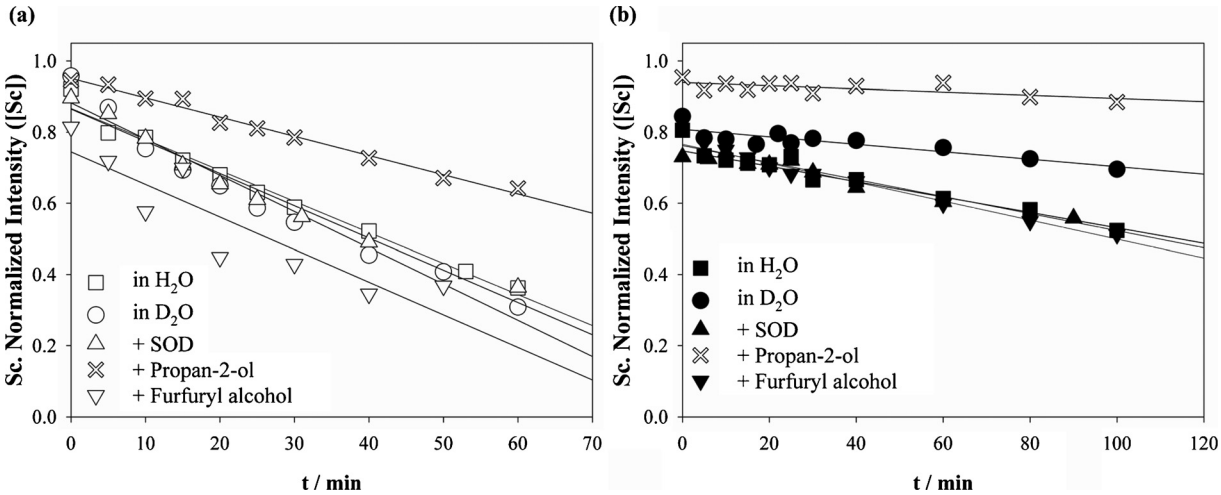


Fig. 5 Representative data showing the evolution of the normalized intensity of the pGEM-3z Sc form as a function of irradiation time. Experiments were performed in harmane (Ho) air-equilibrated aqueous solution, in the presence of different selective ROS scavengers: **(a)** at pH 4.7 and **(b)** at pH 10.2.

supports the hypothesis that the DNA damage photoinduced by Ha follows the SSB model suggested by Kishikawa *et al.*[40]

The data described above are summarized in Table 1. The observed differences between the compounds can be a consequence of many facts, such as: (*i*) a difference in the capability of each βC to produce ROS upon UV-A excitation, (*ii*) a difference in the binding affinity between the alkaloids and the DNA molecule and/or (*iii*) the presence of additional chemical pathways, such as thymidine dimer formation. These hypotheses will be further discussed below.

Role of reactive oxygen species (ROS) in the photocleavage pathways of pGEM®-3z

A very recent quantitative study shows that, in aqueous solution, photoexcited βCs have a very low efficiency to generate ROS.[47] Moreover, we could already demonstrate that ROS are not involved in the DNA relaxation induced by one particular βC, *i.e.* nHo.

In order to test the involvement of ROS in the case of the other βCs, acidic and alkaline experiments were run in the presence and in the absence of selective ROS scavengers. Fig. 5 shows, as an example, the kinetics of Sc DNA relaxation caused by Ho upon UV-A excitation, under different experimental conditions. Table 2 summarizes the d[Sc]/dt values obtained. The experiments carried out in the presence of SOD ($O_2^{\cdot -}$ scavenger), propan-2-ol ($HO^{\cdot}$ scavenger) or furfuryl alcohol ($^1O_2$ scavenger) gave the same results, within the experimental error, as those carried out in the absence of scavengers (see Table 2, entries 1 to 4). These results suggest that the three ROS investigated, albeit present in the reaction mixture, do not participate significantly in the photoinduced DNA relaxation process.

$^1O_2$ is an important oxidizing intermediate in chemical processes and one of the main chemical species responsible for the damaging effects of light on biological systems (photodynamic effects).[48] Although the quantum yields of $^1O_2$ production (Φ_Δ) by βCs in aqueous solutions are generally poor (Φ_Δ = 0.08–0.20),[41,42] and strand breaks are known to be only a minor product in the spectrum of DNA modifications generated by $^1O_2$,[36] the amount of $^1O_2$ generated under our experimental conditions could be enough to induce the DNA photocleavage. Although experiments performed in the presence of furfuryl alcohol suggest that $^1O_2$ has no significant effect on the DNA relaxation, this fact was further substantiated by comparative photolysis in H_2O and D_2O. Due to the longer $^1O_2$ lifetime in D_2O than in H_2O[49] the DNA relaxation should be up to ~16 times faster in D_2O if $^1O_2$ would be involved.

Air-equilibrated H_2O and D_2O solutions of DNA in the presence of a βC were irradiated. The absorbance at 350 nm of both solutions were matched at 0.3. Experiments were run under acidic and alkaline conditions (*i.e.*, pH/pD of 4.4–4.8 and 10.0–10.5, respectively). Electrophoretic analysis showed the

same d[Sc]/dt values, within the experimental error (see Table 2, entries 1 and 5). Only in the case of experiments performed at pH 4.7 was a slight increase in the rate of DNA relaxation observed (*i.e.*, d[Sc]/d$t_{(D_2O)}$ was 1.3 times higher than d[Sc]/d$t_{(H_2O)}$). Therefore, $^1O_2$ is not an important intermediate on DNA photocleavage, neither in acidic nor in alkaline media.[50]

For a given pH condition, a quite similar quantum yield of H_2O_2 and $^1O_2$ production values (Φ_{H2O2} and Φ_{Δ}^{air}, respectively) have been determined for all βCs under study except HaH^+ for which the quantum yields were ~2 times higher.[47] Therefore, differences in the ROS production cannot explain the differences in the yields of DNA damage shown in Fig. 3, in particular in the cases of the *N*-methyl derivatives. Therefore, other factors apart from ROS participation should be taken into account to explain these results (see below).

Interaction between βCs and pGEM®-3z plasmid: spectroscopic studies

The interaction between several βCs and DNA has been investigated.[20,51–53] Although very well performed, all these previous studies refer to quite narrow group of βC not including derivatives such as *N*-methyl-βC. Furthermore, most of these studies have been performed under solvents and pH conditions rather different to those used in the present work. In this context, we decided to examine systematically the βC-DNA interaction dependence on the pH and on the chemical nature of the βC by UV-vis absorption and fluorescence spectroscopy.

UV-vis absorption spectroscopy. The binding strength of both acidic and basic forms of each βC derivative to plasmid pGEM®-3z was analyzed by recording the UV-vis absorption spectra of βCs acidic and alkaline aqueous solution in the presence of increasing amounts of DNA (spectrophotometric titration). Despite the minor changes observed, all βCs studied showed the same behavior to that previously described for nHo.[20] In short, a clear dependence on the pH exists. A representative experiment is displayed in Fig. 6, for the Ho/pGEM®-3z system. In the presence of increasing amounts of DNA the spectra of HoH^+ (pH 4.4) showed small changes accompanied by the appearance of isosbestic points at 358 and 374 nm (inset in Fig. 6a). This fact indicates an interaction between HoH^+ and plasmid DNA in their electronic ground states. On the contrary, the spectra of the neutral form of this alkaloid (pH 10.9) showed a very small increase in the band intensity (see Fig. 6b). However, neither red shift nor isosbestic points were observed, suggesting that the neutral alkaloid molecule has only a negligible interaction with plasmid DNA.

The association constant (K_G) between βCsH^+ and plasmid DNA was evaluated by UV-vis spectrophotometric titration using the Benesi–Hildebrand eqn (2). A representative plot of data for HoH^+/DNA at pH 4.4 showed a linear dependence with the DNA concentration (Fig. 6c). K_G values were obtained from the slope and the intercept of these plots (Table 3). Although high error values are associated to K_G, a clear tendency on the βC interaction can be observed: $nHoH^+ \sim HoH^+ < HaH^+ < N\text{-Me-}nHoH^+ \sim N\text{-Me-}HoH^+$ (see discussion below).

The results shown above confirm that the interaction of βCH^+ (pH 4.4) with DNA is stronger than that observed for the neutral

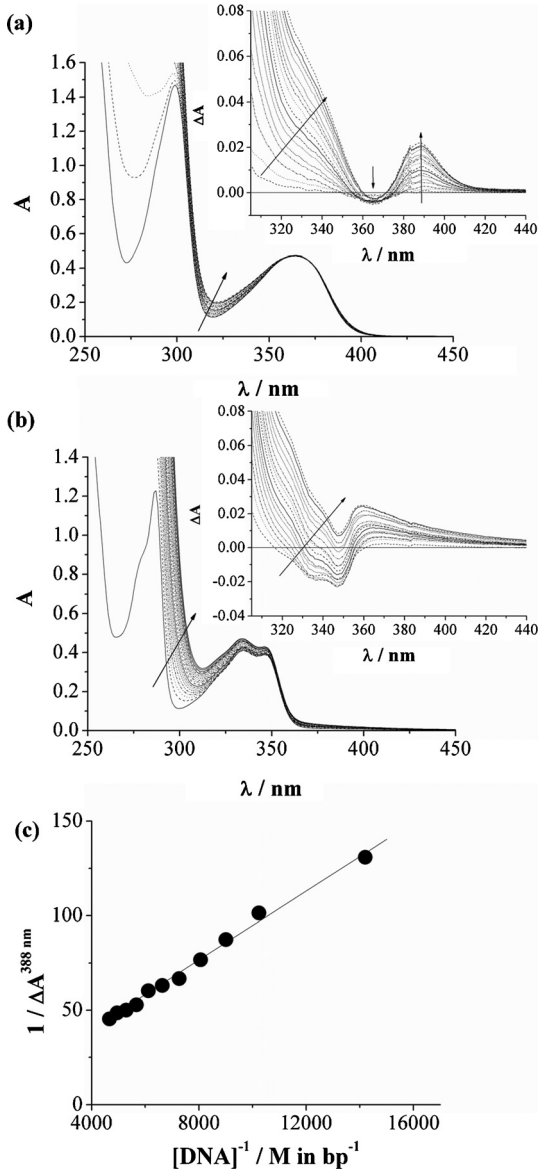


Fig. 6 Absorption spectra of Ho (100 μM) in the presence of increasing amounts of pGEM-3z. Experiments performed: **(a)** in acetic acid–sodium acetate buffer solution (pH 4.4) and **(b)** in K_2HPO_4–KOH buffer solution (pH 10.9). Arrows indicate the increase of DNA concentration. Insets show a magnified view of the corresponding experimental difference (ED) spectra. **(c)** A representative example of the Benesi–Hildebrand plot for the association of HoH^+ with pGEM-3z (pH 4.4).

form of the alkaloid. This is easily explained by taking into account that DNA is a negatively charged molecule and the five βCs studied have a positive net charge under acidic conditions. Moreover, the binding of βCsH^+ with DNA has no significant effect on the UV-vis absorption spectra, indicating that the main binding mode of these alkaloids to DNA might be groove binding instead of intercalation. This fact is in good agreement with the results reported by Taira *et al.*[52]

Fluorescence emission spectroscopy. To further analyze the interaction between βCs and DNA, steady-state and time-resolved fluorescence experiments in the presence of increasing

Table 3 Summary of βCs/DNA interaction parameters: K_G represents the binding constant according to Benesi–Hildebrand equation. K_D and K_S are the K_{SV} values for the dynamic and static quenching, respectively, *i.e.* K_D is equal to $k_q \tau_F^0$ and K_S is the equilibrium constant for complex formation

β-carboline (βC)	K_G		K_S		K_D	
	pH 4.4	pH 10.9	pH 4.4	pH 10.9	pH 4.4	pH 10.9
Ho	$4 (\pm 2) \times 10^2$	nd[a]	3921	2291	541 ± 18	0
nHo	$2 (\pm 1) \times 10^2$ [56]	nd[a]	4207	1376	520 ± 21	0
Ha	$8 (\pm 2) \times 10^2$	nd[a]	9899	4664	328 ± 27	0
N-Me-nHo	$2 (\pm 1) \times 10^4$	nd[a]	7615	6450	250 ± 50	0
N-Me-Ho	$4 (\pm 1) \times 10^4$	nd[a]	13221	12 650	900 ± 38	0

[a] nd = not detectable or measurable.

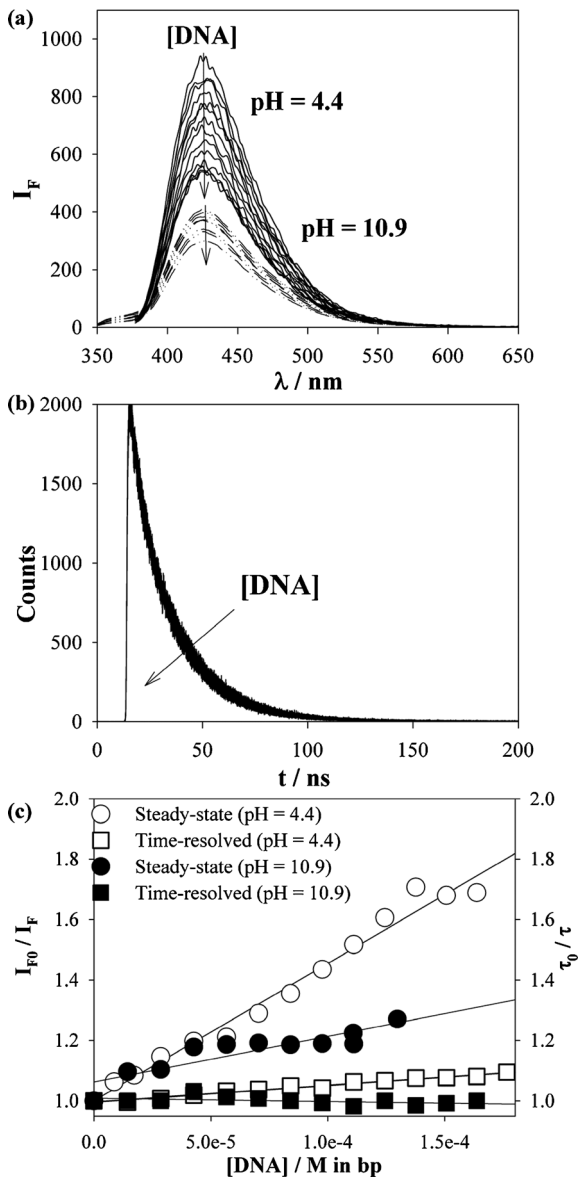
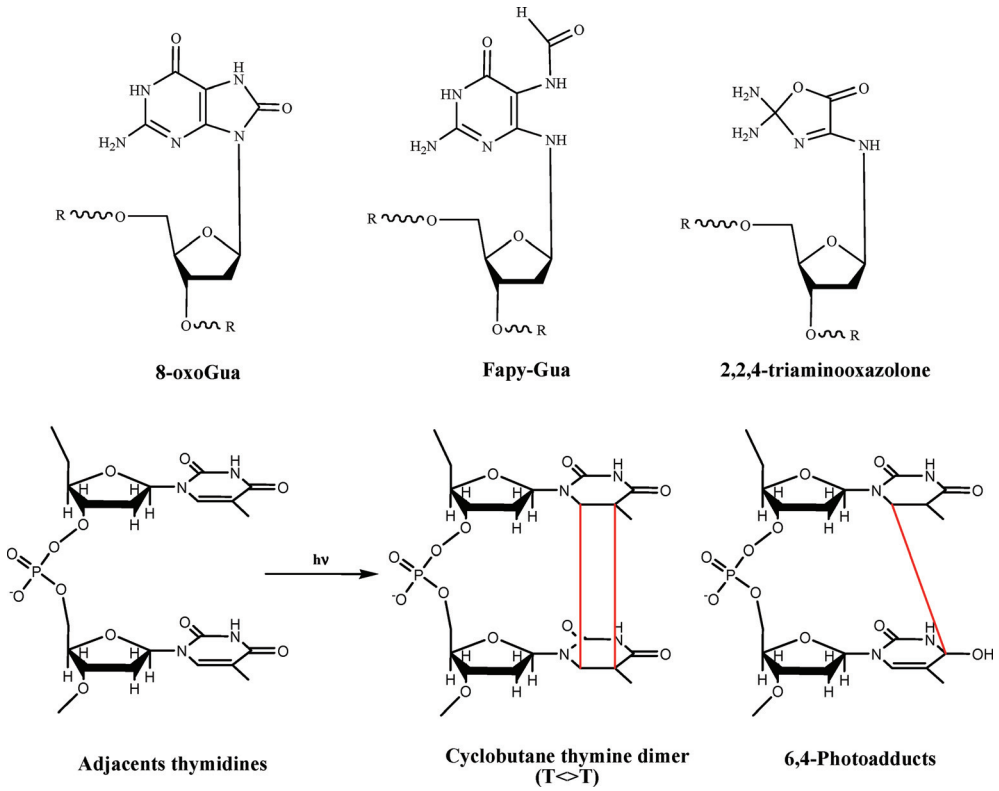


Fig. 7 Quenching of Ho fluorescence by pGEM-3z in air-equilibrated aqueous solutions. **(a)** Steady-state (solid lines: pH = 4.4 and dashed-dotted lines: pH 10.9) and **(b)** Time-resolve (pH 4.4) measurements. The arrows indicate the increase on DNA concentration. **(c)** Stern–Volmer plots of the integrated fluorescence intensity (I_F) and fluorescence lifetime (τ_F).

amounts of pGEM®-3z plasmid DNA were conducted. Fig. 7a shows the results obtained with Ho as a representative case. Under both pH conditions, the increase in the DNA concentration causes a decrease in the fluorescence intensity of βCs. The decrease of the integrated fluorescence intensity (I_F) as a function of the DNA concentration showed linear Stern–Volmer behavior (Fig. 7c). The corresponding slopes ($K_S = \tau_F\ k_q^F$) obtained are listed in Table 3. Bearing in mind the βCs fluorescence lifetime (~ 20 ns),[38,42,43,54] a value of $\sim 10^{11}$–10^{12} M^{-1} s^{-1} can be calculated for the bimolecular quenching constant (k_q^F). This fact suggests a contribution of a static quenching between βCs and pGEM®-3z plasmid (see time-resolved fluorescence experiments below).

The emission spectrum of non-protonated harmane (neutral Ho) has the same shape and the same value of λ_{em}^{MAX} (~450 nm) as the emission spectrum of HoH^+ (Fig. 7a). These results clearly indicate that even in the presence of DNA, the emission observed is due to the protonated form of harmane (HoH^+). The same results were observed for all βCs investigated in this work. This fact strongly suggests that, under both pH conditions, the environment of βC molecules is a polar-protic and/or an acidic medium. Otherwise, the emission band with λ_{em}^{MAX} (~380 nm) should be the only emission band observed.[55] Two facts are well known: the acidic nature of the DNA close environment and also the non-polar environment that a full intercalation would provide to the βC moiety. Thus, from our results comes out that neither the protonated nor the neutral form of βCs are fully intercalated into the DNA double strand. The latter fact is in good accordance with the results reported in our group,[20] suggesting a very low intercalation capability for these βC derivatives. Moreover, fluorescence results are in agreement with those obtained from UV-vis spectrophotometric analysis (see above) indicating that these alkaloids are DNA-groove binders.

First order kinetics were observed for all fluorescence decays of the protonated and the neutral forms of each βC studied, in the presence of p-GEM®-3z DNA. The respective fluorescence lifetimes (τ_F) decreased strongly as a function of the DNA concentration. Typical traces obtained for the quenching of fluorescence of HoH^+ by pGEM®-3z are shown in Fig. 7b. In acidic experiments (pH 4.4), a linear dependence of τ_{0F}/τ_F on the DNA concentration was observed. On the contrary, under alkaline conditions (pH 10.9) the slope of the corresponding Stern–Volmer plot was equal to zero. As an example, the Stern–Volmer analyses of the results obtained in both steady-state and time-resolved experiments for the quenching of the Ho fluorescence



Scheme 2 Structures of the most representative DNA photoproducts.

by pGEM®-3z are shown in Fig. 7c; and values of the corresponding Stern–Volmer constants (K_D) are listed in Table 3.

According to the K_S and K_D values two different points rise to the surface: (*i*) due to the fact that K_D is very small or null (in the case of experiments performed at pH 10.9) the quenching of βCs fluorescence by pGEM®-3z plasmid is mainly or purely static. (*ii*) In terms of the magnitude of K_S values, two main groups of data points are observed: $nHoH^+ \sim HoH^+ \ll HaH^+ \sim$ *N*-Me-$nHoH^+ \sim$ *N*-Me-HoH^+.

These results are in good agreement with those obtained from UV-vis spectrophotometric analysis and explain that Ha and *N*-methyl-derivatives as photosensitizers generate more DNA damage than nHo and Ho. The higher the affinity of a given βC to DNA in the ground state (due to its higher electron density in the aromatic moiety), the more βC-DNA complex formation takes place, yielding higher DNA photodamage.

Characterization of DNA photoproducts

DNA damage by photosensitizers can be mediated by different mechanisms which result in different types of DNA photoproducts. It has been demonstrated that energy transfer from the triplet state of some photosensitizers to pyrimidine bases leads to the formation of pyrimidine dimers (Scheme 2).[57–59] More often, photosensitized oxidations are observed. These can be initiated either directly by the excited states of the photosensitizers (type I mechanism, *e.g.*, *via* electron transfer or hydrogen abstraction) or mediated by ROS such as singlet oxygen (type II mechanism).[60] Both types of reactions can lead to the formation of several DNA photoproducts such as 8-oxo-7,8-dihydroguanine (8-oxoGua), formamidopyrimidines (ring-opened purines), 2,2,4-triaminooxazolone and apurinic-apyrimidinic sites (AP sites), among others (Scheme 2).

Therefore, we wanted to characterize and compare the spectra of DNA photoproducts formed upon photosensitization by different βCs. The absorption and emission spectra of the βCs allow an estimation of the relative energy of the electronic excited states. According to this, the five βCs under study can be divided into two main groups: (*i*) Group I, composed of βCs with electronic excited states of low energy (*i.e.*, nHo, Ho and the corresponding *N*-methyl derivatives) and (*ii*) Group II, represented in our study by Ha, in which the excited states are more rich in energy. In view of this, nHo and Ho, on one hand, and Ha, on the other, were chosen as representative molecules of Group I and II, respectively, to conduct the experiments described below.

DNA damage induced by βCs under cell-free conditions. Supercoiled DNA of bacteriophage PM2 was exposed to a fixed dose of UV-A light (365 nm, irradiation set-up II) in the presence of various concentrations of nHo, Ho and Ha. Subsequently, the numbers of SSB and modifications sensitive to formamidopyrimidine-DNA glycosylase (Fpg protein) from *E. coli*, which recognizes 8-oxoGua, formamidopyrimidines, 2,2,4-triaminooxazolone[61] and AP sites, were quantified.[62] Fig. 8 shows DNA damage in the presence of nHo. The extent of damage increased linearly with the concentration of nHo. Results indicate that products recognized by Fpg are much more frequent than SSB. No significant DNA damage was induced

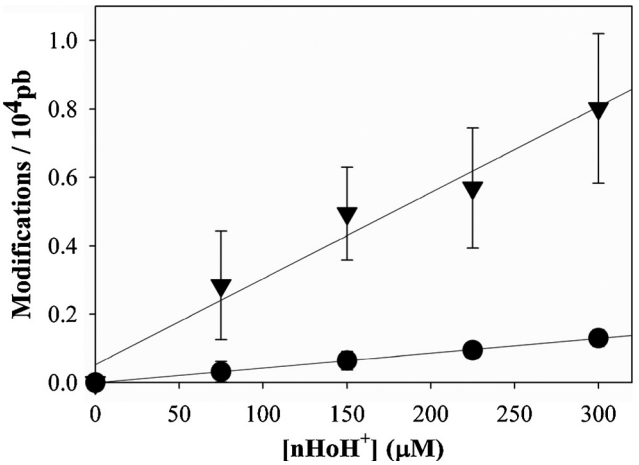


Fig. 8 DNA single-strand breaks (●) and modifications sensitive to Fpg protein (▼) induced in PM2 DNA by exposure to various concentrations of norharmane, in acidic aqueous solution ($nHoH^+$, pH 5.0), to UV-A light. Data are the mean of 3–4 independent experiments (±S.D.).

with and without irradiation (UV-A 365 nm) in the absence of the photosensitizers. Two other βCs investigated, Ho and Ha, showed similar behavior (data not shown).

Photosensitized DNA damage profile. The numbers of SSB and of various types of repair enzyme-sensitive modifications induced by photoexcited βCs are shown in Fig. 9 in the form of DNA damage profiles, which can serve as a kind of fingerprint for the species or mechanism directly responsible for the damage.[63,64] Experiments were performed for three different βCs, under two different pH conditions (5.0 and 7.2).[65]

When $nHoH^+$ and HoH^+ were used as photosensitizers at pH 5.0 the damage profile observed is characterized by a relatively high number of Fpg-sensitive base modifications. As a representative example, Fig. 9a shows the damage profile induced by nHo (Ho showed similar behavior (data not shown)). Sites of base loss, which are specifically recognized by endonuclease IV, and SSB are generated in much lower amounts. Besides, for these two βC alkaloids (nHo and Ho) no cyclobutane pyrimidine photodimers, which, together with certain ("regular") types of sites of base loss are recognized by T4 endonuclease V, were detected. In contrast, the protonated form of Ha (pH 5.0) not only generated relatively high yields of SSB and sites of base loss (detected by endonuclease IV) but also a number of cyclobutane pyrimidine dimers, which are detected as the difference between sites sensitive to the combination of T4 endonuclease V and endonuclease IV (representing pyrimidine dimers plus all sites of base loss) and endonuclease IV alone (Fig. 9c). This difference and therefore the relative yield of pyrimidine dimers is more pronounced at pH 7.2 (Fig. 9d), while the relative yields of SSB and sites of base are reduced.

Kinetic analysis shown in Fig. 3 indicates that the magnitude of DNA damage photosensitized by Ha is much higher than that observed in the case of nHo and Ho. This is also evident from the damage profiles when Ha and nHo are compared at equal absorbance and pH (Fig. 9c and e).

Also from the kinetic analysis shown above it was quite clear that ROS do not have a significant role in the mechanism of DNA damage. However, this conclusion was based on results obtained from experiments monitoring only SSB as a marker of DNA damage. In order to evaluate what happens at the level of the nucleobases the contribution of different ROS on the DNA photoproducts profile was also evaluated. In this regard, in some experiments, superoxide dismutase (SOD) (2 mg ml^{-1}) and/or catalase (315 U ml^{-1}) was added or the H_2O was replaced by D_2O (in the later case, the pD of the buffer was adjusted as it was described elsewhere,[66] the final isotope purity was greater than 96%). These experiments allowed us to investigate the effect of H_2O_2, $O_2^{\cdot -}$ and $^1O_2$ on the DNA photoproduct damage

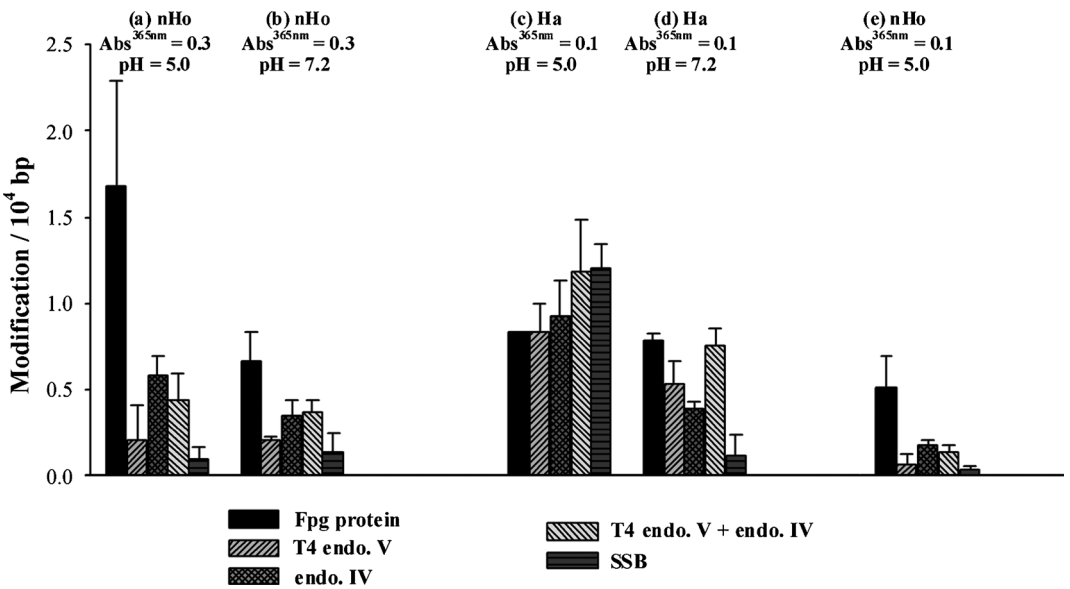


Fig. 9 DNA damage profiles generated by norharmane (nHo) and harmine (Ha) upon irradiation with UV-A under cell-free conditions. Columns indicate the numbers of single-strand breaks (SSB) and various endonuclease-sensitive DNA modifications. They represent the average of 3–4 independent experiments (±S.D.).

Table 4 Effects of SOD, catalase and of D_2O as solvent on the generation of Fpg-sensitive modifications by βCs at pH 7.2

	Relative number of Fpg-sensitive modifications (%) in the presence of:[a,b]			
β-carboline (βC)	SOD	Catalase	SOD + Catalase	D_2O
nHo	107 ± 22	113 ± 44	117 ± 38	75 ± 32
Ha	135 ± 59	121 ± 45	118 ± 32	205 ± 42

[a] Number of Fpg-sensitive modifications observed in the absence of an additive is defined as 100%. [b] Data are means of three independent experiments (± S.D.).

Table 5 Triplet state data for protonated and neutral forms of: nHo, Ho and Ha[a]

	$βCH^+$ (protonated)		βC (neutral)	
β-carboline (βC)	λ_P[b] (nm)	E_T (kJ mol^{-1})	λ_P[b] (nm)	E_T (kJ mol^{-1})
nHo	458	261	410	292
Ho	460	260	404	296
Ha	437	274	396	302

[a] λ_P is the wavelength of the maximum phosphorescence emission band (77 K) and E_T is the triplet energy. [b] Data from refs 69–72.

profile. As is shown in Table 4, participation of these ROS is largely excluded by the lack of influence of catalase, SOD and D_2O. Noteworthy, however, in the case of Ha, a small D_2O effect was observed. The extent of Fpg-sensitive DNA modifications in D_2O was two times higher than the control experiments performed in H_2O. The finding that D_2O affects the generation of Fpg-sensitive sites, but not that of SSB, is in accordance with our previous data indicating that singlet oxygen generates mostly 8-oxoGua and relatively few SSB. The finding also indicates that SSB and 8-oxoGua are generated by photoexcited βCs, at least in the case of Ha, by different mechanisms.

Analysis of thymidine dimer formation by energy transfer from βCs T_1 state. Thymine cyclobutane dimers (T<>T) are the most frequent photoproducts formed following the direct excitation of DNA by UV radiation and are well-known to initiate mutagenic and carcinogenic processes.[57] Besides by direct excitation, this type of damage can also be generated by certain photosensitizers *via* triplet–triplet energy transfer.[57–59] The feasibility of this process is not only linked to the efficiency of triplet formation (Φ_T) of the sensitizer, but also to the relative excited triplet state energies of the donor and acceptor chromophores.[67]

The triplet energy (E_T) of thymine in DNA is a critical parameter and its precise value is markedly different from that of free thymine or thymidine. Moreover, the microenvironments experienced by the different thymine units in DNA are non-equivalent and, therefore, their triplet energies are expectedly different. However, what matters is the minimum E_T (*i.e.*, the E_T "functional" value) required for a given compound to become a potential DNA photosensitizer by triggering T<>T lesions in DNA. The E_T value was recently established to be 267 kJ mol^{-1}.[68,69]

To assess if, from thermodynamic point of view, nHo, Ho and Ha are able to induce T<>T formation in DNA by energy transfer from their triplet excited states (T_1), E_T values of each protonated and neutral form of these βCs were calculated (Table 5).[70,71] The data show that the neutral form of the three alkaloids, but the protonated form of only Ha (HaH^+), have the required E_T to trigger T<>T lesions.[72,73]

In view of this, one would expect photosensitized T<>T formation not only in the case of Ha, but also for all three alkaloids in the experiments performed at pH 7.2, where ~50% of the neutral form of each alkaloid is present in the solution. However, the results shown in Fig. 9 clearly indicate that Ha is the only βC alkaloid producing T<>T dimers. This can be explained by the fact that, in aqueous media, protonation of βCs occurs readily in both singlet and triplet excited states even at neutral pH.[70] Thus, $[βCH^+]$* is the predominant species present upon excitation over the entire pH range investigated in the present work.

Conclusions

In this work we have performed a comparative analysis of the capability of five different βCs to induce DNA damage in the presence of UV-A. A kinetic analysis has been done and the DNA photoproducts formed were characterized.

We have demonstrated that the interaction between βCs with DNA strongly depends on the pH. The protonated forms of the alkaloids ($βCsH^+$) showed a higher interaction, due to coulombic attraction forces, than the neutral forms. Also, a strong pH dependence of the photosensitized DNA relaxation was observed, the generation of DNA SSB was much more pronounced at pH 4.7 than at pH 10.2. The excited states of the protonated forms ($βCH^+$) were found to be responsible for the DNA damage, which is generated mainly through direct electron transfer reactions (type I mechanisms). ROS have no important role.

In addition, this work provides, for the first time, evidences about the strong dependence of the photosensitized DNA rates and also of the photoproducts distribution, on the chemical nature of the βCs investigated. Briefly, three main factors are directly implicated in the extent of the DNA damage observed: (*i*) a difference in the photochemical properties of βCs, (*ii*) a difference in the binding properties of βCs with the DNA and (*iii*) the presence of additional and/or competitive photochemical pathways, *i.e.*, base photo-oxidation and/or thymidine dimer formation.

Furthermore, to better characterize the DNA photoproducts formed, experiments using DNA repair enzymes were carried out. The results show that βCs generate oxidative base modifications, sites of base loss and SSB. The ratio of these lesions depends not only on the nature of the βC, but also on the pH. In addition to these oxidatively generated modifications, cyclobutane pyrimidine dimers were detected in the case of Ha only. Interestingly, this is the only one of the five βCs studied for which the energy of the protonated excited triplet state is calculated to be high enough to allow energy transfer to DNA.

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45 Note that only a fraction of βC goes on with the protonation (*e.g.*, in the case of norharmane (nHo) only a fraction less than 30% of the S_1 of neutral nHo yields [$nHoH^+$]*). The S_1 remaining is deactivated by non-radiative processes.[40–42].
46 The d[Sc]/d*t* value observed at pH 7.4 (*i.e.*, $-4.5\ (\pm\ 0.5) \times 10^{-3}$ NI min^{-1}) was in between those observed at pH 4.8 and 10.2 (*i.e.*, $-8.1\ (\pm\ 0.8) \times 10^{-3}$ NI min^{-1} and $-2.4\ (\pm\ 0.8) \times 10^{-3}$ NI min^{-1}, respectively). In particular, the d[Sc]/d*t* value obtained at pH 7.4 represents ~50–60% of the damage observed at pH 4.7. These results were expected since at pH 7.4 a mixture of both βCH^+ and βC forms are present in the irradiated solution. Taking into account that each acid–base form has a distinctive capability for DNA photocleavage, the extent of the DNA damage is a consequence of the average contribution of both species (βCH^+ and βC). See numerical support described elsewhere.[20].
47 For a given pH condition (*e.g.*, in the pH range 4–5), all βCsH^+ under study show Φ_{H2O2} and Φ_Δ^{air} values of $\sim 0.40 \times 10^{-3}$ and 0.10, respectively. HaH^+ represents an exception showing values of 0.84×10^{-3} and 0.22, respectively.[40,41].
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56 In our previous work, a value of 4.5 (± 0.5) × 10^4 M^{-1} was given for the association constants (K_G) between nHo and DNA. This value seems to be overestimated. Note that, in that case, the K_G value was calculated by looking at the changes on the absorption spectra around 325 nm. Although with an extremely low absorption coefficient (ε) ~325 nm , a non-negligible contribution of the tail of the DNA absorption spectra to the changes observed should be taken into account; mainly, when the intrinsic nHo/DNA interaction changes are particularly small. This fact could provide arguments to explain the overestimation on the K_G value observed previously.
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