Photosensitized cleavage of plasmidic DNA by norharmane, a naturally occurring β-carboline†

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UV-A radiation (320–400 nm) induces damages to the DNA molecule and its components through photosensitized reactions. β-Carbolines (βCs), heterocyclic compounds widespread in biological systems, participate in several biological processes and are able to act as photosensitizers. The photosensitization of plasmidic DNA by norharmane in aqueous solution under UV-A radiation was studied. The effect of pH was evaluated and the participation of reactive oxygen species (ROS), such as hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet -}$) and singlet oxygen ($^1O_2$) was investigated. A strong dependence of the photosensitized DNA relaxation on the pH was observed. The extent of the reaction was shown to be higher in the experiments performed at pH 4.7 than those performed at pH 10.2. As was expected, an intermediate extent of the reaction was observed at physiological pH (pH 7.4). Kinetic studies using ROS scavengers revealed that the chemical reactions between ROS and DNA are not the main pathways responsible for the damage of DNA. Consequently, the predominant mechanism yielding the DNA strand break takes place most probably *via* a type I mechanism (electron transfer) from the single excited state (S_1) of the protonated form of norharmane (^{1}[nHoH$^+$]*). Additional information about the nature of the norharmane electronic excited states involved in the photocleavage reaction was obtained by using the *N*-methyl derivative of norharmane (*N*-methyl-norharmane).

Introduction

Solar radiation induces modifications to genomic DNA and is implicated in the induction of human skin cancers.[1,2] UV radiation is the most mutagenic and carcinogenic component of the solar spectrum that reaches the earth's surface. UV-B radiation (280–320 nm) damages DNA through the direct excitation of the nucleobases.[3] On the other hand, although nucleobases absorb very weakly above 320 nm, UV-A radiation (320–400 nm) may damage DNA through photosensitized reactions.[3,4] This indirect action is mediated by endogenous and/or exogenous photosensitizers which are excited by the UV-A radiation.

The chemical changes in DNA resulting from photosensitized reactions can take place through different mechanisms. It has been demonstrated that energy transfer from the triplet state of some photosensitizers to pyrimidine bases leads to the formation of pyrimidine dimers.[4–6] Photosensitized oxidations also contribute to DNA damage induced by UV-A radiation. These processes involve the generation of radicals (type I mechanism), *e.g.*, *via* electron transfer or hydrogen abstraction, and/or the production of singlet molecular oxygen (type II mechanism).[7]

The nucleobases of DNA are the preferential substrates for type I oxidation.[3,8] Among the DNA bases, guanine (that exhibits the lowest ionization potential) is the preferential target for one electron oxidation reactions, over adenine and pyrimidine bases.[9] Moreover, guanine is the only DNA constituent that significantly reacts with $^1O_2$.[9–12] However, for a given photosensitizer/target molecules pair, it is difficult to evaluate the mechanism of photosensitization involved. In addition, many photosensitizers are able to act through both type I and type II mechanisms.

9*H*-Pyrido[3,4-*b*]indole or norharmane (Fig. 1) is a heterocyclic compound that belongs to an alkaloids family called β-carbolines (βCs). These alkaloids are widespread in biological systems. They are found in many tropical plants as a normal constituent of the seeds,[13] roots,[14] stems[15] and leaves.[16–19] Consequently, they also occur as minor constituents in foods, alcoholic drinks, cigarettes/tobacco smoke, *etc*.[20–22] In mammalian bodies, βC derivatives occur normally in plasma, platelets and urine.[23] Moreover, it seems that some are formed in human body after alcohol intake and smoking.[24] Some βCs are accumulated in tissues such as the skin and the eye.[25]

The participation of several βCs in different photosensitizing processes has been suggested and/or demonstrated. Under UV-A irradiation, norharmane and harmane (1-methyl-9*H*-pyrido[3,4-*b*]indole) are able to induce damage in mammalian cell chromosomes;[26,27] to inactivate bacteria[28,29] and viruses.[30] In addition, it has been proposed that the biological role of some βCs in plants could be related with the defense response by means of phototoxic effects against insects, webworms, *etc*.[31] Despite these facts, the main photobiological role of these alkaloids and the

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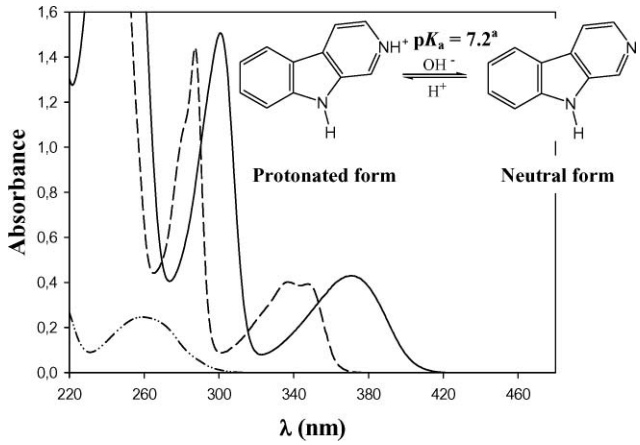

Fig. 1 Absorption spectra of the protonated and neutral form of norharmane (nHoH$^+$ and nHoN, respectively) recorded in air-equilibrated aqueous solutions. Solid line: absorption spectrum of nHoH$^+$ (pH 4.7; 250 μM, optical path length of 0.4 cm); dashed line: absorption spectrum of nHoN (pH 10.2; 250 μM, optical path length of 0.4 cm). Dash-dot-dot line: absorption spectrum of pGEM-3z plasmid DNA (15.5 μg ml^{-1}). Inset: molecular structure of nHoH$^+$ and nHoN and the corresponding acid–base equilibrium observed in the pH range of 4–11. [a]The other functional group of the β-carboline moiety, the nitrogen of the indolic ring, has a pK_a value higher than 13.

mechanisms involved in those processes are, up to now still poorly understood.

Although the photophysics[32–53] and the photochemistry[54,55] of βCs have been extensively studied over the past two decades, only a few studies regarding the photosensitizing properties of these alkaloids have been described.[56,57]

In the context of our studies to elucidate the mechanisms involved in the processes photosensitized by βCs, we describe here the first *in vitro* study of the photosensitized cleavage of plasmid DNA by norharmane upon UV-A irradiation, in both acidic and alkaline aqueous solutions as well as in physiological conditions. Norharmane is a good model compound for aromatic βCs in general due to its chemical structure (see Fig. 1). All full aromatic βCs possess a norharmane-like skeleton moiety, and the difference between them is the nature and position of the substituents. Therefore, the use of norharmane as a photosensitizer appeared to be a reasonable starting point. The effect of pH was evaluated not only on the interaction between norharmane and plasmidic DNA, but also on the photosensitizing properties of βC. The participation of reactive oxygen species (ROS) was also investigated. The contribution of type I (electron transfer) and type II (oxidation *via* $^1O_2$) mechanisms in acidic and alkaline media was analyzed. In addition, the nature of the norharmane electronic excited states involved in the photocleavage reaction was studied by using the *N*-methyl derivative of norharmane (*N*-methyl-norharmane).

Experimental

General

Norharmane (>98%) from Sigma-Aldrich was used without further purification. The method used to synthesize and purify *N*-methyl-norharmane has been published elsewhere.[58] The restriction endonuclease EcoRI and superoxide dismutase were from Invitrogen.

Plasmid pGEM-3z (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 value of A_{260}/A_{280}. The ratio was 1.90, indicating that the content of residual protein should be small. The concentration of the DNA stock solution was quantified by two different methods: (*i*) *Spectrophotometric quantification*: The absorbance of the DNA solution was recorded at 260 nm in a quartz cell of 1 cm path length. The concentration of DNA stock solution was calculated using a molar absorption coefficient of 6600 M^{-1} cm^{-1} yielding a concentration of 1 mM base pair. (*ii*) *Quantification with a fluorescent assay*: Alternatively, plasmidic DNA was quantified by fluorescence by using Quant-iT dsDNA HS assay kit (Invitrogen) following the specification indicated by the manufacturer. The fluorescence emission was measured with a Qubit fluorometer. This assay is useful in cases where contaminants that also absorb at 260 nm are present in the solution. In this case, a concentration value of 0.7 mM base pair was obtained for the DNA stock solution. For a more accurate analysis, the DNA concentrations obtained from both methods were averaged yielding a value of 0.85 mM base pair.

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

Steady-state irradiation

Irradiation set-up. Aqueous solutions containing a mixture of the photosensitizer (norharmane or *N*-methyl-norharmane) and the target molecule (plasmidic DNA at 15.5 μg ml^{-1}) were irradiated, in the presence of air, in 0.4 cm quartz cells at room temperature with a Rayonet RPR lamp emitting at 350 nm (bandwidth ~20 nm) (Southern N.E. Ultraviolet Co.). For comparative reasons, all the experiments were performed using solutions of the photosensitizers with the same absorbance at 350 nm (*i.e.*, the absorbance at 350 nm was matched at 0.3).

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 (Tris 0.05 M; boric acid 0.05 M; EDTA 1 mM) buffer. DNA was visualized with ethidium bromide (Sigma, 0.5 μg ml^{-1}) under UV illumination.

Once electrophoresed, all the stained gels were photographed on an UV transilluminator (Hoefer MacroVue UV-20) using a digital camera KD 120 (Kodak) and the intensity 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 spot was divided by the sum of the intensities of all spots in the same lane.[59] The lesser intercalation of ethidium into supercoiled DNA was taken into account by dividing the corresponding intensity by 0.8.[60] For comparative purpose, the initial relaxation rate of the supercoiled DNA (d[Sc]/dt) was used. This rate was calculated from the slope of the plot of the normalized intensity (NI) of the Sc form *vs.* irradiation time.

Thermal reactions between norharmane and DNA were discarded after control experiments performed by keeping solutions containing the mixture in the dark. These experiments were carried out under different experimental conditions (concentration, pH and time). In another set of control experiments, DNA solutions were irradiated in the absence of norharmane. As is shown in Fig. 2, no electrophoretic change after irradiation was detected, thus discarding direct effects of the radiation on the DNA molecule.

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

Hydroxyl radical ($HO^{\bullet}$) quenching. Mixture solutions containing norharmane and plasmidic DNA were irradiated in the presence of propan-2-ol. The concentration of the alcohol was 50 mM. This compound has a high reactivity with hydroxyl radicals ($HO^{\bullet}$),[61] diminishing its concentration during the experiment. Results of electrophoretic analysis were compared with those obtained in the absence of the selective $HO^{\bullet}$ scavenger.

Superoxide ($O_2^{\bullet -}$) investigation. Mixture solutions containing norharmane and plasmidic DNA, at a given pH (*i.e.*, 4.7 and 10.2) were irradiated in the presence of 100 U/mL of Superoxide Dismutase enzyme (SOD). Results of electrophoretic analysis, were compared with those obtained in the absence of SOD.

Singlet oxygen ($^1O_2$) investigation. *(i) Comparison of continuous photolysis in H_2O and D_2O.* Mixture solutions containing norharmane and plasmidic DNA were prepared in H_2O and D_2O. Couples of both types of solutions containing both the photosensitizer and the target molecule, at the same concentration as those used in previous experiments, were irradiated under identical experimental conditions. The effect of D_2O was evaluated by comparing results of electrophoretic analysis. *(ii) Photolysis in the presences of singlet oxygen scavengers*: For this purpose two different scavengers were used (*i.e.* furfuryl alcohol (Riedel-de Haën) and L-histidine). These compounds have a high reactivity with $^1O_2$.[62] Thus, they greatly reduce the $^1O_2$ concentration during the photolysis. In this group of experiments mixture solutions of norharmane and DNA were irradiated in the presence of scavenger (1 mM). Results of electrophoretic analysis were compared with those obtained in the absence of the $^1O_2$ scavenger.

DNA binding studies

UV-vis spectrophotometric analysis. The interaction of norharmane with pGEM-3z was studied by UV-vis absorption spectrophotometry using the Benesi–Hildebrand equation. The spectra were recorded on a Shimadzu UV-3600 spectrophotometer. Measurements were made in quartz cells of 1 cm optical-path length (105.250-QS Hellma), at room temperature. The cuvette initially containing 100 μL of a 20 μM βC buffered solution was progressively titrated by increasing amounts of plasmid pGEM-3z solution (*i.e.*, from 0 to 30 μM in base pair). 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.

Fluorescence measurements. Steady-state fluorescence measurements were performed using a Perkin-Elmer LS 50B spectrofluorometer. Corrected fluorescence spectra were recorded in a 0.4 × 1 cm path length quartz measurement cell at room temperature. For determining the quenching of fluorescence of norharmane and *N*-methyl-norharmane by plasmidic DNA, emission spectra of each alkaloid solutions (20 μM) were recorded in the absence and in the presence of pGEM-3z plasmid (*i.e.* from 0 to 30 μM in base pair). 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. The fluorescence intensity (I) was obtained by integration of the corrected fluorescence spectra over the entire emission profile.

Results and discussion

Photosensitized cleavage of DNA by norharmane

Air-equilibrated aqueous solutions of norharmane (260 μM) and supercoiled DNA were irradiated with UV-A (350 nm) light for different times. Under these experimental conditions only norharmane was excited (see the corresponding absorption spectra shown in Fig. 1). The reactions were monitored by electrophoresis in agarose gel, which is known to be a very useful tool to detect the conversion of the supercoiled form (Sc) into the relaxed form (Rel) indicating single-strand breaks.

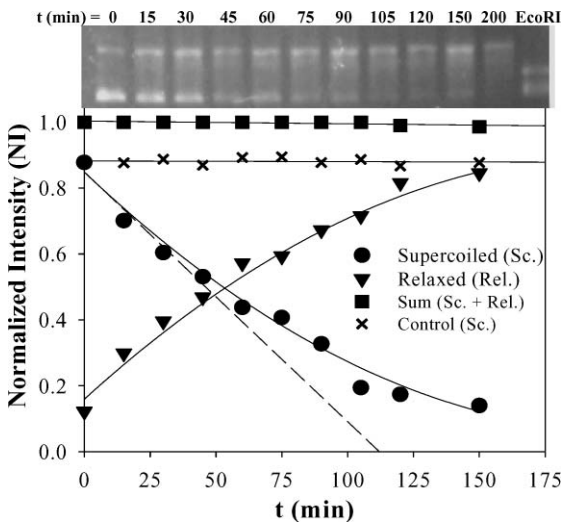

Fig. 2 Evolution of normalized intensity of relaxed (Rel) and supercoiled (Sc) plasmidic forms of pGEM-3z as a function of the irradiation time in the presence of $nHoH^+$ (260 μM, at pH 4.7). Control experiment: DNA solution irradiated in the absence of norharmane (see black crosses). **Inset:** Electrophoretic run: lane 1 to 11 = solution of $nHoH^+$ and pGEM-3z under different irradiation time. Lane 12 = pGEM-3z linearized with the restriction endonuclease EcoRI.

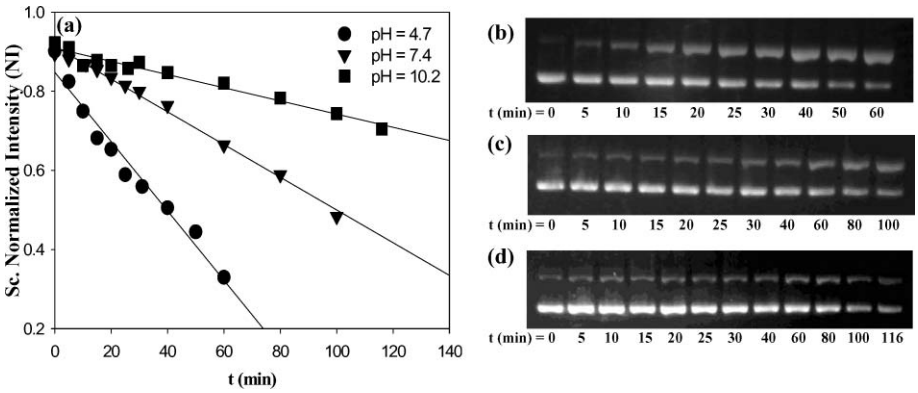

Fig. 3 **(a)** pH dependence on the evolution of normalized intensity of supercoiled (Sc) plasmidic form as a function of the irradiation time, in the presence of norharmane. For each pH-condition, the absorbance at 350 nm was matched at 0.3. In all the cases, the increase in the relaxed form concentration correlated very well with the decrease in the [Sc] (see the ESI†). **(b)**, **(c)** and **(d)** show the electrophoretic runs of the experiments performed at pH 4.7, 7.4 and 10.2, respectively.

In aqueous solution norharmane behaves as a weak acid, showing one dominant acid–base equilibrium over the pH-range 4–11. This equilibrium, which involves the pyridinic nitrogen, is characterized by a pK_a value of 7.2[52] (Fig. 1). It has already been demonstrated that each acid–base form has a very distinctive photochemical behaviour.[54,55] Thus, to assess which role each acid–base form has in the photosensitized cleavage of plasmid DNA, our experiments were performed under two different pH conditions: (*i*) at pH 4.7, where norharmane is present at more than 99% in its protonated form ($nHoH^+$) and (*ii*) at pH 10.2, where the pyridinic nitrogen is almost completely deprotonated and the neutral form of norharmane (nHoN) is the predominant species.

The protonated form of norharmane ($nHoH^+$) as photosensitizer was investigated first. Fig. 2 depicts the photocleavage of supercoiled pGEM-3z plasmid in the presence of $nHoH^+$ (experiment performed at pH 4.7). The agarose gel clearly shows a substantial decrease in the concentration of the Sc form as a function of irradiation time. The kinetic analysis shows that this decrease followed a first order rate law during the first 150 min of irradiation (see circle in Fig. 2). The solid line in Fig. 2 represents the best fit of eqn (1) to the experimental data:

$$[Sc]_t = [Sc]_0 \times [1 - \exp(-kt)] \quad (1)$$

where $[Sc]_0$ and $[Sc]_t$ are the normalized intensity of the band corresponding to the supercoiled form (*i.e.*, the concentration of Sc form) at zero and a given time t, respectively, and k is the apparent first order rate constant. Under our experimental conditions the apparent first order rate constant (k) was found to be 0.010 (± 0.002) min^{-1}. This value is slightly smaller than the k value already published in the literature for other related photosensitizers.[63]

For comparative reasons (see below) we were interested in calculating the initial rate of the disappearance of the Sc form (d[Sc]/dt). Looking at the results described above, a d[Sc]/dt value of $-7.6\ (\pm 0.4) \times 10^{-3}$ NI × min^{-1} was calculated taking into account the points collected up to the first 60 min of irradiation (see dashed line in Fig. 2).[64] To determine a more accurate d[Sc]/dt value, three extra experiments were performed increasing the number of data collected along the first 60 min of irradiation. Fig. 3a shows a representative experiment and the averaged d[Sc]/dt value calculated from these three independent experiments was $-8.2\ (\pm 0.9) \times 10^{-3}$ NI × min^{-1}. Note that the latter value, also listed in Table 1, is in very good agreement with that calculated from the first set of experiments ($-7.6\ (\pm 0.4) \times 10^{-3}$ NI × min^{-1}).

The rate of disappearance of Sc form ($-8.2\ (\pm 0.9) \times 10^{-3}$ NI × min^{-1}) matched with the rate of appearance of the relaxed form ($8.2\ (\pm 0.9) \times 10^{-3}$ NI × min^{-1}) represented in Fig. 3 with dots and downward triangles, respectively. It is interesting to point out that the linearized form of DNA was not observed, even at longer irradiation times (see inset in Fig. 2, lane 11 in the agarose gel showing plasmid linearized by EcoRI). This fact clearly indicates the absence of an independent pathway for double-strand cleavage. Therefore, the relaxation in the supercoiled native DNA conformation photoinduced by $nHoH^+$ follows the single-strand cleave model suggested by Kishikawa *et al.*,[65] where the photocleavage occurs essentially by producing randomly distributed single-strand breaks.

Table 1 Rate of DNA relaxation obtained under different experimental conditions

Entry	Reaction conditions	d[Sc]/dt (NI min^{-1}) at pH 4.7[a]	d[Sc]/dt (NI min^{-1}) at pH 10.2[a]
1	norharmane (in H_2O)	$-8.2\ (\pm 0.9) \times 10^{-3}$	$-2.3\ (\pm 0.5) \times 10^{-3}$
2	norharmane (in D_2O)	$-1.4\ (\pm 0.5) \times 10^{-2}$	$-2.3\ (\pm 0.5) \times 10^{-3}$
3	norharmane + furfuryl alcohol (in H_2O)	$-7.2\ (\pm 0.9) \times 10^{-3}$	$-2.0\ (\pm 0.5) \times 10^{-3}$
4	norharmane + SOD (in H_2O)	$-7.7\ (\pm 0.9) \times 10^{-3}$	$-1.8\ (\pm 0.5) \times 10^{-3}$
5	norharmane + propan-2-ol (in H_2O)	$-7.8\ (\pm 0.8) \times 10^{-3}$	$-1.4\ (\pm 0.5) \times 10^{-3}$
6	*N*-methyl-norharmane (in H_2O)	$-1.5\ (\pm 0.1) \times 10^{-2}$	$-1.4\ (\pm 0.1) \times 10^{-2}$

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

To study the capability of the neutral form of norharmane (nHoN) to photocleavage pGEM-3z plasmid, a similar set of experiments were carried out in alkaline aqueous solutions (pH = 10.2; [nHoN] = 200 μM). Data of a representative experiment are shown in Fig. 3 a and b, and the averaged d[Sc]/dt value obtained was $-2.3\ (\pm 0.5) \times 10^{-3}$ NI × min^{-1}. This value represents the 28% of the damage observed at pH 4.7 (see entry 1 in Table 1), indicating that the photosensitized damage produced by nHoN is lower than that produced by $nHoH^+$.

It is well known that upon excitation, the basic character of the βC pyridinic nitrogen is greatly enhanced. The pK_a* of the acid–base equilibrium between the excited-state of each acid–base form of βC is at least 7 times higher than pK_a.[32,43,54,55] Thus, even in pH 10.2 aqueous solutions, the excited neutral βC species is readily protonated, by rapid proton exchange with the solvent, during the lifetime of its S_1 state. In the particular case of norharmane in aqueous solutions only a fraction less than 30% of the S_1 of nHoN goes on with the protonation yielding $^1[nHoH^+]$*.[54,55] Thus, the photochemical and photophysical behavior of nHoN occurs mainly from the excited-state of the protonated form of norharmane ([$nHoH^+$]*), instead of the excited state of the neutral form. Regarding this point, it is important to note that the results shown in the paragraph above correlate very well with the small fraction of the nHoN that follows a protonation (*i.e.*, d[Sc]/dt observed at pH 10.2 represent the 28% of the damage observed at pH 4.7). This fact suggests that, under both pH conditions, the S_1 state of the protonated form of norharmane ($^1[nHoH^+]$*) is involved in the main step of the photosensitized reaction.

To further substantiate the latter hypothesis, we have extended the DNA photocleavage experiments to other βC derivatives. In particular, a new set of experiments using *N*-methyl-norharmane (*N*-Me-nHo) as photosensitizer were carried out. This particular βC was chosen due to the fact that, upon excitation, almost a 90% of its neutral form follows the fast protonation yielding the corresponding $^1[N\text{-Me-}nHoH^+]$*.[55,66] Therefore, if the DNA photocleavage reaction also occurs from the S_1 state of *N*-Me-$nHoH^+$, then the d[Sc]/dt observed at pH 10.2 would represent ~90% of the damage observed at pH 4.7. Representative experiments are displayed in Fig. 4 showing, under both pH conditions, a substantial DNA photocleavage. The corresponding d[Sc]/dt values are listed in Table 1 (entry 6) and, as can be seen, the rate of DNA relaxation observed at pH 10.2 represents 94% of the corresponding value obtained from the experiments performed at pH 4.7. These results strongly support the hypothesis that the DNA photocleavage reaction occurs from the first excited state of the cationic form of βCs ($^1[\beta CsH^+]$*) (see additional details and discussion below).

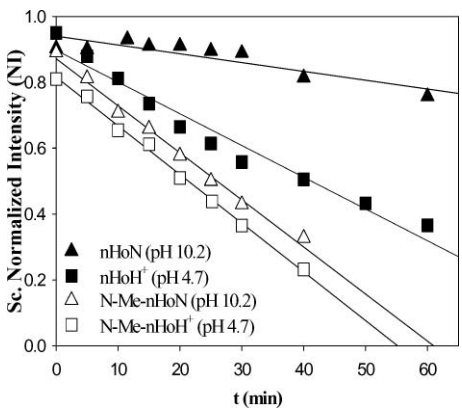

Fig. 4 Evolution of normalized intensity of Sc plasmidic form as a function of the irradiation time, using both acidic and alkaline form of the two photosensitizers: norharmane and *N*-methyl-norharmane.

Role of reactive oxygen species (ROS) in the photocleavage pathways of DNA

In the late eighties, some qualitative studies demonstrated that, upon UV-A irradiation, the βC alkaloids are able to produce ROS, such as singlet oxygen ($^1O_2$), superoxide anion ($^1O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2).[37,67–69] However, recent quantitative studies showed that, in aqueous solution, the efficiency of $^1O_2$ and H_2O_2 production upon excitation is very low.[54,55] Despite this fact, due to the high reactivity of ROS with DNA components, their role in the DNA photocleavage must be analyzed. For this reason, the following experiments were performed (see Table 1):

Singlet oxygen ($^1O_2$) studies. $^1O_2$ 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).[70] Although norharmane in aqueous solutions is a poor $^1O_2$ sensitizer (*i.e.*, quantum yield of $^1O_2$ production (Φ_Δ) of 0.10 and 0.08 at pH 4.8 and 10.2, respectively),[54,55] the amount of $^1O_2$ generated under our experimental conditions would be enough to induce the DNA photocleavage.

In particular, two sets of experiments were carried out: (*a*) *Comparison of photolysis in H_2O and D_2O*. Taking into account the longer $^1O_2$ lifetime in D_2O than in H_2O,[71] the photoreaction of norharmane should be ~16 times faster in D_2O if $^1O_2$ would contribute significantly to the process. Air-equilibrated solutions of DNA in the presence of norharmane in H_2O and D_2O, under both pH/pD conditions, were irradiated under identical conditions. Electrophoretic analysis showed the same d[Sc]/dt values within the experimental error. Note that in the case of experiments performed at pH 4.7 a very slightly increase in the rate of DNA relaxation was observed; *i.e.*, d[Sc]/dt value obtained in D_2O was 1.7 times higher than in H_2O (see Table 1, entries 1 and 2). Taking into account that this small increase observed is faraway from the factor 16 expected for the cases where $^1O_2$ plays the main role, our results are indicating that, under both pH conditions, $^1O_2$ is not involved in the main pathway of DNA photocleavage. Perhaps, this ROS participates in a secondary pathway that is enhanced under certain experimental conditions (*i.e.*, using D_2O as a solvent).

(*b*) *Photolysis in the presences of $^1O_2$ selective scavengers (i.e., furfuryl alcohol and L-histidine)*. Irradiations in the presence of 1 mM of furfuryl alcohol (a selective $^1O_2$ scavenger) were carried out. Under both pH conditions the rate of DNA relaxation was not modified in the presence of this scavenger (see Table 1, entries 1 and 3). Similar results were obtained in the presence of L-histidine (1.5 mM) (results not shown). These results strongly support those described in the previous paragraph, confirming that $^1O_2$, albeit present in the reaction mixture, does not contribute significantly in the DNA relaxation process.

Furthermore, it is important to highlight two additional points that support the hypothesis that $^1O_2$ is not involved in the main

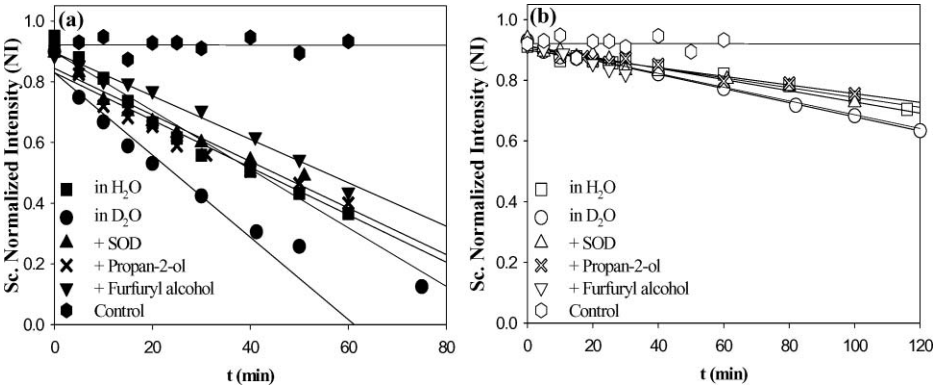

Fig. 5 Evolution of normalized intensity of Sc plasmidic form as a function of the irradiation time. Experiments were performed in air-equilibrated aqueous solutions, in the presence and in the absence of different selective scavengers, at: **(a)** pH 4.7 and **(b)** pH 10.2.

steps of the DNA photocleavage. On the one hand, the efficiency of $^1O_2$ production by each acid–base form of norharmane is quite similar (see above). Therefore, if $^1O_2$ would dominate the photocleavage reaction, then the d[Sc]/dt value observed at pH 10.2 should be similar to that obtained at pH 4.7. On the contrary, the experimental rate of DNA relaxation observed at pH 10.2 was much smaller than the corresponding rate at pH 4.7. On the other hand, in a very recent study[56] García-Zubiri *et al.* have proven for another βC derivative that as a result of its complexation with DNA, the intersystem crossing process became less efficient, decreasing the amount of $^1O_2$ generated by photosesitization.[72] On these facts, the photosensitizing properties of norharmane would involve other routes apart from the $^1O_2$ attack to DNA.

Superoxide anion ($O_2^{\bullet-}$). Experiments in the presence of SOD were carried out. This enzyme catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 and O_2. Thus, the DNA photocleavage reaction should be slower in the presence of SOD if $O_2^{\bullet-}$ would contribute significantly to the process. The results obtained showed that under both pH conditions the relaxation rates observed were quite similar with or without SOD in the solution (see Fig. 5 and Table 1, entries 1 and 4) suggesting that $O_2^{\bullet-}$ does not participate in the main steps of this process.

Hydroxyl radical (HO$^\bullet$). The role of this ROS in the photosensitized processes studied was also discarded. Experiments performed in the presence of propan-2-ol did not show any significant difference with those carried out in the absence of this scavenger (see Fig. 5 and d[Sc]/dt values summarized in Table 1, entries 1 and 5).

Interaction between norharmane and pGEM-3z plasmid. Spectroscopic studies

During the last decade, a few studies on the interaction between βCs and biomolecules have been reported.[56,73–75] In 1997, Taira *et al.*[73] demonstrated for the first time that six βCs (*i.e.*, norharman, harman, harmol, harmine, harmalol and harmaline) intercalate into DNA. The magnitude of intercalation strongly depends on the nature of the substituent and on the planarity of the βC ring, being norharmane the alkaloid that shows the lowest intercalation capability. In a later study,[74] the interaction of harmane with the elemental components of the nucleic acids (nucleobases, nucleosides and mononucleotides) was undertaken. Fluorescence analysis showed that the quenching process is mainly static (*i.e.*, due to the formation of ground state 1 : 1 complexes), although a small dynamic component is also detected in the time-resolved experiments. In a more recent investigation[56,75] García-Zubiri *et al.* have studied the interaction between β-carboline-3-carboxylic acid *N*-methylamide (βCMAM) and several biomolecules (*i.e.*, nucleobases, nucleosides, nucleotides, oligonucleotides and single- and double-stranded DNA). In all the cases analyzed, a 1 : 1 (βCMAM : ligand) complexation was also observed. The complexation between this alkaloid with double-stranded DNA would take place mainly through the guanine base. Hydrogen bonding between the amide group of βCMAM and DNA is believed to facilitate association. Moreover, intercalation does not seem to be the only mechanism of interaction between this dye and DNA.[75]

It is important to highlight that all these studies have been performed under pH conditions very close to the physiological one (*i.e.*, pH ~ 7). It is well known that βCs, specially the full aromatic βCs, show an acid–base equilibrium with a pK_a value ranging between 7 and 8. Therefore, under the pH conditions used in these previous studies a mixture of both acid–base forms (*i.e.*, protonated and neutral forms) of each βC were present in the solution. The protonated form of βC has a positive net charge, and this fact can be the cause of a different mechanism for the βC-DNA interaction. Thus, without any doubt all, these previous studies provide very important information about the interaction of βCs with different biomolecules under physiological conditions, but certainly they do not allow distinguishing of how each acid–base form of βCs interacts with them.

To the best of our knowledge, no studies on the pH dependence of βCs-DNA interaction have been reported previously. In this context, we decided to investigate the norharmane-DNA interaction dependence on the pH medium by recording the UV-vis absorption and fluorescence spectra of βCs acidic and alkaline aqueous solutions in the presence of different amounts of DNA.

UV-vis absorption spectroscopy. The binding strength of both acidic and basic forms of norharmane to plasmid pGEM-3z was analyzed by UV-vis spectrophotometric titration. Despite the minor changes observed, the results showed a clear dependence on the pH (see Fig. 6 and 7). In the presence of increasing DNA amounts, the spectra of nHoH$^+$ (pH = 4.4) showed small changes accompanied by the appearance of isosbestic points at 345 and 393 nm (see Fig. 6). This fact indicates an interaction between

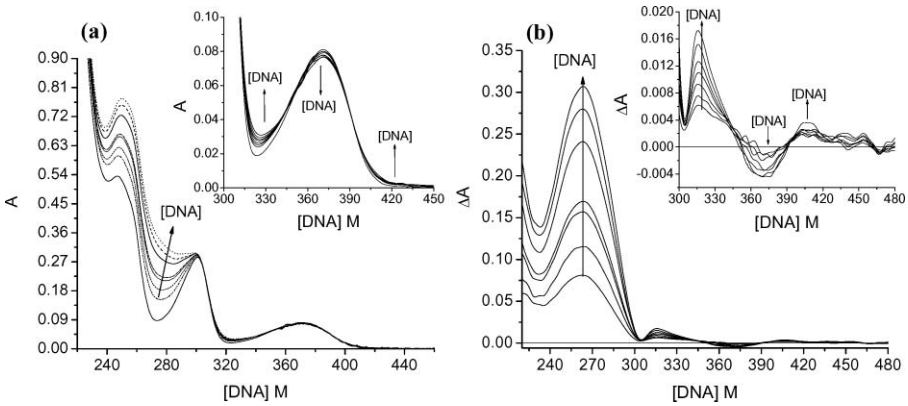

Fig. 6 **(a)** Absorption spectra of $nHoH^+$ (20 μM, at pH 4.4, in acetic acid–sodium acetate buffer solution) in the absence and in the presence of increasing amounts of DNA. The concentration of DNA was (μM in base pair): 0, 8.7, 12.9, 17.2, 21.3, 25.5, 29.6 and 33.7. **(b)** Experimental difference (ED) spectra. Insets show a magnified view of the corresponding spectra.

$nHoH^+$ and plasmidic DNA in their electronic ground states. In this case, a 1 : 1 stoichiometry of the complex was observed with an association constants (K_G) of 4.5 (± 0.5) × 10^4 M^{-1} (see the ESI†). Although small, this value is higher than that reported for βCMAM.[76]

On the contrary, the spectra of the neutral form of norharmane (nHoN, pH = 10.9) showed a very small increase in the band intensity (see Fig. 7). However, neither red shift nor isosbestic points were observed, suggesting that nHoN has an extremely weak interaction (or no interaction) with plasmidic DNA. Unfortunately, a good estimation of K_G could not be done in this case.

In summary, the binding of norharmane with DNA has no significant effect on the UV-vis absorption spectra, indicating that the main binding mode of norharmane to DNA might be a groove binding instead of intercalation. This fact is in good agreement with the results reported by Taira *et al.*[73] In addition, our results showed that the interaction of $nHoH^+$ (pH = 4.4) with the DNA is stronger than that observed for nHoN (pH = 10.9). These results are quite logical because of DNA is a negative charged molecule and norharmane, at pH = 4.4, has a positive net charge.

Fluorescence emission spectroscopy. These steady-state experiments were conducted in order to elucidate which excited state is involved in the interaction of norharmane with DNA. Therefore, corrected emission spectra of both the protonated and neutral forms of norharmane were recorded in the presence and in the absence of plasmid DNA (Fig. 8a).

When considering all the data provided in this section, four key points clearly rise to the surface:

(*i*) Under both pH conditions the increase in the DNA concentration causes a decrease in the fluorescence intensity of norharmane. For the two acid–base form of norharmane studied, the decrease of the integrated fluorescence intensity (I_F) as a function of the concentration of DNA showed linear Stern–Volmer behavior (see Fig. 8a). The slopes ($K_{SV} = \tau_F k_q^F$) obtained were 4827 M^{-1} and 1376 M^{-1} for the experiments performed at pH 4.4 and 10.9, respectively. Taking into account the fluorescence lifetime of each acidic and basic form of norharmane (22 ns and 6 ns, respectively),[44,45] under both pH conditions, for the bimolecular quenching constant (k_q) a value of ~10^{11} M^{-1} s^{-1} can be calculated. These values are larger than the limiting diffusion rate constant (~10^{10} M^{-1} s^{-1}), which is clearly indicating the presence of

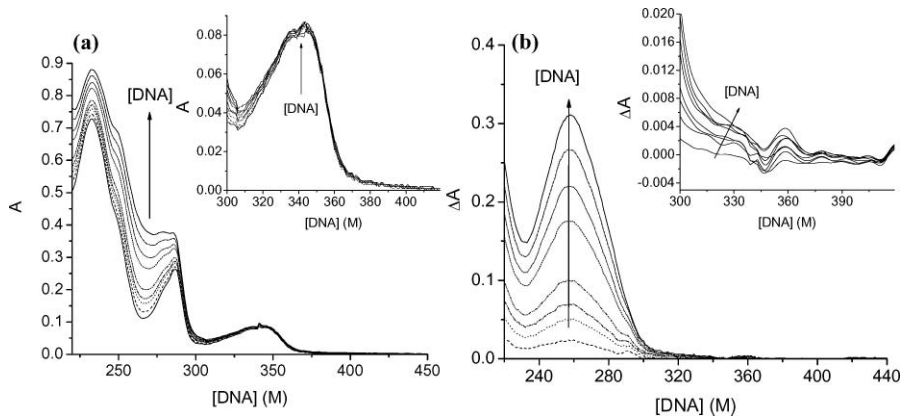

Fig. 7 **(a)** Absorption spectra of nHoN (20 μM, at pH 10.9, in K_2HPO_4–KOH buffer solution) in the absence and in the presence of increasing amounts of DNA. The concentration of DNA was (μM in base pair): 0, 3.5, 8.7, 12.9, 17.2, 21.3, 25.5, 29.6 and 33.7. **(b)** Experimental difference (ED) spectra. Insets show a magnified view of the corresponding spectra.

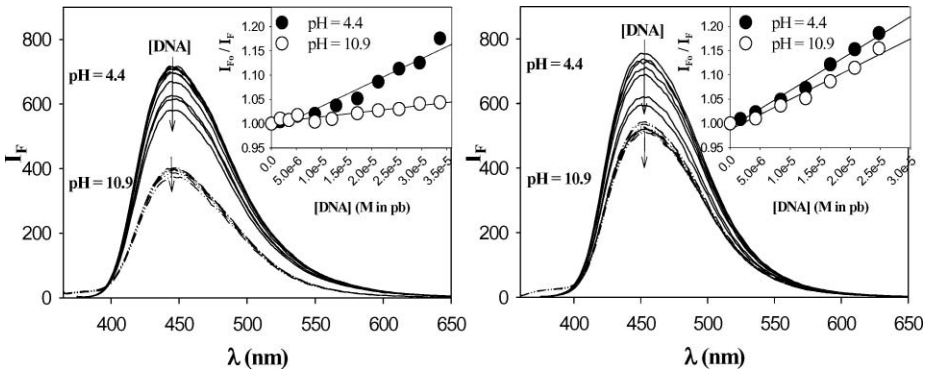

Fig. 8 Quenching of βCs fluorescence by plasmidic DNA, in air-equilibrated aqueous solutions. Corrected fluorescence spectra of: **(a)** norharmane and **(b)** *N*-methyl-norharmane. The arrows indicate the increase of the [DNA]. Insets: Stern–Volmer plots of the integrated fluorescence intensity (I_F).

a static quenching between norharmane and pGEM-3z plasmid. These results are in agreement with those obtained from UV-vis spectrophotometric analysis.

(*ii*) Clearly the extent of the quenching observed at pH 10.9 represents 27% of the quenching observed at pH 4.4. These results are in agreement with the fact that only a fraction less than 30% of the S_1 of nHoN goes on with the protonation yielding ^{1}[nHoH$^+$]*, and with the hypothesis that ^{1}[nHoH$^+$]* is the excited species involved in the interaction with DNA (this point is further supported below).

(*iii*) The emission spectra of βCs in water have been previously described.[55] In brief, the emission spectra of βCs recorded at pH 4.4 show only one emission band with a maximum at ~450 nm. This band has been assigned to the protonated form of the alkaloid (βCsH$^+$). On the other hand, in the fluorescence spectra recorded at pH 10.9, besides the band around 450 nm, an extra less intense emission band, with a maximum at ~380, is also present. The later band has been assigned to the very small fraction of the neutral form of norharmane that remains without protonation. In non-polar and non-protic solvents the band around 380 nm is the only emission band that was detected.[38–40]

Fig. 8a shows that the emission spectrum of nHoN has the same shape and the same value of λ_{em}^{MAX} (448 nm) than the emission spectrum of nHoH$^+$. These results clearly indicate that even in the presence of DNA, the emission observed is due to the protonated form of the norharmane. This fact strongly suggests that, under both pH conditions, the environment of the norharmane molecules is a polar-protic medium. Therefore, from these results it is revealed that neither the protonated (nHoH$^+$) nor the neutral (nHoN) form of norharmane intercalate into the DNA double strand. The latter fact would be in good accordance to the results shown above and with those reported in the literature,[73] suggesting that norharmane shows a very low intercalation capability.

(*iv*) Fig. 8 also shows that, in alkaline solutions where both emission band are present (*i.e.*, at ~380 nm and ~450 nm), only the latter one is quenched by DNA. These results put forward that, even at pH 10.9, the S_1 of βCsH$^+$ (^{1}[βCsH$^+$]*) is the species that interacts with the DNA.

In order to substantiate the hypothesis that the photosensitized reaction occurs from the S_1 electronic excited state of the βC, we decided to extend the quenching studies to the *N*-methylated norharmane derivative (*N*-Me-nHo). This particular βC was chosen due to the fact that the fraction of the excited state that follows protonation is considerably higher than in the case of norharmane (upon excitation almost a 90% of *N*-Me-nHoN yield ^{1}[*N*-Me-nHoH$^+$]*).[55] Therefore, in the case of *N*-methyl-norharmane the extent of the quenching process would be quite similar under both pH conditions.

The data obtained clearly indicate a substantial S_1 deactivation by DNA (see Fig. 8b). For the two acid–base forms of *N*-methyl-norharmane studied, the decrease of I_F as a function of the concentration of DNA also showed a nonlinear Stern–Volmer behaviour. In order to compare the magnitude of the quenching observed under both pH conditions, the slope of the linear part of the Stern–Volmer plots was calculated yielding K_{SV} values of 7615 M^{-1} and 6450 M^{-1} at pH 4.4 and 10.9, respectively. It is easy to note that the slope of the curve obtained from experiments in alkaline solution represents the 84% of the corresponding value obtained from acidic solutions. These results support the hypothesis that the DNA interacts with the first excited state of the protonated form of βCs (^{1}[βCsH$^+$]*). This fact strongly support the hypothesis that in the case of the full aromatic βCs the first singlet excited state of the protonated βCs (^{1}[βCsH$^+$]*) is the excited species that is involved in the interaction and photocleavage of DNA.

Photocleavage of DNA by norharmane under physiological pH conditions

The experiments reported above show a clear pH-dependence on both the interaction βCs-plasmidic DNA and the cleavage of DNA photosensitized by βCs. Although these are *in vitro* studies the evaluation of the photosensitizing properties of norharmane under physiological pH conditions (*i.e.*, at pH = 7.4) would be of importance from a biological point of view.

Kinetic analysis of Sc concentration profiles obtained at pH 7.4 showed that: (*i*) the rate of Sc disappearance matched very well with the rate of appearance of the relaxed form (see the ESI†), following the single-strand cleave model described elsewhere;[65] and (*ii*) the value of the total rate of DNA relaxation was in between those observed at pH 4.7 and 10.2. These results are shown in Fig. 3, where a d[Sc]/dt value of $-4.2\ (\pm 0.5) \times 10^{-3}$ NI $\times$ min^{-1} was obtained. Thus, the d[Sc]/dt value observed at pH 7.4 represents ~50–60% of the damage observed at pH 4.7 (see Table 1, entry 1).

The results described above were expected since at pH 7.4 a mixture of both $nHoH^+$ and nHoN 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 should be a consequence of the average contribution of both species ($nHoH^+$ and nHoN). This fact can be further substantiated by numerical models taking into account both the fraction of the incident light absorbed by each acid–base form of norharmane present in the solution at pH 7.4, and the fact that only 28% of the ^{1}[nHoN]* yields ^{1}[$nHoH^+$]* (see the ESI†).

Conclusions

The current work provides, for the first time, evidences about the strong dependence of the interaction between βCs and DNA on the pH medium. The protonated form of norharmane ($nHoH^+$), in its electronic ground state, shows a higher association or interaction with the DNA molecule than that observed for the neutral form of norharmane (nHoN).

Also, a strong pH dependence of the photosensitized DNA relaxation was observed, being higher the extent of the reaction in the case of experiments performed at pH 4.7 than those performed at pH 10.2. Furthermore, experiments performed under physiological pH conditions (pH 7.4) showed the expected results (*i.e.*, an intermediate effect on the extent of the DNA photocleavage reaction due to a contribution of the fraction of each $nHoH^+$ and nHoN present in the irradiated solution).

In addition, evidences about the mechanism involved in the photosensitized DNA relaxation that takes place in aqueous solution when norharmane acts as photosensitizer are provided. The results suggest that the initial step of the photosensitized DNA cleavage mainly occurs by an electron transfer reaction between DNA and the S_1 state of $nHoH^+$, under the whole pH range studied (pH 4–11). The contributions of other mechanisms, such as damage by reactive oxygen species are only minor or do not take place.

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76 Note that the K_G value obtained for $nHoH^+$ is higher than that reported for β-carboline-3-carboxylic acid *N*-methylamide or βCMAM (363 ± 58, 500 ± 90 and 239 ± 43 M^{-1}, at pH 4, 6 and 9, respectively). The pK_a value of βCMAM is 3.8[77]. In the pH range 6–9, the neutral form of βCMAM is the predominant specie in the solution. Thus, the absence of the positive charge would decrease the interaction with the DNA molecule. On the other hand, at pH 4 ~ 50% of each acid–base form of βCMAM are present. Although in the protonated βCMAM form, the norharmane skeleton plane is twisted by 19.58° with respect to the plane carbon 3 substituent,[77] the availability of the protonated pyridinic nitrogen is not the same as in the case of norharmane.
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