Journal of Photochemistry and Photobiology B: Biology 132 (2014) 66–71

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Intra- and extra-cellular DNA damage by harmine and 9-methyl-harmine

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ARTICLE INFO

Article history:
Received 16 October 2013
Received in revised form 27 December 2013
Accepted 28 January 2014
Available online 13 February 2014

Keywords:
β-carbolines
Photosensitization
Phototoxicity
Cyclobutane pyrimidine photodimers
Micronuclei

ABSTRACT

It is known that β-carbolines are able to produce photosensitized damage in cell-free DNA, but there is little information on their effects on cellular DNA. Therefore, we have analyzed the DNA damage produced by harmine and 9-methyl-harmine under UVA irradiation in V79 cells, together with the associated generation of micronuclei and photocytotoxicity. The results indicate that the most frequent photoproducts generated in the cellular DNA are modified purines such as 8-oxo-7,8-dihydroguanine. Only relatively few single-strand breaks were observed. CPDs were absent, although they were generated in cell-free DNA irradiated under the same conditions. The overall extent of DNA damage in the cells was considerably smaller than the one observed in cell free DNA. The generation of cellular DNA damage was associated with a significant generation of micronuclei and decreased cell proliferation. The data indicate that β-carbolines act as photosensitizers in mammalian cells. The spectrum of DNA modification, and therefore the mechanism of DNA damage generation, differs considerably from that observed with cell-free DNA.

1. Introduction

β-Carboline alkaloids are a group of heterocyclic compounds widespread in nature. They can be extracted from many plants, as they are normal constituents of seeds [1], stems [2] and leaves [3]. Furthermore, β-carbolines are endogenously produced in human and other mammalian tissues, [4] and have been found in plasma, platelets and urine [5]. Extracts from plants of several derivatives have been used as antispasmodics and sedatives since ancient time [6]. In addition, monoamino oxidase inhibition, convulsive or anticonvulsive activity and anxiolytic effects were observed [7,8] and they also act as antimicrobial agents, in particular as antiparasites [9]. Due to the planarity of the main β-carboline ring, these compounds are good DNA intercalators [10–12] having cytotoxic and genotoxic effects in several human tumor cell lines [13–15]. Moreover, the participation of several β-carbolines in photosensitizing processes has been demonstrated. In their excited state, these compounds have the ability to photooxidize deoxyribonucleotides [16], to photoinduce single strand-breaks (SSBs) in cell-free DNA [17,18], chromosome damage in mammalian cells [19] and also to inactivate viruses [20] and bacteria [21].

Recently, the photosensitized damage induced in cell-free DNA by a group of β-carbolines (norharmane, harmane, harmine, 9-methyl-norharmane, 9-methyl-harmane, 9-methyl-harmine) was studied in detail and shown to consist of SSBs, oxidatively generated purine modifications sites of base loss (AP sites), and cyclobutane pyrimidine dimers (CPDs). SSBs and oxidized products were found to be generated by a type I reaction, while CPDs are formed by energy transfer [22]. The extent and type of photosensitized damage depends not only on the nature of the β-carboline substituent, but also on their DNA binding properties and the pH. Although the majority of these compounds are able to produce reactive oxygen species (ROS), such as singlet oxygen, superoxide anion and hydrogen peroxide [23,24], it was shown that these ROS do not play an important role as intermediates in the DNA damage process [18,22].

In this work, we have focused on the analysis of the DNA damage generated by photoexcited harmine and 9-methyl-harmine in V79 cells. The spectrum of DNA modifications generated was

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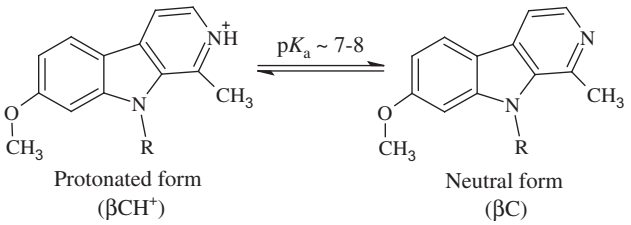

Scheme 1. Structure of harmine (R = H, Ha) and 9-methyl-harmine (R = CH_3, 9-Me-Ha).

compared with that observed under cell-free conditions. The two β-carbolines used in this work are showed in Scheme 1.

2. Experimental

2.1. General

2.1.1. β-Carbolines

Harmine (>98%) from Sigma–Aldrich were used without further purification. The method used to synthesize and purify 9-methyl-derivatives has been published elsewhere [25].

2.1.2. Cells, DNA material and enzymes

Chinese hamster lung fibroblast V79 cells were used for this work. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/l) with L-glutamine, 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). DNA from bacteriophage PM2 (PM2 DNA, 10,000 bp) was prepared according to the method of Salditt et al. [26] Formamidopyrimidine-DNA glycosylase (Fpg protein) was isolated from *Escherichia coli* strain JM105 harboring plasmid pFPG230 [27]. Endonuclease IV, T4 endonuclease V was partially purified from an inducible overproducer (*E. coli* strain A 32480 carrying the plasmid ptacdenV) provided by L. Mullenders, Leiden. All repair endonucleases were tested for their incision at reference modifications (i.e., oxidized purines induced by methylene blue plus light, AP sites by low pH and cyclobutane pyrimidine dimers (CPDs) by UVC) 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 [28].

2.1.3. pH adjustment

The pH of β-carboline derivatives aqueous solutions were 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 (35 wt. % solution in D_2O, 99 atom % D, Aldrich) and NaOD (40 wt. % solution in D_2O, 99+ atom % D, Aldrich) were used. The pD of the solution was adjusted as it was described elsewhere [29], the final isotope purity was greater than 96%. The β-carboline acidic, neutral and alkaline solutions were prepared in acetic acid–sodium acetate (pH 4.8), KH_2PO_4–NaOH (pH 7.4) and $Na_2B_4O_7$–NaOH (pH 10.0) buffers, respectively.

2.2. DNA photoproducts characterization

2.2.1. Irradiation set-up

All samples were irradiated in either a 96 well plate or a culture flask for 20 min on ice with a Philips HPW 125 W lamp emitting at 365 nm (bandwidth ~20 nm), placed at a distance of 10 cm, corresponding to a dose of 30 kJ/m^2.

In the case of cell-free DNA, buffered aqueous solutions (10 mM KH_2PO_4, 50 mM NaCl, pH 7.4) of a β-carboline and PM2 DNA (10 μg/ml) were irradiated. 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. In the case of V79 cells, exponentially growing cells were irradiated in PBSCMF buffer (137 mM NaCl, 2.7 mM KCl, 8.3 mM NaH_2PO_4, 1.5 mM KH_2PO_4, pH 7.4) with different concentrations of β-carboline. After irradiation, cells were washed and fresh medium was added.

2.2.2. Quantification of endonuclease-sensitive modifications

(a) in PM2 plasmid. The DNA relaxation assay used to quantify endonuclease-sensitive modifications (ESSs) and single-strand breaks (SSBs) in PM2 DNA has been described elsewhere [30] and we have recently reviewed its application to characterize the DNA damage induced by photosensitization [31]. It makes use of the fact that supercoiled PM2 DNA is converted by either a SSBs or the incision of a repair enzyme into a relaxed (nicked) form, which migrates separately from the supercoiled 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 in BE1 buffer, (ii) endonuclease IV, 0.3 μg/ml in BE1 buffer and (iii) T4 endonuclease V, 3 μg/ml 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 relaxed and supercoiled forms of the DNA after staining with ethidium bromide allowed calculating the number of SSBs or, if an incubation with a repair endonuclease preceded the gel electrophoresis, the number of SSBs plus ESS. To obtain damage profiles, the data were corrected for the number of modifications in unmodified DNA, and the number of ESSs was obtained by subtraction of the number of SSBs.

(b) in V79 cells. An alkaline elution assay was used to quantify SSBs, oxidatively generated purine modifications sensitive to Fpg and CPDs sensitive to T4 endonuclease V. The protocol followed the method of Kohn et al. [32] with modifications [30,33]. Briefly, the sum of SSBs and DNA modifications sensitive to one of the repair glycosylases was obtained from experiments in which the cellular DNA was incubated with Fpg protein (1 μg/ml) or T4 endonuclease V (0.1 μg/ml) immediately after cell lysis. To quantify SSBs, the incubation was carried out without repair enzyme. The numbers of glycosylase-sensitive modifications were obtained by subtraction of SSBs. The slope of an elution curve obtained with γ-irradiated cells was used for calibration (6 Gy = 1 SSBs/10^6 bp, [32]). The slopes observed with untreated control cells (background levels) were subtracted.

Each repair enzyme used recognizes a different spectrum of DNA modifications [34–36]. Thus, Fpg protein incises at 8-oxo-7, 8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) and also sites of base loss (AP sites). T4 endonuclease V recognizes CPDs and AP sites, while endonuclease IV recognizes only AP sites.

2.3. Phototoxicity assay

V79 cells were irradiated in the presence of different concentration of β-carbolines (see above the irradiation set-up description). For each concentration, two parallel culture flasks were treated. Cells were washed, and one flask was treated with trypsin right after irradiation and cells were counted, while the other flask

was counted after 48 h of incubation in fresh medium (free of β-carbolines). The proliferation factor was calculated then by the ratio of the number of cells counted at 48 h to cells at time 0 (Beckman Coulter Counter Z2).

2.4. Quantification of micronuclei

V79 cells were irradiated with UVA (365 nm) in the presence or absence of β-carbolines in PBSCMF buffer (see above). They were washed and subsequently incubated with fresh medium under culture conditions for 24 h. Approximately, 1×10^5 cells were fixed on a microscope slide by cytospin centrifugation and treated with methanol for 1 h at −20 °C. After staining with bisbenzimide (Hoechst 33,258; 1 μg/ml in PBSCMF for 60 s), 3×1000 cells/flask per experiment were examined in blinded samples for the presence of micronuclei with a fluorescence microscope.

3. Results

β-Carbolines in aqueous solution are weak bases with a pKa ∼ 7, which leads to a mixture of both protonated and neutral species at physiological pH. Although the absorption spectra of protonated and neutral molecules are different, both show a significant absorption at 365 nm, the wavelength used in this work for photoexcitation (Fig. 1). In particular, at pH 7.4, harmine (Ha) and 9-methyl-harmine (9-Me-Ha) show molar absorption coefficient values at 365 nm of 5295 and 3109 M^{-1} cm^{-1}, respectively.

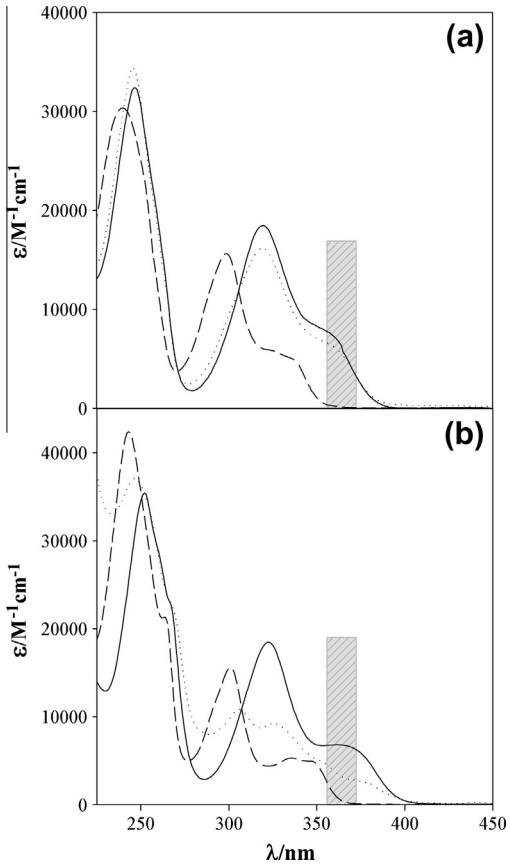

Fig. 1. Absorption spectra of Ha (a) and 9-Me-Ha (b) recorded under different pH conditions: pH 4.8 (solid line), pH 7.4 (dotted line) and pH 10.0 (dashed line).

3.1. Phototoxicity of β-carbolines

To determine the cytotoxicity of photoexcited β-carbolines in V79 cells, proliferation assays were performed. Exponentially growing V79 cells were exposed for 20 min to UVA (365 nm) in the presence of increasing concentrations of Ha or 9-Me-Ha. Subsequently, the influence of the treatments on cell proliferation was determined by cell counting right after damage and after 48 h of incubation in fresh media, free of β-carbolines.

As is shown in Fig. 2, the two β-carbolines caused a quite similar dose-dependent reduction of cell proliferation upon UVA irradiation. On the contrary, no growth inhibition was caused by the irradiation alone (see data point at 0 μM of β-carboline) and in non-irradiated cells pre-incubated with 10 μM of β-carbolines (see open symbols in Fig. 2).

3.2. Characterization of intracellular DNA damage photosensitized by β-carbolines

As it was already demonstrated, photosensitized DNA damage induced by Ha under cell-free conditions consists of Fpg-sensitive base modifications as the main photoproduct [22]. To investigate the extent of damage in V79 cells irradiated with UVA (365 nm) in the presence of various concentrations of Ha and 9-Me-Ha, an alkaline elution assay in combination with repair glycosylases as probes was used. Fig. 3a and b show that both β-carbolines generate relatively high yields of oxidatively generated DNA damage measured as Fpg-sensitive modifications, while SSBs were generated in much lower yields, barely detectable. At the same absorbance (i.e., at the same excited β-carboline's concentration), Ha clearly produces more oxidatively generated DNA damage than 9-Me-Ha.

The spectrum of DNA modifications produced by photoexcited Ha and 9-Me-Ha under cell-free conditions consists not only of SSBs and other oxidatively generated photoproducts but also CPDs [22,37]. To analyze the formation of CPDs in V79 cells, an alkaline elution assay in combination with the repair enzyme T4 endonuclease V was used. Ha was chosen for this experiment because this derivative showed the highest levels of CPDs production in

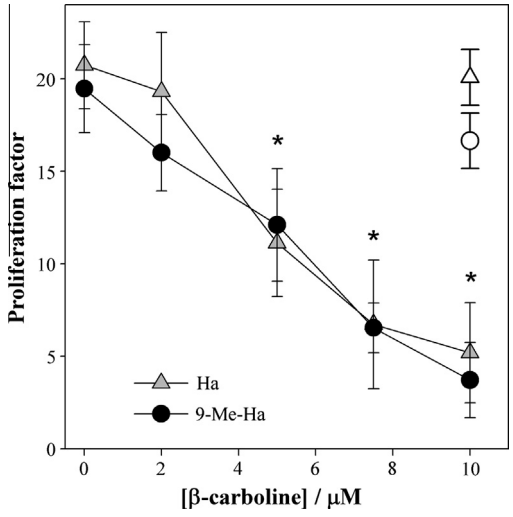

Fig. 2. Phototoxicity of Ha and 9-Me-Ha in V79 cells. Data gives the proliferation factor of treated cells (irradiated with UVA 365 nm; 30 kJ/m^2), incubated for 48 h (see Section 2). Open symbols refer to non-irradiated cells with the highest concentration of β-carbolines tested (10 μM). Data points represent the mean of 2–3 independent experiments (±SD). Statistical differences ($p < 0.05$) are indicated as (*): one-way ANOVA with Dunnett's *t*-test were performed for each compound and compared to the respective controls.

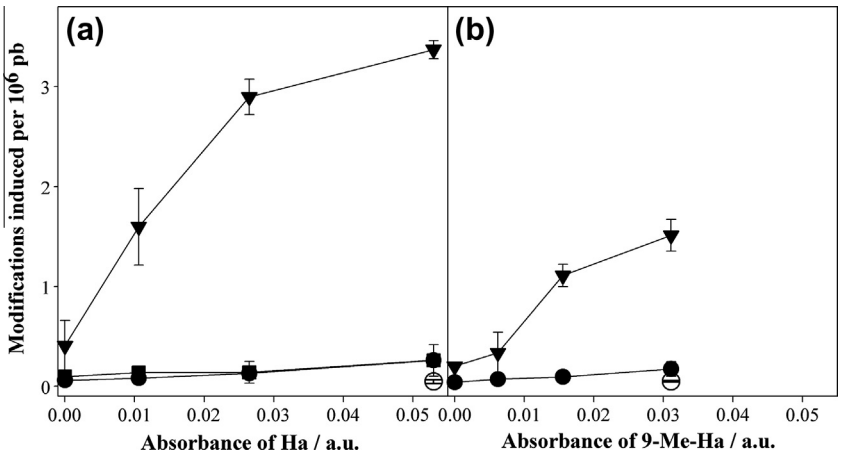

Fig. 3. Concentration-dependence of the generation of DNA modifications in V79 cells by (a) Ha and (b) 9-Me-Ha upon irradiation with UVA (365 nm; 30 kJ/m^2). (●) SSBs, (▼) Fpg-sensitive modifications, (■) CPDs, sensitive to T4 endonuclease V and (○) βC-free irradiated cell. Data points indicate means of three experiments ± S.D.

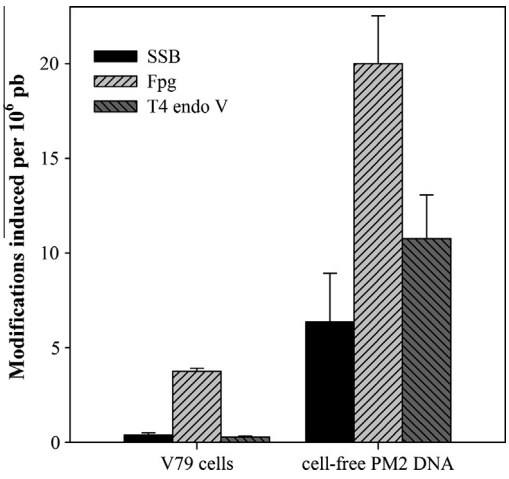

Fig. 4. Comparison of photosensitized DNA damage by Ha (10 μM) under UVA (365 nm; 30 kJ/m^2) irradiation in intracellular conditions or cell-free PM2 DNA.

cell-free DNA experiments. Fig. 4 shows a comparison of the DNA damage spectra measured in cell-free DNA (by the PM2 assay) and in V79 cells (by the alkaline elution technique). The data show that, at the same concentration of Ha, the yield of oxidatively generated DNA damage (Fpg-sensitive modifications) is 5 times higher in cell-free DNA than in V79 cells. Notably, neither SSBs nor CPDs were detected in the cells, while in the cell-free PM2 DNA both lesions were generated in relatively high yield.

3.3. Micronuclei induced by β-carbolines under UVA irradiation

Micronuclei originate as a consequence of different types of DNA damage during mitosis, and they are regarded as a very sensitive indicator of genotoxicity [38]. To quantify the generation of micronuclei by photoexcitation of β-carbolines, V79 cells were cultured for 24 h before exposing to UVA irradiation and Ha. Afterwards, cells were washed and incubated for 24 h in full medium and then fixed on a microscope slide. Fig. 5 shows a significant dose-dependent increase in the number of micronuclei after treatment, confirming the genotoxic potential of the DNA modifications induced by photoexcited of β-carbolines in mammalian cells.

4. Discussion and conclusions

In this work we have shown that Ha and 9-Me-Ha, under UVA irradiation, are able to produce damage in cultured mammalian cells. In particular, in the concentration range evaluated, the two β-carbolines are able to inhibit the proliferation rate of the cell line investigated as well as to induce micronuclei formation. These two

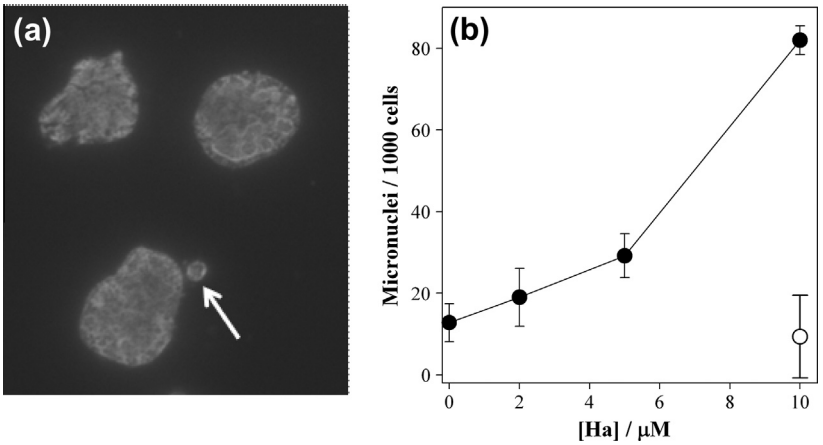

Fig. 5. (a) Section of a fluorescence image obtained from V79 cells after treatment (see Section 2). A micronucleus is marked with the white arrow. (b) Number of micronuclei generated by photosensitization of V79 cells with Ha under UVA irradiation. The open symbol refers to non-irradiated cells exposed to the highest concentration of Ha tested. Data points represent the mean of 2–3 independent experiments (±SD).

effects are a consequence of photoexcitation since no cytotoxic effect is observed in the dark. This is in good agreement with previously published data [9]. Also, DNA damage photoinduced by these two alkaloids was evaluated. The amount of damage produced in intracellular DNA is 5 times smaller than the damage observed in cell-free DNA, most probably due to an efficient quenching of the photoexcited β-carbolines by various cellular constituents (proteins, etc.). The analysis with repair enzymes showed that Fpg-sensitive modifications (i.e., oxidative purine modifications, such as 8-oxoGua, FapyGua and FapyAde, and also AP sites) are the most frequent products generated, while single-strand breaks (SSBs) and cyclobutane pyrimidine dimers (CPDs) were below the limit of detection.

The cellular DNA damage spectrum observed would be consistent with both a type I and a type II damaging mechanism. Based on the results obtained with cell-free DNA, however, the prevalence of a type I mechanism appears more likely. Due to the relatively strong DNA binding of β-carbolines, a close contact between the DNA and the excited photosensitizer, which is required for type I reactions, is ensured. The absence of SSBs could be a consequence of the rapid repair ($t_{1/2}$ generally less than 5 min) that unavoidably takes place during detachment of the cells before damage analysis.

The absence of CPDs is more interesting. Presumably, it is a consequence of the fact that in the case of the β-carbolines the type-I electron transfer reaction, predominantly takes place from the (relatively long-lived) excited singlet state (S_1) rather than from the triplet state (T_1), while the energy transfer, which leads to the formation of CPDs, takes place exclusively from the excited triplet states [37]. Therefore, the relatively low yield of CPDs in the cells can be a consequence of quenching processes by intracellular biomolecules either of T_1 of the neutral form of Ha or S_1, leading to a competitive deactivation pathway or leading to the concomitant decrease on the quantum yield of triplet formation, respectively. Alternatively, the interaction between the photosensitizer and the DNA, which determines the efficiency of the triplet–triplet energy transfer, may be weaker in the cells than under cell-free conditions [39]. Comparisons between DNA damage profiles and the photophysical characteristics of additional photosensitizers will help to understand the subcellular mechanisms.

Acknowledgements

The present work was partially supported by ANPCyT (PICT 2012-0423), CONICET (PIP 00400), UNSAM (E103), UBA (X088), the Deutsche Forschungsgemeinschaft (EP11/10-1) and MinCyT-DAAD-Cooperation Program (DA/11/15). M.V. thanks CONICET for postdoctoral research fellowship. R.E.-B., M.V. and F.M.C. are research members of CONICET. F.M.C. thanks DAAD for the spectrofluorometer Fluoromax4-HORIBA provided in the framework of Equipment Grants for Higher Education and Comparable Scientific and Research Institutions in Developing Countries.

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