Dyes and Pigments 94 (2012) 99–102

Contents lists available at ScienceDirect

Dyes and Pigments

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

The synthesis and characterization of ethylenediamine-modified *Elsinochrome A*

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ARTICLE INFO

Article history:
Received 8 October 2009
Received in revised form
2 March 2010
Accepted 2 March 2010
Available online 12 March 2010

Keywords:
Elsinochrome A
Ethylenediamine-modified
Longer wavelength absorption
Photodynamic therapy
Photosensitizing activity
Photodamage efficacy

ABSTRACT

To overcome the lack of appreciable absorption in the phototherapeutic window (600–900 nm) of the naturally occurring perylenequinonoid pigment, *Elsinochrome A*, which limits its clinical application, an ethylenediamine-modified elsinochrome A, possessing long wavelength absorption (708 nm) was synthesized. Electron paramagnetic resonance and chemiluminescence assays indicated that ethylenediamine-modified *Elsinochrome A* possessed photosensitizing activity and its photodamage efficacy is greater than that of unmodified *Elsinochrome A*.

1. Introduction

As efficient phototherapeutic agents, naturally occurring perylenequinonoid pigments (PQP), including hypocrellins, cercosporin, elsinochromes and hypericin, which have been isolated from fungi, as well as other organisms, have received interest over the past two decades [1–7]. Comparison with currently used photodynamic therapeutic agents, hematoporphyrin derivatives (HPD), PQP possess some advantages, i.e. ready preparation and easy purification relative to HPD, lower aggregation tendency (which decreases the Photodynamic therapy (PDT) efficiency of HPD), high quantum yield of singlet oxygen ($^1O_2$) and significantly reduced normal tissue photosensitivity because of their fast metabolism *in vivo* [8–10]. Now, hypocrellins, an example of a Chinese folk medicine, including hypocrellin A (HA) and hypocrellin B (HB), have been successfully applied in the clinical PDT treatment for some skin diseases, such as white lesions of vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis, without the observation of prolonged normal tissue sensitivity which can occur with the use of HPD [4]. In addition, hypocrellins also have been taken orally as folk medicine in China.

Among these natural perylenequinonoid pigments, elsinochrome A (EA), isolated from *Elsinoe* sp. *I*, is a photosensitive pigment. According to the report by Li et al., comparison of EA with HA and HB the triplet state lifetime of EA (5.2×10^{-6} s) was longer than those of HA (4.5×10^{-6} s) and HB (4.0×10^{-6} s), which contributed to EA displaying a superior $^1O_2$ quantum yield (0.98) taking HB (0.84) as the reference [11–13]. The foregoing data indicated that EA possesses a higher photosensitization activity than hypocrellins and may be a better candidate for photodynamic utilization. Nevertheless, from the viewpoint of clinical applications, the absorption ability in the phototherapeutic window (600–900 nm) of EA must be improved further, because it only absorbs weakly at wavelengths longer than 600 nm [5,14]. Therefore improvement of the absorption profile of EA has become the focus of studies on this molecule.

According to previous work of the present research group [15] and that of Zhao et al., [16–18], an electron-donating amino group, introduced into the peri-hydroxylated perylenequinone ring of PQP, can induce facile intramolecular charge transfer (ICT) between the amino group and the carbonyl group in PQP, and distinctly red shift their absorption spectra.

This paper concerns the improvement of the photodynamic efficacy of EA, via the synthesis of ethylenediamine-modified EA (EDEA; Fig. 1,b). In comparison to previous derivatives of HA or HB, EDEA showed strong absorption at longer wavelength and λ_{max} was shifted to 708 nm. In addition, the ESR spectra indicated that EDEA preserved the good photosensitizing properties of EA.

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doi:10.1016/j.dyepig.2010.03.004

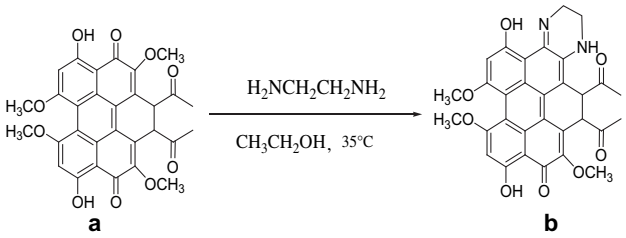

Fig. 1. Pathway for the ethyldiamino-substituted elsinochrome A.

2. Materials and methods

2.1. Chemicals

EA was presented as gift by China pharmaceutical university. Ethylenediamine, 5-dimethyl-1-pyrroline-N-oxide (DMPO), 2,6,6-tetramethyl-4-piperidone (TEMP), 1,4-diazabicyclo[2,2,2]octane (DABCO) and calf thymus deoxyribonucleic acid (CT DNA) were purchased from Sigma–Aldrich used as received. All organic solvents employed were obtained from Beijing Chemical Corporation.

CT DNA was dispersed in PBS buffer solutions (pH 7.4) unless otherwise noted. CT DNA solutions were prepared by dispersing the desired amount of DNA in buffer solution with stirring overnight at temperatures below 4 °C. In the experiments where titration with DNA was required, the DNA solution was sonicated at 0 °C for 10 min, using a Branson probe ultrasonicator. This operation significantly reduced the viscosity of the DNA solutions and permitted more accurate and precise titration. The concentration of CT DNA was expressed as the concentration of nucleotide and was calculated by using an average molecular weight of 338 for a nucleotide and an extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm.

2.2. Spectroscopic measurements

IR spectra were taken on a BIO-RAD FTS 165 grating spectrometer. UV–Vis spectra in solutions were recorded with a Varian-Cary 5000 spectrometer. Mass spectra were recorded with a Varian 3800/2200 MALDI-TOF instrument. Data were presented in *m*/*z* (%) values. ^{1}H NMR spectra were measured with a Bruker AVANCE-400 spectrometer. Electron paramagnetic resonance (ESR) spectra were obtained using a Bruker ESP-300E spectrometer. Elemental analysis was determined using a Vario-Elementar Microcube ELIII.

2.3. Synthesis of EDEA

A mixture of ethylenediamine (5 mL, 74.9 mmol) and EA (150 mg, 0.27 mmol) dissolved in ethanol (50 mL) was heated at 35 °C for 12 h under an argon atmosphere. Upon termination of the reaction, the solution was subjected to column chromatography separation (1.5% KH_2PO_4 activated silica gel column with an eluent of chloroform/methanol in a volume ratio of 25:1) to obtain the desired compound (Fig. 1). EDEA: 35% yield with respect to EA.

FT-IR (KBr, ν_{max}, cm^{-1}): 3423, 2980, 1739, 1612.
^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 16.12 (2H, s), 6.59 (2H, s), 5.18 (2H, s), 5.08 (NH, 1H, m), 4.29 (3H, s), 4.04 (6H, s), 3.40–3.71 (4H, m), 2.03 (6H, s).
MALDI-TOF MS: 555.09 (M+1).
Calculated (Found) for $C_{31}H_{26}N_2O_8$: C, 67.14 (67.27); H, 4.73 (4.76); N, 5.05 (5.07).

2.4. Free radical measurement using EPR

EPR spectra were obtained using a Bruker ESP-300E spectrometer operating at room temperature, and the operating conditions were as follows: microwave bridge, X-band with 100 Hz field modulation; sweep width, 100 G; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; receiver gain, 1×10^5; and microwave power, 5 mW. Samples were injected into the specially made quartz capillaries, and purged with air or oxygen for 30 min in the dark, respectively (the oxygen-saturated systems were sealed while the air-saturated systems were opened to the air), and illuminated directly in the cavity of the EPR spectrometer with a Nd:YAG laser (532 nm, 5–6 ns of pulse width, repetition frequency 10 Hz, 10 mJ/pulse energy). The relative free radical quantum yields were estimated at normalized sample absorbance at 532 nm.

2.5. Chemiluminescence assay

DNA has an intrinsic weak chemiluminescence and upon damage the chemiluminescence is significantly intensified [19,20]. For examining the photodynamic capacity of EDEA, the chemiluminescence assay was utilized to characterize the calf thymus DNA (CT DNA) photosensitized damage by EDEA [21]. In a typical experiment EDEA was added to the air-saturated buffer solution of CT DNA separately. The mixture was then irradiated with light above 470 nm, and the chemiluminescence was recorded after irradiation for 3 min. Meanwhile, the air-saturated buffer solution of CT DNA without EDEA or EA was also irradiated as control experiment.

3. Results and discussions

3.1. Characterization of EDEA

Compared to EA, the absorption spectrum of EDEA shows a significant bathochromic shift in the DMSO solution (Fig. 2). The maximum absorption wavelength of EDEA is shifted from 470 nm ($\lg\epsilon = 4.39$) [13] to 708 nm ($\lg\epsilon = 4.68$) which is much longer wavelength absorbing than any other PQP derivatives. The red shift is evidently the result of the substitution of the carbonyl group and the methoxy function of the EA by the bridged amino group, which greatly increases the absorbance of the resultant EA derivatives in the phototherapeutic window of 600–900 nm. Furthermore the successful synthesis of EDEA was confirmed by the ^{1}H NMR spectrum, mass spectrometry and CHN microanalysis.

3.2. Reactive oxygen generation

The spin-trapping ESR technique was applied to evaluate the ROS generation ability using TEMP and DMPO as spin-trapping

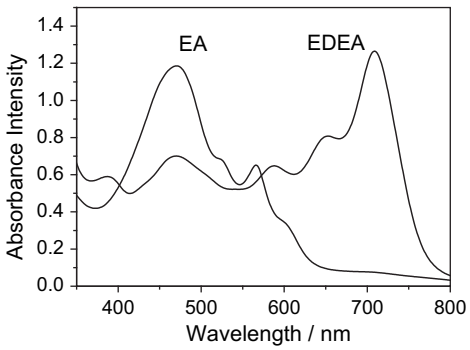

Fig. 2. Absorption spectra of EA and EDEA in DMSO solutions.

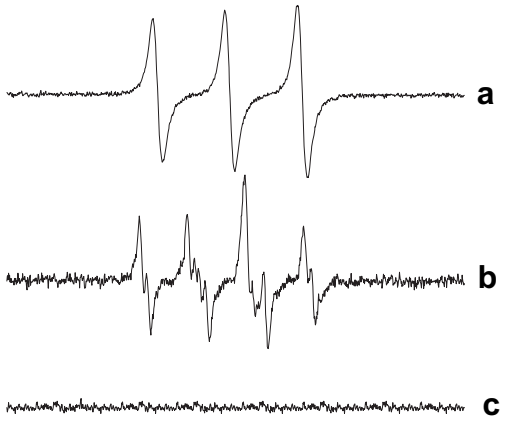

Fig. 3. (a) ESR spectrum obtained upon irradiation of an oxygen-saturated DMSO solution EDEA in the presence of TEMP; (b) ESR spectrum obtained upon irradiation of an air-saturated DMSO solution **3** in the presence of DMPO; (c) The detected baseline signal in the absence of EDEA, oxygen or light at the similar experiment condition with a and b.

agents for $^1O_2$ and $O_2^{-}\bullet$, respectively. When irradiation was carried out in an oxygen-saturated DMSO solution of EDEA in the presence of 20 mM TEMP, three lines with identical intensity and hyperfine coupling constant of 16 G were observed (Fig. 3, curve a) which is a characteristic ESR signal of TEMPO (adduct of TEMP with $^1O_2$). If 1,4-diazabicyclo[2,2,2]octane, a specific scavenger of $^1O_2$, was added, the signal was suppressed efficiently [5]. If DMPO was added, a typical ESR signal attributed to the adduct of superoxide anion radical with DMPO (DMPO-$O_2^{-}\bullet$) appeared upon irradiating the air-saturated DMSO solution of EDEA. This signal is characterized by three hyperfine coupling constants: $\alpha^N = 13$ G, $\alpha_\beta^H = 10$ G, and $\alpha_\gamma^H = 1.5$ G (Fig. 3, curve b) [22]. Efficient quenching by superoxide dismutase, a scavenger of $O_2^{-}\bullet$, supports the assignment of this signal. In addition the control experiments supported the foregoing results and no signals could be detected either in the dark or upon irradiation when any of the sample components were omitted (Fig. 3, curve c). Thus, the ESR spectral results demonstrated that EDEA may generate ROS under irradiation and offers potential to be used as a photosensitizer in PDT.

Furthermore comparison of the DMPO-O_2^{-} signal intensity of the EDEA and EA showed an intensity increase for the EDEA of ca. 2.31 times that of EA (Fig. 4).

3.3. Photodegradation of CT DNA analysis

Fig. 5 shows the relationship between the relative intensity of chemiluminescence and time of the CT DNA solution without and

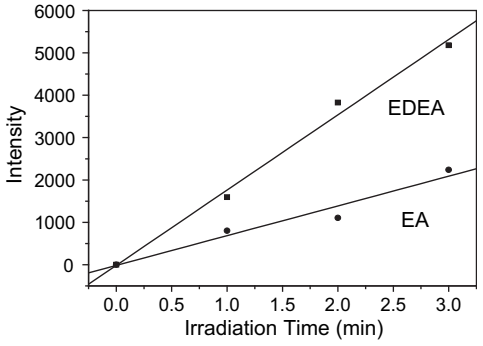

Fig. 4. Dependence of ESR signal intensity of DMPO-superoxide radical adduct on irradiation time for EDEA and EA.

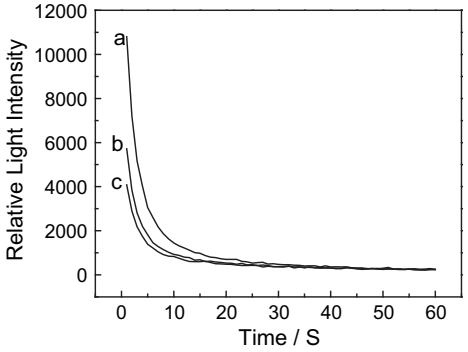

Fig. 5. Chemiluminescent assay for photoinduced CT DNA damage sensitized by EA or EDEA. EDEA + CT DNA (curve a), EA + CT DNA (curve b) and CT DNA control (curve c).

with EA or EDEA. Comparing the chemiluminescence intensity of EA and EDEA systems, it is obvious that the photodamage of CT DNA induced by EDEA was ca. four-fold more severe than that induced by EA under identical experimental conditions.

4. Conclusions

In summary, EDEA, the ethylenediamine-modified elsinochrome A, was synthesized. It exhibits stronger absorbance in phototherapeutic window and greater photodamaging ability than EA, which indicates that EDEA would be a potentially useful candidate in PDT.

Acknowledgement

This research was financially supported by the National Natural Science Foundation of China (No. 20603018).

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