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Xanthone-photosensitized detoxification of the veterinary anthelmintic fenbendazole

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ABSTRACT

Fenbendazole (**1**) is a common veterinary anthelmintic, toxic to water living microorganisms. Fluorescence quantum yields of **1** were found to be 0.11 in acetonitrile, 0.068 in methanol, 0.034 in cyclohexane, and 0.013 in water. The singlet excited state energy was *ca.* 96 kcal mol^{-1} in all solvents. The phosphorescence spectrum of **1** in ethanol at 77 K displayed a maximum at 450 nm, leading to a triplet energy of 75 kcal mol^{-1}. Experimental excited state energies agree well with the results of DFT calculations at the time-dependent B3LYP/6-311+G(d,p)//B3LYP/6-31G(d) level. Laser flash photolysis (LFP) of **1** at 266 nm led to transients absorbing in the 300–700 nm range, ascribed to radical cation $\mathbf{1}^{\bullet+}$, which were also observed upon 355 nm LFP of xanthone (XA) in the presence of **1**. Solar-simulated photolysis revealed XA-enhanced photodegradation of **1** and led to decreased toxicity, as shown by *Daphnia magna* assays.

1. Introduction

Compounds containing the benzimidazole (BZ) moiety have found application as anthelmintic and are effective as anti-nematodal and anti-protozoal agents [1,2]. More interestingly, in the course of investigations on the development of anthelmintic resistance to BZs it has been discovered that they bind specifically and with high affinity to β-tubulin (β-TUB) monomer before dimerization with α-TUB and subsequent blockade of microtubule formation [3,4]; this effect has been exploited for inhibition of cancer cell proliferation by suppressing microtubule dynamics [5]. In this context, the study of TUB–BZ interactions has been a very active area of research, in view of its biological importance.

Unfortunately, many BZs are toxic to humans and animals, so recent studies have focused not only on the elimination of these xenobiotics from the environment, but also on their ecological hazard [6]. Moreover, the extensive use of BZs may leave residues in edible tissues or in animal-derived food, such as milk, egg and meat [7].

Sunlight exposure contributes to the degradation of pesticides and can in principle be used as a tool for environmental remediation. However, little attention has been devoted to the effects of light on BZs. Photochemical studies are limited to the parent molecule and some of its derivatives [8,9], and only recently few studies have appeared on their photo-elimination from the environment [10–12].

Specifically, methyl [5-(phenylthio)-1H-benzimidazol-2-yl]carbamate (fenbendazole, **1**) known commercially as Panacur (Chart 1), is a veterinary biocide extensively used against gastrointestinal parasites in farm animals and pets [13].

Recently the synergistic effect of **1** with an anti-oxidant on the growth of human cancer cells has been reported [14], but in parallel its toxicity to the water living microorganisms has also been proved [6]. By contrast with the widespread utilization of **1**, its photobehavior remains relatively unknown. In this field, the studies on **1** are limited to the degradation process in methanolic solution under simulated sunlight, which leads to formation of two hydrolysis products [15]. However, the involved singlet and/or triplet excited states and other possible transients have not been investigated as yet. With this background, the aim of the present work is the full characterization of photophysical properties of fenbendazole. This knowledge is an essential requirement to check, in a later stage, the possibility of using **1** as a probe for host-guest interaction, such as complexation with cyclodextrin or with tubulin, through fluorescence and/or triplet excited state changes. In addition, recent work has demonstrated the relatively high toxicity of **1** compared with other members of the BZ family [6]; in this context, we wish now

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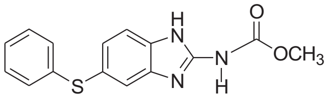

Chart 1. Fenbendazole molecular structure.

to explore the feasibility of using light for detoxification of water polluted with **1,** wich has been evaluated by an established model based on the inhibition of mobility of *Daphnia magna.*

2. Experimental

2.1. Materials and solvents

Fenbendazole (**1**), xanthone (**XA**), S-flurbiprofen, thiabendazole and *tert*-butyl peroxide were purchased from Aldrich. Their purity was checked by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and high performance liquid chromatography (HPLC) analysis. Reagent grade solvent acetonitrile and methanol were purchased from Scharlau and used without further purification. The water was Millipore quality.

2.2. Absorption and emission spectra

Optical spectra in different solvents were measured on a Jasco V-630 spectrophotometer. Emission spectra were recorded on a spectrofluorometer system Jasco FP-8500, provided with an M 300 emission monochromator in the wavelength range of 200−900 nm and are uncorrected. Samples were placed into 10 mm × 10 mm Suprasil quartz cells with a septum cap. Solutions were purged with nitrogen or oxygen for at least 10 min before the measurements. Fluorescence quantum yields were determined using *S*-flurbiprofen as reference ($\Phi_F = 0.27$ at $\lambda_{exc} = 281$ nm in methanol as solvent) [16]. The absorbance of the samples at the excitation wavelength was kept below 0.1. Excitation and emission slits were maintained unchanged during the emission experiments. Fluorescence lifetime measurements were based on a PTI(TM-2/2003) instrument equipped with a H_2/N_2 lamp (50/50, 1.5 ns pulse width) and a stroboscopic detector. All experiments were performed at room temperature (22 °C).

2.3. Laser flash photolysis experiments

The LFP experiments were carried out by using a Q-switched Nd:YAG laser (Quantel Brilliant, 266 or 355 nm, 10 or 14 mJ per pulse, 5 ns fwhm) coupled to a mLFP-111 Luzchem miniaturized equipment. This transient absorption spectrometer includes a ceramic xenon light source, 125 mm monochromator, Tektronix 9-bit digitizer TDS-3000 series with 300 MHz bandwith, compact photomultiplier and power supply, cell holder and fiber optic connectors, fiber optic sensor for laser-sensing pretrigger signal, computer interfaces and a software package developed in the LabVIEW environment from National Instruments. The LFP equipment supplies 5 V trigger pulses with programmable frequency and delay. The risetime of the detector/digitizer is approximately 3 ns up to 300 MHz (2.5 GHz sampling). The laser pulse is probed by a system containing a fiber that synchronizes the LFP with the digitizer operating in the pretrigger mode. All transient spectra were recorded using 10 mm × 10 mm quartz cells with 4 mL capacity, and all were bubbled during 20 min with N_2. Absorbance of the samples was kept between 0.2 and 0.3 at the laser wavelength. All the experiments were carried out at room temperature.

2.4. Computational methods

Density functional theory (DFT) calculations were carried out using the B3LYP [17,18] exchange correlation functional, together with the 6-31G(d) and 6-311+G(d,p) basis sets [19]. Optimizations were carried out using the Berny analytical gradient optimization method [20,21]. Electronic structures of stationary points were analyzed using the Wiberg indices [22,23]. Vertical energies of the singlet and triplet excited states were calculated using the time-dependent (TD-DFT) method [24,25]. Solvent effects of acetonitrile on the excited states were considered by TD-DFT calculation on the gas-phase structures using a self-consistent reaction field (SCRF) [26,27] based on the polarizable continuum model (PCM) of the Tomasi's group [28–30]. All calculations were carried out with the Gaussian 03 suite of programs [31].

2.5. Irradiation procedure

Aerated solutions of **1** in DMSO (5.2×10^{-4} M) were submitted to irradiation in the absence and in the presence of **XA** (5.2×10^{-4} M) using a solar simulator (ABET Technologies Sun 2000). The course of the photoreaction was monitored by means of analytical HPLC, using reverse phase C18 column and methanol-water (50:50, v/v) with 0.6% of ammonia as eluent. The flow rate was 0.8 mL/min, and the detector was a photo diode array (PDA).

2.6. Toxicity assays

The ephippia (dormant eggs) of crustacean *D. magna* were supplied by ECOtest S.L. (Valencia, Spain). The toxicity assays were performed assessing the inhibition of the mobility of *D. magna* according to the standard ISO 6341:1996 [32] procedure. The bioassay uses 24 h old daphnids hatched from the ephippia. Five neonates were placed in 15 mL appropriate containers with 10 mL of test dilution. The assays were conducted in the dark at a constant temperature of 21 ± 1 °C. At the end of the test period (70 h) mobile *D. magna* were counted in each container. Those unable to swim in the 15 s after agitation were considered immobile. All experiments were done in quadruplicate.

Different dilutions were prepared with the standard freshwater, according to mentioned guideline. Standard freshwater was also used as hatching medium.

Assays were conducted with a range of test dilutions in geometric progression plus the controls. Whenever possible, data were used to calculate the EC_{50} (sample dilution required to immobilize 50% of the daphnids after 24 h exposure) by Probit analysis, using the Statistical Analysis System SPSS (version 16.0). Toxicity data were analyzed with one-way analysis of variance (ANOVA) to inspect the effect of treatment time (70 h). The Fisher's least significant difference (LSD) test was used to determine which treatments are statistically significant with respect to the untreated sample. A $P < 0.05$ was taken to indicate statistical significance (STATGRAPHICS PLUS version 5.1).

3. Results and discussion

3.1. Photophysical properties in solution

In a first stage, the absorption properties of fenbendazole (**1**, Chart 1) were studied in different solvents. As shown in Fig. 1, the spectra in MeCN and MeOH are superimposable and display three bands with maxima at 220, 250 and 295 nm. In aqueous solution the long-wavelength band is shifted to the red 17 nm. In acidified water the maxima appear at 230 and 295 nm, whereas in cyclohexane the lowest energy maximum is located at 280 nm. The observed solvent