

## The Photochemical Reactivity of Triplet $\beta$ -Lapachone-3-sulfonic Acid towards Biological Substrates

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A reatividade fotoquímica do ácido 3-sulfônico da  $\beta$ -lapachona (**1**) frente a amino ácidos, bases nucleicas ou nucleosídeos foi determinada empregando a técnica de fotólise por pulso de laser de nanossegundo. A excitação ( $\lambda = 355$  nm) de soluções deaeradas de **1**, em acetonitrila, resultou na formação do seu estado excitado tripleto, o qual foi suprimido eficientemente por L-triptofano, éster metílico de L-triptofano, L-tirosina, éster metílico de L-tirosina e éster metílico de L-cisteína ( $k_q \cong 10^9$  L mol<sup>-1</sup> s<sup>-1</sup>). Para L-triptofano, L-tirosina e seus ésteres metílicos novos transientes foram formados no processo de supressão, os quais foram atribuídos ao par de radicais resultante de uma transferência inicial de elétron do amino ácido, ou dos seus ésteres metílicos, à quinona excitada, seguida por uma transferência de próton rápida. Não foi possível a obtenção de constantes de velocidade de supressão para timina e timidina, em acetonitrila, o que pode ser devido tanto ao caráter  $\pi \pi^*$  de **1** quanto ao baixo valor para a sua energia tripleto. Por outro lado, a supressão de **1** por 2'-deoxaguanosina foi tão eficiente quanto para triptofano ou tirosina ( $k_q \cong 10^9$  L mol<sup>-1</sup> s<sup>-1</sup>). O rendimento quântico para a formação de oxigênio singleto (<sup>1</sup>O<sub>2</sub>) a partir de **1** foi determinado empregando-se estudos de emissão resolvida no tempo na região do infravermelho próximo, tendo-se obtido um valor consideravelmente alto para este rendimento quântico ( $\Phi_\Delta = 0,7$ ).

The photochemical reactivity of  $\beta$ -lapachone-3-sulfonic acid (**1**) towards amino acids, nucleobases or nucleosides has been examined employing the nanosecond laser flash photolysis technique. Excitation ( $\lambda = 355$  nm) of degassed solutions of **1**, in acetonitrile, resulted in the formation of its corresponding triplet excited state. This transient was efficiently quenched by L-tryptophan, L-tryptophan methyl ester, L-tyrosine, L-tyrosine methyl ester and L-cysteine methyl ester ( $k_q \cong 10^9$  L mol<sup>-1</sup> s<sup>-1</sup>). For L-tryptophan, L-tyrosine and their methyl esters new transients were formed in the quenching process, which were assigned to the corresponding radical pairs resulting from an initial electron transfer from the amino acids, or their esters, to the excited quinone, followed by a fast proton transfer. No measurable quenching rate constants could be observed in the presence of thymine or thymidine, in acetonitrile solution, which is probably due to the  $\pi \pi^*$  character of triplet **1** as well as to its low triplet energy. On the other hand, the rate constant obtained when **1** was quenched by 2'-deoxyguanosine is reasonably fast ( $k_q \cong 10^9$  L mol<sup>-1</sup> s<sup>-1</sup>). The quantum efficiency of singlet oxygen (<sup>1</sup>O<sub>2</sub>) formation from **1** was determined employing time-resolved near-IR emission studies upon laser excitation and showed a considerably high value ( $\Phi_\Delta = 0.7$ ).

**Keywords:** laser flash photolysis, *ortho*-quinones, triplet excited state, coupled electron/proton transfer

### Introduction

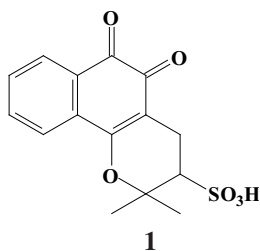
Quinones show several biological and pharmacological activities,<sup>1-9</sup> with their mechanism of action being

related to redox cycling, which leads to the formation of reactive oxygen species that can damage cellular macromolecules.<sup>10,11</sup> The quinone cytotoxicity to many human cancer cell lines is amply recognized,<sup>12-17</sup> acting through inhibition of DNA repair enzymes,<sup>18</sup> inhibition or activation of DNA topoisomerase I,<sup>17,19</sup> induction of

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topoisomerase IIa-mediated DNA breaks,<sup>20</sup> and inhibition of poly(ADP-ribose) polymerase-1.<sup>21</sup> More recently, Boothman and co-workers<sup>23</sup> have clearly demonstrated that  $\beta$ -lapachone, an *ortho*-quinone, activates a novel apoptotic response in a number of cell lines. It was shown that the enzyme NAD(P)H:quinone oxidoreductase (NQO1) substantially enhances the toxicity of  $\beta$ -lapachone in a number of tumor types (*i.e.*, breast, pancreatic, colon, prostate and lung) and during neoplastic transformation.<sup>22</sup>

The characterization and reactivity of triplet  $\beta$ -lapachone-3-sulfonic acid (**1**) has been recently reported by us. Upon laser excitation (266 or 355 nm), **1** leads to the formation of its triplet excited state which shows absorption maxima at 300, 380 and 650 (broad band) nm, with a lifetime of 5  $\mu$ s. Hydrogen abstraction rate constant for the triplet **1** towards 2-propanol or 1,4-cyclohexadiene is quite low ( $10^5 \text{ L mol}^{-1} \text{ s}^{-1}$ ), which has been associated with its  $\pi\pi^*$  character. On the other hand, 4-methoxyphenol or indole quenches the triplet excited state of **1** with a rate constant of  $10^9 \text{ L mol}^{-1} \text{ s}^{-1}$ . Triplet **1** also reacts with electron donors, such as triethylamine, at an almost diffusion-controlled rate, yielding the corresponding long-lived anion radical.



Several mechanisms account for the photosensitization process toward biomolecules.<sup>24-26</sup> For excited carbonyl groups showing  $n\pi^*$  character, Paternò-Büchi reaction between triplet carbonyl and thymine can yield oxetanes.<sup>24</sup> In case the triplet carbonyl compound is higher in energy than the triplet thymine, thymine dimerization can be observed through a triplet-triplet energy transfer process.<sup>25</sup> More general photosensitizing mechanisms can involve either photooxidation of nucleic acid components by the sensitizer, yielding the corresponding radical pair and ultimately leading to sensitizer-protein photobinding, or a triplet-triplet energy transfer to molecular oxygen, resulting in formation of singlet oxygen  $\text{O}_2(^1\Delta_g)$  and other reactive oxygen species, such as superoxide anion, hydrogen peroxide and hydroxyl radical.

In this work we show results of the laser flash photolysis studies on the reactivity of  $\beta$ -lapachone-3-sulfonic acid (**1**) towards biological substrates such as amino acids and their methyl esters, nucleic bases and nucleosides, as well as its ability to form singlet oxygen,  $\text{O}_2(^1\Delta_g)$ .

## Materials and Methods

### Material

The solvent acetonitrile was used as received. Lapachol, 1,2-naphthoquinone, L-tryptophan, L-tryptophan methyl ester, L-tyrosine, L-tyrosine methyl ester, L-cysteine, thymine, thymidine, 2'-deoxyguanosine and perinaphthenone, from Aldrich, were used as received (purity > 99%).

$\beta$ -Lapachone-3-sulfonic acid (**1**) was prepared by drop-wise addition of concentrated  $\text{H}_2\text{SO}_4$  to a suspension of lapachol in  $\text{Ac}_2\text{O}$ , at 20-30 °C. After cooling and filtering, the orange-red crystals were washed with dry ether and recrystallized from ethanol (mp 158-160 °C). Its spectroscopic and spectrometric properties are in full accord with the structure proposed.<sup>27</sup>

### Laser Flash Photolysis

These experiments were carried out using either the 3<sup>rd</sup> ( $\lambda_{\text{exc}} = 355 \text{ nm}$ ) or the fourth harmonic ( $\lambda_{\text{exc}} = 266 \text{ nm}$ ) of a Quantel pulsed Nd:YAG laser. The single pulses were *ca.* 10 ns duration, and the energy was *ca.* 15 mJ pulse<sup>-1</sup>. A Xenon lamp was employed as the detecting light source. The laser flash photolysis apparatus (Luzchem, model mLFP112) consisted of a Xe lamp, a monochromator, and a photomultiplier (PMT) system made up of side-on PMT, PMT housing, and a PMT power supply. The output signal from the Tektronix oscilloscope was transferred to a personal computer for study. Samples were contained in 10 mm  $\times$  10 mm cells made of Suprasil quartz and were deaerated for at least 20 min with oxygen-free nitrogen prior to the experiments. Concentration for the *ortho*-quinone **1** was adjusted to yield an absorbance of *ca.* 0.3 at the excitation wavelength. Stock solutions of quenchers were prepared so that it was only necessary to add microliter volumes to the sample cell in order to obtain appropriate concentrations of the quencher.

Quenching experiments were performed using the 3<sup>rd</sup> harmonic of the Nd-YAG laser ( $\lambda_{\text{exc}} = 355 \text{ nm}$ ) since at this wavelength  $\beta$ -lapachone-3-sulfonic acid (**1**) is the only absorbing species. Rate constants for the reaction of the triplet excited state of **1** with the different quenchers employed in this work were obtained from Stern-Volmer plots,<sup>28</sup> following equation 1.

$$k_{\text{obs}} = k_o + k_q[\text{Q}] \quad (1)$$

where:  $k_o$  is the triplet decay rate constant in the absence of quencher;  $k_q$  is the triplet decay rate constant in the presence

of the quencher and  $[Q]$  is the quencher concentration in  $\text{mol L}^{-1}$ .

### Singlet oxygen measurements

Singlet oxygen generation was monitored through the characteristic phosphorescence at 1270 nm, upon laser excitation of isoabsorptive solutions ( $\text{Abs} = 0.3$ ) of  $\beta$ -lapachone-3-sulfonic acid (**1**) relative to a standard solution of perinaphthenone (quantum yield of 1.0, in acetonitrile).<sup>29</sup> All samples were thoroughly oxygenated before measurement. The quantum yield of singlet oxygen formation was determined from the slope of the linear plots of signal intensity at zero time *versus* laser light intensity, employing equation 2. In all cases acetonitrile was used as solvent. A set of neutral density filters was employed to obtain different laser intensities.

$$\Phi_{\text{sample}} = \frac{I_{\text{sample}}}{I_{\text{perinaphthenone}}} \Phi_{\text{perinaphthenone}} \quad (2)$$

where  $I_{\text{sample}}$  is the emission intensity recorded for the sample,  $I_{\text{perinaphthenone}}$  is the perinaphthenone emission intensity (used as standard) and  $\Phi_{\text{perinaphthenone}}$  is the quantum yield of singlet oxygen formation from perinaphthenone.

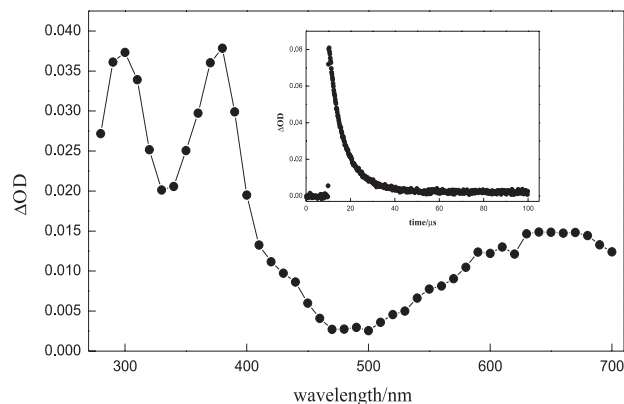
The singlet oxygen luminescence (1270 nm) was detected by means of an Oriel 71614 germanium photodiode ( $5 \text{ mm}^2$ ), which was coupled to the laser photolysis cell in right-angle geometry. A Xe/HCl excimer laser (LEXTRA50 Lambda Physik) was used for excitation at  $\lambda = 308 \text{ nm}$  ( $80 \text{ mJ pulse}^{-1}$ ,  $10 \text{ ns pulse}^{-1}$ ). A 1050 nm cut-off silicon filter ( $5 \text{ mm}$  thick,  $5 \text{ cm}$  diameter) and a 1270 nm interference filter were placed between the diode and the cell. The output current of the photodiode was amplified and fed into a TDS-640A Tektronix oscilloscope *via* a Co-linear 150 MHz, 20 dB amplifier. The output signal from the oscilloscope was treated and studied by means of a personal computer.

## Results and Discussion

### Laser flash photolysis studies

Laser excitation (266 nm) of a deoxygenated acetonitrile solution of **1** leads to the formation of its triplet excited state which shows absorption bands at 300, 380 and 650 nm, as previously reported (Figure 1).<sup>23</sup> This transient decays by first order kinetics and shows a lifetime of 5  $\mu\text{s}$  (Figure 1, inset).

Quenching studies for triplet **1** by amino acids were performed employing L-tyrosine, L-tryptophan, their corresponding methyl esters, and cysteine methyl ester. In all cases, linear quenching plots following equation 1 were



**Figure 1.** Triplet-triplet absorption spectrum for  $\beta$ -lapachone-3-sulfonic acid, in acetonitrile, recorded 1.0  $\mu\text{s}$  after the laser pulse ( $\lambda_{\text{exc}} = 266 \text{ nm}$ ). *Inset:* decay for this transient monitored at 380 nm.

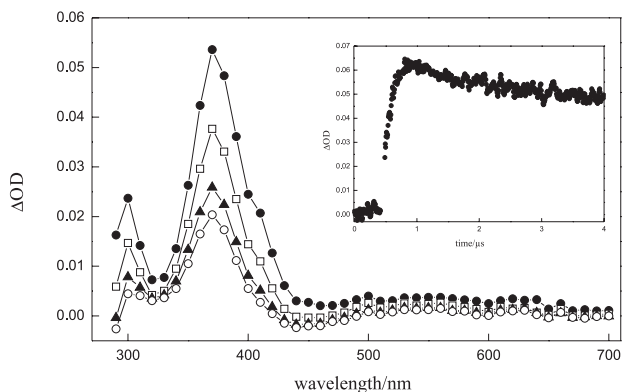
obtained and the resulting quenching rate constants ( $k_q$ ) are of the order of  $10^9 \text{ L mol}^{-1} \text{ s}^{-1}$ , as shown in Table 1. It is worth noting that in the quenching experiments samples were excited at 355 nm (3<sup>rd</sup> harmonic of a Nd-YAG laser), since at this wavelength the only absorbing species is the  $\beta$ -lapachone-3-sulfonic acid.

**Table 1.** Second order rate constants for the quenching of  $\beta$ -lapachone-3-sulfonic acid (**1**) by nucleic acids constituents, in acetonitrile

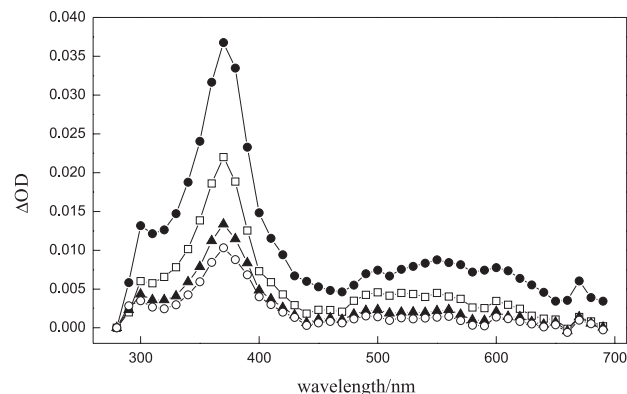
Quencher	$k_q$ ( $\text{L mol}^{-1} \text{ s}^{-1}$ )
L-Tyrosine	$(1.1 \pm 0.1) \times 10^9$
L-Tyrosine methyl ester	$(7.3 \pm 0.6) \times 10^9$
L-Tryptophan	$(3.6 \pm 0.8) \times 10^9$
L-Tryptophan methyl ester	$(3.9 \pm 0.3) \times 10^9$
L-Cysteine methyl ester	$(5.5 \pm 1.0) \times 10^9$
Thymine	a
Thymidine	a
2'-Deoxyguanosine	$(1.3 \pm 0.1) \times 10^9$

<sup>a</sup>no measurable rate constants.

Transient absorption spectra recorded for samples containing  $\beta$ -lapachone-3-sulfonic acid (**1**), in the presence of L-tyrosine, L-tryptophan and their corresponding methyl esters, showed the disappearance of the absorption bands corresponding to its triplet excited state and the formation of new absorption bands at 330 (weak) and 370 (strong) nm (Figure 2). These absorptions were assigned to the semiquinone radical derived from **1** (Scheme 1), which is easily formed when quinones are irradiated in the presence of suitable hydrogen donors. At shorter timescales (400 ns), one can observe that the semiquinone radical ( $\lambda_{\text{max}} = 370 \text{ nm}$ ) grows-in with first order kinetics (Figure 2, *inset*). Due to the strong absorption at 380 nm for the semiquinone radical derived from **1**, we were not able to observe the L-tyrosinyl radical ( $\lambda_{\text{max}} = 380 \text{ nm}$ )<sup>30,31</sup> formed through hydrogen transfer reaction from L-tyrosine or L-tyrosine methyl ester to triplet **1** (Figure 2). On the



**Figure 2.** Absorption spectra for the transient generated upon 355 nm excitation of  $\beta$ -lapachone-3-sulfonic acid, in acetonitrile, in the presence of  $1.3 \times 10^{-4}$  mol L $^{-1}$  tyrosine methyl ester, and recorded 0.2 (●); 12.3 (□); 35.2 (▲) and 70.6 (○)  $\mu$ s after the laser pulse. *Inset:* growth of absorption at 380 nm due to the semiquinone radical generated as above.



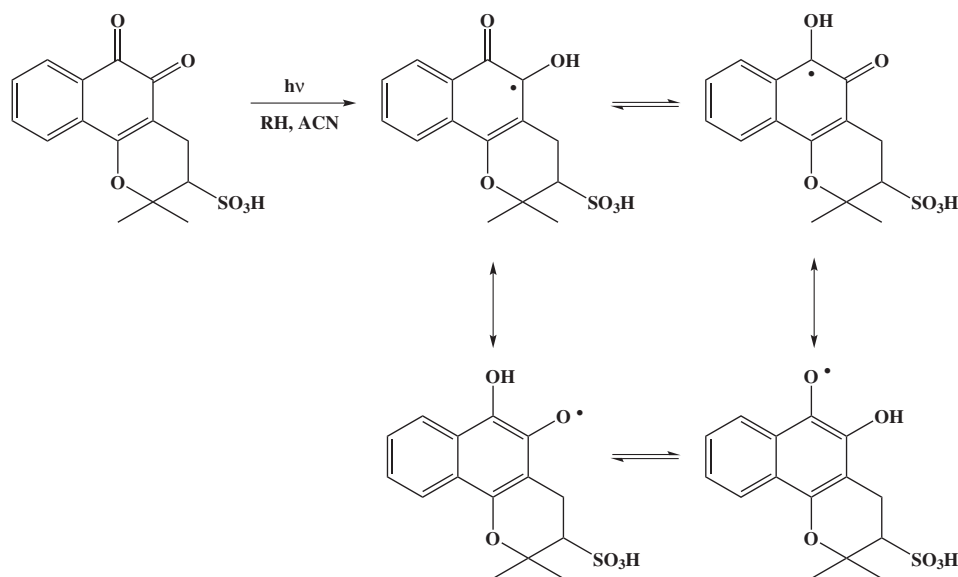
**Figure 3.** Absorption spectra for the transient generated upon 355 nm excitation of  $\beta$ -lapachone-3-sulfonic acid, in acetonitrile, in the presence of  $1.3 \times 10^{-4}$  mol L $^{-1}$  tryptophan methyl ester and recorded 1.4; (□) 10.0; (▲) 35.0 and (○) 70.5  $\mu$ s after the laser pulse.

other hand, when L-tryptophan or its methyl ester were employed as quenchers, the L-tryptophanyl radical, which shows a broad absorption in the 450–550 nm region,<sup>30,31</sup> was easily observed (Figure 3).

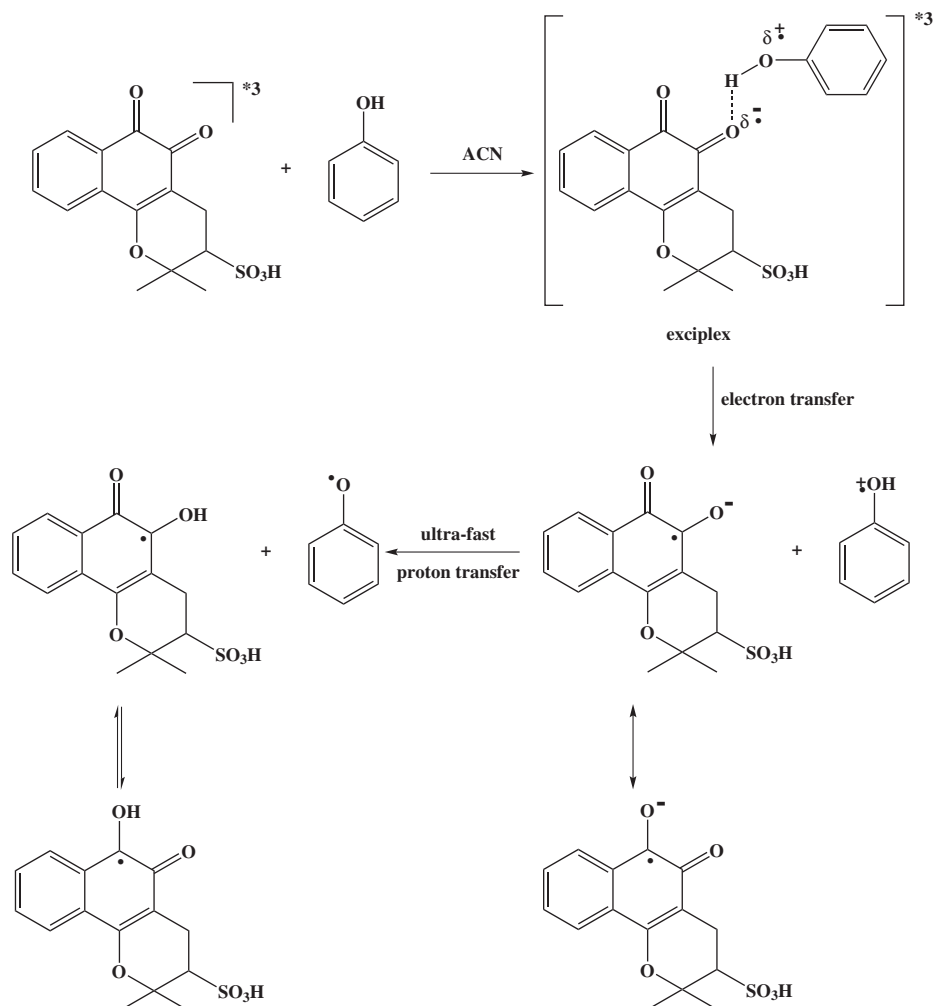
The hydrogen transfer reaction from phenol and indole to triplet excited carbonyls can be usually explained by a mechanism in which, after the formation of an initial hydrogen-bonded triplet exciplex, an electron transfer process is followed by an ultra-fast proton transfer, as exemplified in Scheme 2 for the reaction of triplet **1** with phenol. This mechanism will lead ultimately to the semiquinone-phenoxyl radical pair.<sup>23,32–43</sup> A similar mechanism can be used to explain the high values for the quenching rate constants of triplet **1** by the amino acids L-tryptophan and L-tyrosine and their methyl esters.

L-Cysteine is an amino acid that can mimic the reactivity of thiol-containing enzymes such as topoisomerases. The quenching rate constant of triplet **1** by L-cysteine methyl ester is of the same order of magnitude as that observed for the other amino acids: *ca.*  $(5.5 \pm 1.0) \times 10^9$  L mol $^{-1}$  s $^{-1}$  (Table 1). However, for this amino acid, no new transients could be observed. This could be related to the fact that one of the proposed mechanisms for the cytotoxic action of quinones involves the alkylation of thiol groups on the thiol-containing enzymes.<sup>44</sup> This same mechanism could be operating in the quenching of the triplet excited state of  $\beta$ -lapachone-3-sulfonic acid, which would explain the lack of observation of the semiquinone radical.

As previously reported by our group, triplet **1** has  $\pi\pi^*$  configuration<sup>23</sup> and, as stated before, oxetane formation



**Scheme 1.**



Scheme 2.

in the quenching by thymine of triplet carbonyls is only possible for those compounds having a triplet state with  $n\pi^*$  configuration.<sup>24</sup> Furthermore, since the triplet energy for  $\beta$ -lapachones is relatively low ( $E_T = 46 \text{ kcal mol}^{-1}$  for  $\beta$ -lapachone),<sup>45</sup> triplet-triplet energy transfer from **1** to thymine, leading to its dimerization, is not expected. Indeed, we were not able to measure quenching rate constants for triplet **1** when employing thymine or thymidine as quenchers.

Unlike what was observed for these two nucleic acid constituents, 2'-deoxyguanosine efficiently quenches the triplet excited state of  $\beta$ -lapachone-3-sulfonic acid ( $k_q = (1.3 \pm 0.1) \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$ ) (Table 1). In this case, the semiquinone radical derived from **1** was readily observed, as shown in Figure 4. The broad absorption observed in the 500-600 nm range can be assigned to the 2'-deoxyguanosyl radical, since it displays absorption bands at 315, 380 and 540 nm,<sup>46,47</sup> with its first two bands probably being overlapped by the semiquinone radical absorptions, which has strong bands at the same wavelengths.

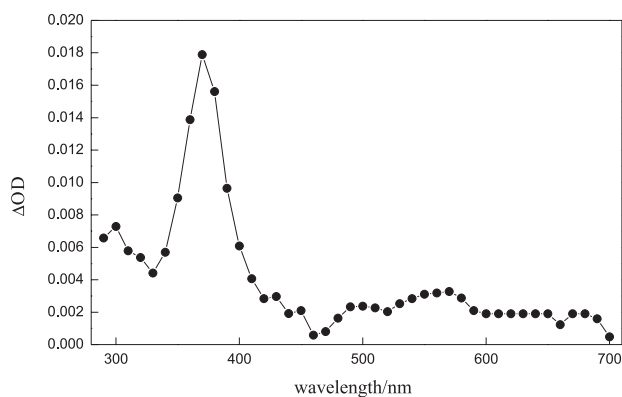


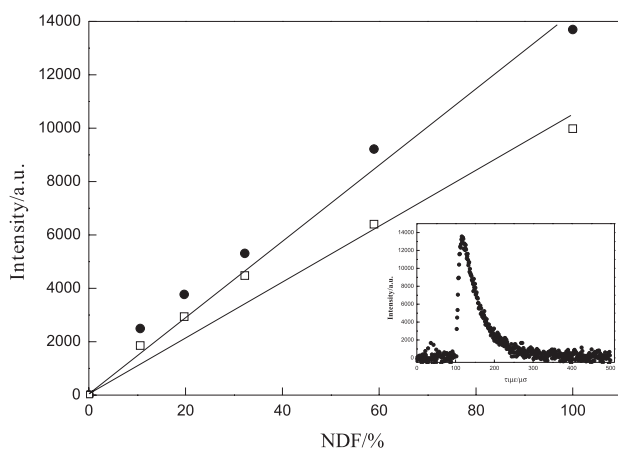
Figure 4. Absorption spectrum for the transient generated upon 355 nm excitation of  $\beta$ -lapachone-3-sulfonic acid, in acetonitrile, in the presence of  $1.0 \times 10^{-4} \text{ mol L}^{-1}$  2'-deoxyguanosine and recorded 35  $\mu\text{s}$  after the laser pulse.

The above results indicate that the photochemical behavior of  $\beta$ -lapachone-3-sulfonic acid (**1**) towards biological substrates is similar to that observed for  $\beta$ -lapachone and *nor*- $\beta$ -lapachone, which was recently

reported by us.<sup>43</sup> Thus, it is easy to conclude that the presence of the sulfonic acid group in **1** does not affect the photoreactivity of the *ortho*-quinone chromophore.

### Singlet oxygen formation

Figure 5 shows plots for the singlet oxygen phosphorescence intensity versus energy dependence for oxygenated solutions of perinaphthenone and  $\beta$ -lapachone-3-sulfonic acid in acetonitrile, from which a quantum efficiency of singlet oxygen formation  $\Phi_{\Delta}$  of 0.7 was obtained. This high value is fully in accord with earlier suggestions<sup>48-53</sup> that a  $\pi\pi^*$  triplet is required for highly efficient singlet oxygen formation. Similar values were reported for other  $\beta$ -lapachones.<sup>43</sup> A decay of 70  $\mu$ s was measured for singlet oxygen phosphorescence generated by energy transfer from the lapachone **1** to oxygen (inset of Figure 5), which is similar to that previously observed when acetonitrile was employed as the solvent.<sup>54</sup>



**Figure 5.** Plots for singlet oxygen phosphorescence emission versus laser intensity for oxygen saturated solutions of  $\beta$ -lapachone-3-sulfonic acid ( $\square$ ) and perinaphthenone ( $\bullet$ ) (standard for comparison  $\Phi_{\Delta} = 1$ ), in acetonitrile ( $\lambda_{\text{mon}} = 1270$  nm). Inset: decay of singlet oxygen phosphorescence monitored at  $\lambda = 1270$  nm.

### Conclusions

In conclusion, it was shown that  $\beta$ -lapachone-3-sulphonic acid (**1**) is able to act as photosensitizer for the one-electron oxidation of L-tryptophan, L-tyrosine and their methyl esters, as well as of 2'-deoxyguanosine. Furthermore, L-cysteine methyl ester also is an efficient quencher of triplet **1**, but may be acting by a different mechanism. Besides, efficient singlet oxygen formation was measured for this  $\beta$ -lapachone derivative ( $\Phi_{\Delta} = 0.7$ ). These results clearly demonstrate that **1** is able to photosensitize biological substrates by both type I and type II mechanisms, with its reactivity being similar to other  $\beta$ -lapachones, as already reported in the literature.

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