PCCP

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: V. Rizzi, I. Losito, A. Ventrella, P. Fini, A. Fraix, S. Sortino, A. Agostiano, F. Longobardi and P. Cosma, *Phys. Chem. Chem. Phys.*, 2015, DOI: 10.1039/C5CP03615A.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/pccp

View Article Online
DOI: 10.1039/C5CP03615A

Journal Name

RSCPublishing

ARTICLE

Rose Bengal-photosensitized oxidation of 4-thiothymidine in aqueous medium: evidence for the reaction of the nucleoside with singlet state oxygen

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

Vito Rizzi[a], Ilario Losito[a,b], Andrea Ventrella[a], Paola Fini[c], Aurore Fraix[d], Salvatore Sortino[d], Angela Agostiano[a,c], Francesco Longobardi[a] and Pinalysa Cosma[a,c,*]

The photoreactivity of 4-thiothymidine (S^4TdR) under visible light in the presence of Rose Bengal (RB), acting as a photosensitizer, was investigated in aqueous solutions at pH 7 and 12, using UV-Vis, FTIR-ATR and ^{1}H-NMR spectroscopic techniques,time resolved absorption spectroscopy and ElectroSpray Ionization Mass Spectrometry (ESI-MS). Evidences for the generation of thymidine (TdR) as the main product, after one hour of irradiation, were obtained from UV-Vis data, thatsuggested4-thiothymidinephotodegradation to be faster at basic pH, and confirmed by FTIR-ATR and ^{1}H-NMR data. Clues for the presence of a further product, likely corresponding to a dimeric form of S^4TdR,were obtained from the latter techniques. Beside indicating the presence of thymidine, ESI-MS and MS/MS spectra of the reaction mixturesenabled the identification of the additional product as aS-S bridged covalent dimer of 4-thiothymidine. The concentration of the dimeric species could be estimated with the aid of ^{1}H-NMR data and was found to be lower than that of thymidine in pH 7 reaction mixtures and almost negligible in pH 12 ones. From a mechanistic point of view, time-resolved absorption spectroscopy measurements provided direct evidences that the formation of the two products cannot be ascribed to a photoinduced electron transfer involving S^4TdR and the excited triplet state of RB. Rather, their generation can be interpreted as the result of a bimolecular reaction occurring between singlet state oxygen ($^1O_2$), photogenerated by RB, and S^4TdR, as demonstrated by the direct detection of $^1O_2$ through IR luminescence spectroscopy. More specifically, a sequential reaction pathway, consisting in the generation of an electrophilic hydroxylated form of S^4TdR and its subsequent, rapid reaction with S^4TdR, was hypothesized to explain the presence of the S-S bridged covalent dimer of 4-thiothymidine in the reaction mixtures. The described processesmakeS^4TdR an interesting candidate to the role of molecular probe for the detection of$^1O_2$under different pH conditions.

Introduction

Light has played a therapeutic role for humans for many centuries. Indeed, ancient Egyptian, Indian and Chinese populations used the exposure to sun to treat a variety of diseases, including vitiligo, psoriasis, cancer and even psychosis[1]. Among modern versions of such a therapeutic approach, Photo Dynamic Therapy (PDT), *i.e.* the combination of a light source with a photosensitizing agent (PS) and endogenous molecular oxygen, has emerged as a therapy for cancer and for hyperproliferative, ophthalmic and dermatologic diseases in the last 30 years andis currently feasible in several medical institutions around the world[2]. Nucleic acid bases containing a sulphur atom instead of an oxygen one have been the object of a considerable interest in the field of PDT for several years, due to their reactivity with Reactive Oxygen Species (ROS) generated upon interaction between molecular oxygen and light-excited photosensitizing agents[3-7]. Among such sulphur-containing nucleobases, the potential of 4-thiothymidine (S^4TdR) in the PDT-based treatment of cancer, inflammatory conditions, viral diseases and in the therapy of organ transplant patients has emerged from the literature[8]. In particular, the S^4TdR incorporation into DNA suggests that it might act synergistically with non lethal doses of UVA to kill selectively hyperproliferative or cancerous skin cells[9]. Although the increase of cellular sensitivity to UVA radiation has not been completely elucidated, the process seems to start with the absorption of radiation by the molecule and the consequent generation of cytotoxic species (Type I photosensitization)[10,11]. Additionally, the energy absorbed by sulphur-containing bases may be transferred to molecular oxygen to generate Singlet State Oxygen ($^1O_2$) as ROS, leading to a Type II photosensitization reaction. $^1O_2$has been identified as the main ROS generated upon UVA irradiation of sulphur-containing bases; as an example, in the case of 6-thioguanosine (6-TG) Type II photosensitization has been indicated

ARTICLE View Article Online DOI: 10.1039/C5CP03613A Journal Name

as the main mechanism by which the base exerts its photochemical effects[9,12-15].

When visible light is used in PDT no significant absorption by sulphur-containing bases occurs, thus they cannot be regarded as photosensitizing agents but rather as probes/quenchers, able to detect the presence in solution of ROS eventually generated by visible light-absorbing PSs deliberately added to the system. Starting from these considerations, an investigation on the ability of S^4TdR to act as a quencher for $^1O_2$ generated by the interaction between molecular oxygen and visible light-absorbing PSs has been recently undertaken in our laboratory. As a preliminary part of this study, the stability of aqueous S^4TdR solutions irradiated with visible light (400-700 nm) generated by a neon lamp, at room temperature, has been demonstrated for irradiation times as long as two hours[8]. This result has confirmed the possibility of using S^4TdR to probe the presence of ROS, in particular $^1O_2$, generated upon irradiation of visible light-absorbing PSs. Rose Bengal (RB), a xanthene dye, has been chosen as a good representative of such PSs, since it is one of the most used photosensitizers in visible light-based PDT, due to its high water solubility, high $^1O_2$ quantum yield (Φ_Δ) and low rate of photo-degradation[16,17]. The key step in PDT involving RB is the energy transfer from the lowest excited triplet state of the dye to molecular oxygen (Type II pathway), resulting in $\Phi_\Delta$values ranging between 0.76 and 0.86[18]. On the other hand, the generation of hydroperoxyl radicals or superoxide ion radicals ($HO_2^{\bullet}/O_2^{\bullet-}$) by electron transfer (Type I pathway) cannot be excluded, although the value 0.2 estimated for the quantum yield of this process is still matter of debate[18].

In the present paper a study, based on different spectroscopic techniques (UV-Vis, ^{1}H-NMR, FTIR-ATR) and including time-resolved absorption spectroscopy, of the reactions occurring in S^4TdR aqueous solutionscontaining also Rose Bengal, when irradiated with visible light, will be presented. In particular, since the pKa of S^4TdR has been recently estimated[8] to be close to 9, the behavior observed at pH 7 and 12 will be described, in order to emphasize the eventual differences in reactivity due to the presence of neutral or anionic S^4TdR, respectively. Additionally, the identity of the main by-products ofS^4TdRgenerated at pH 7 and 12 will be confirmed using ElectroSpray Ionization Single and Tandem Mass Spectrometry(ESI-MS, MS/MS).

Experimental

Chemicals

4-thio(deoxy)thymidine was purchased from Carbosynth (Compton, Berkshire, UK). Rose Bengal, thymidine (TdR), and LC-MS grade water and methanol (used as solvents for ESI-MS analyses) were purchased from Sigma-Aldrich (Milan, Italy). The same commercial source was also chosen for the following chemicals: NaOH, employed to adjust the S^4TdR solution pH at 12; KH_2PO_4 and KOH, used to prepare pH 7 buffer solutions; concentrated HCl, used to neutralize basic solutions of S^4TdR just before proceeding with the infusion for ESI-MS analysis; NaN_3, adopted as a $^1O_2$ quencher; D_2O and trimethylsilyl-propionic-2,2,3,3-d4 acid (TSP), used in ^{1}H-NMR analyses. All the employed chemicals, having 99+% purity, were used as received.

S^4TdR and RB stock solutions (respective concentrations: 10^{-2} M and 10^{-3} M) were prepared in doubly distilled water or, in the case of ESI-MS analyses, in LC-MS grade water, and stored in the dark at -20°C when not in use. A NaN_3 stock solution (0.8 M concentration) was prepared in doubly distilled water.

UV-Vis spectroscopy measurements

Two series of solutions, characterized by different S^4TdR/RB molar ratios, were prepared in doubly distilled water, at pH 7 or 12, for UV-Vis spectroscopy measurements. In particular, solutions containing S^4TdR at a 8×10^{-4} M or 2×10^{-4} M concentration and RB at a 5×10^{-4} M concentration were considered. Spectra were recordedin a 200–800 nm range, at a 1 nm/s scan rate, using a Varian CARY 5 UV-Vis-NIR spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA). Due to the relatively high concentrations adopted for S^4TdR and RB, a cuvette with a 1 mm path length was used to keep absorbance below reasonable values. Measurements in the presence of NaN_3 (at 10mM concentration) were also performed, at pH 7 or 12, to evaluate eventual variations in the reactivity of S^4TdR due to the possible quenching of singlet state oxygen by sodium azide.

^{1}H-NMR spectroscopy measurements

Aqueous solutions containing S^4TdR 2×10^{-4} M or 8×10^{-4} M and RB 5×10^{-4} M, prepared at pH 7 and 12, were subjected to ^{1}H-NMR analyses. For the sake of comparison, analyses were also performed on a 8×10^{-4} M TdR solution, at pH 7 and 12.

An appropriate volume of a stock solution of TrimethylSilylPropanoic acid (TSP) in D_2O was always added to get a final TSP concentration of 10^{-3} M in a H_2O/D_2O (90/10, v/v) mixture; TSP was used as the internal reference, whereas the small percentage of D_2O in the solvent mixture was necessary to enable the lock procedure. An appropriate volume (600 µL) of the prepared mixture was transferred into a 5 mm NMR tube (WilmadLabglass Inc., Vineland, NJ, USA) before analysis. ^{1}H-NMR measurements were performed by a Bruker AVANCE III 700 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), equipped with a 5 mm 1H/D-BB probe head, with z-gradient, automated tuning and matching accessory, and a BTO-2000 accessory for temperature control. The routines included in the TOPSPIN 3.0 software (Bruker BioSpin GmbH, Germany) were used to perform tuning and matching, locking and shimming, and to optimize the NMR conditions. After these procedures, the one-dimensional sequence called “noesygppr1d” (Bruker library) was carried out, allowing the partial suppression of the intense water signal at 4.77 ppm, by a presaturation scheme. In particular, noesygppr1d applies a continuous RF wave during the relaxation delay, causing the saturation of the selected signal before the application of the observation pulse (the mixing time is a short fixed time); the application of a spoil gradient allows eliminating undesired magnetization, so leading to a cleaner spectrum. For the noesygppr1d experiments performed in this work, the parameters were set as follows: time domain size TD = 64k, spectral width SW = 20.5 ppm, acquisition time AQ = 2.28 s, number of scans NS = 64, dummy scans DS = 4, receiver gain RG = 4, dwell time DW = 34.8 µs, pre-scan delay DE = 6.50 µs, relaxation delay D1 = 10.0 s, mixing time D8 = 0.01 s, delay for homospoil/gradient recovery D16 = 0.0002 s, frequency of the proton channel O1 = 3289.8 Hz, 90° high power pulse duration P1 = 7.45 µs, power level for 90° pulse PLW1 = -11.76 dB, power level for presaturation PLW9 = 50.80 dB, homospoil/gradient pulse duration P16 = 1000 µs.

Journal Name View Article Online DOI: 10.1039/C5CP03615A ARTICLE

FTIR-ATR spectroscopy measurements

Solutions containing S^4TdR 2×10^{-4} M or 8×10^{-4} M and RB 5×10^{-4} M, prepared at pH 7 and 12, were subjected to FTIR-ATR analysis. FTIR ATR spectra were obtained also for a 8×10^{-4} M TdR solution and for a 5×10^{-4} M RB solution, at pH 7 and 12.FTIR-ATR spectra were recorded in a 550–4000 cm^{-1} range, using a Fourier Transform Infrared spectrometer 670-IR (Agilent Technologies Inc., Santa Clara, CA, USA), whose resolution was set to 4 cm^{-1}. 32 scans were summed for each acquisition. Before each analysis an aliquot (50 μL) of the solution of interest was deposited on the surface of the ATR device and the solvent was left to evaporate slowly before proceeding with the acquisition of spectra.

ElectroSpray Ionization Mass Spectrometry (ESI-MS) Measurements

ESI-MS measurements in positive polarity were focused on the same S^4TdR/RBmolar ratios and pH values explored during ^{1}H-NMR and FTIR-ATRmeasurements. In this case the solutions were prepared in LC-MS grade water.

Before proceeding with the analysis the solutions were diluted 1:1 (v/v) with LC-MS grade methanol, in order to improve the electro-nebulization efficiency during the ESI process. The S^4TdR/RB solutions at pH 12 were preliminarily neutralized, using an appropriate volume of concentrated HCl, so that the ionization conditions could be the same as those occurring for pH 7 reaction mixtures. The resulting solutions were infused, at a 10μL/min flow rate, into the ESI interface of a LCQ 3D-Ion Trap Mass Spectrometer (Thermo Scientific, West Palm Beach, FL, USA), using the syringe pump included into the spectrometer.ESI-MS and MS/MS acquisitions were sequentially performed during each infusion experiment. The following values were adopted for the main ESI interface and ion optics parameters: sheath gas flow rate, 60 (a.u.); auxiliary gas flow rate, 0 (a.u.); spray voltage, 6 kV; capillary temperature, 190°C; capillary voltage, 15 V; tube lens offset, 10 V; octapole 1 offset, −2.5V; lens voltage,−16V; octapole 2 offset, −5.5V; octapole RF amplitude, 400 V_{p-p}. These values were obtained by optimizing a set of parameters originally provided by the instrument manufacturer for positive ion ESI-MS measurements at low flow rates, so that the main signal related to 4-thio(2' deoxy)thymidine was maximized.

ESI-MS *full scan* acquisitions were performed in a 50−1000 m/z range. ESI-MS/MS acquisitions were performed on targeted precursor ions (all singly charged), which were isolated in the 3D-Ion trap using a 1 m/z units-wide window including only the main isotopologue of each precursor ion. Isolated precursor ions were fragmented using a collisional energy corresponding to the 35% of the maximum value, i.e. to a 1.75 V peak-to-peak amplitude for the excitation voltage applied to the 3D ion trap end-caps. Such a value enabled an almost complete fragmentation of all the selected precursor ions. MS/MS spectra were acquired in a m/z range comprised between the *Low Mass Cut Off* value automatically set by the spectrometer (related to the m/z ratio of the precursor ion) and a m/z value 10 units higher than the one of the precursor ion.

The *Xcalibur* software (Thermo Scientific) was used to control the LCQ spectrometer during the acquisitions and to perform data elaboration. The *Mass Frontier* 1.0 software (HighChem Ltd., SlovakRepublic) was adopted for the simulation of MS/MS fragmentations, useful to identify as many product ions as possible for each precursor ion.

Time-resolved absorption spectroscopy

All the samples were excited with the second harmonic of Nd–YAG Continuum Surelite II–10 laser (532 nm, 6 ns FWHM), using quartz cells with a path length of 1.0 cm. The excited solutions were analyzed by a Luzchem Research mLFP–111 apparatus with an orthogonal pump/probe configuration. The probe source was a ceramic xenon lamp coupled to quartz fiber-optic cables. The laser pulse and the mLFP–111 system were synchronized by a Tektronix TDS 3032 digitizer, operating in pre-trigger mode. The signals from a compact Hamamatsu photomultiplier were initially captured by the digitizer and then transferred to a personal computer, controlled by Luzchem Research software operating in the National Instruments LabView 5.1 environment. The solutions were deoxygenated by bubbling with a vigorous and constant flux of pure nitrogen (previously saturated with solvent). In all of these experiments, the solutions were renewed after each laser shot (in a flow cell of 1 cm optical path), to prevent probable auto-oxidation processes. The sample temperature was 295 ± 2 K. The energy of the laser pulse was measured at each shot with a SPHD25 Scientech pyroelectric meter.

Quantum yields for the triplet formation (Φ_T) were determined by using optically matched solution at the excitation wavelength of RB in the absence and in the presence of S^4TdR. The top ΔA of the triplet signal from each sample was plotted as a function of the laser intensity. In this case the initial part of each set of data points is proportional to the product $\Phi_T \times\varepsilon_{T\text{-}T}$, where Φ_T and $\varepsilon_{T\text{-}T}$ are the quantum yield of the triplet state and its molar absorption coefficient, respectively. By taking into account that all solutions are almost optically matched at the excitation wavelength and that no differences in $\varepsilon_{T\text{-}T}$ are expected in the presence of S^4TdR, Φ_T values may be directly estimated by the different slopes (π) of the straight-lines obtained from the linear portion of the plots, *via* the simple equation:

$$\Phi_T = \Phi_{T(RB)}\pi / \pi_{(RB)}$$

Detection of $^1O_2$by IR luminescence

Steady-state emission of $^1O_2$ in the NIR region was recorded with a Fluorolog-2 Mod. 111 spectrometer, equipped with a InGaAs detector maintained at −196 °C. The samples were illuminated orthogonally at 540 nm, using the monochromatic radiation of the fluorimeter as excitation source. D_2O was used as a solvent for $^1O_2$ luminescence measurements to take advantage of the larger radiative constant and longer lifetime with respect to H_2O. Φ_Δ values were determined by using optically matched solution at the excitation wavelength of RB and 5,10,15,20-tetrakis(4-sulfonatophenyl)-21H,23H-porphyrin (TPPS) in D_2O as a standard (Φ_Δ = 0.6) through the following equation:

$$\Phi_\Delta = \Phi_{\Delta(s)}\, A/A_{(s)}$$

where $\Phi_{\Delta(s)}$is the $^1O_2$ quantum yield of the standard and A and $A_{(s)}$ are the areas of the IR phosphorescence spectra of RB and of the standard, respectively.

Photo-oxidation experiments

Photo-oxidation experiments were performed by exposing, for increasing times, aqueous solutions (pH 7 or 12) containing S^4TdR and RB to the radiation emitted by an artificial neon lamp, whose emission had been previously assessed to occur mainly between 400 and 700 nm and with a power surface density of 60mW/cm^2. 1 hour was chosen as the maximum irradiation time.

ARTICLE View Article Online DOI: 10.1039/C5CP03613A Journal Name

Results and discussion

UV-Vis spectroscopy data

As a preliminary step of the study based on UV-Vis spectroscopy, spectra of S^4TdR/RB 8×10^{-4} M/5×10^{-4} M aqueous solutions at pH 7 and 12, not exposed to the neon lamp radiation, were acquired and are compared in Figure 1a.As already observed in aqueous solutions containing only the nucleoside[8],a significant shift of the S^4TdR absorption maximum occurred at basic pH. On the other hand, the main spectral features related to RB, *i.e.* a maximum absorption band at 549 nm and a shoulder at about 510 nm, already reported in the literature[19], remained substantially unchanged with pH. It is worth noting that the intensity of the cited shoulder has been correlated to the self-aggregation of RB[20]. The relatively low ratio observed between the absorbance values related to the shoulder and tothe maximum (*ca.*0.37) suggests that no significant RB aggregation is present under the above experimental conditions.The independence of RB absorption on the solution pH is consistent with data reported by Miller [21] for a pH range 5-12 and suggests that, in the absence of S^4TdR, a nearly constant value of the $^1O_2$ quantum yield should be obtained upon RB irradiation (*vide infra*),in accordance with data reported by Nowakowska *et al.*[22]. When the S^4TdR/RB mixtures were irradiated a significantly higher reactivity was observed for S^4TdR at pH 12, compared to pH 7 (see Figure 1b). Moreover, the decrease of the S^4TdR absorption band at 321 nm, clearly observed at basic pH, was accompanied by a concomitant absorbance increase at 270 nm, with the generation of an isosbestic point (marked by a grey circle in Figure 1b). This spectral evolution is in excellent agreement with our previous results on 4-thiothymidine photo-stability[8] and indicatesthymidine as the most relevant photodegradation product generated under these pH conditions.As evidenced by the inset in Figure 1b, the decrease of S^4TdR absorbance (at 337 nm) was less pronounced at pH 7.Accordingly, the increase of absorbance at 270 nm, due to generated TdR, was less evident. Nonetheless, the presence of an absorption band due to TdR at that wavelength was confirmed after an appropriate subtraction of the absorbance due to a minor band of RB, located between 250 and 300 nm and easily detectable in Figure 1a spectra.

As for RB, a slight decrease in its maximum absorbance was observed upon irradiation (see Figure 1b), suggesting an almost negligible degradation of the photosensitizer. This aspect will be re-considered later in the paper.

In order to draw more detailed information on the mechanism leading to the generation of thymidine, the photodegradation of thyothimidine was further investigated using time-resolved absorption spectroscopy and singlet oxygen detection based onnear-infraredluminescence.

Time-resolved absorption spectroscopy and $^1O_2$ detection

The excited triplet state of a PS is the key transient intermediate for the photosensitization of $^1O_2$ and its effective generation is thus crucial for the photodynamic action. Laser flash photolysis with nanosecond time-resolution is a powerful tool for obtaining spectroscopic and kinetic features of excited triplets of many PS, since these transient species exhibit very intense absorptions in the UV-Vis region and possess lifetimes falling in the microsecond time regime. Figure 2a shows the transient absorption spectra recorded at different delay times with respect to a 532 nm laser excitation of aqueous solutions of RB at pH 7. The transient spectrum observed 5 μs after the pulse shows the typical features of the excited triplet state of RB, with positive, non-structured absorptions above 550 nm and below 500 nm, respectively, and a bleaching due to the ground-state absorption[23].

The time evolution of the transient absorption reveals that no new species are formed concurrently to the triplet decay, under our experimental conditions, ruling out any possible formation of semi-reduced and semi-oxidized radicals (whose maxima are expected to be located at 420 and 460 nm, respectively) through typical self-quenching reactions. The triplet state decays mono-exponentially with a triplet lifetime of *ca.* 50 μs (see the inset in Figure 2a). This spectroscopic and kinetic scenario is basically the same at pH 12 (see Supporting Information, Figure S1).

Furthermore, since the solutions are optically matched at the excitation wavelength, the similar intensity of the observed transient spectra accounts for similar quantum yields of the triplet state (Φ_T) values at the two different pH values.

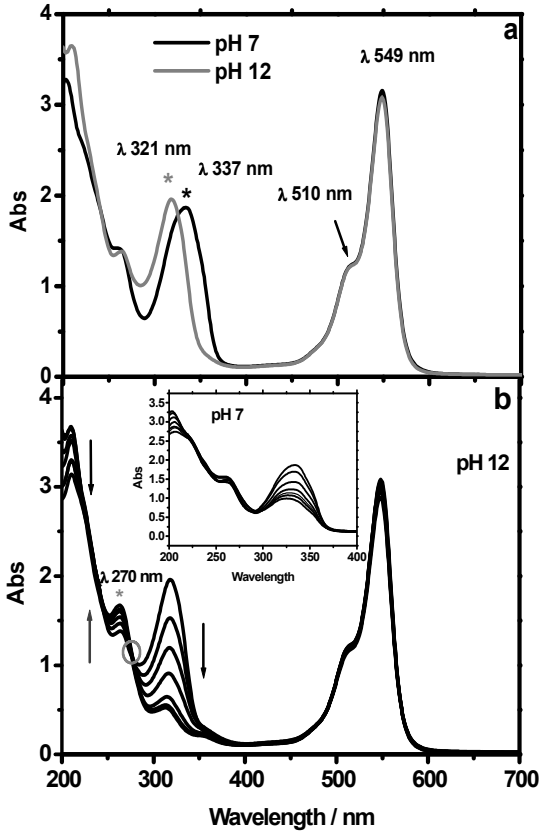


Figure 1.a) Comparison between the UV-Vis absorption spectra obtained for pH 7 (black line) and pH 12 (dark grey line) aqueous solutions containing 8×10^{-4}M S^4TdR and 5×10^{-4} M RB. **b)** Comparison between UV-Vis absorption spectraobtainedfor the same solution irradiated for up to 1 hour at pH 12 using a neon lamp (emission between 400 and 700 nm). *Inset:* Comparison between the UV-Vis absorption spectra (shown only between 200 and 400 nm) obtained for the 8×10^{-4} M S^4TdR/5×10^{-4} M RB solution irradiated for up to 1 hour at pH 7. In both cases the reported spectra were recorded at 10 minute intervals.

Journal Name
View Article Online
DOI: 10.1039/C5CP03615A ARTICLE

The effects of S^4TdR on the efficiency of population of the RB triplet state, as well as on its deactivation dynamics, were also explored in detail at both neutral and basic pH values.Figure 2b shows the laser intensity dependence of the top ΔA of the triplet absorption, monitored at 600 nm in the absence or in the presence of S^4TdR, at neutral or basic pH. The behaviour observed is typical of a one-photon process, such as the generation of the lowest triplet state. In this case, the initial part of each set of data points is proportional to the product $\Phi_T \times \varepsilon_{T\text{-}T}$, where $\varepsilon_{T\text{-}T}$is the molar absorption coefficient of the triplet state (see experimental). By taking into account that all solutions are almost optically matched at the excitation wavelength and that large changes in the $\varepsilon_{T\text{-}T}$ are fairly unlikely, the very similar set of points obtained suggests that S^4TdR does not influence the efficiency of population of the triplet state of

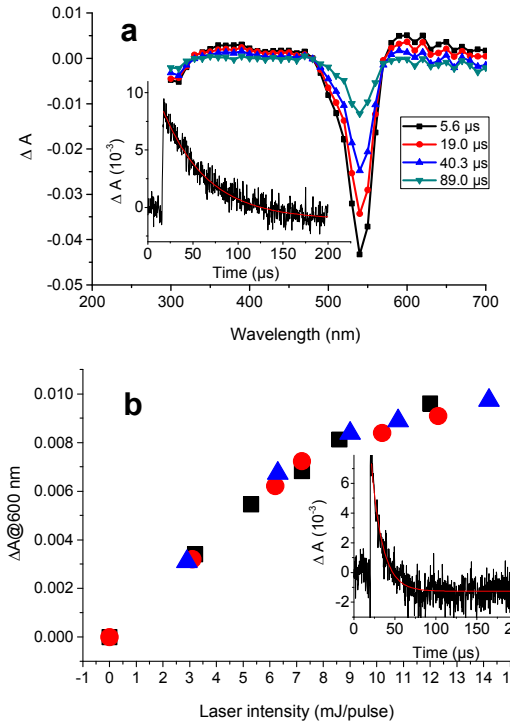


Figure 2.(a) Transient absorption spectra recorded at different delay times with respect to 532 nm laser excitation of N_2-saturated aqueous solutions of RB at pH 7 (E_{532} ~ 10 mJ/ pulse, pulse width ≈ 6 ns). The inset shows the decay trace monitored at 600 nm and the related first-order fitting. **(b)** Laser intensity dependence of the △A at 600 nm taken 0.1 μs after the laser pulse for N_2-saturated aqueous solutions of RB at pH 7in the absence (■) and in the presence of S^4TdR 8×10^{-4} M(●) and at pH 12 in the presence of the same amount of S^4TdR (▲).The inset shows the decay trace monitored at 600 nm and the related first-order fitting in the presence of S^4TdR 8×10^{-4} M at pH 12.

RB. In contrast, S^4TdR affects the decay of the RB triplet at both neutral and basic pH (see the inset in Figure 2b). In particular, we estimated a bimolecular quenching constant $k_{q(S^4TdR)} < 10^8$ $M^{-1}s^{-1}$ at pH 7 and 12. However, this quenching process cannot be attributed to a photoinduced electron transfer involving the reduction of RB and oxidation of S^4TdR. Indeed, in accordance with the endoergonicity of this reaction (a positive △G value can be estimated on the basis of the energy of the RB triplet and on the reduction potentials of RB and S^4TdR), the spectral evolution of the RB triplet in the presence of S^4TdR does not reveal the formation of the reduced form of RB expected at 420 nm (see Supporting Information, Figure S2).

Energy transfer from the triplet state of RB to molecular oxygen results in the concomitant photogeneration of $^1O_2$. Near-infrared luminescence is the most suitable technique to demonstrate unequivocally the generation of$^1O_2$. This species, in fact, exhibits a typical luminescence signal at 1270 μm[24]. Figure 3 shows the diagnostic phosphorescence spectrum of $^1O_2$photogenerated by RB at pH 7 and 12, respectively. Taking into account that the solutions are optically matched at the excitation wavelength, the similar area of the spectra obtained at pH 7 and 12 in the absence of S^4TdR lead us to conclude that the $^1O_2$ quantum yield (Φ_Δ) of RB is basically independent on pH. A value for Φ_Δ = 0.75 was obtained, in accordance with literature[21,22].This is in excellent agreement with

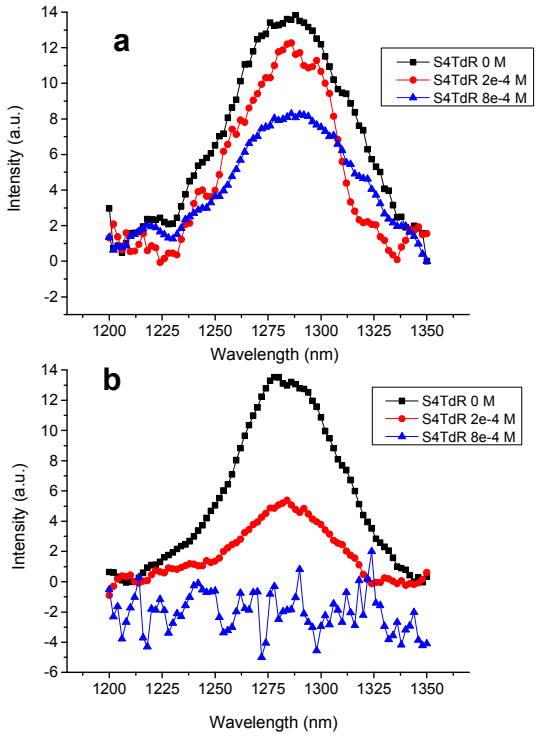


Figure 3. Singlet oxygen luminescence detected in D_2O solutions of RB in the absence and in the presence of S^4TdR, at two different concentrations, at pH 7 (**a**) and 12 (**b**). λ_{exc}= 540 nm.

the very similar quantum yield found for the formation of the triplet precursor. Addition of S^4TdR had a remarkable and pH-dependent effect on the luminescence signal of $^1O_2$. In fact, the phosphorescence intensity decreased as a function of the concentration of S^4TdR and the effect was much more pronounced atpH 12, where the $^1O_2$ signal was basically totally suppressed at the highest concentration used.

In principle, one may ask if these results may reflect an influence of S^4TdR on the efficiency of RB triplet, on its decay in competition with molecular oxygen, or both. However, this is not the case. In fact we have demonstrated that the Φ_T values are independent on the concentration at both pH. Moreover, taking into account that thequenching constant of RB triplet by oxygen is diffusion controlled ($k_\Delta^{O_2} \approx 2\times10^9$ M^{-1} s^{-1}), the concentration of O_2 in water

ARTICLE View Article Online DOI: 10.1039/C5CP03613A Journal Name

medium is 2.4×10^{-4} M, and the constant of RB triplet quenching by S^4TdR estimated above is $< 10^8$ M^{-1} s^{-1}, it can be safely concluded that almost 100% of RB triplet is quenched by molecular oxygen under aerobic conditions,even at the highest concentration of S^4TdR used. Therefore, the luminescence quenching observed in Figure 3 can be reasonably attributed to a chemical process, responsible for the formation of S^4TdR oxidation products.

FTIR-ATR spectroscopy measurements

FTIR-ATR, ^{1}H-NMR and ESI-MS measurements were performed on irradiated S^4TdR/RB mixtures in order to confirm the presence of thymidine, clearly indicated by UV-Vis data, and to detect eventualfurther compounds arising from S^4TdR photodegradation but not exhibiting a peculiar absorption band in UV-Vis spectra. In a preliminary step of the FTIR-ATR study, spectra were acquired on the RB 5×10^{-4} M solution at pH 7 and 12 and are reported in Figures S3a and S3d of the Supporting Information, respectively.

In both cases the comparison between spectra recorded before (time 0) and after 1 hour of irradiation with the neon lamp adopted during this studyis shown. Bands at 1340, 1450 and 1545 cm^{-1}, formerly ascribed to C=C stretching[25], and the one at 1600 cm^{-1}, likely due to C=O groups, observed at pH 7 / time 0, are characteristic of xanthene dyes. A remarkable change of the spectral features occurred at pH 12, where the 1450 cm^{-1} band became larger, shifted to 1425 cm^{-1} and significantly more intense than the others. This effect could be due to the more extended conjugation resulting from the basic pH. Not surprisingly, a general increase of transmittance occurred after irradiating the RB solution for 1 hour (dark grey lines in Figures S3a and S3d), suggesting that a slight RB bleaching took place, as also observed in the UV-Vis spectra (Figure 1b), likely accompanied by variations in electron delocalization in the case of pH 12.

FTIR-ATR spectra observed for the S^4TdR/RB reaction mixtures, as those reported in Figures S3b and S3e, were quite complex, due to the superposition with S^4TdR spectral features, recently described for the same system in the absence of RB[8]. The main S^4TdR-related bands, located at 1625 and 1690 cm^{-1} and ascribed to C_2=O and C_5=C_6 stretching, respectively[8], were clearly visible for the non-irradiated solution at pH 7 (black line in Figure S3b). When the non-irradiated S^4TdR/RB mixture at pH 12 was considered no significant modifications were present with respect to the RB spectrum (black line in Figure S3e), since the main absorption band of S^4TdR at that pH was located exactly at the same wavenumber (1425 cm^{-1}) as the main RB band[8]. After 1 hour of irradiation of the mixture containing 8×10^{-4} M S^4TdR and 5×10^{-4} M RB at pH 7 the valley between the characteristic bands of S^4TdR appeared much less evident (grey line in Figure S3b), suggesting the presence of a contribution due to the main band of TdR (1690 cm^{-1} at pH 7, see Figure S3c). At pH 12, after 1 hour of irradiation (grey spectrum in Figure S3e), the increase of absorption at 1600 and 1650 cm^{-1}, i.e. the wavenumbers related to the TdR bands not superimposed to those due to RB or residual S^4TdR at that pH (see Figure S3f), suggested that thymidine had been generated from S^4TdR. Consequently, FTIR-ATR data confirmed TdR to be the main product of S^4TdRphotoxidation both at pH 7 and pH 12. However, some minor variations occurring in the FTIR-ATR spectra after 1 hour of irradiation of the S^4TdR-RB solutions suggested the presence of a further reaction product. In particular, a slight transmittance decrease was observed at wavenumbers lower than 1000 cm^{-1}, especially at pH 7 (see the grey spectrum in Figures S3b and S3e). Interestingly, absorptions below 1000 cm^{-1} have been often reported for the C-S and S-S stretching, although their detection is generally difficult, due to the low molar absorption coefficients[26]. The generation, as a minor product, of a covalent S-S bridged dimer formed by two S^4TdR molecules could be responsible for those spectral features. It is worth noting that thisspecies has been already observed after long term exposure of S^4TdR to the same light source adopted during the present investigation but in the absence of RB[8].

FTIR-ATR data qualitatively similar to those described so far were obtained for the S^4TdR/RB$2\times10^{-4}/5\times10^{-4}$ M reaction mixtures at pH 7 and 12 (data not shown).

^{1}H-NMR spectroscopy measurements

The ^{1}H-NMR spectrum obtained before irradiation (0 h) for the S^4TdR/RB $8\times10^{-4}/5\times10^{-4}$ M aqueous solution at pH 7 is reported in Figure 4. As already explained, a considerable reduction of the intense signal due to the water protons(4.77 ppm) was achieved during this study by using the pulse sequence reported in the experimental section, thus avoiding the need for a deuterated solvent. The main signals related to S^4TdR (each marked by an S followed by the indication of the corresponding hydrogen, numbered as in the S^4TdR structure reported in Figure 4), were assigned according to Zhang *et al*[27]and have been recently discussed in detail in Ref. 8. The further signal detected in the spectrum, labeled simply as R, was attributed to the RB molecule[28],using, as a reference, the spectrum acquired, under the same instrumental conditions, for a RB 5×10^{-4} M solution at pH 7 (data not shown).

As shown in Figure 4, several new signals appeared in the spectrum after 1 hour of irradiation (1 h). Some of them, labeled with a T,were easily related to TdR (see the numbering of hydrogen atoms in the TdR structure reported in the bottom part of Figure 4) through a comparison witha spectrum obtained, under the same experimental conditions, for a thymidine solution containing RB (data not shown). Not surprisingly, most of the signals related to the deoxyribose ring ofS^4TdR and TdR were very close, even superimposed; indeed, the replacement of an oxygen atom with a sulphur one on the nucleoside basecan influence the chemical shift of the deoxyribose protons onlyslightly. Interestingly, two additional signals (indicated by dotted arrows in Figure 4) were observed, close to S^4TdR ones, in the spectrum obtained after 1 h of irradiation at pH 7 and have been emphasized in the two spectral magnifications reported in Figure 4.Their correlation with a further compound having a thiothymidine-like (S-*Like*) structure could be hypothesized, based on their chemical shifts, 8.085 ppm and 2.060 ppm, respectively. Indeed, signalsclose to these shifts were detected in the ^{1}H-NMR spectrum of S^4TdR before irradiation and assigned to its6-H and 7-CH_3 protons, respectively (see the 0 h spectrum in Figure 4). Moreover, the ratio of the integral areas related tothe two new signalswas in excellent agreement with the 1:3 proportion predicted between the 6-H and the 7-CH_3 protons of S^4TdR. It is also worth noting that the chemical shift variation(from 7.77 to 8.085 ppm)observed forthe new 6-H proton suggested the presence of a structure with an enhanced aromatic character, in which carbon 6 perceived a higher deshielding effect with respect to its counterpart in S^4TdR. The presence of a S^4TdR dimer, already suggested by FTIR-ATR data, would be compatible with this finding. It is also worth noting that the weak additional signals observed in the 1 h spectrum, with chemical shifts close to those related to some of the ribose ring protons of S^4TdR and TdR (i.e., 3'-H, 4'-H and 5'-H), are likely related to the ribose rings protons of the dimeric species.

The ^{1}H-NMR spectrum obtained for the pH 7 reaction mixture initially containing 2×10^{-4} M S^4TdR and 5×10^{-4} M RB, before and after 1 hour of irradiation, showed spectral features similar to those

View Article Online
DOI: 10.1039/C5CP03615A

obtained in the case of the more concentrated S⁴TdR solution. When the reaction mixtures at pH 12 were considered, the most remarkable difference, with respect to pH 7, was related to the chemical shift ofthe 6-H proton of S⁴TdR.Indeed, the latter was detected at 7.61 ppm, i.e. slightly up-field shifted withrespect to the pH 7 reaction mixture. This result could be explained by considering the effect exerted by the negative charge introduced in the thiothymine ring upon deprotonation of the N-H group (see the structures reported for deprotonated S⁴TdR in Ref. 8).Indeed, resonance forms with a negative charge located on the electronegative carbonylic oxygen atoms could become more important for the description of the molecule in this case. Consequently, the 6-H proton would be linked to a slightly less aromatic carbon, thus perceiving a lower deshielding effect, compared to the same proton at pH 7.

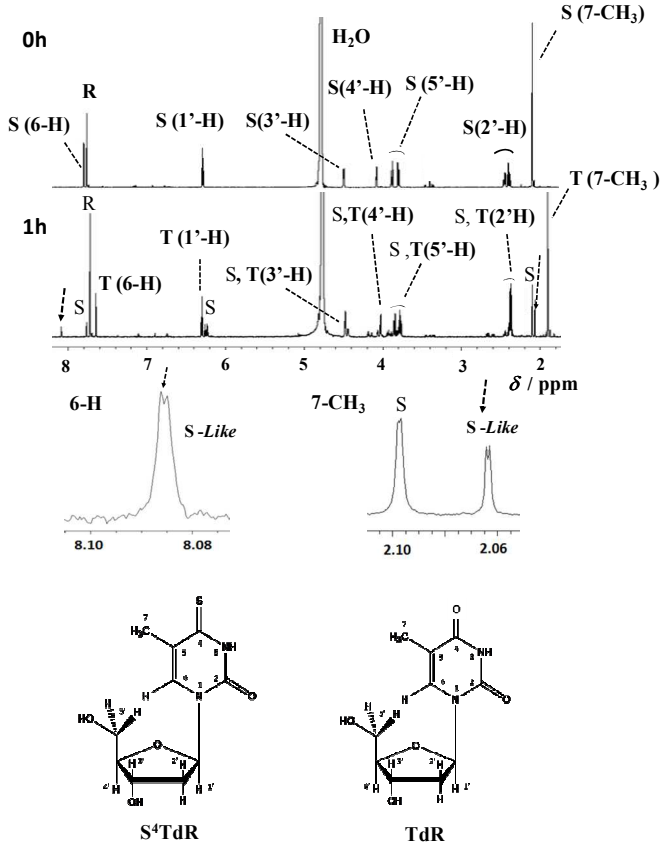


Figure 4. ^{1}H-NMR spectra obtained for a pH 7 aqueous solution containing 8×10^{-4}M S⁴TdR and RB 5×10^{-4}M before (**0 h**) and after one hour of irradiation (**1 h**) with neon light (emission between 400 and 700 nm). Signals are labeled as follows: **R**= Rose Bengal, **S**= 4-thiothymidine and **T** = thymidine; molecular structures of 4-thiothymidine (left) and thymidine (right), as present at pH 7, i.e. as neutral forms, are reported in the bottom part of the figure to clarify the hydrogen atoms numbering. Note that only one of the possible tautomers is reported for each compound.
The dotted black arrows in the 1 h spectrum indicate signals assigned to 6-H (8.085 ppm) and 7-CH_3 (2.060 ppm) protons related to a reaction by-product having a S⁴TdR-like structure (**S-*Like***), putatively corresponding to a dimer of S⁴TdR. Magnifications of such signals are also reported in the lower part of the figure.
Note that, for the sake of clarity, the labels of S(6-H), S(1'-H) and S(7-CH_3) protons have been abbreviated as S in the NMR spectrum related to the 1 h reaction mixture.

Signals assignable to TdR appeared in the ^{1}H-NMR spectra relevant to the S⁴TdR/RB $8\times10^{-4}/5\times10^{-4}$ M reaction mixture after 1 hour of irradiation also at pH 12, but those related to residual S⁴TdR were weak in this case (data not shown). This finding confirmed the higher reactivity of S⁴TdR at basic pH, already inferred from UV-Vis and FTIR-ATR data. Interestingly, the signals detected at 8.085 ppm and 2.06ppm, potentially related to dimeric S⁴TdR,were recognized also in the spectrum obtained at pH 12 but they were much weaker than those detected for the pH 7 reaction mixture, thus suggesting the presence of a much lower concentration of the hypothesized dimeric species at basic pH.Due to the even lower concentration of this product, those signals could not be detected at all in the spectrum related to the S⁴TdR/RB 2×10^{-4} /5×10^{-4} M solution at pH 12 after 1 h of irradiation.

ElectroSpray Ionization-Mass Spectrometry (ESI-MS) measurements

Direct information on the nature of thiothymidine products was searched for throughESI-MS analysis of the S⁴TdR/RB reaction mixtures before and after one hour of irradiation, at pH 7 and 12. For the sake of comparison the concentrations already considered for spectroscopic measurements (i.e. 5×10^{-4} M for RB and either 8×10^{-4} or 2×10^{-4} M for S⁴TdR) were adopted. Just before the ESI-MS analysis an aliquot of eachanalyzed reaction mixture was withdrawn and diluted 1:1 (v/v) with LC-MS grade methanol; afterwards the diluted mixturewas infused into the ESI-MS spectrometer using its syringe pump, operated at a flow of 10 μL/min. As explained in our recent paper[8], the cationization effect due to the Na^+ ions originally contained in the S⁴TdR batch was exploited to give thiothymidine and its products a positive charge, since deliberate protonation through acidification with formic acid resulted in the generation of further by-products, not related to the photo-oxidation process[8]. In order to reproduce ionization conditions similar to those relevant to pH 7 solutions, reaction mixtures at pH 12 were preliminary neutralized using an appropriate volume of concentrated HCl.

The ESI-MS spectra obtained for the S⁴TdR/RB $8\times10^{-4}/5\times10^{-4}$ M reaction mixture at pH 7, before and after 1 hour of irradiation, are reported in Figure 5. If the spectrum obtained before irradiation is compared with that acquired, under the same conditions, for a solution containing only S⁴TdR 8×10^{-4} M (see Figure 7 in Ref. 8) it is evident that cationization by Na^+ was so prevailing in this case that only the signal due to sodiated S⁴TdR (m/z 281.1) was detected, whereas the one related to protonated S⁴TdR (expected at m/z 259.1) was absent. The m/z 281.1 ion was accompanied by the main product ion of sodiated S⁴TdR, i.e. the fragment at m/z 165.0, arising from the detachment of a dehydro-deoxyribose (ddr) molecule (-116 m/z units shift), occurring through H migration from the deoxyribose ring to the ^{1}N of the thiothymine ring[8]. Such a process is not too demanding from an energetical point of view, thus it can occur already during the ESI process and/or the ion transport towards the mass analyzer. Additionally, the previously observed[8] non covalent sodiated dimeric ion of S⁴TdR (m/z 538.8) and two further S⁴TdR-related ions, namely the mono deprotonated/doubly sodiated monomeric species (m/z 303.1) and the doubly deprotonated/triply sodiated non covalent dimer (m/z 583.1), *i.e.* adducts arising from an internal charge compensation, were observed. It is likely that such adducts are formed directly during the ESI process, rather than in solution, and that the remarkable incidence of cationization by Na^+ (compared to solutions not containing RB) can be ascribed to the increase in the Na^+ concentration due to the introduction of the sodium salt of RB in the mixture. As far as RB is concerned, its detection in positive polarity was difficult, as expected, since the

View Article Online
DOI: 10.1039/C5CP03613A
Journal Name

molecule does not bear functional groups suitable for positive charging. Nonetheless, as shown in the top panel of Figure 5, the first of the four characteristic signals detected for RB in the 950-1050 m/z range (the relative abundance scale was expanded in this range by a factor 10 to make the signals more easily observable) was represented by the protonated form of RB (m/z 974.6), *i.e.* by the $[RB+H]^+$ ion. The further signals were related to the mono-sodiated (m/z 996.4), the deprotonated/doubly-sodiated (m/z 1018.4) and the doubly deprotonated/triply-sodiated (m/z 1040.3) adducts of the dye. As shown in the inset to the top panel of Figure 5, the experimental isotopic pattern for the m/z 974.6 ion was in excellent agreement with that expected for the $[RB+H]^+$ ion, characterized by a peculiar abundance of M+2, M+4 and M+6 isotopologues, due to the presence of several Cl atoms in the RB molecular structure. A similar accordance was observed for the other three RB-related signals.

A remarkable change in the ESI-MS spectrum was observed after 1 hour of irradiation, as shown in the bottom panel of Figure 5. Indeed, the signal due to the $[S^4TdR + Na]^+$ ion was much lower than the one detected before irradiation and the spectrum was dominated by a signal at m/z 537.0, i.e. the m/z ratio already ascribed to the S^4TdR S-S bridged covalent dimer in our previous paper on thiothymidine[8]. The observation, already in the MS spectrum, of signals at m/z 421.0 and 305.1, related to one or two losses of dehydro-deoxyribose from this dimer[8], respectively, and detected also in the MS/MS spectrum acquired for the m/z 537.0 ion during the present investigation (data not shown), confirmed the identity of the latter (see the molecular structure in Figure 5). Consequently, ESI-MS data were in accordance with thegeneration of a covalent dimerof S^4TdR already

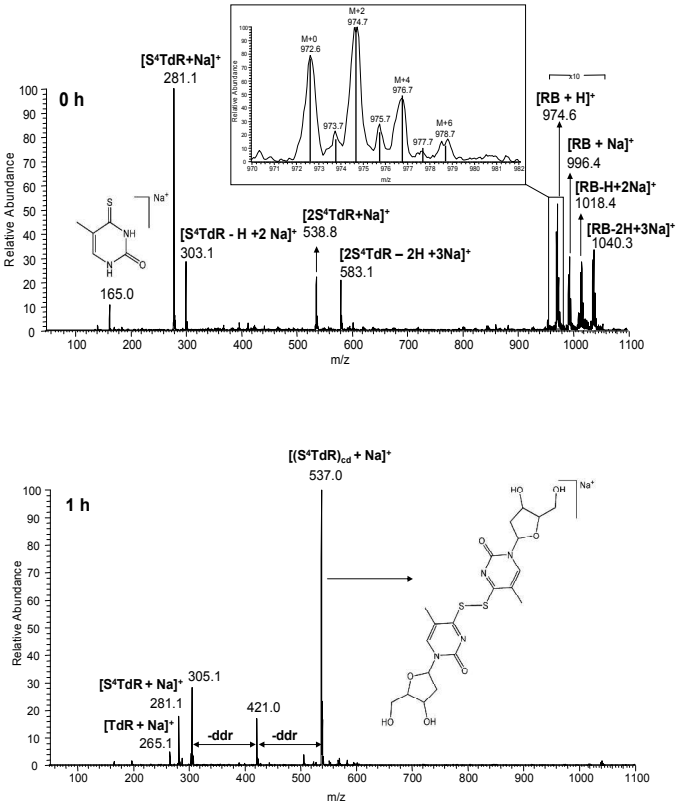


Figure 5. Comparison between ESI-MS positive ion spectra obtained for an aqueous solution containing 8×10^{-4} M S^4TdR and 5×10^{-4} M of RB at pH 7 before (**0 h**) and after irradiation for **1 h** with neon light (emission between 400 and 700 nm). See the text for details about peak assignments. The *ddr* label indicates a dehydro-deoxyribose neutral loss.

suggested by FTIR-ATR and ^{1}H-NMR spectra of the reaction mixture at pH 7 after one hour of irradiation. It is worth noting that the absence of features related specifically to dimeric S^4TdR in the UV-Vis spectra shown in Figure 1bis not surprising, since the thymine base, responsible for the S^4TdR UV absorption, is part of the dimeric species structure. Further considerations about this aspect will be made later.

The ESI-MS spectrum recorded for the 1 h reaction mixture at pH 7 provided an evidence also of the generation of TdR, that was detected as a sodiated adduct (signal at m/z 265.1 in the lower spectrum of Figure 5), like S^4TdR and its dimer. The identification of TdR was confirmed by the MS/MS spectrum obtained for the m/z 265.1 ion, characterized by the presence of a unique, intense signal related to the typical loss of dehydro-deoxyribose (data not shown). The presence of thymidine as a product of the RB-mediated photo-oxidation of 4-thiothymidine, already indicated by UV-Vis, FTIR-ATR and NMR spectraandrelated to the occurrence of a Type II mechanism[29-31], was thus confirmed by ESI-MS. On the other hand, differently from the indications provided by FTIR or NMR spectra,the dimeric species seemed to prevail over thymidine in thereaction mixture, at least in terms ofESI-MS response. This incongruence could be explained by a particularly high cationization yield of the S^4TdRdimer, due to the presence of two deoxyribose rings on its molecule, since these rings are those expected to promote primarily the Na^+-mediated cationization. In other words, the ratio between dimeric S^4TdR and thymidine concentrationsis likely quite lower than thatobserved between their ESI-MS responses.Unfortunately, no standard is available for the S-S bridged dimer of S^4TdR, thus its sodiation yield could not be verified directly and then compared with that of TdR. In order to clarify this aspect the ^{1}H-NMR signals assigned to7-CH_3 protons of S^4TdR, its dimer (*S-like*) and TdR in the 1h spectrum reported in Figure 4 were reconsidered. Starting from the respective integral areas and considering that the number of 7-CH_3 protons in a dimeric S^4TdR molecule is double than that relevant to S^4TdR and TdR molecules, the following concentration ratios were estimated for the 8×10^{-4} MS^4TdR/5×10^{-4} MRB reaction mixture at pH 7, after 1 h of irradiation: S^4TdR/TdR/dimeric S^4TdR = 1/2.7/0.28. A comparison of these ratios with those inferred from the signals in the 1 h spectrum of Figure 5 emphasizes the striking enhancement of the ESI-MS response occurring in dimericS^4TdR. Actually, S^4TdR itself experienced a higher cationization yield compared to TdR, its signal (m/z 281.1) being three times higher than that of TdR (m/z 265.1), although its concentration was estimated to be three times lower. The presence of the sulphur atom on the molecular structure seems then to promote the cationization by Na^+ ions.

The NMR-based approach described for the relative quantitation of residual S^4TdR and its two products was adopted also for the mixture initially containing S^4TdR 2×10^{-4} Mand RB 5×10^{-4} M after 1 h of reaction.A detailed view of the corresponding 1H-NMR spectrum, including the 7-CH_3 proton signals, is reported in Figure S4 of the Supporting Material.The following concentration ratios were estimated from the integral areas of signals shown in Figure S4: S^4TdR/TdR/dimeric S^4TdR = 1/1.5/0.26. It can be thus inferred that while the concentration ratio between S^4TdR and its dimer remained substantially unchanged, a lower amount of TdR was generated when using a 2×10^{-4} M initial concentration of S^4TdR. This result was confirmed by the corresponding ESI-MS spectrum (see Supporting Information, Figure S5).

View Article Online
DOI: 10.1039/C5CP03615A ARTICLE

ESI-MS data confirmed also the different evolution of the photo-oxidation reactionin mixtures at pH 12. Indeed, as shown in Figure 6, relevant to the S^4TdR/RB $8\times10^{-4}/5\times10^{-4}$ M mixtureafter 1 h of irradiation, the signal due to dimeric S^4TdR was much less relevant in this case, whereas TdR was the dominating product. Considering the extreme sensitivity of the adopted ESI-MS approach towards dimeric S^4TdR,a very low concentration can be inferred for the latter in this case, thus it is not surprising that the signals due to the dimeric species were very weak in the corresponding ^{1}H-NMR spectrum.

On the other hand, the thymidine concentration was so relevant that, in spite of its lower cationization yield, the signal of its sodium adduct was more intense than that of residual S^4TdR. Moreover, signals related to non covalent sodiated dimers involving either two TdR molecules (m/z 507.0) or a TdR and a S^4TdR molecule (m/z 522.9) were observed in the ESI-MS spectrum (see Figure 6). The rate of generation for TdR seemed to be even faster when the S^4TdR/RB $2\times10^{-4}/5\times10^{-4}$ M solution was considered, since its signal was the only detectable, along with that of its non covalent dimer, in the spectrum obtained after one hour of irradiation, whereas the signals due to residual S^4TdR and to its dimer were absent (data not shown).

As far as RB is concerned, it is worth noting that no significant signal related to possible degradation by-products of Rose Bengal was observed in the ESI-MS spectra obtained after 1 h of irradiation at both pH values. This result seems to confirm that provided by UV-Vis spectroscopy, suggesting a quite limited RB degradation (see Figure 1b).

As a final comment to ESI-MS analyses, it is important to emphasize that no signal was ever detected for the hydroxylated form of S^4TdR, namely a sulphenylic derivative, often reported among possible singlet oxygen-induced productsof 4-thiothymidine and proposed as an intermediate of the pathway leading to further oxidized by-products[30-32]. Actually, hydroxylated derivatives have been reported to be hardly detectable also in the case of other sulphur-containing species, in particular thioguanine[32-33]. This circumstance supports the hypothesis that they are short-lived intermediates of $^1O_2$-induced photoxidation processes[34,35]. Actually, an hydroxylated form of S^4TdR has been detected by ESI-MS during our recent investigation on the photostability of S^4TdR under visible light in the absence of RB (see Figure 8 in Ref. 8), thus its detection is possible under the ESI-MS conditions adopted during the present investigation. Since the S-S bridged covalent dimer of S^4TdR was detected also in that case, hydroxylated S^4TdR might represent a transient structure evolving towards dimeric S^4TdR, by reaction with unmodified S^4TdR[34-37]. However, in the present case, the rate of this further transformation of hydroxylated S^4TdR is likely so high that it does not accumulate significantly in the reaction mixture.

Hypotheses on the reaction mechanism

After clarifying the identity of photo-oxidation products and estimating their relative concentrations, along with that of residual 4-thiothymidine, mechanistic evaluations were made on the reactivity of the nucleoside. At this aim, the already described UV-Vis spectra,registered at 10 min intervals, were reconsidered on aquantitative basis. The attention was focused on reaction mixturesinitiallycontaining a 8×10^{-4} M concentration of S^4TdR (see spectra in Figure 1b). In particular, the absorbance values registered at 337 nm, for pH 7, and 321 nm, for pH 12, were exploited to follow the degradation of the nucleoside. First, the values were corrected for the residual absorption due to RB, that was estimated from itsabsorbance at 549 nm, once the molar absorption coefficients ratios $\varepsilon_{337}/\varepsilon_{549}$ and $\varepsilon_{321}/\varepsilon_{549}$ had been evaluated, at pH 7 and 12, respectively, from the UV-Vis spectrum of RB 5×10^{-4} M. Corrected absorbance valuesobtained before (A_0) and after (A) a certain irradiation time, up to 1 h, were used to calculate A/A_0 ratios, that were then plotted as a function of time. Plots obtained for the S^4TdR 8×10^{-4} M/ RB 5×10^{-4} M mixture at pH 7 (black circles) and 12 (white circles) are compared in Figure 7a.

At a first glance a faster S^4TdR degradation was observed at pH 12, with a significant reduction of the nucleoside concentration after 1 h of irradiation. These indications are in excellent agreement with those previously obtained by the other techniques adopted during this study. However, it is important to point out that the corrected absorbance at 337/321 nm could be influenced also by the absorption due to the S^4TdR dimeric form. Indeed, the significant structural similarity existing between the latter and the monomeric nucleoside suggests that their UV spectra could be similar. Moreover, the proximity of the absorbance maxima due to S^4TdR and to its S-bridged dimer, arising from photodegradation under UVA radiation, has been already hypothesized by Reelfs *et al*.[9]

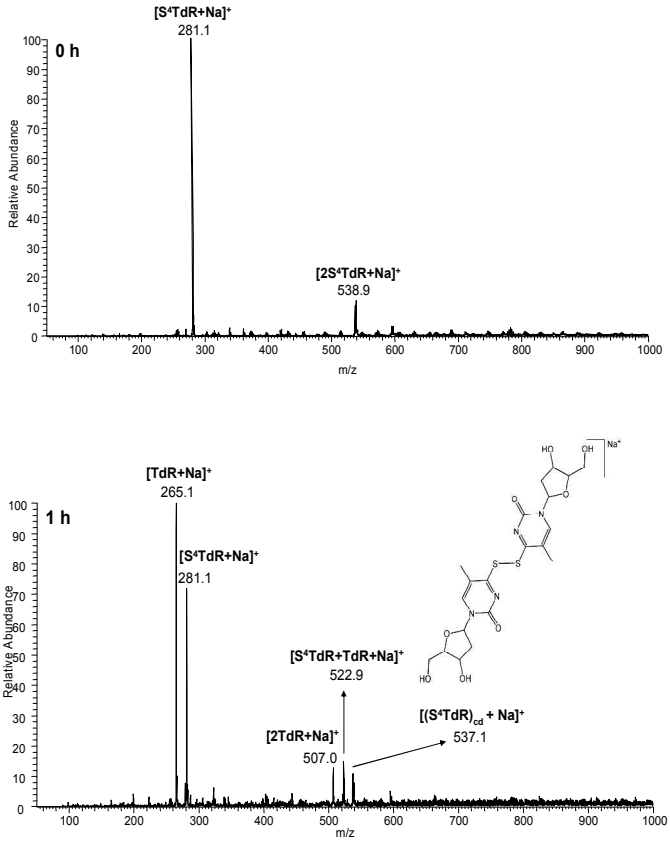


Figure 6.Comparison between ESI-MS positive ion spectra obtained for an aqueous solution containing 8×10^{-4} MS^4TdR and 5×10^{-4} M of RB at pH 12 after the neutralization of the solution with concentrated HCl, before (**0 h**) and after irradiation for **1 h** with neon light (emission between 400 and 700 nm). See the text for details about peak assignments. The cd subscript indicates the covalent dimer of S^4TdR.

A careful examination of data relevant to the pH 7 reaction mixture, reported in Figure 7a as black circles, confirmed the hypothesis. In fact, the A/A_0 ratio observed after 1 h of irradiation was slightly lower than 0.5, whereas the expected ratio, calculated from the

View Article Online
DOI: 10.1039/C5CP03613A
Journal Name

residual S^4TdR concentration, as estimated from NMR data and from the mass balance on S^4TdR, should have been lower than 0.25. The effect was not present at pH 12, as expected, since NMR and ESI-MS data showed that the concentration of dimeric S^4TdR was negligible after 1 h of irradiation at this pH.

Additional data reported in Figure 7a were obtained from special experiments, i.e., photo-oxidation reactions carried out in the presence of sodium azide (NaN_3), a known water-soluble physical quencher for $^1O_2$[21,38,39], at a 10 mM concentration. Both at pH 7 (black triangles) and at pH 12 (white triangles) the presence of NaN_3 in the reaction mixture led to a slight decrease of the S^4TdRphotodegradation rate. Thiseffect supported data previously described in this paper, suggesting the occurrence of a Type II mechanism[10,11,21] as the main oxidation pathway of S^4TdR in the presence of RB as a photosensitizer. The role played by $^1O_2$ was confirmed also by an additional experiment, performed at pH 7 but using as a solvent D_2O, that is well known to increase the lifetime of $^1O_2$[40]. As shown by black squares in Figure 7b, the degradation rate was so increasedinD_2Othat the absorbance due to residual S^4TdR became quite low already after 30 minutes of reaction. Interestingly, the low final absorbance suggested the generation of a negligible concentration of dimericS^4TdR. This hypothesis was confirmed directly by the ESI-MS spectrum of the same reaction mixture, in which the cluster of signals related to sodiated adducts of dimeric S^4TdR, resulting from multiple H/D exchanges, exhibited a very low intensity, compared to the cluster related to TdR (data not shown).

A similar result was obtained when the pH 7 reaction mixture containing 10 mM NaN_3 was analyzed by ESI-MS after one hour of irradiation. In fact, although the ESI-MS spectrum was complicated by the presence of a series of signals due to $[(NaN_3)_nNa]^+$ cluster ions, the absence of a response related to dimeric S^4TdR could be easily ascertained.

Starting from the data described in this and in the previous sections of the paper and frompathways previously proposed for similar systems, involving a phenol[21] or a thiobase[32], a possible reaction scheme was hypothesized and is reported as Scheme 1.In its upper section the scheme is focused on RB in its excited triplet state (^{3}RB), generated after absorption of visible radiation by ground state RB (^{0}RB) and subsequent intersystem crossing of excited singlet state RB (^{1}RB). The rate of this process can be expressed as $\phi_T I_a$, where ϕ_T represents the quantum yield of triplet formation andI_a is the flux of absorbed photons per unit of reaction volume. Once generated,^{3}RB can be potentially involved in several processes, for example physical quenching(rate constant k_Q^{RB}), relaxation (rate constant k_D) and/or electron transfer with $^1O_2$ (rate constant k_Q^{O2})[21]. However, in accordance with our experimental results, the most relevant process, in terms of S^4TdR photo-oxidation, is the chemical quenching by triplet state oxygen, leading to reactive singlet state oxygen (rate constant expressed in Scheme 1 as k_Δ^{O2},with a quantum yield, ϕ_Δ). As shown in Scheme 1, singlet state oxygen ($^1O_2$) can decay to triplet state oxygen by relaxation (rate constant k_d) or be potentially involved also in a chemical reaction with RB (rate constant k_r^{RB}),thus generating RB by-products, although the relevance of such process was found to be negligible in this system.

The most important pathway, in terms of S^4TdR photo-oxidation, is then represented by the $^1O_2$ interaction with S^4TdR, described in the lower part of Scheme 1, divided into separate sections, one for each pH value investigated. In particular, $^1O_2$ might undergo a physical quenching mediated by S^4TdR, in its neutral or anionic form, according to the solution pH (rate constants K'_q and K'_{q^*}, respectively), or be involved intwo different chemical reactions, leading, ultimately, to the oxidation products described in the present investigation. One of these reactions can generate TdR (rate constants indicated in Scheme 1 as $k_r^{S4TdR}$and $k_{r^*}^{S4TdR-}$, at pH 7 and 12, respectively), the other leads to a S-S bridged S^4TdR dimer, likely passing through an intermediate represented by sulphenyl S^4TdR (rate constants k_Q^{S4TdR} and $k_{Q^*}^{S4TdR}$, at pH 7 and 12, respectively).

By analogy with studies involving thiolic compounds[31,32], an attack of singlet state oxygen to the C=S double bond of S^4TdR can be hypothesized during the process. In fact, it is known that the $^1O_2$ π^* orbital can react with filled π or n orbitals of thiocompounds, in turn able to produce TdR or TdR and an hydroxylated form of these compounds, respectively, involving the generation of a hydroperoxide as an intermediate [31,32]. More specifically, $^1O_2$ could react either with the sulphur atom of S^4TdR, generating a sulphur-hydroperoxide intermediate (n-attack), or with the carbon atom in position 4, generating a carbon-hydroperoxide intermediate (π-attack). According to the cited literature[31,32], the former would evolve first to the sulphenylic form of S^4TdR and then to TdR, the latter mainly to TdR. The generation of the S^4TdR dimeric species,

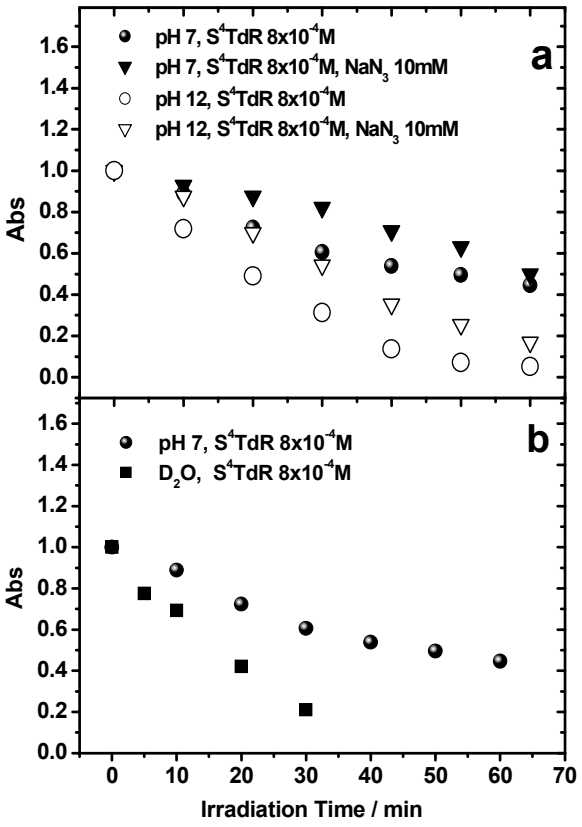


Figure 7.Evolution of the ratio between the S^4TdR maximum absorbance (337 nm for pH 7, 321 nm for pH 12) registered at a certain time of irradiation (A) and that obtained before irradiation (A_0) of a S^4TdR 8 $\times 10^{-4}$ M solution: **a)** as such, at pH 7 (●) and 12 (○) or in the presence of NaN_3 10 mM at pH 7 (▼) and pH 12 (▽); **b)** at pH 7 in H_2O (●) or in D_2O (■);

likely arising from the reaction between sulphenylic –S^4TdR and unreacted S^4TdR[41], seems almost negligible at pH 12, also due to the fast rate of the process leading to TdR, that rapidly subtracts S^4TdR, required to form the dimer. This result is in accordance with those reported by Ramnath *et a.*[30,31]. In particular, the reactivity of the anionic form of S^4TdR is expected to be higher due to its better

Journal Name

View Article Online
DOI: 10.1039/C5CP03615A ARTICLE

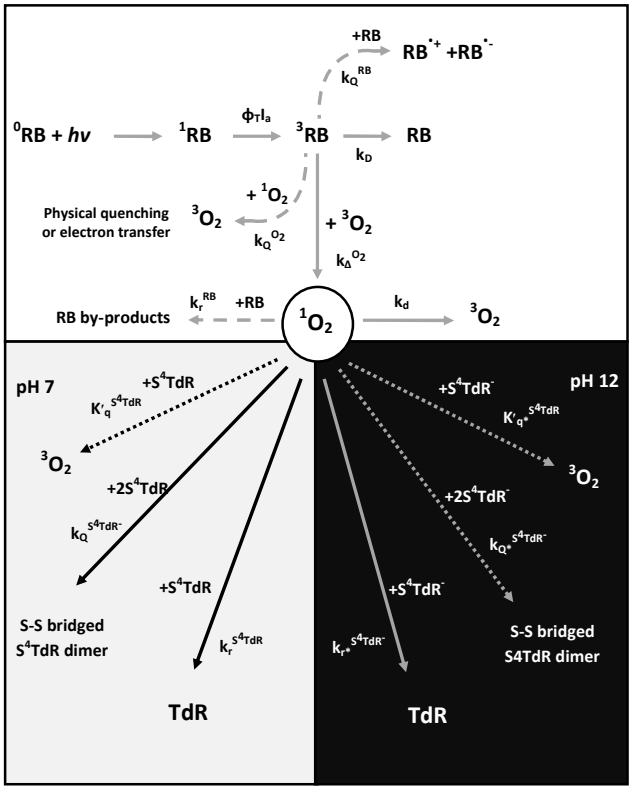
electron delocalization[8], whose effect on reactivity is similar to one induced by the presence of electron-releasing groups on the structure of nucleosides. When pH 7 mixtures are considered the generation of TdR is clearly slower than that observed at pH 12, thus the process leading to dimeric S^4TdR becomes more relevant. Nonetheless, the higher rate related to TdR generation can be appreciated if the lifetime of singlet oxygen is increased, e.g. by using D_2O instead of H_2O as solvent.

Conclusions

The synergic use of three spectroscopic techniques (UV-Vis, FTIR-ATR and ^{1}H-NMR), of time-resolved absorption spectroscopy and of ElectroSpray Ionization-Single and Tandem Mass Spectrometry (ESI-MS and MS/MS) enabled a careful study of the reactivity of 4-thiothymidine (S^4TdR) under visible light excitation in aqueous solution at pH 7 and 12 and in the presence of Rose Bengal, acting as a photosensitizer.Clear indications on the generation of thymidine as a product were obtained by all the spectroscopic techniques and also by ESI-MS. Due to a very favorable yield of cationization, the latter enabled the unambiguous detection of an additional product, namely the S-S bridged covalent dimer of S^4TdR, whose presence was suggested also by some FTIR-ATR and ^{1}H-NMR spectral features. Quantitative estimates based on UV-Vis and ^{1}H-NMR data showed that the dimeric species concentration waslower than that of thymidine at pH 7 and was almost negligible at pH 12.

Time-resolved absorption spectroscopy measurements supported the hypothesis that singlet state oxygen, generated from triplet state oxygen through the intervention of triplet state RB, could be the key *in situ* reactant for the generation of both products. The overall degradation rate for the nucleoside was found to be quite higher at basic pH, likely due to a faster reaction of its anionic form with singlet state oxygen. Moreover, a lower reactivity was observed when a singlet state oxygen quencher, sodium azide, was added to the reaction mixture. On the contrary, the degradation rate became much higher when using D_2O as reaction solvent, thus increasing the singlet state oxygen lifetime.

Consequently, data obtained during the present investigation indicate S^4TdR as an excellent chemical quencher for singlet state oxygen,both at neutral and at basic pH.



Scheme 1. Reaction pathways proposed to explain the reactivity of 4-thiothymidine in Rose Bengal-containing aqueous solutions (pH 7 or 12) upon exposure to visible radiation. Dashed lines were referred to secondary pathways. See text for details.

Acknowledgements

This study was supported by the PRIN-MIUR 2010-2011 (Prot. 2010C4R8M8) funding program entitled: "Architetture ibride multifunzionali basate su biomolecole per applicazioni nel campo della sensoristica, della conversione di energia e del biomedicale". We gratefully acknowledge the skilfull and excellent technical assistance of Mr. Sergio Nuzzo.

Notes and References

[a]Università degli Studi "Aldo Moro" di Bari, Dip. Chimica, Via Orabona, 4- 70126 Bari, Italy.

[b]Centro Interdipartimentale SMART, Via Orabona, 4- 70126 Bari, Italy.

[c]Consiglio Nazionale delle Ricerche CNR-IPCF, UOS Bari, Via Orabona, 4- 70126 Bari, Italy.

[d]Laboratory of Photochemistry, Department of DrugSciences, University of Catania, Viale Andrea Doria 6, I-95125 Catania, Italy

*pinalysa.cosma@uniba.it

1 R. Ackroyd, C. Kelty, N. Brown and M. Reed, *J. Photochem. Photobiol*., 2001, **74**, 656-669.

2 B.W. Henderson, S.M. Waldow, T.S. Mang, W.R. Potter, P.B. Malone and J.G. Levy, *Semin. Oncol*., 1994, **21**, 4-10.

3 I. Penn, *Transpl Proc. XI.,*1979, **5**, 1047–1051.

View Article Online
DOI: 10.1039/C5CP03613A
Journal Name

4 L.J. Kinlen, A.G.R Sheil, J. Peto and R. Doll. *Br. Med. J.,*1979, **8**, 1461–1466.

5 J. Aarbakke, G. Janka-Schaub and G.B. Elion, *Trends Pharmacol. Sci.,* 1997, **18**, 3–8.

6 M.V. Relling and T. Dervieux, *Nat. Rev. Cancer.,* 2001, **1**, 99–108.

7 P. Karran, *Br. Med. Bull*., 2006, **79-80**, 153–170.

8 V. Rizzi, I. Losito, A. Ventrella, P. Fini, A. Agostiano, F. Longobardi and P. Cosma, *RSCAdv.,* 2014, **4**, 48804-48814.

9 O. Reelfs, P. MacPherson, X. Ren, Y.Z. Xu, P. Karran and A. Young, *Nucleic Acids Res.,* 2011, **39**, 9620-9632.

10 R. Belalia, S. Grelier, M. Benaissa and V. Coma, *J. Agric.,FoodChem.*, 2008, **56**, 1582–1588.

11 T. Dai, B.B. Fuchs, J.J. Coleman, R. Prates, A. Astrakas, T.G. St.Denis, M.S. Ribeiro, E. Mylonakis, M.R. Hamblin and G.P. Tegos, *Frontiers in Microbiology, Fungi and Their Interactions,* 2012,**3**, 1-16.

12 P. O'Donovan, C.M. Perrett, X. Zhang, B. Montaner, Y.Z. Xu, C.A. Harwood, J.M. McGregor, S.L. Walker, F. Hanaoka and P. Karran, *Science.*, 2005, **309**, 1871–1874.

13 X. Zhang, G. Jeffs, X. Ren, P. O'Donovan, B. Montaner, C.M. Perrett, P. Karrana and Y.Z. Xu, *DNA Repair.*, 2007, **6**, 344–354.

14 X. L. Ren, F. Li, G. Jeffs, X. H. Zhang, Y. Z. Xu and P. Karran, *Nucleic Acids Res.*, 2010, **38**, 1832−1840.

15 Y. Z. Zhang, X. C. Zhu, J. Smith, M. T. Haygood and R. M. Gao, *J. Phys. Chem. B*, 2011, **115**, 1889−1894.

16 K. Gollnick, T. Franken, G. Schade and G. Dorhofer, *Ann.N. Y. Acad. Sci*., 1970, **171**, 89–107.

17 P. Bilski, A.G. Motten, M. Bilskaa and C.F. Chignell, *Photochem. Photobiol.,* 1993, **58**, 11–18.

18 D.C. Neckersa and O.M. Valdes-Aguilera, *Adv. Photochem*., 1993, **18**, 315–394.

19 P. Fini, L. Catucci, M. Castagnolo, P. Cosma, V. Pluchinotta and A. Agostiano, *J. Incl. Phenom. Macrocycl*., Chem. 2007,**57**, 663–668.

20 P. Fini, R. Loseto, L. Catucci, P. Cosma and A. Agostiano, *Bioelectrochem.*, 2007,**70**, 44–49.

21 J.S. Miller, *Water Research*, 2005, **3**, 412–422.

22 M. Nowakowska and M. Kępczynski, *J. Photochem. Photobiol. A: Chem.,* 1998, *116*, 251–256.

23 L. Flamingni, *J. Chem. Soc. Faraday Trans.*, 1994, **90**, 2331-2336

24 F. Wilkinson, W. P. Helman and A. B. Ross, *J. Phys. Chem. Ref. Data* 1993, **22**, 113

25 P.P. Hankarea, A.V. Jadhava, R.P. Patila, K.M. Garadkara, I.S. Mullab and R. Sasikalac, *Scholars Research Library Archives of Physics Research*, 2012, **3(4),** 269-276.

26 B. H. Stuart. Infrared Spectroscopy: Fundamentals and Applications (Wiley, Analytical Technique in the Sciences) 2004.

27 X. Zhang, J. Wang and Y.Z. Xu, *Magn. Reson. Chem*., 2013, **51**, 523-529.

28 V.P. Batistela, D.S. Pellosi, F.D. De Souza, W.F. Da Costa, S.M. De Oliveira Santin, V.R. De Souza, W. Caetano, H.P.M. De Oliveira, I.S. Scarminio and N. Hioka, *Spectrochimica Acta Part A*. 2011, **79**, 889–897.

29 K.P. Prasanthkumar, C.H. Suresh and C.T. Aravindakumar, *J. of physic. Org. chem.,* 2013, **26**, 510-516.

30 N. Ramnath, V. Ramesch and V. J.C.S. Ramamurthy, *Chem. Comm*., 1981,112-114 Published on 01 January 1981 on http://pubs.rsc.org |doi:10.1039/C39810000112.

31 V. Ramesch, N. Ramnath, V. Jayathertha Rao and V. Ramamurthy, *J. of Photochem.*, 1982,**18**, 109 – 115.

32 X. Zhang, G. Jeffs, X. Ren, P. O'Donovan, B. Montaner, C.M. Perrett, P. Karran and Y.Z. Xu, *DNA repair*., 2007,**6**, 344–354.

33 W.S. Allison, *Accounts of Chemical Research*.,1976,**9**,293-299.

34 K. Goto, M. Holler, R. Okazaki, *J. Am. Chem. Soc*., 1997 ,**119**, 1460-1461.

35 N.J. Kettenhofen, M.J. Wood, *Chem. Res. Toxicol*.,2010, **23**,1633–1646

36 C.C. Winterbourn, D. Metodiewa, Free Rad. Biol. & Med., 1999,**27 (Nos. ¾)**, 322–328

37 V. Gupta, K.S. Carroll,*Biochimica et BiophysicaActa*. 2014, **1840**, 847–875.

38 S. Criado, S.G. Bertolotti and N.A. Garcia, *J. Photochem. Photobiol. B: Biol.*, 1996, **34**, 79–86.

39 F. Wilkinson, W.P. Helman and A.B. Ross, *J. Phys. Chem. Ref. Data.*, 1993, **22**, 113–262.

40 B.M. Cellamare, P. Fini, A. Agostiano, S. Sortino and P. Cosma, *PhotochemPhotobiol*., 2013,**89**, 432-441.

41 C.C. Winterboun, D. Metodiewa, *Free Radical Biology & Medicine*, 1999, 27, Nos. ¾, 322–328.