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Riboflavin as a sensitiser in the photodegradation of tetracyclines. Kinetics, mechanism and microbiological implications

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Abstract

Kinetic, mechanistic and microbiological studies focus on the visible-light-promoted photoreactions that can take place when tetracycline derivatives (TccD) are in the presence of riboflavin (Rf), and on the consequences of these phototransformations. We found that the TccD doxycycline, methacycline, chlorotetracycline, demeclocycline, oxytetracycline and the parent compound tetracycline, quenched riboflavin singlet and triplet (^{3}Rf*) excited states in solution. Within the sub-mM range of concentrations of TccD only the quenching of ^{3}Rf* was significant. As a result the species $Rf^{\cdot -}$ was generated through an electron transfer event from TccD ground state. Further, experimental evidence supported the generation of superoxide radical anion ($O_2^{\cdot -}$). In a parallel process singlet molecular oxygen ($O_2(^1\Delta_g)$) was produced by energy transfer from ^{3}Rf* to dissolved oxygen. Kinetic evidence indicates that doxycycline, methacycline, chlorotetracycline, demeclocycline and oxytetracycline are photodegraded through both $O_2^{\cdot -}$ and $O_2(^1\Delta_g)$ mechanisms, whereas tetracycline is comparatively less photodegradable through a dominant $O_2^{\cdot -}$-mediated process. It was also observed that the antibiotic activity of tetracycline decreases in a parallel fashion with the photodegradation of the drug.

Keywords: Photodegradation; Photooxidation; Riboflavin; Singlet oxygen; Superoxide radical anion; Tetracyclines

1. Introduction

The photochemical degradation of pharmaceutical compounds, especially under daylight irradiation, has received particular attention in the last decades [1,2]. Although the photoreaction could give rise to products with different, null or even undesirable bioactivity, most drugs with pharmaceutical relevance are transparent to daylight. However, the presence of other compounds able to absorb environmental light, the so-called photosensitised reactions, may affect the stability of pharmaceutical drugs. This is the case of tetracycline derivatives (TccD), transparent to visible light, in the presence of the yellow-green pigment riboflavin (Rf, a vitamin B_2 component), as reported by Leeson and Weidenheimer [3]. Although, these authors observed the chemical loss of tetracycline (Tcc) in the presence of Rf, air and visible light, the kinetic and mechanistic details of the processes involved in the photodegradation of Tcc have not yet been investigated. Since the vitamin and TccD can occupy common locations in complex biological structures, kinetic information about visible-light-photopromoted interactions between these compounds can help to understand the behaviour of Rf-generated oxidative species in general, the potential in vivo or in vitro photoreactions on TccD in particular, and the propensity of such processes to occur under given environmental conditions. Besides, mechanistic information on oxidative processes may be of great interest in pharmaceutical and medical fields, to interpret possible photoreactions in the presence of photosensitisers that are able to absorb daylight.

Riboflavin has been postulated as a viable sensitiser for the in vivo photooxidative degradation of naturally relevant or

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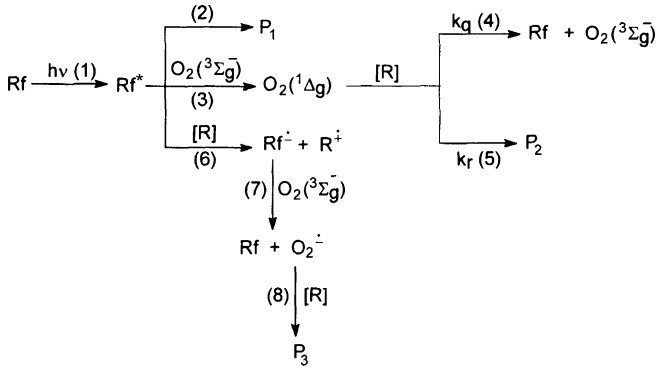
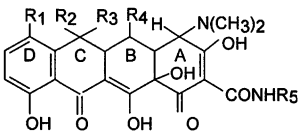


Scheme 1. Possible photoprocesses involved in the Rf-sensitised photooxidation of TccD.



	R_1	R_2	R_3	R_4	R_5
Tetracycline	H	CH_3	OH	H	H
Chlorotetracycline	Cl	CH_3	OH	H	H
Oxytetracycline	H	CH_3	OH	OH	H
Doxycycline	H	CH_3	H	OH	H
Demeclocycline	Cl	H	OH	H	H
Methacycline	H	$=CH_2$	H	OH	H

Scheme 2. Structure of several tetracyclines.

externally-added substrates in different organisms [4,5], by means of processes, that, in a simplified representation, are depicted in Scheme 1. Upon absorption of light (1) the pigment can decompose (2) or, in the presence of air and a reactant R (TccD in the present case) could act via the generation of singlet molecular oxygen ($O_2(^1\Delta_g)$) (3) and further physical (4) or chemical (5) interaction of the oxidative species with R, and/or directly oxidise a suitable substrate by electron abstraction (6) and/or via radical species generate superoxide radical anion (7) which can react with R (8). Rf* represents either electronically excited singlet (^{1}Rf*) or triplet (^{3}Rf*) states of Rf, $O_2(^3\Sigma_g^-)$ is ground state molecular oxygen, and P_1–P_3 are possible photoproducts of Rf and/or R.

The proclivity of TccD to photodecompose is well known. Several works have been published in relation to their phototoxic effects, UV-promoted photochemical oxidation and eosin- and Rose Bengal (RB)-sensitised photooxidations, in which the TccD photodegradation is promoted by direct or by dye-sensitised irradiation [6–8].

The aim of the present study was to analyse the behaviour of the TccD (Scheme 2) tetracycline (Tcc), doxycycline (Dox), methacycline (Met), chlorotetracycline (Chl), demeclocycline (Dem) and oxytetracycline (Oxy), under visible light in the presence of the natural pigment Rf in order to obtain information about the kinetic and mechanistic aspects and the microbiological consequences in the photodegradation of TccD.

2. Experimental

2.1. Materials

Tetracycline, doxycycline, methacycline, chlorotetracycline, demeclocycline, oxytetracycline, riboflavin, sodium azide (NaN_3) and superoxide dismutase (SOD) were purchased from Sigma Chem. Co (USA). The solvents employed were the mixture of water (triply distilled)–methanol (HPLC quality, from Sintorgan (Argentina)) (70:30, v/v), methanol (MeOH) and deuterated methanol (MeOD) from Aldrich. Chloramphenicol 98% was from Aldrich. *Staphylococcus aureus* ATCC 29213 was a gift from Dr. Osvaldo Córdoba.

2.2. Methods

Fluorescence lifetimes were measured with a time-correlated single photon counting technique on an Edinburgh FL-9000CD instrument. In both cases, excitation and emission wavelengths were 445 and 515 nm, respectively. A classical Stern–Volmer treatment of the data was applied using the expression $^1\tau_0/^1\tau = 1 + {}^1k_q\,{}^1\tau_0[\text{TccD}]$, where $^1\tau$ and $^1\tau_0$ are the respective fluorescence lifetimes in the presence and in the absence of TccD.

Ground state absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer. In all cases quartz cells of 1 cm path-length were employed.

Stationary aerobic photolysis of aqueous solutions containing TccD (0.2–0.5 mM) and Rf (0.02 mM) was carried out in a PTI unit, provided with a high pass monochromator and 150 W Xe lamp, irradiating with 440 ± 10 nm, or in a home-made photolyser for non-monochromatic irradiation (150 W quartz-halogen lamp). In this case cut-off filters (400 nm) ensured that the light was only absorbed by the sensitiser.

Rf aerobic and anaerobic photodecomposition rates were determined by the evaluation of the initial slopes of Rf consumption (decrease of absorbance at 445 nm) vs irradiation time.

The $O_2(^1\Delta_g)$-sensitiser RB was employed at concentrations corresponding to absorbance at 532 nm in the range 0.4–0.5. $O_2(^1\Delta_g)$ lifetimes were evaluated in the absence (τ_0) and in the presence (τ) of TccD, and the ratio τ_0/τ was plotted as a function of the quencher concentration, according to a simple Stern–Volmer treatment, using the expression $\tau_0/\tau = 1 + k_t\tau_0$ [TccD]. The determination of k_t was made in MeOD due to the enlargement of the $O_2(^1\Delta_g)$ lifetime in this solvent [9].

The rate constant of the chemical reaction of each TccD with $O_2(^1\Delta_g)$ (step (5) in Scheme 1), generated through Rose Bengal sensitisation ($Abs_{560} \cong 0.5$) was determined in water–MeOH (70:30, v/v) by means of the method introduced by Foote and Ching [10] and assuming that this reaction is the only way for oxygen consumption, with a 1:1 stoichiometry (moles of TccD consumed:moles of $O_2(^3\Sigma_g^-)$ consumed).

The irradiation device has been described elsewhere [11]. Cut-off filters ensure that the light was only absorbed by the sensitiser RB. The ratio of the slope of the first-order plot for oxygen uptake, measured employing a specific electrode Orion 97-08, versus the irradiation time ($slope_Q$), and the slope of a similar plot for a reference compound ($slope_{Ref}$) of known k_r value (k_{rRef}), both at identical concentrations, is equal to the ratio k_r/k_{rRef}. The reference used was the amino acid tryptophan with a reported $k_{rRef} = 6 \times 10^6\ M^{-1}\ s^{-1}$ in MeOH [12]. Oxygen consumption with time by tryptophan or the TccD was unambiguously first-order, at least through three half-lives.

The Rf-sensitised oxygen photoconsumption rates of TccD solutions (0.5 mM) and Rf (0.02 mM) were determined by the evaluation of the initial slopes of oxygen consumption vs irradiation time, employing a specific oxygen electrode (Orion 97-08).

The agar-well diffusion method was used in the microbiological determinations [13]. Nutrient agar plates were inoculated with the standard strain *S. aereus* ATCC 29213. The inoculum was prepared from broth cultures containing 10^8 UFC/ml.

The wells were made in each of these plates using a sterile cork borer. About 100 μl each of aqueous solutions at pH 7 of Tcc 3 mM, before and after Rf-sensitised photoirradiation was added into the wells using sterilised dropping pipettes.

Solvent controls and chloramphenicol 2 μg/ml were used as reference standards.

The plates were incubated at 37 °C for 24–48 h, and the diameters of the inhibition zones were recorded.

3. Results

3.1. Visible-light-promoted degradation of riboflavin and tetracyclines

The Rf-sensitised photoirradiation of air-equilibrated aqueous solutions of each TccD ca. 0.5 mM produces qualitatively similar changes in the whole absorption spectrum of the mixture which reflects the addition of chemical changes in both, TccD and Rf. No spectral changes were observed in the absence of light. Fig. 1 shows the case of Dem. Also the respective absorption spectra of Rf and Dem were included in the figure for comparative purposes.

3.2. Oxygen consumption by the photoirradiated system tetracyclines–riboflavin

Oxygen consumption upon visible photoirradiation was detected in methanolic solutions containing Rf (ca. 0.05 mM) and TccD (0.5 mM). The rate of oxygen uptake was strongly reduced in the comparative irradiations of: (a) a solution of Rf (0.05 mM) in the absence of TccD and (b) a mixture of TccD (0.5 mM), Rf (0.05 mM) and NaN_3 (1 mM); it was slightly reduced when a mixture of TccD (0.5 mM), Rf (0.05 mM) and the enzyme SOD (1 mg/100 ml) was photolysed. The case of Chl is shown as a typical example (Fig. 2).

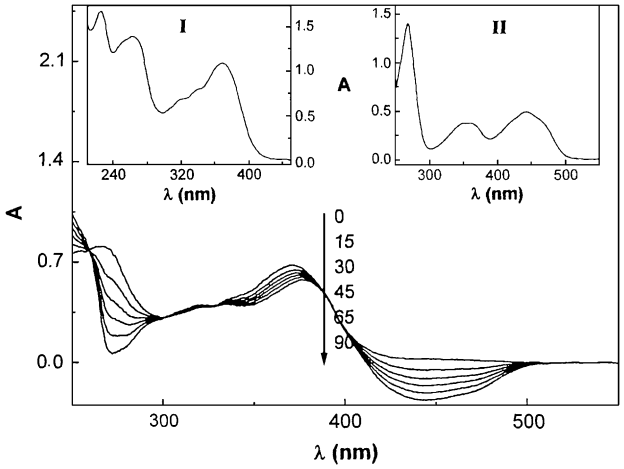


Fig. 1. Spectral changes of methanolic solutions of demeclocycline (ca. 0.1 mM) plus Rf (ca. 0.05 mM) vs Rf (ca. 0.05 mM) as a function of irradiation time. Numbers on the spectra represent irradiation time, in minutes, with wavelength higher than 400 nm. Insets I and II show spectra of methanolic solutions of Rf and demeclocycline included for comparative purposes.

Sodium azide is a well known selective physical quencher of $O_2(^1\Delta_g)$ with a reported k_t value of $2.3 \times 10^8\ M^{-1}\ s^{-1}$ in MeOH [9]. Under these experimental conditions the lifetime of $O_2(^1\Delta_g)$ is reduced from 10 μs to approximately 0.2 μs, due to the presence of NaN_3, making negligible, in practice, any $O_2(^1\Delta_g)$-mediated oxidation. Regarding the experiments employing SOD, similar concentration of the protein has been previously used as an efficient quencher in $O_2^{\cdot-}$-mediated photooxidations [14–16].

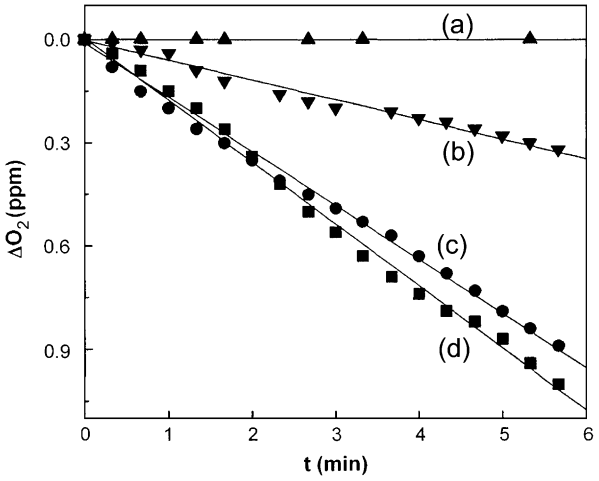


Fig. 2. Oxygen consumption as a function of irradiation time with wavelength higher than 400 nm, of solutions of Rf (ca. 0.05 mM) (a); Rf (ca. 0.05 mM) plus chlorotetracycline 0.5 mM plus sodium azide (1 mM) (b); Rf (ca. 0.05 mM) plus chlorotetracycline 0.5 mM and the enzyme SOD (1 mg/100 ml) (c) and Rf (ca. 0.05 mM) plus chlorotetracycline 0.5 mM (d).

3.3. Inhibition of riboflavin photodegradation by tetracyclines

We observed that both the anaerobic and the aerobic photodegradation rates of Rf, a process that is well known to occur from ^{3}Rf* (anaerobically) [4] and/or from ^{3}Rf* plus autosensitisation via $O_2(^1\Delta_g)$ (aerobically) [17], suffer a decrease in the presence of each one of the TccD studied herein, for concentrations in the range of 0.1 mM. The graphical representation of the anaerobic runs is shown in Fig. 3, for the cases of Tcc and Oxy.

All the pieces of experimental evidence, shown in Sections 3.1–3.3, clearly indicate an interaction between TccD and electronically excited singlet (^{1}Rf*) and/or triplet (^{3}Rf*) states of Rf, globally represented in Scheme 1 by Rf*.

3.4. Quenching of riboflavin fluorescence by tetracyclines

The fluorescence properties of Rf in MeOH are well known [4]. TccD quenched the emission of ^{1}Rf* with a rate constant ^1k_q (process (9)) in the order of $10^9\ M^{-1}\ s^{-1}$ (Table 1), as determined through time-resolved methods (see Section 2).

$$^1\mathrm{Rf}^* + \mathrm{TccD} \xrightarrow{^1k_q} \mathrm{Rf} + \mathrm{TccD}\ \text{or products} \quad (9)$$

A τ_0 value of 5.75 ns obtained for the lifetime of ^{1}Rf* in MeOH, is in excellent agreement with literature data [18]. The Stern–Volmer plots for Chl and Oxy are shown in Fig. 4.

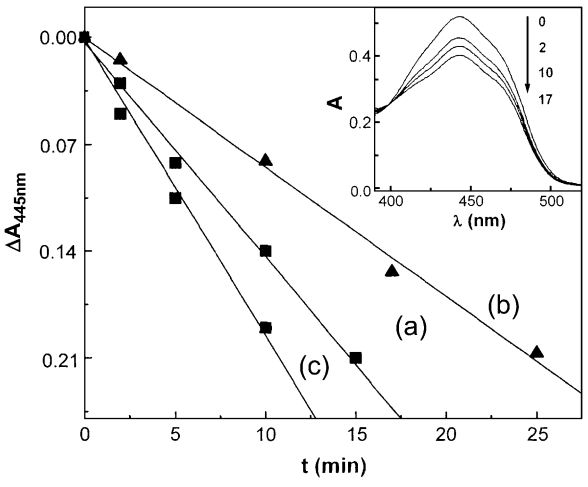


Fig. 3. Decrease in the absorbance of the Rf absorption band of 445 nm as a function of irradiation time, with wavelength higher than 400 nm, in methanolic solutions, under N_2 atmosphere. Rf (ca. 0.05 mM) (a); Rf (ca. 0.05 mM) plus oxytetracycline 0.5 mM (b) and Rf (ca. 0.05 mM) plus tetracycline 0.5 mM. Inset: absorption spectrum of Rf (ca. 0.05 mM) in MeOH upon photoirradiation with wavelength higher than 400 nm in N_2 atmosphere. Numbers on the spectra represent irradiation time, in minutes.

Table 1
Rate constants in MeOH for the quenching of ^{1}Rf* (^1k_q, $M^{-1}\ s^{-1}$) by tetracyclines, apparent rate constants for the quenching of ^{3}Rf* ($^3k_{qapp}$, $M^{-1}\ s^{-1}$) by tetracyclines, relative rates of oxygen photoconsumption (V_{-ox} (rel)) upon Rf-sensitisation, rate constants for the reactive quenching of $O_2(^1\Delta_g)$ (k_r, $M^{-1}\ s^{-1}$) and rate constants for the overall quenching of $O_2(^1\Delta_g)$ (k_t, $M^{-1}\ s^{-1}$) in MeOD

TccD	$^1k_q \times 10^{10}$	$^3k_{qapp} \times 10^9$	V_{-ox} (rel)	$k_t \times 10^6$	$k_r \times 10^6$	k_r/k_t
Tcc	1.30	9.3	0.09	<0.05	<0.01	
Dox	0.27	2.7	1	1.4	1.4	1
Met	0.22	ND	0.67	2.3	2.3	1
Chl	0.21	1.8	0.94	1.6	1.5	~1
Dem	0.20	ND	0.62	2.5	1.5	0.6
Oxy	0.33	5.9	0.47	2.1	1.1	~0.5

ND: not determined due to photoproducts absorption at the observation wavelength.

3.5. Determination of the apparent bimolecular rate constant ($^3k_{qapp}$) for the quenching of ^{3}Rf* by tetracyclines

The rate constant ^3k_q, accounting for the quenching of ^{3}Rf* by TccD (process (10)), could not be determined by laser flash photolysis, employing the available laser described in Section 2, due to the strong absorption of TccD at 355 nm, the excitation wavelength of Rf employing the second harmonic of the laser emission.

$$^3\mathrm{Rf}^* + \mathrm{TccD} \xrightarrow{^3k_q} \mathrm{Rf} + \mathrm{TccD}\ \text{or products} \quad (10)$$

As said in Section 3.3, it is known that anaerobic photodegradation of Rf under visible light irradiation predominantly proceeds through the triplet state [4,5] and the rate of the process can be estimated by absorption spectroscopy from the absorbance decrease at 445 nm (Fig. 3, inset). Comparative irradiations of N_2-saturated methanolic solutions of Rf under identical experimental conditions, in the absence and in the presence of TccD showed that this rate decreased in the presence of TccD in the range 0.1–0.01 mM (Fig. 3). At the said TccD concentrations, no fluorescence quenching of Rf occurs,

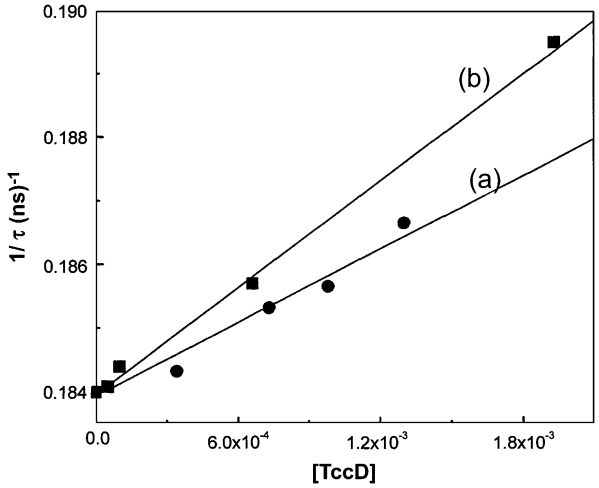


Fig. 4. Stern–Volmer plots for the time-resolved quenching of Rf fluorescence in MeOH by chlorotetracycline (a) and by oxytetracycline (b).

and the experimental data strongly support the idea that a long-lived triplet state, intermediate in the photolysis of Rf, can be quenched by relatively very low TccD concentrations. Hence, the data here were evaluated through a simple Stern–Volmer treatment: $V_0/V = 1 + {}^3k_{qapp} \times {}^3\tau_0 \times [TccD]$, where V and V_0 are the respective velocities of Rf photobleaching at 446 nm in the presence and absence of TccD, ${}^3\tau_0$ is the ${}^3Rf^*$ lifetime, ${}^3k_{qapp}$ represents the apparent rate constant for the process of quenching of ${}^3Rf^*$ (process (10), being ${}^3k_q = {}^3k_{qapp}$) and the ${}^3\tau_0$ value was 12 μs in MeOH which was taken from our previous reports [19].

3.6. Quenching of $O_2(^1Ag)$ by tetracyclines

The rate constant k_t (addition $k_q + k_r$, processes (4) and (5)) for the overall interaction of TccD with $O_2(^1\Delta_g)$ was determined in MeOD, employing TRPD and RB as sensitisers (Fig. 5). RB, the most frequently employed dye in $O_2(^1\Delta_g)$ reactions [20] generates this oxidative species with high quantum yields 0.81 in methanol [21] and was chosen as a sensitiser in order to avoid possible absorption from the TccD at the excitation wavelength of RB (532 nm). k_t values determined by TRPD depend neither on the type of sensitiser nor on potential interactions of the substrate with excited states of the sensitiser involved in $O_2(^1\Delta_g)$-generation.

The k_t values obtained are shown in Table 1. Only a slight decrease in the $O_2(^1\Delta_g)$ lifetime (τ_0, see Section 2) was observed through the TRPD experiments in the presence of ca. 10 mM Tcc. Hence a rate constant value of $k_t \leq 1 \times 10^5\ M^{-1}\ s^{-1}$ was quoted for this TccD.

The k_r values (step (5)) were determined employing an actinometric method in water–MeOH (70:30, v/v) (Table 1). Results for Dox and Met shown in Fig. 5, inset, indicate that with exception of Tcc all TccD were photooxidisable by reaction with the species $O_2(^1\Delta_g)$.

3.7. Evolution of the antibiotic power of tetracycline upon photoirradiation with visible light in the presence of riboflavin

The impact of the photoirradiation of the system Tcc (ca. 0.1 mM)–Rf (0.05 mM) on their antibiotic power, was tested. Fig. 6 shows a representation of the normalised diameter of the bacteriostatic inhibitory *halo* after (H) and before (H_0) photolysis and the normalised absorbance value of the maximum of Tcc absorption band at 320 nm, after (A) and before (A_0) photolysis, both as a function of photoirradiation time. Results clearly show a decrease in the bacteriostatic activity of Tcc as the concentration of the antibiotic decreases, due to the phototransformation.

4. Discussion

4.1. General

An initial coarse evaluation of the results indicates that the Rf-sensitised aerobic irradiation of TccD produces chemical transformations clearly observable in the range of the spectral TccD absorption. Oxygen uptake experiments show the occurrence of photoprocesses in which reactive oxygenated species, generated directly or indirectly by ${}^1Rf^*$ and/or ${}^3Rf^*$, take part. In parallel, the microbiological activity of TccD is decreased due to the phototransformations.

From these results it is possible to extract important information on potential damage of TccD due to exposure to environmental light in the presence of Rf. It consists in the identification of the oxygenated reactive species involved in the reaction with TccD, the extent to which each species participates in the overall event and a measure of the specific damage, in terms of evolution of the therapeutic effect produced by the photodegradation. In some way this information should warn about possible abnormal or undesired effects

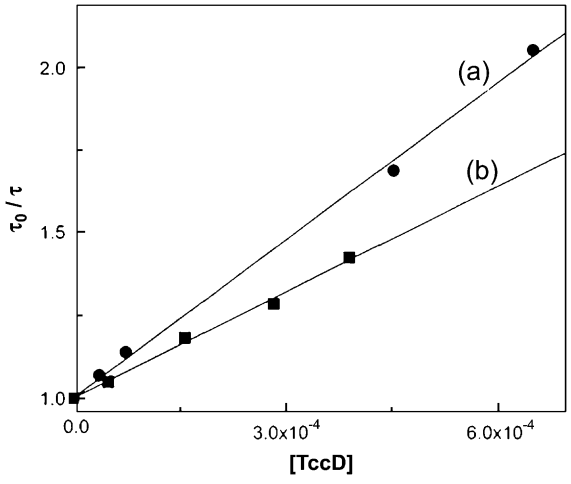


Fig. 5. Stern–Volmer plots for the quenching of $O_2(^1\Delta_g)$ by tetracycline (a) and oxytetracycline (b), in MeOD, determined by means of time-resolved detection of $O_2(^1\Delta_g)$ phosphorescence at 1270 nm. Inset: first-order plots for oxygen uptake upon visible light irradiation by doxycycline (a) and methacycline (b), in water–MeOH (70:30, v/v).

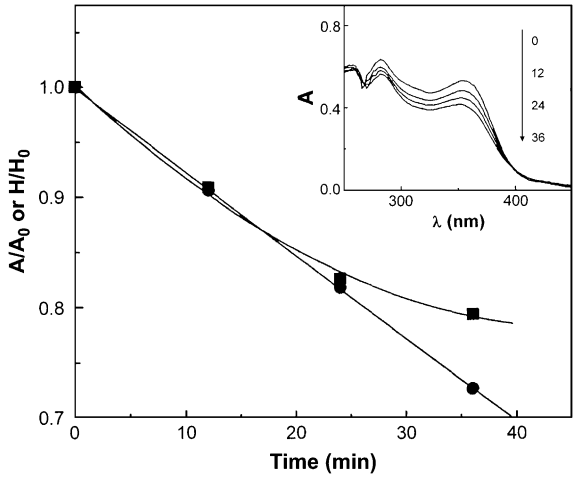


Fig. 6. Normalised diameter of the bacteriostatic inhibitory *halo* after (H) and before (H_0) photolysis of solutions of Rf plus tetracycline and normalised absorbance value of the maximum of tetracycline absorption band at 320 nm, after (A) and before (A_0) photolysis as a function of photoirradiation time, in minutes.

upon application of the drugs, in the presence of photosensitisers such as Rf, and should allow the implementation of preventive actions.

4.2. Photochemical degradation

In spite of the high value – close to the diffusion limit – of the rate constant for the interaction ^{1}Rf*–TccD (^1k_q, process (9)), concentrations of TccD, much higher than those employed in our photolysis experiments, are necessary to produce a noticeable quenching of ^{1}Rf*. Hence, we can assume that employing concentrations of TccD in the sub-mM range, the population of ^{3}Rf* is not affected.

It is known that ^{3}Rf* in solution generates both $O_2(^1\Delta_g)$ (process (3)) and $O_2^{\cdot-}$ (process (11), not included in Scheme 1), upon visible light irradiation with reported quantum yields of 0.48 and 0.009, respectively [22].

$$^1\mathrm{Rf}^* + O_2(^3\Sigma_g^-) \longrightarrow \mathrm{Rf}^{\cdot-} + O_2^{\cdot-} \quad (11)$$

Regarding the participation of $O_2(^1\Delta_g)$ as responsible for oxygen consumption, in a previous paper [8] we demonstrated that TccD are relatively good quenchers of $O_2(^1\Delta_g)$ in alkaline medium (k_t in the order of $10^8\ M^{-1}\ s^{-1}$), the phenolic moiety being mainly responsible for the interaction. In the present case, the relatively strong inhibition of oxygen consumption exerted by NaN_3, confirms the reactive interaction TccD–$O_2(^1\Delta_g)$. In non-alkalinised MeOD solutions, the rate constants (k_t and k_r in the order of $10^6\ M^{-1}\ s^{-1}$, Table 1) are considerably reduced as compared to those obtained in aqueous solutions in the presence of KOH [8].

Tcc practically does not react with $O_2(^1\Delta_g)$, whereas the other substituted TccD scarcely interact with the oxidative species. Nevertheless, the k_r/k_t ratio, considered as a measure of the actual effectiveness of the degradative process through a $O_2(^1\Delta_g)$-mediated reaction [23], is relatively high, indicating that most of the collisions TccD–$O_2(^1\Delta_g)$ lead to reaction, Dox, Chl and Met being the most photooxidisable compounds. Although Rf has been reported as a quencher of $O_2(^1\Delta_g)$ with $k_{t\mathrm{Rf}} = 6 \times 10^7\ M^{-1}\ s^{-1}$ [17], under work conditions most of the reactive oxygen species generated should be intercepted by TccD, given that $k_t[\mathrm{TccD}] \gg k_{t\mathrm{Rf}}[\mathrm{Rf}]$. Likewise, given the relatively low k_t values of TccD, these compounds will compete unfavourably towards the species $O_2(^1\Delta_g)$ in the potential presence of similar concentrations of other moderate $O_2(^1\Delta_g)$ quenchers, with rate constants k_t in the order of 10^7–$10^8\ M^{-1}\ s^{-1}$.

Turning to the oxygen consumption experiments, the decrease in the rate of oxygen uptake due to the presence of SOD (Fig. 2) indicates the participation of $O_2^{\cdot-}$ in the photooxidative process of TccD. Although in the direct generation of $O_2^{\cdot-}$ by electron transfer from ^{3}Rf* to $O_2(^3\Sigma_g^-)$ must be considered negligible due to the extremely low values for the quantum yield of process (11), in the presence of TccD process (6) could proceed, and subsequently the species $O_2^{\cdot-}$ could be formed through electron transfer (process (7)). This sequence, with high efficiency of $Rf^{\cdot-}$ production, has been described already for other phenolic derivatives [24,25]. The high rate constant values obtained for step (6) and the inhibitory effect of SOD in oxygen uptake experiments, strongly suggest that $O_2^{\cdot-}$ generated by step (7) also oxidises TccD (process (8)). Reaction (7) at the same time regenerates ground state Rf, a crucial step in living organisms in which it is well known that $O_2^{\cdot-}$ is a key intermediate in the oxygen redox chemistry [26].

The predominance of a given process (oxidation of TccD via either $O_2^{\cdot-}$ or $O_2(^1\Delta_g)$) will depend on the competition between $O_2(^3\Sigma_g^-)$ and TccD for the quenching of ^{3}Rf*. It is currently accepted that the quenching of ^{3}Rf* by $O_2(^3\Sigma_g^-)$ occurs with an approximate rate constant k_{ET} of 1/9 of the difussional value [27]. Employing a value for 1/9 k_{ET} of $1.2 \times 10^9\ M^{-1}\ s^{-1}$ in MeOH [28], a mean value of $5 \times 10^9\ M^{-1}\ s^{-1}$ for $^3k_{qapp}$ and a concentration of 2.1 mM for the dissolved $O_2(^3\Sigma_g^-)$ in air-saturated MeOH [29] it arises that under work conditions (typically [TccD] = 0.5 mM) processes (3) and (6) are equivalent for all TccD excluding Tcc. For TccD step (10), i.e. $^3k_q[\mathrm{TccD}] \gg k_{ET}[O_2(^3\Sigma_g^-)]$ clearly prevails. In other words, for Dox, Met, Chl, Dem and Oxy $O_2(^1\Delta_g)$ and $Rf^{\cdot-}$ are formed in a similar extent, whereas for Tcc $O_2(^1\Delta_g)$ production is largely overcome by the generation of the species $Rf^{\cdot-}$.

The rate constant values for the quenching of ^{1}Rf*, shown in Table 1, increase in a parallel fashion to those corresponding to the quenching of ^{3}Rf*. This fact could arise from the oxidation potential values of the respective TccD (not available in the literature), assuming that the interaction with both electronic excited states of the pigment is mediated by an electron transfer mechanism. The species $Rf^{\cdot-}$ could also be generated from ^{1}Rf. Semireduction of Rf from its singlet state was previously proposed in order to explain the Rf-photoinitiated vinyl polymerisation [18]. Nevertheless, this process is not viable in the present case because, as pointed out at the beginning of this section, it would require TccD concentrations higher than those employed in our photoirradiation experiments.

4.3. Microbiological activity

The microbiological results for the Tcc, the parent compound for the series of TccD herein studied, are straightforwardly interpretable. The normalised rate for the loss of microbiological activity perfectly parallels the normalised photodegradation rate up to approximately 15% degradation, as observed in Fig. 6. Any contribution to absorbance by photoproducts, at the observation wavelength, could produce an apparent decrease in the photodegradation rate.

4.4. Main remarks

The irradiation of Rf with visible light, under aerobic conditions, in the presence of TccD triggers a cascade of photoprocesses that include the interaction of electronic excited states of the pigment with TccD and $O_2(^3\Sigma_g^-)$, and the generation of reactive oxygen species and radical Rf species. As

a consequence, both Rf and TccD are photooxidised by means of $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$. Nevertheless the above-mentioned interactions protect Rf which is photodegraded to a lesser extent than in the absence of TccD.

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