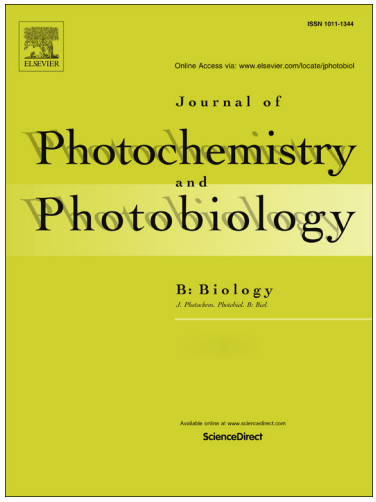

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PII: S1011-1344(14)00341-8
DOI: http://dx.doi.org/10.1016/j.jphotobiol.2014.11.004
Reference: JPB 9875

To appear in: *Journal of Photochemistry and Photobiology B: Biology*

Received Date: 7 August 2014
Revised Date: 11 November 2014
Accepted Date: 18 November 2014

Please cite this article as: E. Reynoso, M.B. Spesia, N.A. García, M.A. Biasutti, S. Criado, Riboflavin-sensitized Photooxidation of Ceftriaxone and Cefotaxime. Kinetic Study and Effect on *Staphylococcus aureus*, *Journal of Photochemistry and Photobiology B: Biology* (2014), doi: http://dx.doi.org/10.1016/j.jphotobiol.2014.11.004

Riboflavin-sensitized Photooxidation of Ceftriaxone and Cefotaxime. Kinetic Study and Effect on *Staphylococcus aureus.*

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Abstract

Trace amounts of the widely used β-lactam antibiotics (Atbs) in waste water may cause adverse effects on the ecosystems and contribute to the proliferation of antibiotic-resistant bacteria. On these grounds, kinetic and mechanistic aspects of photosensitized degradation of Ceftriaxone (Cft) and Cefotaxime (Ctx), have been studied in pure water by stationary and time-resolved techniques. Additionally, possible implications of these photoprocesses on the antimicrobial activity of the Atbs have also been investigated.

Photoirradiation of aqueous solutions of Cft and Ctx produces the degradation of both Atbs in the presence of Riboflavin (vitamin B2), a well known pigment dissolved in natural aquatic systems. The process occurs through Type I and Type II mechanisms, with effective prevalence of the former. The participation of $O_2^{\bullet-}$, $^{\bullet}OH$ and $O_2(^1\Delta_g)$ is supported by experiments of oxygen consumption carried out in the presence of specific scavengers for such reactive oxygen species.

Microbiological assays exhibit a parallelism between the rate of Cft and Ctx photodegradation and the loss of their bactericidal capacity on *Staphylococcus aureus* strains. Results contribute to both understanding kinetic and mechanism aspects of the degradation and predicting on natural decay of Atbs waste water-contaminants.

Keywords: Antibiotics; Riboflavin; photosensitization; photodegradation; reactive oxygen species; microbiological assays

Abbreviations

Atb, Antibiotics

CAT, Catalase

Cft, Ceftriaxone

Ctx, Cefotaxime

D_2O, Deuterium oxide

H_2O_2, Hydrogen peroxide

KH_2PO_4, Potassium phosphate monobasic

K_2HPO_4, Potassium phosphate dibasic

MHA, Mueller Hinton agar

NaN_3, Sodium azide

NaOH, Sodium hydroxide

NB, nutrient broth

$O_2(^1\Delta_g)$, Singlet molecular oxygen

$O_2^{\bullet-}$, Superoxide radical anion

$^{\bullet}OH$, hydroxyl radical

PN, Perinaphthenone

RB, Rose Bengal

Rf, Riboflavin (vitamin B2)

ROS, Reactive oxygen species

SOD, Superoxide dismutase

SPC, Time-correlated single photon counting

TRPD, Time resolved phosphorescence detection

1. Introduction

Several pharmaceutical substances have been recognized as pollutants due to their persistence and bioaccumulation in the environment, provoking negative effects in aquatic or terrestrial ecosystems [1-3]. Most antibiotics do not undergo metabolic inactivation and are inevitably discharged into the environment [4-7]. Furthermore, most antibiotics are non-biodegradable and remain, at trace levels, in water systems even after the conventional wastewater treatments [8-12]. The presence of antibiotics in aquatic systems could contribute to the proliferation of strains of bacteria that are resistant to important classes of antibiotics [13,14].

Therefore, the development of different methods, capable of removing antibiotic residues, has received special attention in the last years [15-19]. In this context, the photosensitized-degradation constitutes a useful environmentally friendly possibility for antibiotic contaminants.

Antibiotics, generally transparent to daylight, could be degraded through photosensitizing action of compounds, present in trace amounts which are able to absorb environmental light [20,21]. In these cases, the only requirement in the aquatic medium containing the antibiotic and dissolved oxygen is daylight, and an adequate daylight-absorbing impurity. This generates electronically excited molecules which may initiate a cascade of photoprocesses producing highly reactive species. This scheme is largely fulfilled in several aquatic environments and, under aerobic conditions, different reactive oxygen species (ROS) could be generated by interaction of dissolved molecular oxygen ($O_2(^3\Sigma^-_g)$) with the electronically excited states of molecules which are present in the

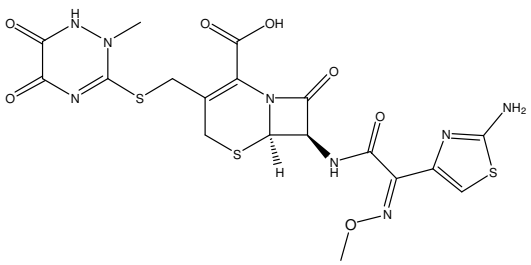
medium. Antibiotics can be degraded by these photogenerated species and consequently may reduce their specific activity [22-24] and their accumulation in aquatic ecosystems.

Some sensitizers are naturally present in the water of rivers, lakes and seas [25]. Riboflavin (Rf, vitamin B2) represent only a small fraction of sensitizers in aquatic environments. However, upon visible light irradiation and especially in the presence of electron-donating compound, the contribution in the generation of ROS by this pigment is very efficient as compared to other natural sensitizers, such as humic acid substances [26]. For this reason, Riboflavin has been postulated as a possible sensitizer for photooxidative decay of different types of organic contaminants [27-29].

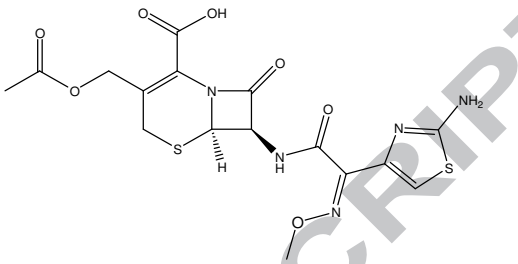
β-lactam derivates belong to the most widely employed antibiotics in human and veterinary medicine [5,8,9,30]. Such widespread usage inevitably leads to the excretion of considerable amounts of these antibiotics into water systems [8,9,31]. In this context, for the present study we have selected two β-lactam antibiotics (Atbs), Ceftriaxone (Cft) and Cefotaxime (Ctx) (Scheme 1). Both bactericidal antibiotics cause the death of sensitive microorganisms in the logarithmic growth phase by inhibition of the last stage in the synthesis of bacterial cell wall [32,33]. These antibiotics currently administered in treatments of multiple infections caused by *Staphylococcus aureus* [34,35].

The purpose of this work was to evaluate of kinetic and mechanistic aspects of Rf-sensitized photodegradation of Cft and Ctx in pure water. In parallel, possible implications of the processes in their antimicrobial activity was evaluated *in vitro*. This knowledge could help in the predicting the potential

impact of β-lactam antibiotics in aquatic ecosystems and programming of the natural decay of these pollutants.



Ceftriaxone (Cft)



Cefotaxime (Ctx)

Scheme 1. Chemical structures of Ceftriaxone (Cft) and Cefotaxime (Ctx)

2. Experimental

2.1. Materials

Ceftriaxone disodium salt hemi(heptahydrate) (Cft), Cefotaxime sodium salt (Ctx), Riboflavin (Rf), Perinaphthenone (PN), tryptophan, sodium azide (NaN_3), catalase from bovine liver (CAT) and superoxide dismutase from bovine erythrocytes (SOD) were purchased from Sigma Chem. Co (USA). D-Mannitol was provided by Sigma-Aldrich Argentina S.A. Deuterated water (99.9 %), KH_2PO_4, $Na_2HPO_4.12H_2O$ and NaOH were obtained from Aldrich.

The culture media used were purchased from Laboratorios Britania S.A., Buenos Aires, Argentina. These media were Mueller Hinton agar (MHA: meat infusion 300 g; peptone casein acid 17.5 g; starch 15 g; agar 15 g; distilled water 1000 ml) and nutrient broth (NB: meat extract 3 g; soy peptone 5 g; NaCl 8 g; distilled water 1000 ml). Both media were autoclaved at 121 ℃ for 20 min.

Staphylococcus aureus (S. aureus) DM1 and DM2 strains were isolated from human nosocomial infection in Rio Cuarto, Córdoba, Argentina. The isolates belong to the Microbiology Department bacterial collection of the National University of Rio Cuarto, Cordoba, Argentina.

2.2. Methods

2.2.1. Steady-state photolysis

Stationary aerobic photolysis of aqueous solutions containing Atb and the sensitizer (Rf, A_{445nm} = 0.5 or PN, A_{365nm} = 0.5) were carried out in a home-made photolyser [36]. This is provided with a quartz-halogen lamp (OSRAM XENOPHOT HPLX 64640, 150w-24 V G35, OSRAM Augsburg, Germany). The light source was equipped with an appropriate cut-off filters (<420 nm> for experiments with Rf and <320 nm> for experiments with PN) in order to remove radiation below 420 nm or 320 nm and to ensure that the light was only absorbed by the sensitizers. The light was passed through a water filter and focused on a hermetically sealed reaction cell with a specific oxygen electrode (Orion 97-08). The solutions were continuously stirred.

From oxygen uptake *vs*. irradiation time plots, the initial slopes were determined in order to evaluate the rates of oxygen consumption by Atb.

In order to investigate the eventual involvement of ROS, spectral evolution of Atb and experiments of oxygen uptake in the absence and in the presence of different additives with ROS-scavenging capacity were made.

The reactive rate constant of deactivation of $O_2(^1\Delta_g)$ by Atbs, k_r, was quantified by means of the method introduced by Foote and Ching [37]. The synthetic dye-sensitizer PN was used as $O_2(^1\Delta_g)$-generator [38]. Assuming that

the reaction of $O_2(^1\Delta_g)$ with the substrate is the only way of oxygen consumption, the ratio of the slope of the first-order plot for oxygen consumption by Atbs and by a reference compound (with a k_{rRef} value known), under identical conditions, is equal to the ratio k_r/k_{rRef}. In all cases, conversions lower than 10% were employed in order to avoid possible interference from photoproducts.

All the experiments were performed in aqueous solutions at pH 7.4 and with freshly prepared solutions.

Ground state absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer.

2.2.2. Time resolved phosphorescence detection of $O_2(^1\Delta_g)$ (TRPD)

The overall rate constant of deactivation of $O_2(^1\Delta_g)$ by Cft and Ctx, k_t, was determined by time resolved phosphorescence detection of $O_2(^1\Delta_g)$ (TRPD). Briefly a Nd:YAG laser (Spectron Laser System, SL400) was used as excitation source. The output at 355 nm was employed to excite the sensitizer PN, and the emitted radiation at 1270 nm was detected at right angles using an amplified Judson J16/8Sp germanium detector, after passing through appropriate filters. The output of the detector was coupled to a digital oscilloscope Agilent Technologies DSO 6012 A and to a personal computer for the signal processing. Usually, 6-8 shots were needed for averaging, so as to achieve a good signal to noise ratio, from which the decay curve was obtained [39]. Air-saturated solution of PN with absorbance at the laser wavelength of *ca.* 0.2 was employed. The decay kinetics was first order in all cases. The experiments were made in D_2O as a solvent instead of H_2O, in order to enlarge the lifetime of $O_2(^1\Delta_g)$ [40]. $O_2(^1\Delta_g)$ lifetimes were evaluated in the presence (τ) and absence

(τ_0) of Atbs, and the data were plotted as a function of concentration of Atbs, according to a simple Stern-Volmer treatment

$$\tau_0 / \tau = 1 + k_t \tau_0 [Atb] \qquad \text{Eq. 1}$$

2.2.3. Stationary and time-resolved fluorescence

For the stationary Rf fluorescence experiments, a Hitachi F-2500 spectrofluorometer was employed.

Fluorescence lifetimes were measured using a time-correlated single photon counting technique (SPC) on an Edinburgh FL-9000CD instrument equipped with a PicoQuant sub nanosecond pulsed LED emitting at 450 nm. The emission wavelength was 520 nm. The quenching rate constant of ^{1}Rf* by Atb, ^1k_q, was graphically determined by the classical Stern-Volmer expression.

$$1 / {}^1\tau = 1/ {}^1\tau_0 + {}^1k_q [Atb] \qquad \text{Eq. 2}$$

Where $^1\tau$ and $^1\tau_0$ are the experimentally determined lifetimes of ^{1}Rf* in the presence and absence of Atb, respectively.

Experiments were performed in air equilibrated aqueous solutions at 25±1°C.

2.2.4. Laser flash photolysis experiments

Argon-saturated Rf aqueous solutions were photolysed using a laser flash photolysis apparatus. A nanosecond Nd:YAG laser system (Spectron Lasser System, SL400) at 355 nm was the excitation source, employing a

150W Xenon lamp as analyzing light. The apparatus has been already described [28,41]. The disappearance of excited triplet state of Rf (^{3}Rf*) was monitored from the first-order decay of the absorbance at 670 nm, a zone where the interference from other possible species is negligible. The triplet decay was measured at low Rf concentration (typical 0.02 mM) and at low enough laser energy, to avoid self-quenching and triplet-triplet annihilation. The ^{3}Rf* lifetimes were determined in the presence ($^3\tau$) and absence ($^3\tau_0$) of the quenchers, Atbs. The data was plotted according to a Stern-Volmer treatment in order to obtain the bimolecular rate constant, ^3k_q.

$$1/{}^3\tau = 1/{}^3\tau_0 + {}^3k_q\,[Atb] \qquad \text{Eq. 3}$$

The transient absorption spectra of Rf (0.02 mM) in the presence and absence of Atbs (*ca.* 0.05 mM) were determined in Argon-saturated aqueous solutions, using a laser flash photolysis apparatus, described above.

Quantum yields of $^{\bullet}$RfH generation ($\Phi_{\bullet RfH}$) were determined in aqueous solutions, relative to the triplet yield of Rf in methanol (Φ_T=0.6) [42,43] employing the expression:

$$\Phi_{\bullet RfH} = [(OD_T\,\varepsilon_R) / (OD_R\,\varepsilon_T)]\,\Phi_T \qquad \text{Eq. 4}$$

where OD_T is the absorbance of ^{3}Rf* in methanol at 670 nm immediately after the laser pulse and OD_R is the absorbance of $^{\bullet}$RfH in aqueous solution at 570 nm. ε_T is the molar absorption coefficient of ^{3}Rf* in methanol (1.1 x10^4 M^{-1}s^{-1}) [42] and ε_R is the molar absorption coefficient of $^{\bullet}$RfH in aqueous solution (5.1

$x10^3$ M^{-1} s^{-1}) [44].

2.2.5. Microbiological tests

The bactericidal activity of Cft and Ctx solutions was evaluated at different photolysis times employing a modification of Kirby–Bauer methodology [45]. This qualitative microbiological assay enables the evaluation of the ability of the Atb to retain or lose their specific activity.

Solutions of Cft (0.7 mM) and Ctx (0.6 mM) were prepared in buffer at pH 7.4 containing the sensitizer. Mueller Hinton agar (MHA) in Petri plates were seeded with *S. aureus* (DM1 and DM2 strains). Then, filter paper discs impregnated with the antibiotic without irradiation and irradiated at different times were placed on MHA plates and incubated at 37°C for 24 h. A paper filter disc impregnated with the sensitizer was used to verify the antimicrobial activity by itself. The inhibitory activity was evaluated measuring the diameter of clear zone around the disks before and after photolysis. In all cases, the experiments were carried out in triplicate.

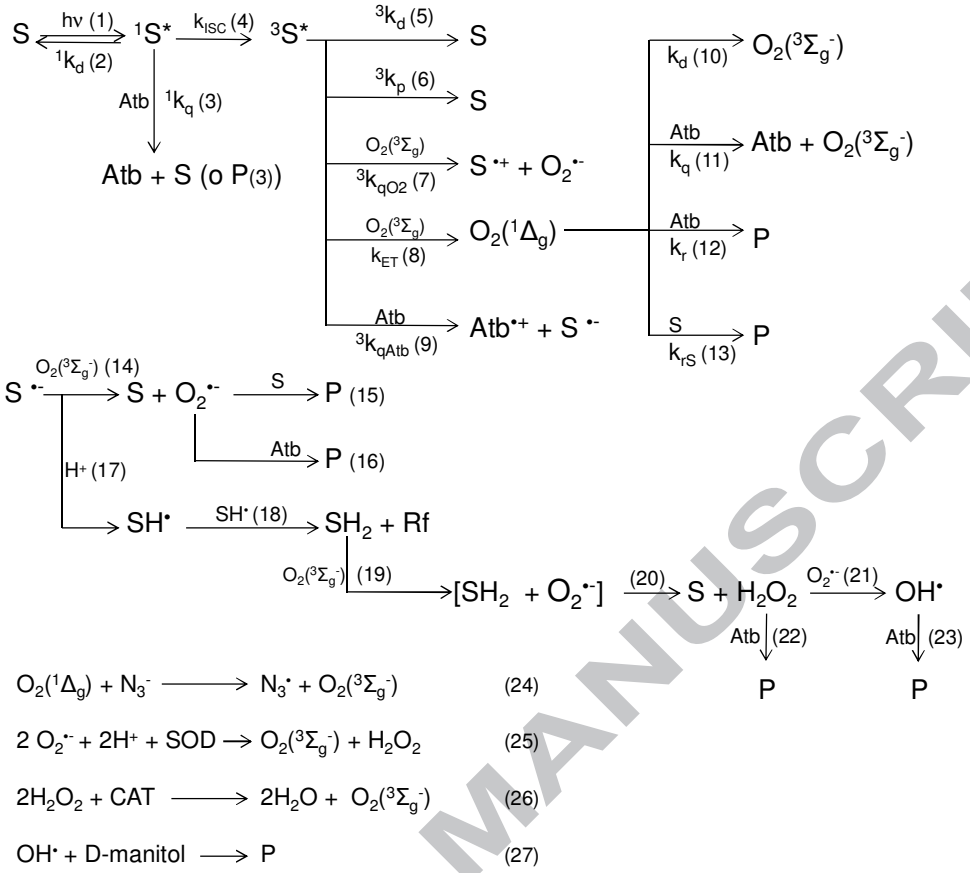
In order to confirm these results, the number of microorganisms, expressed as colony forming units per milliliter (CFU/mL), was determined by the spread plate technique as described below.

S. aureus DM2 strain were grown aerobically at 37°C with stirring (100 rpm) in nutrient broth overnight. Aliquot (~20 µL) of this culture was aseptically transferred to 50 mL of fresh medium (nutrient broth). This microbial suspension was distributed in Pirex® brand culture tubes (13 mm x 100 mm) and the Cft-sensitizer solution, before and after photolysis, was added from a stock solution of 100 mg/L in water. Bacterial cultures grown under the same conditions but

without Cft served as controls. All tubes were incubated with stirring at 37°C for 6 h in order to observe the effect. After that, cellular suspensions were serially diluted with buffer at pH 7.4. Each solution was spread on nutrient agar and the number of colonies formed after 18-24 h incubation at 37 ºC was counted. This experiment was repeated separately three times.

3. Results and Discussion

Scheme 2 shows the set of reactions employed for interpretation and discussion of the results. It depicts a generic photosensitized process in which the absorption of visible light promotes the dye-sensitizer (S) to the electronically excited singlet ($^1S^*$) and triplet ($^3S^*$) states (processes (1) and (4)). $^3S^*$ can transfer energy to ground state oxygen ($O_2(^3\Sigma_g^-)$) in the aerated solution, generating $O_2(^1\Delta_g)$ (process (8)). These species can decay by collision with solvent molecules (process (10)), and can interact physically (process (11)) and/or chemically (process (12)) with a photooxidizable substrate, in the present case, Atbs, with overall rate constant $k_t = k_q + k_r$. Typical electron donors can also transfer an electron to $^3S^*$, giving rise to the respective semi reduced ($S^{\bullet-}$). This species can generate different ROS in the presence of $O_2(^3\Sigma_g^-)$ than in further steps when they can react with Atbs.



Scheme 2. Possible processes involved in the sensitized photooxidation of the antibiotics and reactions of reactive oxygen species with specific scavengers. S represents the sensitizer and Atb the antibiotics.

3.1. Photosensitized degradation of Atb

The visible-light photoirradiation of the system Atb / Rf (A_{445nm} = 0.5) in pH 7.4 air saturated aqueous solution, produced modifications in the absorption spectra of Atbs and the dye, indicating chemical changes in both compounds.

Fig. 1 (main) and Inset I show the spectral evolution of 0.05 mM Cfx/Rf *vs.* Rf and 0.03 mM Cft/Rf *vs.* Rf, respectively, under the same conditions. In

parallel, oxygen consumption by both Atb was detected.

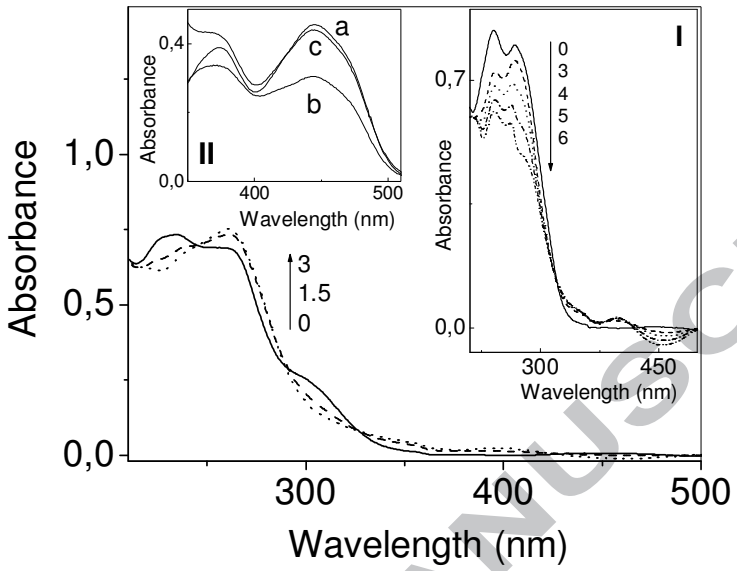


Figure 1. Spectral evolution of 0.05 mM Cefotaxime + Riboflavin (A_{445nm} = 0.5) *vs.* Riboflavin (A_{445nm} = 0.5) upon visible-light photoirradiation. **Inset I:** Spectral evolution of 0.03 mM Ceftriaxone + Riboflavin (A_{445nm} = 0.5) *vs.* Riboflavin (A_{445nm} = 0.5) upon visible-light photoirradiation. **Inset II**: Spectral changes upon visible-light photoirradiation of: **a:** Riboflavin, non irradiated; **b:** Riboflavin 2 min irradiation; **c:** Riboflavin in the presence of 0.5 mM Ceftriaxone, 2 min irradiation. All the experiments were made in Argon-saturated aqueous solutions.

It is known [46] that the anaerobic photodegradation of Rf under visible-light irradiation predominantly proceeds through ^{3}Rf*, and the rate of the process can be deduced from the absorbance decrease of the 445-nm band. Fig. 1, Inset II shows comparative irradiations of Argon-saturated aqueous

solutions of Rf (*ca.* 0.02 mM) in the presence and absence of 0.5 mM Cft. The presence of Cft produced a decrease in the rate of Rf consumption, suggesting the occurrence of a quenching process of ^{3}Rf* by Cft. Similar results were found with Ctx (data not shown).

The above mentioned experimental evidence indicates that the interactions between Atb y Rf could include the participation of electronically excited states of Rf and/or ROS, generated from these states.

In order to elucidate the nature of ROS involved in Rf-photosensitized process, experiments of oxygen uptake in the presence and absence of additives with ROS-scavenging capacity NaN_3, SOD, CAT and D-Mannitol were made [40,47-50].These scavengers have been already employed in similar concentrations to confirm/discard the participation of $O_2(^1\Delta_g)$, $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ respectively, in a given oxidative process [27,51,52]. It is known that the salt NaN_3 is a physical quencher of $O_2(^1\Delta_g)$ with a rate constant of $3 \times 10^8\ M^{-1}s^{-1}$ in aqueous solution, whereas the enzyme SOD dismutates the species $O_2^{\bullet-}$, through reaction (24). CAT decomposes H_2O_2 (reaction (25)) and D-Mannitol reacts with the species $^{\bullet}OH$ with a rate constant $k_q = 1.9 \times 10^9\ M^{-1}s^{-1}$ (reaction (26)) [50,51].

Fig. 2, Inset I shows runs of oxygen uptake by Cft/Rf, in the presence and absence of ROS-scavengers. For each Atb, experiments were performed in triplicate and the runs differed by less than 3%. In comparative irradiations, the rates of oxygen consumption by both Atbs were decreased in the presence of 1.0 mM NaN_3, SOD (1mg/100mL) and 10.0 mM D-Mannitol. These results suggest the involvement of $O_2(^1\Delta_g)$, $O_2^{\bullet-}$ and $^{\bullet}OH$, respectively, as possible reaction pathways. Fig. 2 also includes the spectral evolution of the system Cft/Rf in the absence and in the presence of ROS-scavengers.

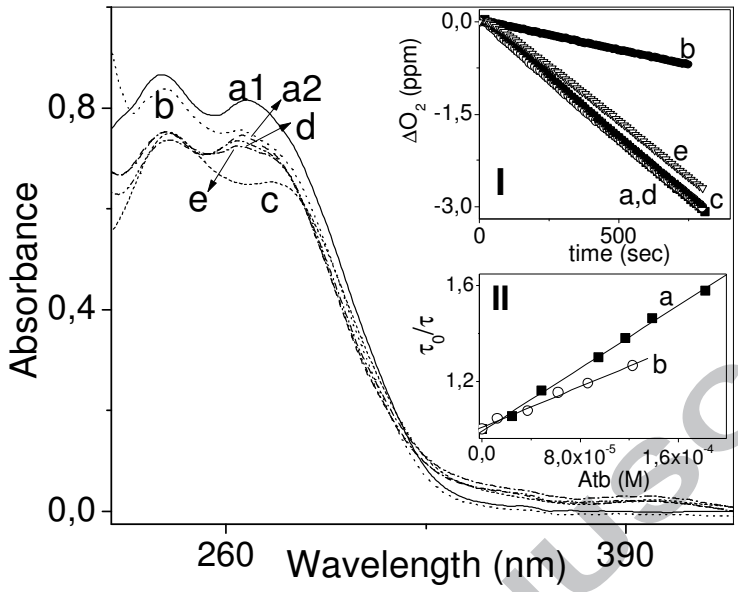


Figure 2. Spectral changes upon visible-light photoirradiation of **a1:** Riboflavin (A_{445nm} = 0.5) + 0.03 mM Ceftriaxone *vs.* Riboflavin (A_{445nm} = 0.5), non irradiated; **a2:** Riboflavin (A_{445nm} = 0.5) + Ceftriaxone *vs.* Riboflavin (A_{445nm} = 0.5), 15 min irradiation; **b:** Riboflavin (A_{445nm} = 0.5) + 0.03 mM Ceftriaxone + sodium azide 1 mM; **c:** Riboflavin (A_{445nm} = 0.5) + 0.03 mM Ceftriaxone + 1 mg/100 ml superoxide dismutase; **d:** Riboflavin (A_{445nm} = 0.5) + 0.03 mM Ceftriaxone + 1 mg/100 ml Catalase; **e:** Riboflavin (A_{445nm} = 0.5) + 0.03 mM Ceftriaxone + 10 mM D-Mannitol. All spectra in the presence of ROS-scavengers were taken after 15 min irradiation *vs.* Riboflavin (A_{445nm} = 0.5) + the corresponding ROS-scavenger under identical conditions. **Inset I:** Profiles of oxygen uptake as a function of irradiation time of **a:** Riboflavin (A_{445nm} = 0.5) + 0.5 mM Ceftriaxone; **b:** Riboflavin (A_{445nm} = 0.5) + 0.5 mM Ceftriaxone + sodium azide 1 mM; **c**: Riboflavin (A_{445nm} = 0.5) + 0.5 mM Ceftriaxone + 1 mg/100 ml superoxide dismutase; **d:** Riboflavin (A_{445nm} = 0.5) + 0.5 mM Ceftriaxone + 1 mg/100 ml Catalase; e: Riboflavin (A_{445nm} = 0.5) + 0.5 mM

Ceftriaxone + 10 mM D-Mannitol. **Inset II:** Stern Volmer plots for the quenching of $O_2(^1\Delta_g)$-phosphorescence emission by **a:** Ceftriaxone; **b:** Cefotaxime.

It is known [40], that Rf generates $O_2(^1\Delta_g)$ (reaction (8)) and $O_2^{\bullet-}$ (reaction (7)) with a quantum yield of 0.49 and 0.005, respectively, indicating that the direct generation of $O_2^{\bullet-}$ by electron transfer from $^3Rf^*$ to O_2 (reaction (7)) can be neglected.

In order to isolate the contribution of $O_2(^1\Delta_g)$ in the Rf-photodegradation of Atbs, it was quantified employing PN as sensitizer, an exclusive $O_2(^1\Delta_g)$-generator [38]. No experimental evidence for the interaction between ground and electronically excited state of PN and the Atbs was found.

The reactive contribution of $O_2(^1\Delta_g)$ was evaluated by the Foote and Ching method [37], as described in the experimental section. As reference compound tryptophan was used, with $k_{rR} = 3.4 \times 10^7\ M^{-1}s^{-1}$ in aqueous solution [53]. Table 1 includes k_r values (process (12)).

Table 1: Rate constants for the reactive (k_r) and overall (k_t) quenching of singlet oxygen by Ceftriaxone (Cft) and Cefotaxime (Ctx); k_r/k_t ratios; rate constants for the quenching of electronically excited singlet state of Riboflavin (^1k_q); rate constants for the quenching of electronically excited triplet state of Riboflavin; quantum yield of generation of $^{\bullet}$RfH ($\Phi_{\bullet RfH}$). Solvent: aqueous solution pH 7.4.

	k_r x10^{-7} $M^{-1}s^{-1}$	k_t x10^{-7} $M^{-1}s^{-1}$	k_r/k_t	^1k_q x10^{-7} $M^{-1}s^{-1}$	^3k_q x10^{-7} $M^{-1}s^{-1}$	$\Phi_{\bullet RfH}$
Cft	0.97 ± 0.05	6.80 ± 0.03	0.14	460 ± 23	230 ± 11	0.16
Ctx	0.76 ± 0.04	5.80 ± 0.03	0.13	320 ± 16	210 ± 10	0.17

Table 1 shows the overall quenching rate constants values for deactivation of $O_2(^1\Delta_g)$ by Atbs, k_t, (sum of k_q plus k_r, processes (11) and (12), respectively, Scheme 2), graphically determined by means of a Stern-Volmer treatment (Fig. 2, Inset II).

The analysis of isolated k_t, k_q and k_r values does not provide much information about the photosensitized-reactions. Nevertheless, the evaluation of the k_r/k_t ratio is a simple and useful approach, which can be envisaged as the fraction of the overall interaction $O_2(^1\Delta_g)$-substrate that leads to effective chemical transformation. Table 1 also includes k_r/k_q ratios. These values show that both Atbs have a high component of O_2 ($^1\Delta_g$)-physical deactivation and low photodegradation efficiency by this oxidative species.

The results strongly suggest that under visible light irradiation the interaction Rf-Atb includes the participation of electronically excited states of the dye and the species $O_2(^1\Delta_g)$, $O_2^{\bullet-}$ and $^{\bullet}$OH formed from these states.

Following, a systematic kinetic study of the electronically excited states of Rf in the presence of Atbs was carried out.

3.2. Interaction of Atb with electronically excited states of Rf

In air-equilibrated aqueous solution, Rf shows a fluorescence emission band centered at 520 nm with a fluorescence quantum yield reported [46,54] of 0.25. In the presence of > 3 mM Atb, a decrease in the intensity of the steady-state emission of ^{1}Rf* was detected but the shape of the emission spectrum did not change. In parallel, the lifetime of ^{1}Rf* in the presence ($^1\tau$) and absence ($^1\tau_0$) of different concentrations of Atb was evaluated (SPC technique). The fluorescence decay of Rf in aqueous solution was monoexponencial with a value $^1\tau_0$ of 4.9 ns, in excellent agreement with previous published data [44,55]. Table 1 shows the values of the rate constants ^1k_q (process (3)), which were graphically determined through a classical Stern-Volmer treatment (data not shown). The quenching of ^{1}Rf* by Atbs was only observed at relatively high concentrations of the substrates (> 3 mM). Hence, this process was not significant under the conditions employed in the photosensitization experiments (*ca.* 0.5 mM).

The interaction ^{3}Rf*-Atb (process (9)) was studied through laser flash photolysis experiments in saturated-Argon aqueous solution.

Addition of Atb to solution of Rf reduces the ^{3}Rf* lifetime, demonstrating the occurrence of an interaction with the electronically excited triplet state of the pigment. As before, a Stern-Volmer treatment of the ^{3}Rf* quenching yielded the bimolecular rate constants ^3k_q (Table 1). The values of ^3k_q, close to diffusional limit, indicate that ^{3}Rf* is effectively deactivated by Cft and Ctx.

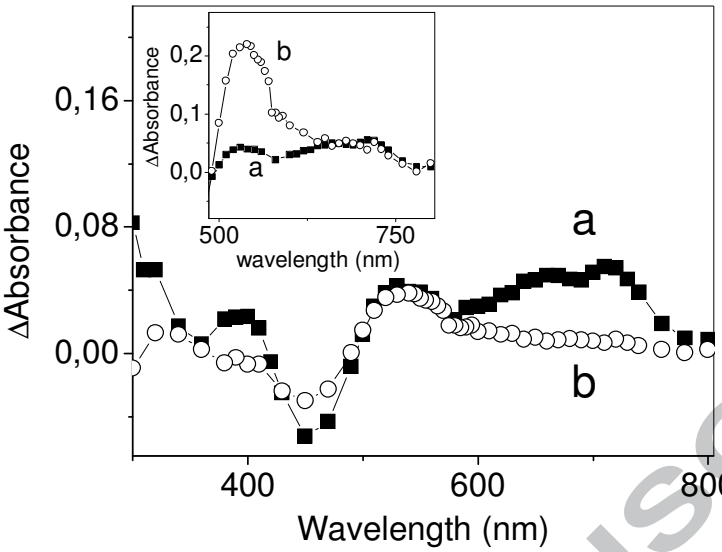


Figure 3. Transient absorption spectra of **a:** 0.02 mM Riboflavin and **b:** 0.02 mM Riboflavin + 0.05 mM Cefotaxime taken at 1 and 15 μs after the laser pulse, respectively, in Argon-saturated aqueous solutions. **Inset:** Transient absorption spectra of **a:** 0.02 mM Riboflavin (1 μs) and **b:** 0.02 mM Riboflavin + 0.05 mM Cefotaxime (15 μs) normalized at 670 nm, in Argon-saturated aqueous solutions

In order to further explore the type of interaction ^{3}Rf*-Atb involved, transient absorption spectra of 0.02 mM Rf was recorded in the presence and absence of Atbs, in saturated-Argon aqueous solution (Fig. 3). Transient absorption spectrum of Rf taken at 1 μs after the laser pulse is in good agreement with spectra for the species ^{3}Rf*, previously published [43,44,56] Under identical conditions but in the presence of 0.05 mM Atb (*ca.* 60 % ^{3}Rf* is deactivated by Atb), the spectrum (taken at 15 μs after the laser pulse) shows an important decay in the 600-750 nm zone. In Fig. 3, inset, the transient

absorption spectrum of Rf (1 μs) is compared in the presence of Ctx (15 μs), normalized at 670 nm. The appearance of long-lived absorption in the 500-600 nm regions is due to the formation of the neutral Rf radical ($^{\bullet}$RfH), as has been reported by others researchers [56].

To confirm the participation of $^{\bullet}$RfH, the quantum yield ($\Phi_{\bullet RfH}$) of generation of this species was determined in the presence of the individual Atb, according to Eq. 4 (Table 1).

Flash photolysis results indicate that the quenching of ^{3}Rf* by Atb is due to an electron transfer process towards the dye, with the concomitant production of $Rf^{\bullet-}$ (process (9)). The interaction of $Rf^{\bullet-}$ with $O_2(^3\Sigma_g^-)$ could generate the reactive species $O_2^{\bullet-}$ through process 14. At pH 7.4, $Rf^{\bullet-}$ is protonated ($pK_a = 8.3$) and the radical $^{\bullet}$RfH is formed (process (17)) [57,58]. It is known that the bimolecular decay of $^{\bullet}$RfH proceeds through a disproportionation reaction yielding Rf and fully reduced Rf (RfH_2) (process (18)) [59,60]. In the presence of $O_2(^3\Sigma_g^-)$, RfH_2 is reoxidised to $RFH_2^{\bullet+}$ and $O_2^{\bullet-}$, and finally Rf and H_2O_2 (process 19 and 20) while H_2O_2 together with $O_2^{\bullet-}$ could give rise to $^{\bullet}$OH (process 21). Some of these different ROS can react with Atbs or with the sensitizer.

Results suggest that the Rf-photosensitized degradation of Cft y Ctx can occur *via* a combination of Type I and Type II mechanisms. The predominance of one of them will depend on the competition between $O_2(^3\Sigma_g^-)$ and Atb for ^{3}Rf*.

It is currently accepted [61] that, in general, the quenching of ^{3}S* by $O_2(^3\Sigma_g^-)$ to produce $O_2(^1\Delta_g)$ occurs with a rate constant k_{ET} of 1/9 of the diffusional value. A kinetic analysis of laser flash photolysis data for Rf, considering a rate constant k_{ET} *ca.* 7 x 10^8 $M^{-1}s^{-1}$ in water [62], a saturation

value of $O_2(^3\Sigma_g^-)$ in water under normal atmospheric pressure [63] of 0.2 mM and 0.5 mM Atb shows that ^3k_q [Atb] is *ca.* 8-fold higher than k_{ET} $[O_2(^3\Sigma_g^-)]$. So, the deactivation of ^{3}Rf* occurs with neat prevalence *via* radical species (Type I mechanism) with the participation of $O_2^{\bullet-}$ and $^{\bullet}$OH, as evidenced by the experiments with specific ROS-scavengers.

3.3. Microbiological tests

It has been demonstrated [64,65] that Rf, under UVA irradiation (365 nm), has antimicrobial properties against common pathogens such as *S. aureus, P. aeruginosa* and *S. epidermidis*. For this reason, the sensitizer RB was used instead of Rf in these microbiological assays. RB is, exclusively, an $O_2(^1\Delta_g)$-generator [66] and it has been employed as sensitizer in several microbiological tests in order to evaluate the bactericidal activity in a parallel fashion with the photodegradation of different drugs. It has been demonstrated that the presence of RB does not affect *B. subtilis, P. aeruginosa* and *S. aureus* strains [22-24].

Microbiological activity of Atb, in the presence of RB in sensitizing concentrations, was evaluated by measuring the inhibition halo diameter before (H_0) and after photolysis (H). The results were expressed as a relative index of bactericidal activity (H/H_0) *vs.* irradiation time (Fig. 4). This figure shows a decrease of the bactericidal activity of Cft and Ctx for DM1 and DM2 strains. This effect is generated by the degradation of both Atb as a consequence of the photosensitized process. The decomposition of Cft and Ctx leads to the loss of bactericidal capacity. On the other hand, this result may suggest that, the

possible photoproducts generated do not have antimicrobial activity when studied on *S. aureus* strains, under experimental conditions.

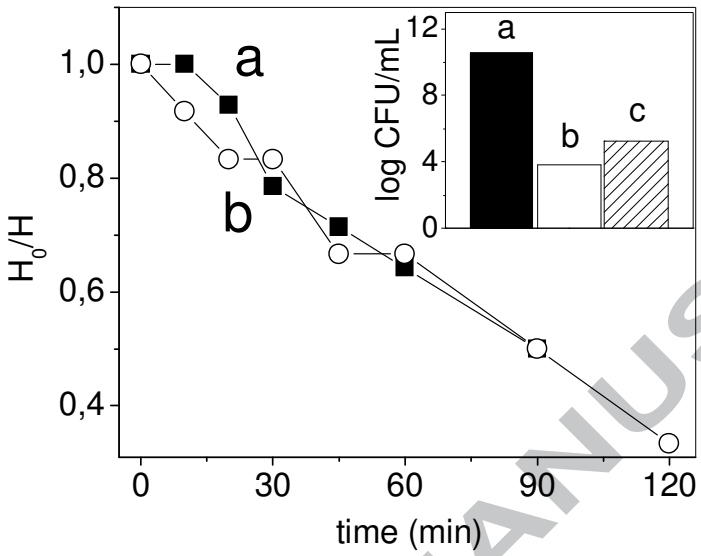


Figura 4. Relative normalized diameter of the bactericidal inhibitory activity of 1 mM Ceftriaxone, in the presence of Rose Bengal, upon **a:** *S. aureus* DM1 and **b:** DM2. H_0 and H represent the inhibitory halo diameter at t=0 (H_0) and at different photolysis times (H), respectively. **Inset:** Effect of 100 mg/L Ceftriaxone **b:** before and **c:** after Rose Bengal-sensitized photolysis on *S. aureus* DM2 strain; **a:** cellular control without Ceftriaxone.

In parallel, the solution containing only RB was irradiated under identical conditions and the microbiological test was performed, but in the absence of Atb. The results show that RB does not affect the strains *S. aureus* studied (data not shown). This evidence confirms that the decrease of the inhibitory halos after photolysis is only a consequence of the photodegradation of Atbs.

Similar results were obtained in $O_2(^1\Delta_g)$-mediate photooxidations, with amoxicillin and cephalexin in the same bacteria [24]. Other families of antibiotics such as tetracycline and sulfa drugs suffer photo-oxidation with the consequent loss of antimicrobial power against *B. subtilis*, *P. aeruginosa* and *S. aureus* [22-24]. Wammer *et al.* used a bacterial assay to determine loss of antimicrobial activity of Sulfa-drugs [67] and three fluoroquinolones [68] under direct photolysis. They found that the all the sulfa-drugs studied and norfloxacin and ofloxacin photoproducts are inactive; however, enrofloxacin photoproducts do retain significant antibacterial activity.

Fig. 4, inset displays the effect of Cft on *S. aureus* DM2 strain growth. This figure shows a decrease in the number of microorganisms treated with 100 mg/L of Cft with respect to *S. aureus* culture without treatment. This diminution of ~ 7 log in relation to culture control was expected because of the bactericidal effect of this antibiotic. On the other hand, after the Cft photolysis its antimicrobial activity was affected, ~5 log decrease is observed, compared with 7 log decrease in CFU/mL when the Cft is intact. These results prove the loss of bactericidal effect by Cft on the studied strain, as a consequence of photosensitized-degradation. Thus, confirming the result observed in the assay previously described.

4. Conclusions

The Rf-photosensitized degradation of Cft and Ctx, in pH 7.4 aqueous solution pH 7.4, occurs through Type I and Type II mechanisms, with prevalence of the former.

Laser flash photolysis results demonstrate that $^3Rf^*$ is efficiently

deactivated by Atbs with the concomitant production of $^{\bullet}$RfH. The neutral radical triggers a cascade of ROS-generating photoprocesses.

Oxygen uptake experiments in the presence of ROS-scavengers species show that $O_2^{\bullet-}$, H_2O_2 y $^{\bullet}$OH are generated *via* ^{3}Rf* through Type I mechanism. Cft and Ctx do not react with H_2O_2. $O_2(^1\Delta_g)$ is also produced in the Rf-sensitized process. Both Atbs interact with this species mainly through a physical process. Microbiological assays show parallelism between the rate of photodegradation of Cft and Ctx and the loss of their bactericidal capacity. The photodegraded Atbs do not maintain any inhibitory activity on *S. aureus* strains. The fact that similar results are obtained for both Atbs, despite the different side chain, strongly suggests that the reactive target could be the common structure present in both molecules.

Acknowledgements

Financial support from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (SECyT UNRC), both from Argentine, is gratefully acknowledged.

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Highlights

Ceftriaxone and Cefotaxime are photodegradated in the presence of Riboflavin

Ceftriaxone and Cefotaxime *are photodegradated principally via* Type I mechanism

The photooxidation of *Ceftriaxone* and Cefotaxime yield loss of bactericidal capacity

The photodegraded antibiotics do not maintain inhibitory activity on *S.aureus* strains