On the photooxidation of the multifunctional drug niclosamide. A kinetic study in the presence of vitamin B_2 and visible light

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Objectives: The multifunctional drug niclosamide (NSD), extensively employed therapeutically, is a frequent pollutant of surface waters. Considering the environmental importance of photodegradative processes for this type of contaminant, the kinetic and mechanistic aspects of the possible visible-light-mediated photooxidation of NSD were studied under naturalistic conditions.

Methods: The visible-light absorber riboflavin (vitamin B_2) was employed as a photosensitizer. The vitamin can usually be found in natural waters and is the most common endogenous photosensitizer in mammals.

The interaction of NSD with electronically excited states of Rf and with photogenerated reactive oxygen species (ROS) was evaluated through conventional UV spectroscopy, laser flash photolysis, time-resolved phosphorescence detection of singlet molecular oxygen ($O_2(^1\Delta_g)$), and polarographic dosage of dissolved oxygen.

Results: Ground state NSD quenched the long-lived triplet excited state of Rf ($^3Rf^*$) and the photogenerated ROS ($O_2(^1\Delta_g)$) and superoxide radical anion ($O_2^{\bullet-}$). As a result, NSD was photooxidized. The rate constants for the interaction NSD–$O_2(^1\Delta_g)$ are particularly low, in the order of 10^6/M/s, although the whole interaction is attributable to a pure reactive process. The $O_2(^1\Delta_g)$ quenching was faster in alkaline medium, favored by the ionization of the NSD phenolic group.

Under Rf-photosensitization, NSD was degraded very much more rapidly than phenol, the latter being considered a paradigmatic water-contaminant model compound.

NSD may behave as an antioxidant in bio-environments, as demonstrated employing the photooxidizable amino acid tryptophan as a relevant biological target.

Discussion: The results indicate that a $O_2^{\bullet-}$-mediated process is the main route for the Rf-sensitized photooxidation of NSD. Photodegradation of the biocide in the presence and absence of phenol and tryptophan was quantitatively evaluated, discussed, and interpreted in terms of competitive quenching processes of $^3Rf^*$, $O_2(^1\Delta_g)$, and $O_2^{\bullet-}$ by the substrates.

Keywords: Niclosamide, Photodegradation, Photooxidation, Reactive oxygen species, Riboflavin

Introduction

Niclosamide (NSD) is a multifunctional drug normally found as natural surface waters due to its widespread use as a biocide.[1] NSD's chemical structure and the respective absorption spectra for the molecular and OH-ionized species are shown in Fig. 1. The drug is incorporated into wastewaters mainly through animal excretion and anthropogenic industrial activities. The toxicity of NSD to several non-target fish species and snails has been demonstrated.[2,3] Some years ago, the interaction of NSD with DNA was reported. The study suggested that NSD-toxicity can be caused by this interaction, after reductive activation.[4]

However, although some research works emphasize the toxicity of NSD, others attribute a very low level of noxious health effects on mammals.[5] In parallel, several beneficial biomedical properties have been assigned to the tenicide: a number of recent studies have established anticancer activities of the drug,

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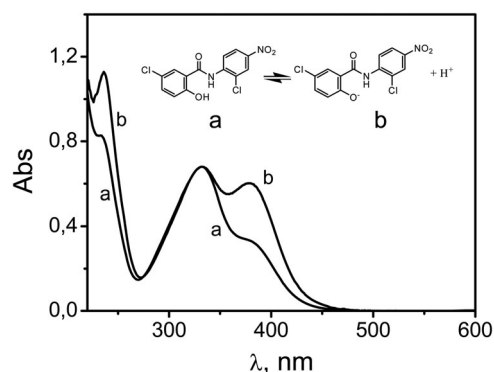
Figure 1 Acid-base equilibrium of NSD species: (a) NSD in MeOH, (b) NSD in MeOH plus 50 mM NaOH. The spectra were normalized at 330 nm.

both *in vitro* and *in vivo* models.[6] Furthermore, NSD possesses significant activity toward tuberculosis and as a chemotherapeutic drug against trypanosomiasis.[7]

The variety of properties mentioned makes NSD a particularly interesting target for stability studies. The results of these experiments would be useful not only in the depuration of natural waters contaminated with NSD, but also for the prevention of NSD degradation when used as a health-beneficial drug.

In 2003, Dawson[8] reviewed the processes of NSD's fate in environmental conditions. His work points out that NSD breaks down in natural waters through hydrolysis, microbial degradation, and direct photolysis. Mainly, it was established that degradation under laboratory conditions is highly dependent on microbial activity, suggesting that hydrolysis plays a limited role in the degradation of NSD.

A very recent study from Zaazaa *et al.*[9] goes forward in the thermal degradation of NSD through an interesting kinetic and mechanistic study in alkaline solution. A novel double divisor-ratio-spectra method allowed determination of NSD in the presence of its degradation products, the mutagenic metabolites 2-chloro-4-nitro aniline and 5-chloro salicylic acid.[10] Under established pseudo-first-order conditions, the degradation half-life of NSD at 80°C was 8.35 hours in 0.8 M NaOH methanolic solution.

The photochemical fading of NSD was initially observed by Strufe and Gönnert[11] in 1962, upon irradiation with ultraviolet light. Some relevant quantitative data collected in Dawsońs review are half-lives of *ca.* 42 hours and *ca.* 7 days for NSD in solution, exposed to direct sunlight and upon photoirradiation in the range 290–405 nm, respectively.[12]

During manufacturing, storage, or even after therapeutic administration, NSD might, in the presence of native photosensitizers, be able to generate potentially aggressive agents, such as reactive oxygen species (ROS). The same hypothesis is valid for the cases when the drug is a contaminant of surface waters. According to our knowledge, no studies have

specifically examined this possibility. In this context, the objective of the present work was to evaluate the stability of NSD under irradiation conditions similar to those frequently found in nature, in the presence of riboflavin (Rf, vitamin B_2), a common accompanying substance that could eventually act as a native photogenerator of ROS.[13–15] Rf was chosen as a photosensitizer in order to mimic natural scenarios. The vitamin constitutes one of the most important endogenous visible-light-absorbing photosensitizers in mammals. From the environmental point of view, Rf is present as traces in natural watercourses and water bodies.

Experimental
Materials
NSD (5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide), superoxide dismutase from bovine erythrocytes (SOD), and Rf were from Sigma. Rose Bengal (RB), furfuryl alcohol (FFA), monodeuterated methanol (CH_3OD), bromoxynil, and NaOH were from Aldrich (Milwaukee, WI, USA). Sodium azide (NaN_3) was from Merck. All these compounds were used as received.

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

Stationary photolysis in the visible range was carried out with a home-made photolyzer provided with two blue LEDs (467 ± 30 nm) or two green LEDs (510 ± 45 nm) employed for Rf-containing and RB-containing solutions, respectively.

Irradiation at 254 nm of matched absorbances of NSD and bromoxynil (the actinometer) in MeOH and MeOH plus 0.05 M NaOH was achieved with an Osram Germicide lamp (15 W) in a 1×1 cm fluorescence quartz cuvette provided with a magnetic stirrer.

Continuous photolysis
The reactive rate constant, k_r, for the chemical reaction of $O_2(^1\Delta_g)$ (see further process (11)) was determined by a known method,[16] using the expression $\text{slope}/\text{slope}_R = k_r\,[\text{NSD}]/k_{rR}\,[R]$. The rate constant for the photooxidative step of a reference compound, R, at similar concentration, must be known. Slope_R and slope are the respective slopes for the first-order plots of oxygen consumption by the reference compound and by the substrate, under sensitized irradiation. The reference R employed was FFA, with a reported k_{rR} value in MeOH of 2.1×10^7/M/s.[17] Oxygen consumption in MeOH was monitored with a 97-08 Orion electrode, employing RB as

sensitizer. It was assumed that the reaction of $O_2(^1\Delta_g)$ with NSD is the only path for oxygen uptake.

Laser flash photolysis experiments

Argon-saturated aqueous solutions of Rf 0.04 mM were irradiated with a flash photolysis (LFP) apparatus. The disappearance of ^{3}Rf*, generated by a 355 nm laser pulse, was monitored by the first-order decay of the solution absorbance at 670 nm. At this wavelength, interference from other species is negligible.

A ns Nd:YAG laser system (Spectron) at 355 nm was used for excitation, employing a 150-W Xenon lamp as source for the analyzing light. The detection system comprised a PTI (Photon Technology International) monochromator and a red-extended photomultiplier (Hamamatsu R666). Details of acquisition, monitoring, and handling of the decay ^{3}Rf* signal have been described elsewhere.[18]

NSD presents a relatively strong absorption at 355 nm ($\varepsilon_{355} \sim 9700/M/cm$), which is the excitation wavelength employed to generate triplet excited Rf. This condition enables the determination of the rate constant for the quenching of triplet excited Rf by NSD since a very low concentration of the quencher (Abs$_{355} \sim 0.01$ as an upper limit) was enough to obtain a good Stern–Volmer representation. Nevertheless, it was not possible to observe any transient species derived from triplet excited Rf. In this case, a relatively high NSD concentration is required to reach the quenching of a significant fraction of the excited species.

Time-resolved phosphorescence detection of $O_2(^1\Delta_g)$

The overall quenching rate constant for the deactivation of $O_2(^1\Delta_g)$ by NSD (k_t, the sum of k_q plus k_r, processes (10) and (11), respectively, see later) was determined using a previously reported system. MeOD, instead of MeOH, was used as solvent in order to enlarge the lifetime of $O_2(^1\Delta_g)$. A lifetime of 21 μs has been obtained for $O_2(^1\Delta_g)$ in MeOD in the absence and in the presence of NaOH 0.05 M.

Results and discussion

Photosensitized irradiation

The spectral changes produced upon photoirradiation of a methanolic solution of Rf 0.035 mM plus NSD 0.45 mM with light of $\lambda > 450$ nm are shown in Fig. 2. NSD is transparent at the irradiation wavelength. For comparative purposes, the absorption spectrum of the sensitizer alone also is shown in Fig. 2. The mentioned spectral changes, in both NSD and the sensitizer, could be attributed to photo-transformations. The absorbance change in the
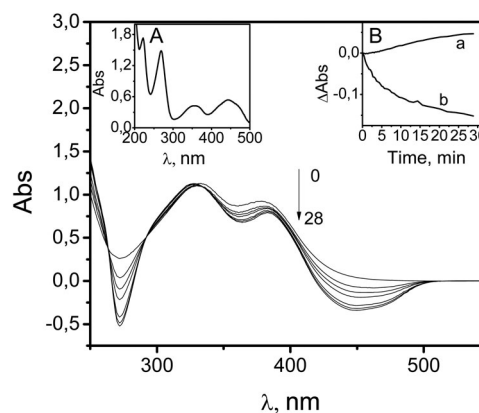

Figure 2   Changes in the UV-vis absorption spectrum of a methanolic solution of 0.05 mM Rf plus 0.03 mM NSD upon photoirradiation, taken vs. 0.05 mM Rf in the same solvent. Inset (A): absorption spectrum of 0.05 mM Rf in MeOH shown for comparative purposes. Inset (B): monitoring of the absorption changes at 378 nm upon photoirradiation of: Rf 0.05 mM in MeOH (a); the main figure (b).

photolyzed mixture at 378 nm, as a function of irradiation time, is shown in inset A of Fig. 2 (trace b). When only the sensitizer was photoirradiated, under identical experimental conditions, the representation of these absorbance changes shows a totally different time profile (trace a). This observation indicates that both components of the photolyzed mixture are being transformed.

Photoirradiation of the Rf-NSD methanolic mixtures, under the described experimental conditions, gave rise to oxygen uptake. A slight decrease in the rate of oxygen consumption (ROC) could be observed in the presence of 2 mM sodium azide, whereas in the presence of 1 μg/ml SOD, the ROC was greatly reduced. The salt and the protein are frequently employed to confirm/discard the respective participation of the ROS singlet molecular oxygen ($O_2(^1\Delta_g)$) and superoxide anion radical ($O_2^{\bullet-}$) in a given process.[19] Results are shown in the bar diagram of Fig. 3, where there is also evident a small ROC when the sensitizer alone is photolyzed.

It is known that NaN$_3$ deactivates $O_2(^1\Delta_g)$ with a rate constant value $k_t = 2.2 \times 10^8/M/s$ in MeOH (symbols of the rate constants are defined below). From a simple calculation through the Stern–Volmer equation, it arises that a decrease of *ca.* 85% must be expected for the lifetime of $O_2(^1\Delta_g)$ in a solution of the mixture NSD–NaN$_3$ as compared to the $O_2(^1\Delta_g)$ lifetime in the presence of NSD alone. On this basis, the slight decrease in the ROC observed in Fig. 3 due to the presence of 1 mM NaN$_3$ strongly suggests the existence of at least a second efficient pathway for oxygen consumption, generated upon Rf-photoirradiation and possibly represented by the reaction NSD–$O_2^{\bullet-}$.
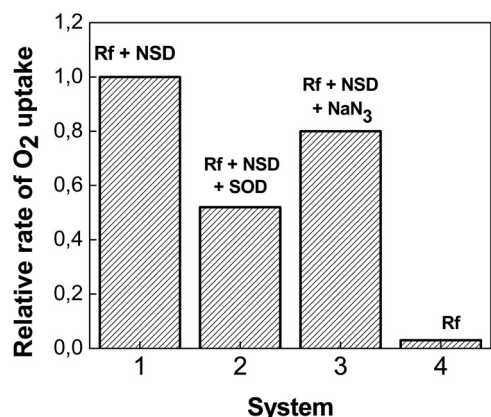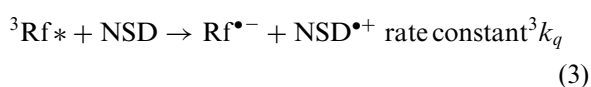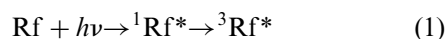
Figure 3 Bar diagram for the relative rates of oxygen uptake for 0.5 mM NSD + Riboflavin ($A_{445} = 0.4$) as a function of photoirradiation time in MeOH, in the absence (1) and in the presence of 1 μg/ml SOD (2); and 1 mM NaN$_3$ (3). Bar (4) corresponds to the relative rate for riboflavin ($A_{445} = 0.4$) in MeOH.

## The interaction of NSD with electronically excited states of Rf

Experimental results indicate a possible interaction of NSD with electronically excited states of Rf and/or with photogenerated ROS. The quenching of singlet excited Rf (^{1}Rf*) by NSD can be disregarded. Since a lifetime of *ca.* 5 ns has been reported for ^{1}Rf*, a NSD concentration in the sub-mM range, similar to those employed in the present work, is not enough to significantly intercept the excited singlet species of the vitamin.

It is known that, under visible-light irradiation, the anaerobic photodegradation of Rf predominantly proceeds through its electronically excited triplet state (reaction (1)).[20] The rate of the process can be estimated by monitoring the absorbance-decay of the absorption band centered at 445 nm. Comparative irradiations of Ar-saturated aqueous solutions of Rf in the presence and in the absence of 0.5 mM NSD indicated that this rate is increased in the presence of NSD by *ca.* 18% (data not shown). This finding strongly suggests a quenching process of the electronic excited triplet state of Rf (^{3}Rf*).

In aerated solutions, reactions (4) and (5) may occur, leading to generation of O$_2^{\bullet-}$ (reaction 6).

$$\mathrm{Rf} + h\nu \rightarrow {}^1\mathrm{Rf}^* \rightarrow {}^3\mathrm{Rf}^* \qquad (1)$$

$$^3\mathrm{Rf}* \rightarrow P(2) \qquad (2)$$

$$^3\mathrm{Rf}* + \mathrm{NSD} \rightarrow \mathrm{Rf}^{\bullet-} + \mathrm{NSD}^{\bullet+} \text{ rate constant} \, {}^3k_q \qquad (3)$$

$$\mathrm{Rf}^{\bullet-} + \mathrm{H}^+ \rightarrow \mathrm{RfH}^{\bullet} \qquad (4)$$

$$2\mathrm{RfH}^{\bullet} \rightarrow \mathrm{Rf} + \mathrm{RfH}_2 \qquad (5)$$

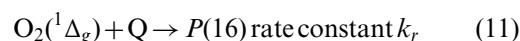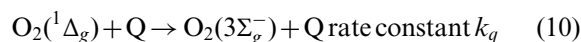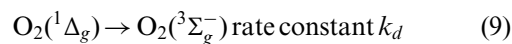$$\mathrm{RfH}_2 + \mathrm{O}_2(^3\Sigma_g^-) \rightarrow \mathrm{RfH}_2^{\bullet+} + \mathrm{O}_2^{\bullet-} \qquad (6)$$

Subsequently, the species O$_2^{\bullet-}$ may react with NSD (step (7)) as suggested by both the SOD-dependent oxygen consumption shown in Fig. 3 and the spectral changes observed in Fig. 2.

$$\mathrm{O}_2^{\bullet-} + \mathrm{NSD} \rightarrow P(7) \text{ rate constant } k_9 \qquad (7)$$
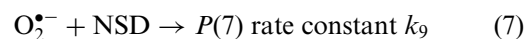
The energy transfer step (8) is known to proceed in aerated solutions, with a reported quantum yield of 0.47 in MeOH. This reaction could be competitive with the reaction pathway (3),

$$^3\mathrm{Rf}^* + \mathrm{O}_2(3\Sigma_g^-) \rightarrow \mathrm{Rf} + \mathrm{O}_2(^1\Delta_g) \text{ rate constant } k_{\mathrm{ET}} \qquad (8)$$
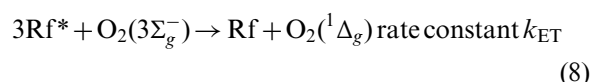
The oxidative species can decay *via* the solvent-dependent process (9) or interact with NSD through physical and/or the reactive processes depicted by steps (10) and (11), respectively.

$$\mathrm{O}_2(^1\Delta_g) \rightarrow \mathrm{O}_2(^3\Sigma_g^-) \text{ rate constant } k_d \qquad (9)$$

$$\mathrm{O}_2(^1\Delta_g) + \mathrm{Q} \rightarrow \mathrm{O}_2(3\Sigma_g^-) + \mathrm{Q} \text{ rate constant } k_q \qquad (10)$$

$$\mathrm{O}_2(^1\Delta_g) + \mathrm{Q} \rightarrow P(16) \text{ rate constant } k_r \qquad (11)$$

The presence of NSD in the sub-mM concentration range produces a decrease in the ^{3}Rf* lifetime (process (3)). A rate constant value $^3k_q = 9.3 \times 10^9$/M/s was obtained, by LFP, for the quenching of ^{3}Rf* by NSD through the Stern–Volmer treatment shown in Fig. 4. It has been reported by other authors and by ourselves that ^{3}Rf* is quenched by substituted phenols through an electron transfer process, represented by reaction (3), with rate constant values ^3k_q in the order of 10^9/M/s.[21,22] In these cases, a positive identification of the protonated species RfH$^{\bullet}$ (reaction (4)) was made through LFP experiments, strongly suggesting the occurrence of reaction (3). This might be the case in the quenching of ^{3}Rf* by NSD. Nevertheless, we were not able to
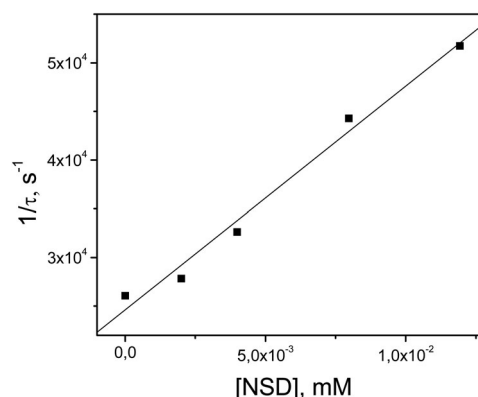

Figure 4 Stern–Volmer plot for the quenching of ^{3}Rf* by NSD in MeOH. The ordinate axis represents the inverse of ^{3}Rf* lifetime in the presence of different NSD concentrations.

confirm the participation of the radical species involved in step (3) due to experimental limitations, as stated above.

The interaction of NSD with $O_2(^1\Delta_g)$

The rate constants for the quenching of $O_2(^1\Delta_g)$ by NSD were evaluated. In order to avoid possible inter-actions of the substrate with $^3Rf^*$ or with ROS aside from $O_2(^1\Delta_g)$ photogenerated upon Rf photoexcita-tion, instead of the vitamin the well known $O_2(^1\Delta_g)$-sensitizer RB was employed. The xanthene dye is one of the most used photosensitizers in $O_2(^1\Delta_g)$ reactions. It produces the oxidative species, in MeOH, with a quantum yield of 0.81.[23] Visible-light irradiation of RB ($Abs_{549} = 0.5$) plus 0.025 mM NSD in methanolic solution produces absorbance changes in the wavelength range of NSD absorption (Fig. 5, inset). The presence of NSD in the sub-mM concentration range quenches the $O_2(^1\Delta_g)$ phosphorescence emission, as detected by TRPD experiments. This experiment unambiguously demonstrates the existence of the interaction NSD–$O_2(^1\Delta_g)$, which may be physical in nature (process (10)) and/or chemical (reactive, process (11)). It has been reported often that the inter-action $O_2(^1\Delta_g)$ with different phenols occurs through processes highly dependent on the ionization degree of the phenolic OH groups.[24] Hence, the rate constant k_t, accounting for the overall $O_2(^1\Delta_g)$-deactivation by NSD, was determined in pure MeOD and in MeOD/0.05 M NaOH.

The rate constant k_r was determined monitoring oxygen consumption upon photoirradiation of RB-NSD mixtures, by means of an already described actinometric method. Results indicate $k_t = 5.1 \pm$

$0.8 \times 10^5/M/s$ in MeOD; $k_r = 5.0 \pm 0.4 \times 10^5/M/s$ in MeOH; $k_t = 8.7 \pm 0.1 \times 10^6/M/s$ in MeOD plus 0.05 M NaOH and $k_r = 8.6 \pm 0.1 \times 10^6/M/s$ in MeOH plus 0.05 M NaOH. Experimental data for the determination of k_t and k_r values are shown in Fig. 5.

It is known that k_r/k_t values represent a measure for the effectiveness of the degradation pathway in a $O_2(^1\Delta_g)$-mediated process. At both pH values tested in these experiments, and within the estimated error, the calculated $k_r/k_t \sim 1$ indicates a high efficiency in the $O_2(^1\Delta_g)$-mediated degradation by the tenicide, in spite of the low values obtained for the absolute rate constants.

To our knowledge, the only report on NSD oxi-dation was published in 2003 by Alemu *et al.*[25] The oxidation proceeds in a similar way to a series of Schiff bases, which present structures analogous to NSD structure. The substrate initially gives a radical cation, which is deprotonated and further oxidized to form a di-radical cation that, after losing another proton, gives a cyclic product.

Kinetic-mechanistic evaluation of NSD photooxidation

The feasibility of the electron transfer process between NSD and Rf (reaction (3)) can be evaluated in thermodynamic terms through the Gibbs free energy for electron transfer, $\Delta_{ET}G_0 = E_{0(NSD/NSD+)} - E_{0(Rf/Rf-)} - E_{Rf^*} + C$. A value of 0.75 V was reported for $E_{0(NSD/NSD+)}$, the electrode potential of the donor, in aqueous solution. $E_{0(Rf/Rf-)}$ is the standard electrode potential of Rf, the acceptor (-0.80 V), E_{Rf^*} is the $^3Rf^*$ energy (2.17 eV), and C is the coulombic energy term (-0.06 V).[26] The obtained $\Delta_{ET}G_0$ value of -0.68 V constitutes evi-dence for the thermodynamic feasibility of process (7). As a consequence, the species $O_2^{\bullet-}$ could be formed through processes (4)–(6), provided that process (7) is kinetically competitive with $O_2(^1\Delta_g)$ generation, step (5). Since $^3k_q = 9.3 \times 10^9/M/s$ for the NSD and $k_{ET} = 1.2 \times 10^9/Me$ in MeOH (i.e., 1/9 of the diffusional value),[27] for the same concen-trations of NSD and dissolved $O_2(^3\Sigma_g^-)$ it can be inferred that the rate value for the generation of both $RfH^{\bullet} -$ the initial $O_2^{\bullet-}$ precursor $-$ is *ca.* eight times faster than the corresponding one for $O_2(^1\Delta_g)$, and will be the prevailing oxidation pathway. In addition to the relatively low k_r value exhibited by NSD and the limited inhibitory effect of NaN_3 on the ROC (Fig. 3), this finding implies a less impor-tant role for the $O_2(^1\Delta_g)$-oxidative pathway as com-pared to the $O_2^{\bullet-}$-mediated oxidation, under Rf photosensitization.
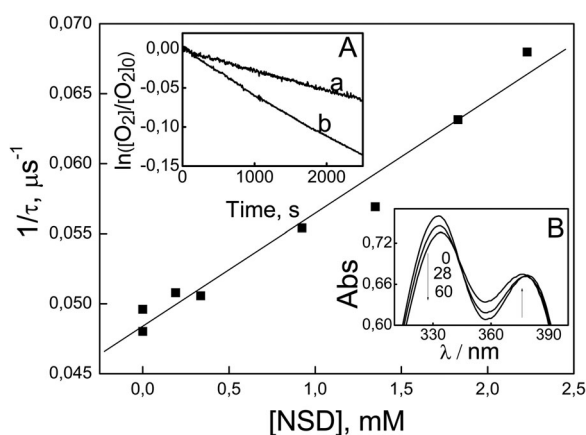

Figure 5   Stern–Volmer plot for the quenching of $O_2(^1\Delta_g)$ by NSD in MeOD plus 50 mM NaOH. Inset (A): first-order plot for oxygen uptake upon visible-light irradiation by solutions containing RB ($A_{549} = 0.32$) plus: 0.5 mM FFAc (a) and 0.5 mM NSD (b), in MeOH plus 50 mM NaOH. Inset (B): spectral evolution of 0.04 mM NSD plus RB $A_{557} = 0.54$ vs. RB $A_{557} = 0.54$ upon photoirradiation (cutoff 400 nm) in MeOH plus 50 mM NaOH.

Comparative experiments on the photooxidation of NSD in the presence of phenol and tryptophan

In parallel experiments and for comparative purposes, the ROC by a photoirradiated solution of 0.035 mM Rf plus 0.45 mM phenol (PHE) or plus 0.45 mM of tryptophan (Trp) were evaluated. The latter was performed in the absence and in the presence of 0.45 mM NSD. The ROC by the different mixtures can be taken as a measure of their respective photodegradabilities under established experimental conditions.

PHE is considered as a paradigmatic pollutant model in the context of surface waters contamination, and the amino acid (AA) participate in photooxidative damages of proteins through photodynamic oxidation.[28] In both cases, Rf plays the role of a naturally occurring ROS-photogenerator. Results are shown in Fig. 6.

The rate of oxygen uptake by the system Rf + NSD is practically three-fold higher than that observed for the mixture Rf + PHE (Fig. 6). This result suggests a higher photodegradability for NSD in a hypothetical contaminated medium. It has been previously established that under Rf-photosensitization the molecular form of PHE is degraded mainly through a $O_2^{\bullet-}$-mediated mechanism. The reported $^3k_q = 4.8 \times 10^8/M/s$ for reaction (3) in MeOH,[29] with PHE instead of NSD, indicates that the rate for the initial step for $O_2^{\bullet-}$ generation is *ca.* 20 times slower than the corresponding one for NSD. It is also known that in methanolic solution PHE quenches $O_2(^1\Delta_g)$ by an exclusive physical interaction with an extremely poor rate constant value $k_t = 2.5 \times 10^4/M/s$.[30] In synthesis, all the experimental data related to potential interaction of PHE with Rf-photogenerated ROS justify the observed lower reactivity of PHE as compared to the tenicide reactivity.

The observed ROC for Trp is a little higher than the respective one exhibited by NSD under identical conditions (Fig. 6). Besides, the photooxidation rate of the AA plus NSD is very much lower than the simple addition of the respective ROCs by the individual substrates in the presence of Rf. This can be due to a differential contribution of the individual mixture components to the whole oxidative process. The overall mechanism may be affected by the interaction of either the initial by-products generated upon photo-irradiation or by interactions of the substrates with the electronically excited states of the sensitizer that generate ROS.

The photodynamic action on aromatic AAs is well known.[31] Mainly Type II ($O_2(^1\Delta_g)$-mediated) and Type-I (radical-mediated) reactions are the reported mechanisms responsible for degradation of Trp under Rf-photosensitization.[32] A rate constant value $k_t = 6 \times 10^6/M/s$ in MeOH has been reported for the interaction $O_2(^1\Delta_g)$–Trp.[33] It is also known that Trp quenches ^{3}Rf* (reaction (3) with Trp instead of NSD) with ^3k_q values in the range of the diffusion limit.[34] For Trp, the primary products are the reduced flavin radicals (RfH$^\bullet$) and oxidized radicals of the AA. The overall reaction observed was conversion of dissolved $O_2(^3\Sigma_g^-)$ to $O_2^{\bullet-}$. On this basis, the apparently complex photoprocesses in the system NSD + Trp + Rf could be simplified by considering a scheme that includes quenching of ^{3}Rf* by Trp and NSD, production of $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$ and interaction of the generated ROS with the oxidizable substrates. The ROC from NSD remains as a low fraction of the overall contribution by the mixture, but the rate for isolated Trp and for the mixture are not significantly different. This effect could be attributed to the quenching of ^{3}Rf* by NSD and Trp. The process would start a Type I mechanism. As Type II is the dominant route in the Rf-sensitized photooxidation of Trp,[35] the quenching of the excited triplet flavin by NSD would decrease the stationary concentration of $O_2(^1\Delta_g)$ with the concomitant reduction of the overall ROC by the mixture. In a biological medium, the role exerted by NSD can be interpreted as a sort of photoprotection against vitamin B$_2$-mediated oxidation of a Trp-containing proteinaceous environment.

Direct photolysis of NSD

To our knowledge, no quantitative information on the evaluation of direct UV photolysis of NSD has been reported. Hence, considering the direct photoirradiation as a possible alternative for NSD degradation, the quantum yield for this process, upon 254 nm-irradiation ($phi_{r(254)}$), was determined in MeOH and MeOH plus 0.05 M NaOH. The phenolic derivative bromoxynil was used as an actinometer, for which a
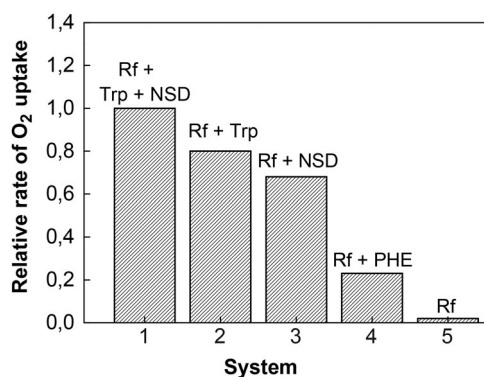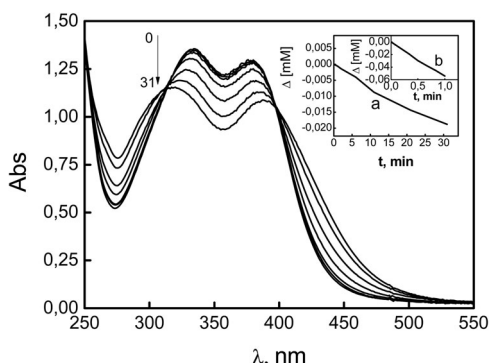

Figure 6 Bar diagram for the relative rates of oxygen uptake as a function of photoirradiation time (cut-off 400 nm) for 0.035 mM Riboflavin + 0.45 mM NSD + 0.45 mM Trp (1); 0.035 mM Riboflavin + 0.45 mM Trp (2); 0.035 mM Riboflavin + 0.45 mM NSD (3); 0.035 mM Riboflavin + 0.45 mM PHE (4). Bar (5) corresponds to the relative rate for 0.035 mM Riboflavin in MeOH.

Figure 7   Evolution of the absorption spectrum of 0.08 mM NSD in MeOH plus 50 mM NaOH upon photolysis at 254 nm. Numbers on the spectra represent irradiation time, in minutes. Inset (a): molar consumption as a function of irradiation time for 0.08 mM NSD in MeOH plus 50 mM NaOH monitored at 380 nm. Inset (b): molar consumption as a function of irradiation time for 0.16 mM BXN in H$_2$O (the actinometer) monitored at 285 nm.

$\phi_{r(254)} = 0.18$ has been reported, in neutral water. The $\phi_{r(254)}$ values of 0.0010 ± 0.0004 and 0.0013 ± 0.0004 were obtained for NSD in MeOH in the absence and in the presence of 0.05 M NaOH, respectively. Typical results are shown in Fig. 7.

This kind of energetically expensive treatment, with an input of *ca.* 470 kJ/Einstein, could be of interest in case of deposits of residues of the tenicide for which a rapid degradation is required. Nevertheless, results indicate a low photodegradation efficiency for NSD, independent of the different ionization degree of the phenolic OH group.

Final remarks
NSD is photooxidized in methanolic solution by visible-light irradiation in the presence of Rf as sensitizer. Experimental evidence strongly suggests the participation of the ROS $O_2^{\bullet -}$ and $O_2(^1\Delta_g)$. Alkaline medium highly favors the $O_2(^1\Delta_g)$-mediated oxidative pathway, due to the ionization of the phenolic group of NSD.

In comparative terms and under Rf photosensitization, NSD is degraded very much faster than phenol, the latter being considered a paradigmatic water-contaminant model.

In the context of a possible role of NSD in biological environments, mutual interactions between NSD, Trp, and Rf-electronically excited states, under Rf-photosensitized irradiation, induce photoprotection of the AA against photodynamic degradation.

Highly energetic direct photoirradiation produces NSD degradation with a quantum yield of 0.001.

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