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Studies of the photooxidant properties of antibacterial fluoroquinolones and their naphthalene derivatives

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We synthesized and determined the production of reactive oxygen species (ROS) as $^1O_2$, $\cdot^-O_2$, $\cdot OH$, H_2O_2 during the photolysis with UV-A light of three antibacterial quinolones and their naphthyl ester derivatives. Singlet oxygen and ROS dose-dependant generation from norfloxacin (**1**), enoxacin (**2**), ciprofloxacin (**3**) and their respective naphthyl ester derivatives **4**–**6** were detecting in cell-free systems by the histidine assay and by luminol-enhanced chemiluminescence (LCL). Both the electronic absorption and emission spectra were quantified and their photostability determined. The antibacterial activity in darkness and under irradiation of compounds **4**, **5** and **6** was tested on *E. coli* and compared with their parent drugs.

1. Introduction

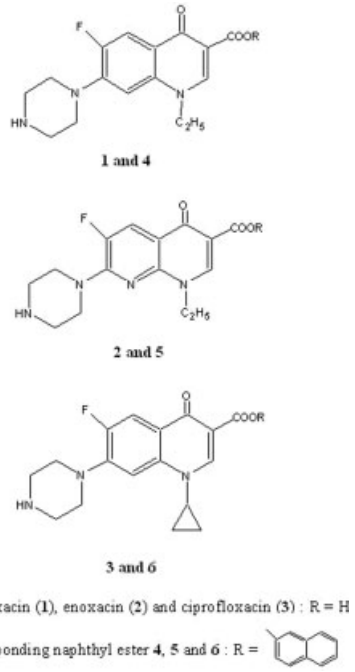
This work on ROS photogeneration by antibacterial drugs and new alternatives for photoinactivation of micro-organisms, takes as a model the family of compounds known as quinolones, which exert their pharmacological activity interacting with the bacterial gyrase affecting the bacterial replication mechanism. Fluoroquinolones are important synthetic antimicrobial agents and they have been widely used as therapeutic agents for general bacterial infectious diseases. Also, they can induce phototoxicity as a significant side effect. This property has been conclusively demonstrated in the last 14 years through investigations based on their photodegradation mechanisms and *in vitro* tests (Cárdenas et al. 1991; Vargas et al. 1994; Vargas and Rivas 1997; Vargas et al. 2003a; Fasani et al. 1998, 1999, Ferguson et al. 1988; Klecak et al. 1997). A large number of studies have been reported to elucidate the mechanism of phototoxicity of fluoroquinolones. It has been reported that carbon centered radical ($^{\cdot}C$), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$) are generated during photodegradation of fluoroquinolones in neutral aqueous solutions. The substituted fluoroquinolones at the 8-position by fluorine, generates a high degree of $^1O_2$ and $^{\cdot}OH$ under photoirradiation. Such reactive species were associated with DNA damage. Some fluoroquinolones are photo-labile compounds and the phototoxicity might arise from its photodegradation products (Przybilla et al. 1990; Ferguson 1995; Sánchez et al. 2005).

The studies on the pharmaceuticals compounds **1**–**3** gave positive results regarding photochemical mechanisms of decomposition, intermediates, photoproducts, especially those formed in the presence of oxygen, in a way that has been as to proposed a scale of relative phototoxic activity based on photohemolysis and reactions with human serum albumin (HSA) monitored by means of fluorescence and other spectroscopic techniques (Kawabe et al. 1989; González-Jimenez and García-Cantalejo 2002). There are several reports about light-related adverse effects of fluoroquinolones. Besides phototoxicity these effects are photoallergy and in some cases photomutagenicity and photocarcinogenesis as well. The mechanism underlying the phototoxic effect has not been clarified so far, although it is known that some structural factors affect phototoxicity (e.g. it is increased by a halogen atom in position 8) leading to serious limitatious of their use. On the other hand, those phototoxic undesirable effects, suggest that those same effects can be used for the inactivation of pathogen agents.

Photodynamic antimicrobial chemotherapy is the delivery of a non-toxic photosensitizer to the site of a microbial infection. When taken up by the pathogen, illumination of the photosensitizer by light at an appropriate wavelength can lead to inactivation of the pathogen through the production of highly reactive free radical species, which induce oxidative damage to lipid, proteins and DNA/RNA, and/or adduct formation between the photosensitizer and these microbial biomolecules (Wainwright 1998). Currently, the major use of photodynamic antimicrobial chemotherapy is in the disinfection of blood products, particularly for viral inactivation, although more clinically-based protocols are being developed, e.g. in the treatment of oral infection. The technique has been shown to be effective *in vitro* against bacteria (including drug-resistant strains), yeasts, viruses and parasites. Photodynamic antimicrobial chemotherapy is also proposed as a potential,

low-cost approach to the treatment of locally occurring infectious (Phoenix and Harris 2006).

We designed a study with these antibacterial drugs and with a synthesized ester derivative, trying to elucidate and to quantify the photooxidant properties or their ability of producing reactive oxygenated species ($^1O_2$, ·OH, H_2O_2) by means of histidine and chemiluminescence assay (Martínez et al. 1998; Marutani et al. 1993; Zoltan et al. 2007; Vargas et al. 2008). On the other hand, the synthesis of a quinolone homologous naphthyl ester derivative was carried out with the intention of increasing photostability and fluorescence quantum yield and in this way, giving the quinolones better properties of electron and/or energy transfer when they are subjected to irradiation. This would generate in these compounds, besides their antibacterials properties, a new behavior and make them suitable photosensitizes in bacterial culture media.



Structures of the investigated antibacterial quinolones and their naphthyl ester derivatives

2. Investigations and results

2.1. Primary photophysical properties of naphthyl ester derivates

The UV spectra of fluoroquinolones **1**, **2** and **3** of the selected working wavelength for detection and comparison showed extinction coefficient values ($\varepsilon_{\lambda max}$ in absorbance units $\times$ L mol^{-1} cm^{-1}) for norfloxacin (**1**) 16.4×10^3 at λ_{max} 271 nm, for enoxacin (**2**) 9.7×10^3 at λ_{max} 264 nm and for ciprofloxacin (**3**) 83.7×10^3 at λ_{max} 275 nm. These data were very similar to those reported in the literature (Drevensek et al. 2002; Samanidou et al. 2003; Suhagia et al. 2006). The absorption spectra of the naphthyl ester derivates showed two main bands (224 and 272 nm) with a molar extinction coefficient ($\lambda_{max} = 272$ nm) $\varepsilon_{max} = 15.475 \times 10^3 \pm 120$ for naphthyl ester norfloxacin (**4**), ($\lambda_{max} = 266$ nm) $\varepsilon_{max} = 38.992 \times 10^3 \pm 280$ for naphthyl ester enoxacin (**5**) and ($\lambda_{max} = 271$ nm) $\varepsilon_{max} = 12.499 \times 10^3 \pm 140$ for naphthyl ester ciprofloxacin (**6**).

Fluorescence emission was found to be pH dependent. The fluorescent quantum yields (Φ_F) of the compounds **1, 2** and **3** were 0.12, 0.014, 0.10 (Bilski et al. 1996; Sortino et al. 1998; Albini and Monti 2003). Frequently employed standard values for Φ_F in aqueous media are 0.31 for rhodamine B and 0.58 for quinine sulphate (Magde et al. 1999; Shimadzu Corporation 1995). The quantum yield of fluorescence at pH 7, the more important bands of the absorption and emission spectra of compounds **4**–**6** are summarized in the Table.

A study on the photostability of compounds **4**, **5** and **6** followed spectrophotometrically, showed that under irradiation with visible light and UV-A for 5 h, these compounds turned out to be very stable. While under UV-B irradiation and for the same period of time, their photodegradation was little significant. Comparatively these ester compounds turned out to be more photostable that the drugs **1**, **2** and **3**.

2.2. Generation and detection of reactive oxygen species

Triplet state is most often responsible for phototoxicity together with singlet oxygen and other oxygen transients they can initiate. Measuring singlet oxygen yield gives a good estimate of the yield of triplet formation without knowing their nature.

2.2.1. Histidine assay

Singlet oxygen quantum yield: The singlet oxygen quantum yield is a key property of a photosensitizing agent. This quantity is defined as the number of $^1O_2$ molecules generated for each photon absorbed by a photosensitizer. Quantum efficiency is an equivalent term. The production of $^1O_2$ by photosensitization involves four steps: (1) Absorption of light by the photosensitizer; (2) formation of the photosensitizer triplet state; the quantum yield of this process is the intersystem crossing efficiency or triplet yield; (3) trapping of the triplet state by molecular oxygen within its lifetime; (4) energy transfer from the triplet state to molecular oxygen; the experimental value of this parameter is usually unity for those agents in which the fluor-

Table: Positions (nm) of principal bands in the absorption (1.0×10^{-5} M) and fluorescence spectra (1.0×10^{-7} M) for different excitation wavelengths and the quantum yields of fluorescence for the compounds 4-6

Compound (Φ_F)	Absorption nm (Intensity)	Fluorescence nm (Intensity $\times 10^3$)	
4 (0.390)	224 (0.60) 272 (0.52) 320 (0.22)	$\lambda_{exc} = 330$	439 (247), 660 (638), 746 (16)
5 (0.131)	221 (0.58) 266 (1.29) 335 (0.74)	$\lambda_{exc} = 330$	376 (127), 660 (694), 747 (25)
6 (0.493)	223 (0.35) 271 (0.42) 323 (0.20)	$\lambda_{exc} = 330$	434 (143), 621 (834), 692 (24)

Aerobic conditions and phosphate buffer pH 7.2–7.4). Errors in quantum yields are typically within 8%

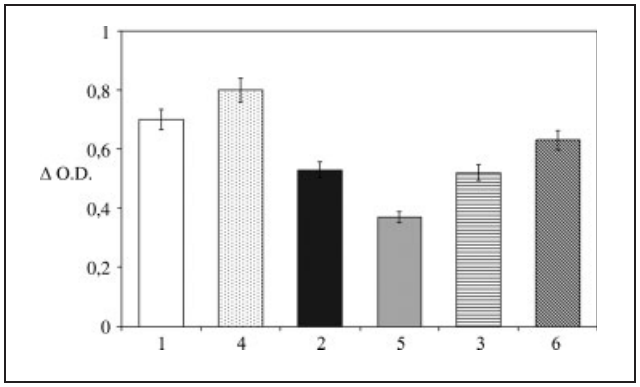


Fig. 1: Influence of the time of irradiation on the bleaching of *p*-nitrosodimethylaniline in the histidine-naphthyl ester derivate system at 440 nm. Δ O.D. represents the difference in optical density of irradiated sample of compounds **1**–**6**

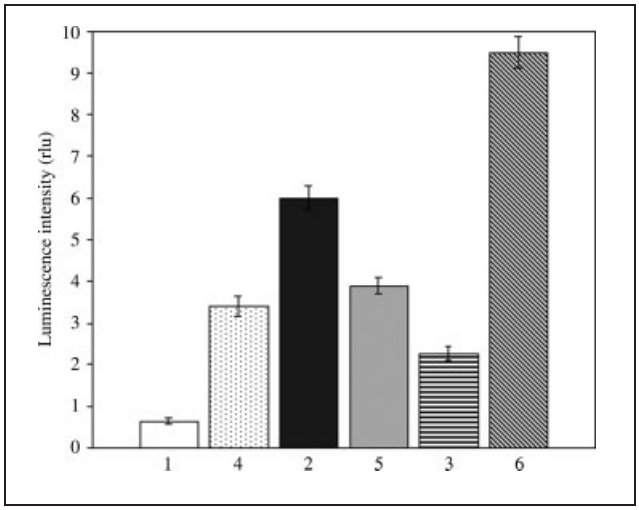


Fig. 2: Generation of chemiluminescence (luminol – NADPH) taken place after irradiation of norfloxacin (1), enoxacin (2), ciprofloxacin (3) and their naphthyl ester derivatives 4, 5 and 6. Data are the mean and SEM, $n = 4$, $p < 0.05$ vs. control; analysis of variance). The controls in the darkness didn't show some signal. Value of 10 relative unit of light = 2.44×10^{-2} ppm H_2O_2

escence is not quenched by oxygen. Virtually all measurements of singlet oxygen quantum yield are scaled to a reference substance. Frequently employed standard values of this in aqueous media are 0.79 for Rose Bengal and 0.52 for methylene blue. The published values of ΦΔ show considerable variations with the solvent, reaction conditions, and the measurement technique (Zoltan et al. 2007; Redmond and Gamlin 1999).

Compounds **1** to **3** are capable of producing singlet oxygen when they are irradiated with UV-A and visible light in the presence of molecular oxygen. This fact can be confirmed by trapping with histidine. We use a simple and sensitive spectrophotometric method for the detection of $^1O_2$ as produced by different sensitizing dyes in neutral air saturated aqueous solutions. The reaction between histidine and $^1O_2$ results in the formation of a trans-annular peroxide. The presence of the latter compound may be detected by bleaching the p-nitrosodimethylaniline at 440 nm. Singlet oxygen alone cannot cause the bleaching of the latter compound. No bleaching occurs in the mixture of histidine and p-nitrosodimethylaniline without singlet oxygen (Kraljic and El Mohsni 1978). In order to control the reaction, we observed no measurable loss of the p-nitrosodimethylaniline in the absence of histidine. We conclude that an oxidation of histidine (which is susceptible to singlet oxygen attack) is produced through photoexcitation of the compounds **4** to **6** acting as a singlet oxygen sensitizer (type II mechanism) (see Fig. 1).

For an estimate of the efficiency of singlet oxygen formation by compounds **4** to **6** photoexcitation, we compared them with the reported data for the same reaction using Rose Bengal. The quantum yield of singlet oxygen generation for Rose Bengal is $\phi(^1O_2) = 0.79$ (Redmond and Gamlin 1999; Gandin and van de Vorst Lion 1983). This value can be used as a standard to determine the quantum yield singlet oxygen for **4**: $\phi(^1O_2) = 0.092 \pm 0.001$, **5**: $\phi(^1O_2) = 0.042 \pm 0.001$ and **6**: $\phi(^1O_2) = 0.072 \pm 0.001$. For their parent drugs norfloxacin (**1**) $\phi(^1O_2) = 0.081 \pm 0.001$, enoxacin (**2**) $\phi(^1O_2) = 0.061 \pm 0.001$ and ciprofloxacin (**3**) $\phi(^1O_2) = 0.060 \pm 0.001$ under the same experimental conditions (Albini and Monti 2003).

The histidine model should be regarded simply as a test for oxygen dependent photosensitized damage to cellular protein. The compounds **4** to **6** at several concentrations were less efficient than the drugs **1**, 2 and **3** for photooxidation of histidine which is susceptible to singlet oxygen attack.

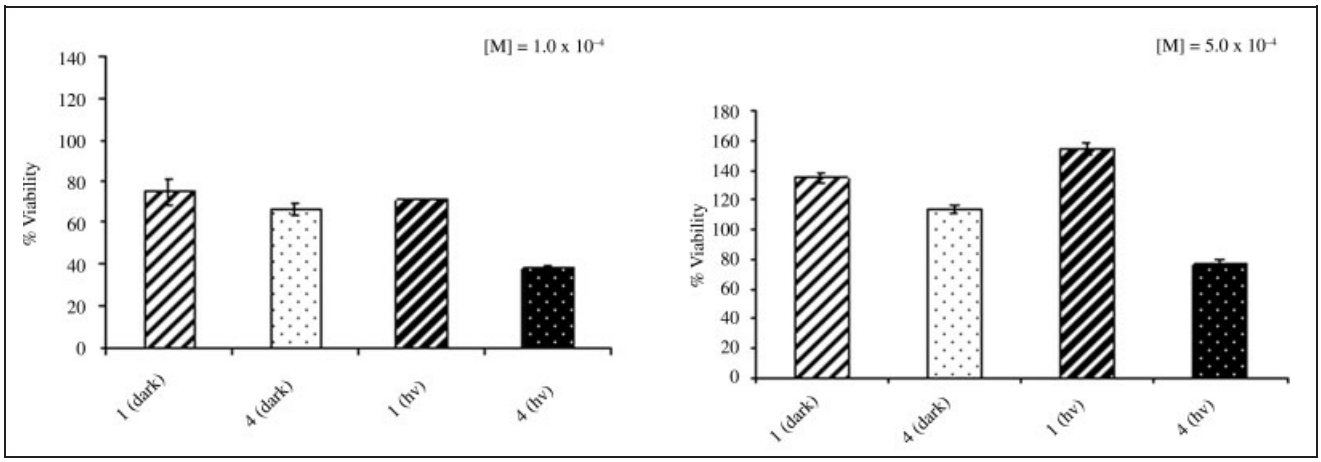


Fig. 3: Microbial cell (*E. coli*) viability assay in presence of compounds **1** and **4**, based on quantification of the ATP present measuring by luminescence after 30 min irradiation at two different concentrations

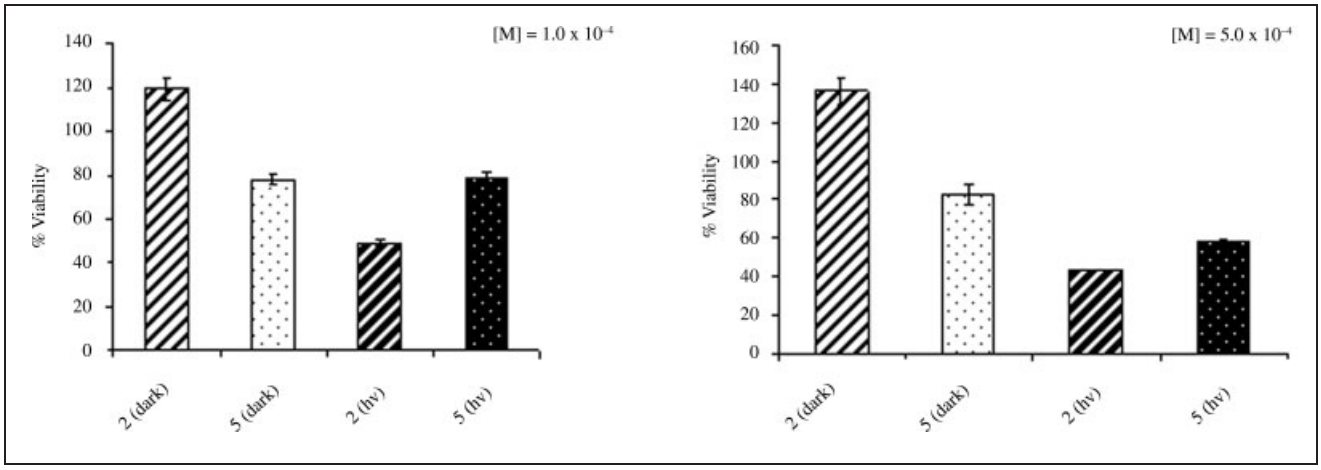


Fig. 4: Microbial cell (*E. coli*) viability assay in presence of compounds **2** and **5**, based on quantification of the ATP present measuring by luminescence after 30 min irradiation at two different concentrations

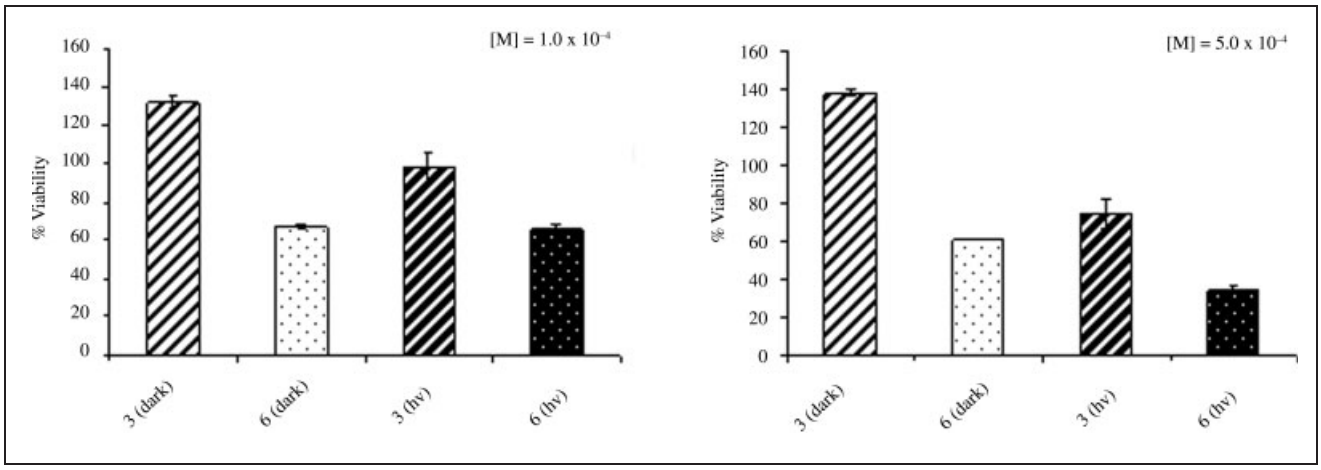


Fig. 5: Microbial cell (*E. coli*) viability assay in presence of compounds **3** and **6**, based on quantification of the ATP present measuring by luminescence after 30 min irradiation at two different concentrations

2.2.2. Chemiluminescence assay

The photochemical study of the compounds **1** to **6** opens new perspectives to establish their activity mechanisms and phototoxicity. From an absolute quantification of hydrogen peroxide generated after the irradiation of compounds **1** to **6** at similar concentrations of 1.5×10^{-4} M in the presence of NADH, we obtained the following results: compound **1** (norfloxacin) generated 0.0976×10^{-2} ppm of H_2O_2 (relative yields) (2%), compound **2** (enoxacin) generated 1.27×10^{-2} ppm (26%), compound **3** (ciprofloxacin) $= 0.44 \times 10^{-2}$ ppm (9.0%) and their ester derivatives **4** $= 0.73 \times 10^{-2}$ ppm (15%), **5** $= 0.85 \times 10^{-2}$ ppm (17.5%), and **6** $= 2.20 \times 10^{-2}$ ppm (45%) (Fig. 2). One value of 20 relative unit of light (rlu) corresponds to 4.88×10^{-2} ppm of H_2O_2.

2.3. Photoinduced antibacterial activity on Escherichia coli

Only at concentrations of 1.0×10^{-4} M an enhanced antibacterial activity against *Escherichia coli* was observed by irradiation (1 h) of the naphthyl esters of norfloxacin (**4**). This compound showed the highest generation of peroxidic species detected by means of chemiluminescence (Figs. 2–5). The compounds **5** and **6** did not show any photo-activity at this concentration but at 5.0×10^{-4} M all the compounds showed an enhanced antibacterial activity under irradiation.

3. Discussion

The degree to which a fluorquinolone is photolabile is modulated by further substituents (e.g. electron donating groups limit the electrophilicity and thus decrease the efficiency of the aromatic photosubstitution, or a halogen atom in position 8 makes the molecule quite photolabile), such drugs must possess positive characteristics sufficient to counterbalance this adverse effects. With the incorporation of the naphthyl ester group to these molecules we have been able to give them a high photostability.

The fluorescent quantum yields (Φ_F) of the compounds **4**, **5** and **6** were higher than those of **1**, **2** and **3**. We could also observe that the Φ_F values of these compounds under oxygen, were lower than those carried out under argon, presumably because of quencher effects of the oxygen on the fluorescent intensity of these compounds. Compounds **4** and **6** showed a relatively higher quantum yield of singlet oxygen $\phi(^1O_2) = 0.092$ and 0.072 respectively compared with their parent drugs **1** (0.081), and **3** (0.060). These naphthyl ester derivatives present an enhanced antibacterial activity on *E. coli* under irradiation, while otherwise compound **5** showed lower activity on *E. coli* under irradiation than compound **2**, with $\phi(^1O_2) = 0.042$ and 0.061, respectively. These results indicate that a mechanism of photosensitization type II promulgates the antibacterial activity by irradiation of these three compounds. It is also remarkable, that the generation of peroxide species

(Fig. 2), which is very marked for compound **6**, would also, in this case, contribute a lot to the photo-induced antibacterial properties, or in the same way, of a higher phototoxic character. Previously, the formation of H_2O_2 has been observed during the photolysis of some fluoroquinolones and is attributed to oxygen trapping of carbene-like intermediates generated by defluorination (Martinez et al. 1997). Those results suggest that H_2O_2 can be a common product in the photolysis of fluoroquinolones and provides an alternative mechanistic route of photodegradation. However, H_2O_2 is generated by the naphthyl ester derivatives via photosensitization without degradation of the irradiated compound. In this work we proof that a dioxygen related action operates on the observed photoinduced antibacterial effects, since the naphthyl ester derivatives **4**, **5** and **6** activate oxygen, forming both singlet oxygen and superoxide anion.

It is important to emphasize that in the lapse of irradiation time, some minimum inhibitory bacterial activity was not observed in the darkness. These facts are of major significance for the study of the photodynamic action and make these compounds promising candidates as photodynamic therapy agents. *In situ* production of singlet oxygen could be the principle mechanism for tumour destruction in application of photodynamic therapy employing these novel water soluble compounds. Their photobiological properties are deserving further investigation.

4. Experimental

4.1. Chemicals

Norfloxacin (**1**), enoxacin (**2**) and ciprofloxacin (**3**), horseradish peroxidase (HRP), rhodamine B, superoxide dismutase (SOD), LB Broth medium and nicotinamide adenine dinucleotide reduced (NADH) and Rose bengal were purchased from Sigma (St. Louis, MO, USA). Their purity was 99.2% as determined by ^{1}H NMR-spectroscopy (Bruker Aspect 3000, 300 MHz) and UV-Vis spectrometry (Lambda35 spectrophotometer, Perkin Elmer). The fluorescence spectra and the quantum yields were registered with a Shimadzu RF 1501 spectrofluorophotometer and PerkinElmer LS Luminescence spectrometer. 3-Aminophthalhydrazide (Luminol), isoluminol, and ferrous sulfate heptahydrate were purchased from Aldrich (Milwaukee, USA). All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

4.2. Synthesis of naphthyl ester quinolone derivatives

Esterification of norfloxacin (**1**), enoxacin (**2**) and ciprofloxacin (**3**) with β-naphthol was carried out by passing a flow of dry and gassy HCl through an equimolar dispersion of compound **1** and β-naphthol (3.0×10^{-3} mol) in CH_2Cl_2 at the reflux temperature during 1h. The solid filtrate washed with dichloromethane was dissolved in cold water and taken to pH 8.9–9.2 (accurately), where a solid precipitated in the cold overnight.

The corresponding naphthyl ester (**4**); 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinenaphthyl ester ($C_{26}H_{24}FN_3O_3$, mol wt 445.48), yield: 0.430 g of white needles (32 %), m.p. 236–237 °C. IR (KBr): 3419, 1626, 1489, 1385, 1269, 1030, 931, 825, 741 cm^{-1}.

1H NMR-(300 MHz, $CDCl_3$): δ = ppm 8.65 (s, 1H, H-13), 8.07 (d, 1H, $J_{9\text{-}F}$ = 9.20, H-9), 8.00 (m, 1H, H-23), 7.94 (m, 1H, H-22), 7.80 (m, 1H, H-30), 7.40–7.19 (m, 4H, H naphthyl-26, 27, 28, 29), 6.30 (d, 1H, $J_{16\text{-}F} = 6.20$, H-16), 4.31 (c, 2H, $J_{31-32} = 6.88$, H-31), 3.36–3.78 (m, 8H, H piperazin-6, 5, 3, 2), 2.29 (s, 1H, H-4), 1.57 (t, 3H, J_{32-31} = 6.88, H-32). ^{13}C NMR-(300 MHz, $CDCl_3$): δ = 176.94 (CO-11), 167.20 (CO-19), 159.40 (C-15), 155.20 (C-8), 149.20 (C-21), 147.77 (C-13), 146.38 (C-7), 137.09 (C-25), 137.00 (C-15), 131.00 (C-24), 127.50 (C-30), 127.52 (C-27), 125.50 (C-29), 125.00 (C-28), 120.50 (C-10), 120.48 (C-22), 118.00 (C-26), 112.94 (C-9), 108.37 (C-12), 105.02 (C-16), 99.75 (C-16), 51.06 (C-2, 6), 49.66 (C-31), 45.84 (C-3, 5), 14.39 (32).

$C_{26}H_{24}FN_3O_3$

The corresponding naphthyl ester (**5**); 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-[1,8]naphthyridine-3-naphthyl ester ($C_{25}H_{23}FN_4O_3$, mol wt 446.47), yield: 0.461 g of white needles (34 %), m.p. 239–240 °C. IR (KBr): 3439, 1624, 1485, 1378, 1293, 1183, 1147, 1029, 944, 733, 621, 542 cm^{-1}.

1H NMR-(300 MHz, $CDCl_3$): δ = ppm 8.35 (d, 1H, $J_{23-22} = 9.00$, H-23), 8.05 (s, 1H, H-11), 7.90 (d, 1H, $J_{22-23} = 9.00$, H-22), 7.80 (m, 1H, H-30), 7.61 (d, 1H, $J_{26-22} = 2.50$, H-26), 7.48 (m, 1H, H-28), 7.40 (d, 1H, $J_{15\text{-}F} = 9.60$, H-15), 7.38 (m, 1H, H-27), 7.12 (m, 1H, H-29), 4.60 (c, 2H, $J_{17-18} = 6.80$, H-17), 3.00–2.80 (m, 8H, H piperazin-6, 5, 2, 3), 2.20 (s, 1H, H-4), 1.38 (t, 3H, $J_{18-17} = 6.80$ H-18). ^{13}C NMR-(300 MHz, $CDCl_3$): δ = 176.00 (CO-13), 166.20 (CO-9), 160.00 (CO-19), 150.00 (C-21), 148.70 (C-7), 147.00 (C-11), 146.50 (C-16), 139.00 (C-25), 133.00 (C-23), 131.05 (C-24), 127.50 (C-30), 127.00 (C-27), 126.40 (C-29), 125.60 (C-28), 120.00 (C-22), 119.70 (C-15), 118.02 (C-26), 112.20 (C-14), 107.60 (C-12), 48.00 (C-2, 6), 47.10 (C-17), 45.88 (C-3, 5), 14.90 (C-18).

$C_{25}H_{23}FN_4O_3$

The corresponding naphthyl ester (**6**); 1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-naphthyl ester ($C_{27}H_{24}FN_3O_3$, mol wt 457.50), yield: 0.380 g of white needles (31 %), m.p. 280–282 °C. IR (KBr): 3439, 1624, 1485, 1378, 1183, 1147, 1029, 944, 733, 621, 542 cm^{-1}.

1H-NMR-(300 MHz, $CDCl_3$): δ = 8.77 (d, 1H, $J_{9\text{-}F}$ = 8.70, H-9), 8.35 (d, 1H, $J_{23-22} = 9.60$, H-23), 8.20 (s, 1H, H-13), 8.00 (d, 1H, $J_{22-23} = 9.60$, H-22), 7.80 (m, 1H, H-30), 7.60 (d, 1H, $J_{26-22} = 2.60$, H-26), 7.50 (m, 1H, H-28), 7.40 (m, 1H, H-27), 7.20 (m, 1H, H-29), 6.30 (s, 1H, H-16), 3.30–2.70 (m, 8H, H piperazin-2, 6, 3, 5), 2.50 (s, 1H, H-4), 2.38 (dt, 1H, $J_{31-32} = J_{31-33} = 5.00$, H-31), 2.00 (m 2H, H-32), 1.90 (m, 2H, H-33). ^{13}C NMR-(300 MHz, $CDCl_3$): δ = 175.50 (CO-11), 160.10 (C-19), 156.10 (C-8), 150.00 (C-21), 143.00 (C-7), 142.20 (C-13), 138.05 (C-25), 137.00 (C-15), 132.8 (C-23), 131.42 (C-24), 127.60 (C-30), 127.47 (C-27), 126.48 (C-29), 125.50 (C-28), 120.00 (C-22), 116.62 (C-26), 116.45 (C-10), 109.80 (C-12), 107.25 (C-9), 106.50 (C-16), 51.30 (C-2, 6), 45.00 (C-3, 5), 28.45 (C-31), 7.50 (C-32, 33). Analysis, $C_{27}H_{24}FN_3O_3$, calculated: %C 70.88, %H 5.29, %N 9.18; found: %C 71.04, %H 4.98, %N 9.09.

The mass spectra of compounds **4, 5** and **6** did not show a parent radical ion, instead only ions of a retro-ester process were detected. The base peak results from elimination of $^{\bullet}OC_{10}H_7$, as well as of $^{\bullet}COO\ C_{10}H_7$ respectively.

4.3. Photolysis

Solutions of compounds **1**–**6** were irradiated at room temperature for 2 h in H_2O (0.120 g, 0.335 mmol in 100 ml) with an illuminator Cole Palmer 41720-series keeping a distance of 10 cm between the lamp surface and the solution, with a emission maximum in UV-A 320–400 nm (3.3 mW/cm^2, 45.575 Lux/seg) as measured with a model of UVX Digital Radiometer at 25 °C under oxygen atmosphere, as also with a Rayonet RPR 200 with 16 UV-lamps RPR-3500 (spectral density 300–400 nm). The course of the reaction was followed by UV-Vis spectrophotometry using a Milton-Roy 3000 instrument and also a Lambda650 spectrophotometer Perkin Elmer. The photochemical experiments were also followed by GC-Mass spectroscopy (Carlo Erba/Kratos MS25RFA) and HPLC (Water Delta Prep 4000 equipped with a 3.9 × 300 mm, 10 μm Bondapak C18 column using a CH_3CN/H_2O binary solvent system).

4.4. Quantum yields

Quantum yields of fluorescence were determined for compounds **4**, **5** and **6**. The relative quantum yields of fluorescence at room temperature were determined either by comparing the corrected fluorescence intensity of compounds **1**–**3** in H_2O with that of rhodamine B (at a concentration of 1.0×10^{-6} M fluorescence quantum yield, 0.31) or with that of quinine bisulfate in 0.05 M H_2SO_4 (fluorescence quantum yield, 0.55) (Magde et al. 1999; Calvert and Pitts 1966; Shimadzu Corporation 1995).

In the product quantum yields determination the photolysis was allowed to proceed to less than 10% product formation to minimize light absorption by the photoproducts and additional products from side reactions. The photon flux incident on 3 ml of solution in quartz cuvettes of 1 cm optical path was measured by means of a ferric oxalate actinometer and it was of the order of 10^{15}–10^{16} quanta s^{-1}.

4.5. Singlet oxygen ($^1O_2$) generated in cell-free systems; histidine test

Indirectly, singlet oxygen was detected by photosensitized degradation of L-histidine (Lovell 1990). The latter was measured in the presence of 0.25, 0.50, 1.0 and 1.5×10^{-5} M of compounds 4, 5 and 6 and compared with their parent drugs 1, 2 and 3. These solutions were mixed with an equal quantity in volume of l-histidine solution in concentrations ranging from 0.60 to 0.74 mM in phosphate buffer 0.01, pH 7.4. Samples of these mixtures were irradiated with an illuminator Cole Palmer 41720-series keeping a distance of 10 cm between the lamp surface and the solution at 25 °C, with a emission maximum in UV-A 320–400 nm (3.3 mW/cm^2, 45.575 Lux/seg) at time intervals from 60 to 180 min. The respective controls were protected from light. The histidine degradation was determined by a colorimetric by bleaching of *p*-nitrosodimethylaniline (Kraljic and El Mohsni 1978). The quantum yield of singlet oxygen generation for Rose Bengal is $\phi(^1O_2) = 0.76$ (Redmond and Gamlin 1999). This value can be used as a standard to determine a relative quantum yield of the new compounds (Zoltan et al. 2007).

4.6. Chemiluminescence (CL) generated in cell-free systems; H_2O_2-induced CL

Chemiluminescence (CL) was generated in cell-free systems; H_2O_2-induced CL (as a blank): H_2O_2 (3.5 mM) was added to phosphate buffered saline solution (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.2) and luminol (250 μM, prepared daily in 2 M NaOH and diluted with PBS). The quinolone-induced CL at different concentrations was dispensed after irradiation using an illuminator Cole Palmer 41720-series keeping a distance of 10 cm between the lamp surface and the solution, varying the time periods of exposure at 25 °C, with a emission maximum in UV-A 320–400 nm (3.3 mW/cm^2, 45.575 Lux/seg) in presence of NADH. The generated CL at 25 °C was measured continuously for 5 min in a Luminoskan Ascent luminometer (ThermoLabsystems, Finland) in a 96-well Thermo Labsystems Microtiter plate (Lundqvist and Dahlgren 1996; Vargas 2003b; Yildiz et al. 1998). The emitted light was recorded as luminescent units at 20s intervals during 20min. All results were expressed as percentages of the control (areas under the luminescent units (rlu), relative light units vs. time curves).

4.7. Antibacterial activity

The antibacterial assay was carried out using *Escherichia coli* (ATCC 8739) and their proliferation and viability were obtained by means of chemiluminescence using BacTiter-Glo Microbial Cell (Promega, USA). The compounds used **1**–**6** were prepared in H_2O/ethanol (90 : 10) at a concentration of 1.0×10^{-4} M. We took into account that different bacteria have different amounts of ATP per cell, and values reported for the ATP level in cells vary considerably. Factors that affect the ATP content of cells such as growth phase, culture medium, and presence of metabolic inhibitors, can influence the relationship between cell concentration and luminescence (Stanley 1986). The antibacterial photoactivity was carried out under irradiation with visible light during 1h with an illuminator Cole Palmer 41720-series as in Section 4.3.

The BacTiter-Glo Microbial Cell Viability Assay is a homogeneous method to determine the number of viable bacterial cells in culture based on quantification of ATP present. ATP is an indicator of metabolically active cells. The homogeneous assay procedure involves the addition of a single reagent (BacTiter-Glo® Reagent) directly on bacterial cells in LB Broth medium and the measurement of luminescence. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of living cells in culture.

The recorded luminescence signals (Luminescence (R.L.U.) (relative light units) represent the mean of three replicates for each measurement. The signal-to-noise ratio was calculated: S : N = [mean of signal mean of background]/standard deviation of background.

A direct relationship (linear correlation) exists between luminescence measured with the BacTiter-Glo® Microbial Cell Viability Assay and the number of cells in culture over five orders of magnitude. Values represent the mean ± S.D. of 4 replicates for each cell number. The luminescent signal from 50 *Escherichia coli* cells is greater than three standard deviations above the background signal resulting from serum supplemented medium without cells. There is a linear relationship ($r^2 = 0.99$) between the luminescent signal and the number of cells from 0 to 50,000 cells per well.

4.8. Statistical treatment of results

At least three independent experiments were performed except where indicated otherwise. The quantification of results is expressed as a mean ± SD standard deviation (SD) is obtained from 3–4 observations. The level of significance accepted was $p \leq 0.05$.

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