Photoreaction and Active Oxygen Generation by Photosensitization of a New Antibacterial Fluoroquinolone Derivative, Orbifloxacin, in the Presence of Chloride Ion

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The photoreaction of orbifloxacin (ORFX), a fluoroquinolone derivative having a fluorine at the 8 position, was investigated in aqueous solution containing chloride ion to determine its photochemical properties. In the presence of chloride ion, ORFX was found to convert into a photoproduct substituted by chlorine at the 8 position (8-Cl ORFX) under irradiation by ultraviolet-A (UVA) light. Although the formation rate of 8-Cl ORFX increased greatly with increase in chloride ion concentration, the apparent photodegradation rate of ORFX in aqueous solution was not varied by the presence of the ion. This implies that the dissociation of the C–F bond at the 8 position is the rate-limiting step in the photodegradation of ORFX. The active oxygen generated by the photosensitization of ORFX were determined by measuring the bleaching rate of *p*-nitrosodimethylaniline and the reduction rate of cytochrome c. Their generation was inhibited by sodium azide and superoxide dismutase but not by D-mannitol. The photoreaction between ORFX and chloride ion also inhibited their generation. The mechanism is believed to be the photochlorination at the 8 position competing with the reaction between free radical and oxygen.

Key words active oxygen; chloride ion; fluoroquinolone; photochlorination; photodegradation; orbifloxacin

Several quinolone antibacterials are easily photodecomposed[1–4] and generate active oxygen species in the presence of light and oxygen.[5–8] Active oxygen species induced by quinolones have been postulated to be responsible for the observed phototoxic reaction.[9,10]

Especially, the fluoroquinolones having a fluorine at the 8 position (8-F quinolone) have unique characteristic photochemical properties compared with other quinolones. Several reports have described the generation of active oxygen from 8-F quinolones under irradiation with ultraviolet-A (UVA) light.[5,6,11] These reports show that the generation rate of active oxygen induced by the photosensitization of 8-F quinolones is much larger than other quinolones. The generating mechanism of the active oxygen from 8-F quinolones through the photodegradation process has not yet been identified, however.

Previous reports[12,13] described that orbifloxacin (ORFX), which is a fluoroquinolone derivative having fluorine at the 8 position, was easily photodecomposed in aqueous solution, and dissociation of the C–F bond at the 8 position was an important reaction for the photodegradation of ORFX.

This account reports that ORFX is converted to a photoproduct substituted with a chlorine at the 8 position by its photoreaction in aqueous solution containing chloride ion, and that ORFX generates active oxygen in the photodegradation process. The generation of active oxygen by the photosensitization of ORFX was measured by determining the bleaching rate of *p*-nitrosodimethylaniline (RNO) and measuring the reduction rate of cytochrome c.

Experimental

Materials ORFX (1-cyclopropyl-5,6,8-trifluoro-1,4-dihydro-7-(*cis*-3,5-dimethyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid) and enoxacin (ENX) were prepared in our laboratories. Ciprofloxacin (CPFX) and norfloxacin (NRFX) were extracted from the commercial drug products. The chemical structures of quinolones used in this study are shown in Fig. 1. Superoxide dismutase (from bovine erythrocyte, 3650 units/mg) and horse-heart cytochrome c were purchased from Wako Pure Chemical Industries, Ltd. All other chemicals used in this experiment were of reagent grade.

Photodegradation Study ORFX was dissolved in 0.02 M phosphate buffer (pH 7.3) containing 0 to 200 mM of sodium chloride (NaCl), and these solutions (0.1 mM of ORFX) were used as the sample solutions. Twenty-five ml of each of these sample solutions was put into a Pyrex test tube with a stopper, and the exposure tests were performed at room temperature by irradiating the solutions at a distance of approximately 200 mm with a UVA light (FL20S BL, Toshiba Electric Co., Ltd., Tokyo) for various periods of time. At appropriate time intervals, the sample solutions were tested and the residual amounts of ORFX were determined by HPLC.

The HPLC system consisted of a Shimadzu LC-9A pump, an SIL-6A auto-injector, an SPD-2A UV spectrophotometric detector, a C-R5A Chromatopak and a reversed-phase column (Develosil ODS-7, 250 × 4 mm i.d.). A mixture of 0.1 M citrate buffer (pH 3.5)–methanol–dioxane (84 : 12 : 5) was used as the mobile phase at a flow rate of 1.2 ml/min. The column eluent was monitored at 290 nm.

Preparative HPLC The preparative HPLC consisted of a Waters 600F pump equipped with a Waters 600E system controller, a Waters 170 sample loader, a Waters fraction collector and a Shimadzu SPD-6A UV spectrophotometric detector. A Develosil ODS-7 column (7 μm, 250 × 8 mm i.d.) was used. A mixture of 0.1 M citrate buffer (pH 3.5)–

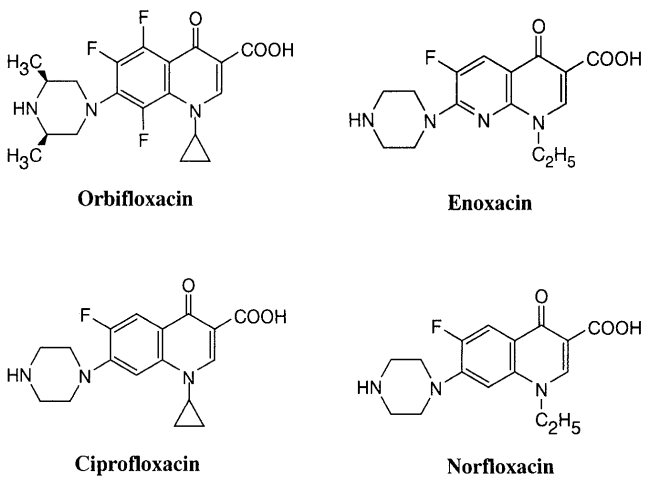

Fig. 1. Chemical Structures of Quinolones

methanol–dioxane (84 : 12 : 5) was used as the mobile phase at a flow rate of 4.0 ml/min. The column eluent was monitored at 290 nm.

Isolation of Photoproduct 1 ORFX was dissolved in 1 M of NaCl solution and this solution (0.1 mM ORFX) was exposed to a UVA light for 1 h. The sample solution after irradiation was fractionated by preparative HPLC and photoproduct 1 was isolated.

Mass Spectrometry Electron-impact mass spectrum (EI-MS) was obtained with a Hitachi M-80B mass spectrometer using a direct inlet system.

NMR ^{1}H-NMR spectrum at 300 MHz and ^{19}F-NMR spectrum at 272 MHz were obtained in DMSO-d_6 using a Varian XL 300 NMR spectrometer.

Measurement of RNO Bleaching RNO bleaching was determined by the method reported to detect hydroxyl radical.[14] Quinolones were dissolved in 0.02 M phosphate buffer (pH 7.3) containing 0 to 200 mM of NaCl and 0.05 mM RNO, and the solutions (0.1 mM each of quinolone) were used as sample solutions. The effects of radical scavengers on the bleaching of RNO were examined by dissolving 100 mM of D-mannitol (a hydroxyl radical scavenger) or 10 mM of sodium azide (NaN_3, a singlet oxygen ($^1O_2$) scavenger) in the sample solutions. Twenty-five ml of each solution was irradiated with UVA light at a distance of approximately 100 mm (CPFX and NRFX) or 200 mm (ORFX and ENX). At appropriate time intervals, the rate of RNO destruction was determined spectrophotometrically by the optical density (OD) at 440 nm before and after irradiation.

Bleaching of RNO induced by photoirradiated ORFX was also measured by the histidine plus RNO method reported as a detection method of $^1O_2$.[15] L-Histidine (10 mM) was dissolved in 0.1 mM of ORFX buffer solution (pH 7.3) with 0.05 mM of RNO, and RNO bleaching under irradiation was measured following the procedure described above.

Determinations of RNO bleaching were made in triplicate with less than 5% of relative standard deviations for all measurements.

Measurement of Superoxide Anion Superoxide anion (O_2^-) was determined by the cytochrome c reduction method modified by Wagai and Tawara.[6] The sample solutions containing 0.033 mM of cytochrome c and 0.033 mM of ORFX were prepared by dissolving them in 0.02 M phosphate buffer without NaCl or containing 200 mM NaCl. The two reference solutions were prepared by dissolving 1000 units of superoxide dismutase (SOD) in 3.0 ml of each sample solution. Three ml of each sample and reference solution was put into a 4.5-ml quartz cell, placed 200 mm from UVA light and irradiated for 12 min at an intensity of 2.3 μW/cm^2. Cytochrome c reduction was determined by measuring the absorbance at 550 nm. ΔE_{mM} (ferrocytochrome c minus ferricytochrome c) at 550 nm was taken at 18.5.[16] Determinations of O_2^- were performed in triplicate.

Measurement of Radiation Dose The radiation dose measured 100 and 200 mm from the light was 8.1 and 2.3 μW/cm^2 respectively, using a Topcon UVR-254 UV Radiometer.

Results and Discussion

Photoreaction of ORFX with Chloride Ion HPLC chromatograms obtained from the photodegradation studies of ORFX solution without NaCl and containing 200 mM of NaCl are shown in Fig. 2. A new photoproduct, eluted at the retention time of 7.0 min, was observed when the ORFX solution containing chloride ion was irradiated with UVA light. ORFX is known to photodecompose to several photodegradation products,[13] the main products being identified as 5,6-difluoro-1,4-dihydro-7-(*cis*-3,5-dimethyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid (photoproduct 2) and 7-(2-aminopropylamino)-1-cyclopropyl-5,6-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (photoproduct 3). The formation of photoproduct 1 in aqueous solution containing 200 mM of NaCl was not observed under light-resistant condition. Photoproduct 1 was isolated using preparative HPLC, and identified as 8-chloro-1-cyclopropyl-5,6-difluoro-1,4-dihydro-7-(*cis*-3,5-dimethyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid by EIMS, ^{1}H-NMR and ^{19}F-NMR spectroscopy: EIMS *m/z*: 411 (M^+), 341 ($M-C_4H_8N$), 297 ($M-CO_2-C_4H_8N$). ^{1}H-NMR (DMSO-d_6) δ: 1.21 (6H, d, J=6.0 Hz, $-CH_3\times2$), 0.84—1.35 (4H, m, cyclopropyl), 3.00—3.40 (6H, m, piperazinyl), 4.32 (1H, m, cyclopropyl), 8.88 (1H, s, 2-H). ^{19}F-NMR (DMSO-d_6) δ:

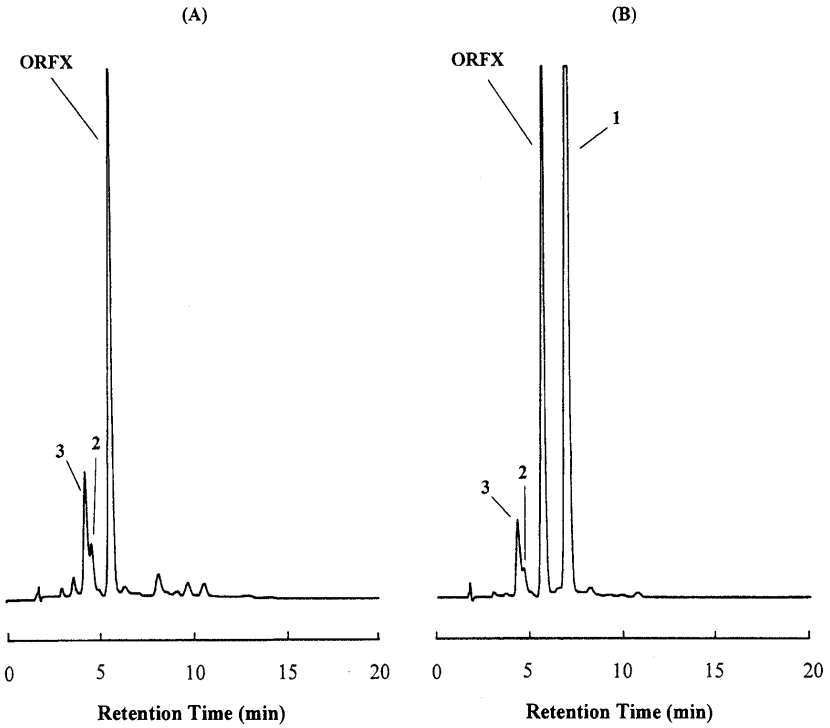

Fig. 2. HPLC Chromatograms of Photodegraded Samples of ORFX in the Absence (A) and the Presence (B) of 200 mM NaCl after UVA Irradiation for 60 min

1, photoproduct 1; 2, photoproduct 2; 3, photoproduct 3.

−137.10 (1F, s, 5-F), −147.01 (1F, d, J=17.0 Hz, 6-F).

The time courses of the photodegradation of ORFX in pH 7.3 phosphate buffers without NaCl and containing 100 and 200 mM of NaCl are shown in Fig. 3. Under all experimental conditions, ORFX photodegraded rapidly when exposed to UVA and the photodegradation was found to follow the apparent first-order kinetics.

In this experiment, the formation rate of photoproduct 1 increased proportionally with the increase in chloride ion concentration, while the formation rate of photoproduct 2, photoproduct 3 and other unknowns decreased with increasing concentration. This indicates that these other photoreactions of ORFX are inhibited when NaCl is added to the medium.

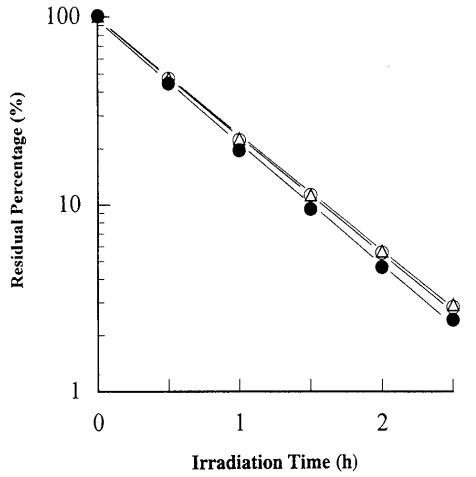

Fig. 3. Time Course of the Photodegradation of ORFX in pH 7.3 Phosphate Buffer Containing NaCl and without NaCl

●, without NaCl; △, 100 mM NaCl; ○, 200 mM NaCl.

The photodegradation mechanism of ORFX was described in a previous report (Fig. 4).[13] In the initial step of the ORFX photoreaction the fluorine atom seems to be eliminated by irradiation with UVA light and a carbon radical is produced at the 8 position. Then, the protons located at a similar distance from the carbon radical at the 8 position, the hydrogen at the 2 position of the cyclopropyl group or the hydrogen at the 3 position of the dimethylpiperazinyl group, seems to be abstracted by the carbon radical at the 8 position. It is hard to think that a direct homolysis of the C–F bond occurs by irradiation. The dissociation mechanism of this bond at the 8 position remains unknown.

The fact that the photoreaction of ORFX in the presence of chloride ion yields photoproduct 1 indicates the reaction between carbon radical and chloride ion at the 8 position. The photochlorination at the 8 position may compete with the abstraction of hydrogen atom from the substituents by carbon radical.

The rate constants for the photodegradation of ORFX in pH 7.3 phosphate buffer solution containing 0 to 200 mM of NaCl are shown in Table 1. Although the formation rate of photoproduct 1 increased with rising chloride ion concentration, no effect of the ion on the photodegradation rate of ORFX was observed. These results also suggest

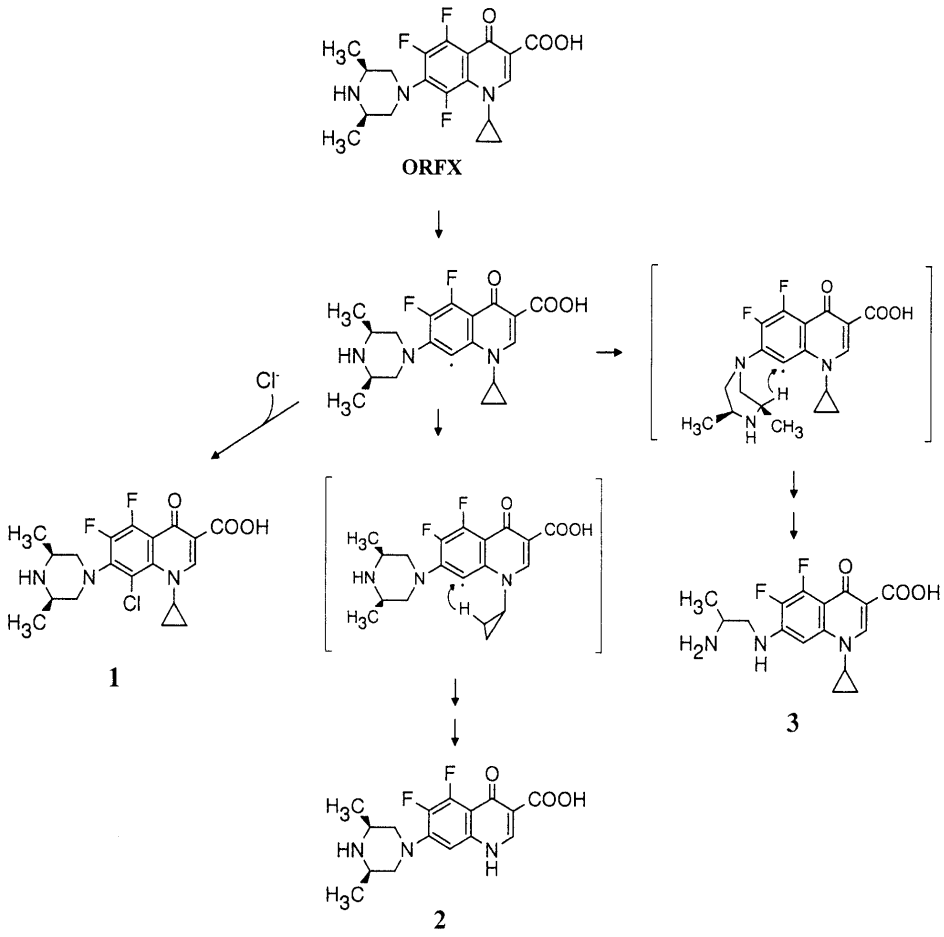

Fig. 4. Postulated Mechanism for the Photodegradation of ORFX

Table 1. Rate Constants for the Photodegradation of ORFX in pH 7.3 Phosphate Buffer Containing Various Concentrations of NaCl

NaCl Concentration (mM)	Rate constant (h^{-1})
0	1.47
50	1.45
100	1.42
200	1.43

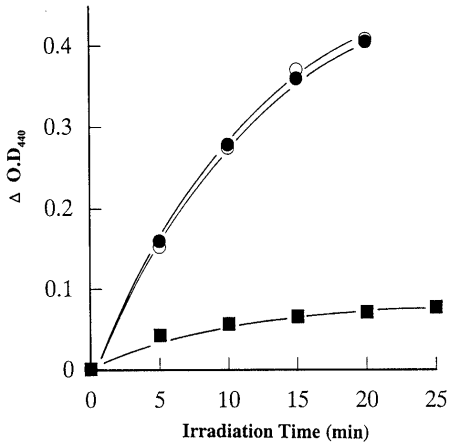

Fig. 5. The Repression Effect of Radical Scavengers on the RNO Bleaching by Photosensitization of ORFX in pH 7.3 Phosphate Buffer

●, control; ■, 10 mM NaN_3; ○, 100 mM D-mannitol.

that the dissociation of the C–F bond at the 8 position is the rate-limiting step in the initial step of the photoreaction of ORFX.

Repression Effect of Chloride Ion on the RNO Bleaching The effect of radical scavengers on the RNO bleaching by photosensitization of ORFX in pH 7.3 phosphate buffer is shown in Fig. 5. The photodestruction of RNO proceeds in parallel with the progress of the photoreaction of ORFX, indicating that ORFX efficiently generates active oxygen in the photodegradation process. The bleaching of RNO was not detected in either the RNO solution without ORFX after UVA irradiation or in the ORFX solution with 0.05 mM of RNO before irradiation.

The effect of a hydroxyl radical scavenger, D-mannitol, and of a $^1O_2$ scavenger, NaN_3, on the bleaching of RNO induced by ORFX and UVA are shown in Fig. 5. Addition of the D-mannitol to the ORFX solution did not affect the rate of bleaching, while the bleaching was inhibited to about 20% or lower when NaN_3 was added to the solution. These results imply that $^1O_2$ is responsible for the RNO bleaching.

Kraljic and El Mohsni reported that the histidine plus RNO method is a sensitive and selective test to determine the presence of $^1O_2$ in aqueous solution.[15] They also said that $^1O_2$ does not react chemically with RNO, and that bleaching can be induced by using $^1O_2$ acceptor such as an imidazole derivative which results in the formation of an endoperoxide intermediate capable of inducing the RNO bleaching. However, bleaching of RNO by photosensitization of ORFX was detected both 10 mM of histidine and contained no histidine, and the differences in the results obtained from the two bleaching methods

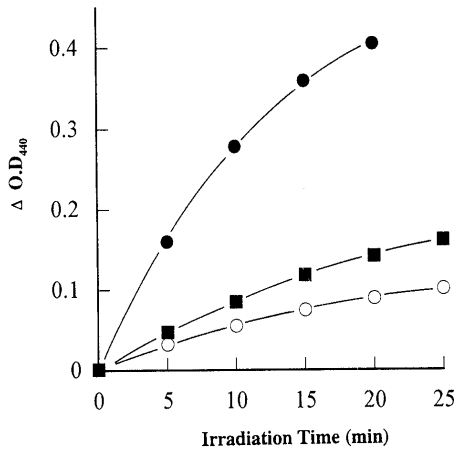

Fig. 6. Repression Effect of Chloride Ion on the RNO Bleaching by Photosensitization of ORFX in pH 7.3 Phosphate Buffer without NaCl or Containing NaCl

●, without NaCl; ■, 100 mM NaCl; ○, 200 mM NaCl.

Table 2. Effect of Chloride Ion on the RNO Bleaching Induced by Photsensitization of Quinolone

Quinolone	Δ OD at 440 nm (Mean ± S.D.)	
	Without NaCl	200 mM NaCl
ORFX[1]	0.404 ± 0.002	0.088 ± 0.003
ENX[2]	0.093 ± 0.002	0.095 ± 0.002
CPFX[3]	0.079 ± 0.001	0.081 ± 0.001
NRFX[3]	0.069 ± 0.002	0.068 ± 0.001

Samples were irradiated by UVA light: 1) for 20 min at a distance of approximately 200 mm, 2) for 150 min at a distance of approximately 200 mm, 3) for 180 min at a distance of approximately 100 mm.

were very small. When sample solutions were irradiated for 15 min, Δ OD at 440 nm was 0.358 ± 0.004 in the presence of 10 mM of L-histidine, compared with a value of 0.358 ± 0.002 in its absence. The inhibitory effect of NaN_3 on the bleaching of RNO was also observed under both conditions: when sample solutions were irradiated for 15 min, Δ OD at 440 nm was 0.058 ± 0.001 in the presence of 10 mM of L-histidine, and 0.067 ± 0.004 in its absence. The reaction between ORFX and $^1O_2$ may yield an endoperoxide which can bleach the RNO.

As shown in Fig. 6, the rates of the RNO bleaching induced by photosensitization of ORFX in pH 7.3 phosphate buffer varied with the concentrations of NaCl in the solutions. In the presence of 200 mM of NaCl, the bleaching rate was decreased to about 20%. This repression effect of chloride ion on the bleaching was dependent on the ion concentration. However, this repression effect of chloride ion was not observed on the RNO bleaching induced by photosensitization of CPFX, NRFX or ENX none of which has fluorine at the 8 position (Table 2).

The mechanism of the blocking of RNO bleaching by chloride ion is speculated to be competition of the photochlorination at the 8 position with the reaction between ORFX radical and oxygen.

Repression Effect of Chloride Ion on the O_2^- Generation Photoirradiation of ORFX solution with cytochrome c led to the reduction of the latter, and this reduction was

inhibited by adding SOD to the ORFX solution, thus indicating the generation of O_2^- in photoirradiated ORFX solution. When the ORFX solution without NaCl was irradiated for 12 min, O_2^- generated by photosensitization of ORFX amounted to $31 \pm 5\,\mu$mol per 1 mol of the derivative. In the solution containing 200 mM of NaCl, the apparent amount of O_2^- generated was $29 \pm 3\,\mu$mol per mol. These results indicate that chloride ion does not inhibit the O_2^- generation by photosensitization of ORFX.

The photochemical properties of 8-F quinolone differ from those of other quinolones.[5,6,13] The differences are assumed to be attributable to generation of carbon radical at the 8 position. By spin trapping ESR method was demonstrated that hydroxyl radical, $^1O_2$ and carbon radical were generated in the photodegradation process of 8-F quinolones such as sparfloxacin and lomefloxacin.[11]

It is interesting that the chloride ion is effective in blocking the RNO bleaching by photosensitization of ORFX. Human serum contains 96—110 mEq/l of chloride ion, so the generation of active oxygen by ORFX photosensitization may be inhibited under physiological conditions. Further detailed investigations are required to elucidate the photoreaction of 8-F quinolone in the presence of chloride ion. However, the relevant properties of 8-F quinolone in the presence of chloride ion must be considered when the photochemistry of this quinolone is investigated in biological materials.

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