Transient species of several fluoroquinolones and their reactions with amino acids

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ABSTRACT

The photochemistry of several fluoroquinolone antibiotics (FQs), including enoxacin (ENX), norfloxacin (NFX), and ciprofloxacin (CPX), have been investigated in neutral aqueous solution by laser flash photolysis and pulse radiolysis. Transient absorption spectra of FQs were observed and transient species were assigned. The absorption maxima (λ_{max}) of triplet states of ENX, NFX and CPX were located at 520 nm, 620 nm and 610 nm, respectively. The radical anions of FQs were characterized by $\lambda_{max} = 370$ nm. Reactions of the FQ triplet states with amino acids (tyrosine [TyrOH], tryptophan [TrpH], glycine [Gly] and cysteine [Cys]) were investigated in different conditions. All three FQ triplet states can oxidize TrpH and TyrOH. The FQ radical anions and oxidized radicals of TrpH (Trp•) and TyrOH (TyrO•) were directly observed. Under aerobic conditions, the photooxidation of TrpH and TyrOH involves both Type I and Type II mechanisms. There were no distinct quenching reactions of the FQ triplet states by Gly and Cys in N_2-saturated aqueous solutions.

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1. Introduction

Fluoroquinolone antibiotics are a class of compounds widely used as broad-spectrum antimicrobial agents in clinical treatment [1–3], but these drugs are also well known to exhibit phototoxic, photomutagenic and photocarcinogenic properties [4,5], often causing undesirable side effects when patients are exposed to light, especially at UV-A wavelengths. A series of FQs have been investigated to determine their potential phototoxicity as photosensitizers of proteins and DNA [6–9]. Photodamage of these macromolecules is primarily the result of the FQs in their excited state, which are highly oxidizing species. Reactive oxygen species are formed in various photoprocesses of FQs [10]. Especially those FQs bearing a further fluorine atom at position 8, undergo unimolecular fragmentation to yield aryl cations that play important roles in photodamage of biological macromolecules [10–15]. At the same time, the excited states of FQs can activate O_2, forming both singlet oxygen and superoxide anion, so a dioxygen related effect operates [16]. For this reason, 6-monofluoroquinolone derivatives are more phototoxic in aerobic conditions than in anaerobic conditions [6].

Over the past decade, many studies have been devoted to understanding the exact mechanisms of phototoxic reactions induced by FQs. The photochemistry of a series of FQs has been reported [10,17–19]. However, the mechanisms underlying phototoxic reactions are not yet known. Researchers have extended these studies, but only for a deeper understanding of FQ photophysical and photochemical properties, with some unexpected reactions being reported.

The most interesting photoprocess of FQs is that of heterolytic defluorination, because of the strength of the aromatic C–F bond (dissociation energy *ca.* 120 kcal/mol) that is involved. The photoprocess of defluorination occurs through two different pathways:

(1) The triplet states of 6-monofluoroquinoloes defluorinate through a photosubstitution reaction of the fluorine atom by OH^-, such as ENX, NFX and CPX. The quantum yield of the defluorination reaction is 0.13 (ENX), 0.06–0.007(CPX and NFX) [20–22].
(2) 6,8-Difluoroquinolone derivatives are much more sensitive than 6-monofluoroquiolones because of the addition of the second fluorine atom at position 8. When these derivatives are excited, difluoroquinolones defluorinate at position 8, with formation of an aryl cation that plays an important role in the phototoxicity process [10–15]. The aryl cation exists in equilibrium between singlet ground state and triplet ground state [13].

Apart from photocleavage of the C–F bond, other photoreactions of FQs occur, such as decarboxylation [22,23] and side-chain degra-

dation [10,21]. Some hypotheses have been presented to explain these photoreactions, but more evidences are needed to confirm their validity.

A precondition of deeper understanding of the mechanism of phototoxic reactions is the exact knowledge of FQ photochemistry. Excited states, especially triplet states, singlet oxygen and superoxide anion play important roles in the photodamage process induced by photosensitizers. Study of the kinetics of ^{3}FQs* and reactions with O$_2$ could yield a more in depth understanding of the phototoxic properties of FQs. A deeper understanding of their reactions with amino acids could also provide clues regarding the phototoxic reactions of FQs with proteins. Detailed reports of the absorption of FQ excited states are now available. However, details of the kinetics of FQ excited states or of the transient absorption spectra of various radicals that are generated during the photoprocess are still lacking. More research into these aspects could provide further direct evidence in support of hypotheses regarding the various photoreactions.

In the present paper, we report our research concerning the photochemical properties of FQs and their reactions with amino acid. Laser flash photolysis (LFP) was used to generate FQ triplet states and to monitor their time behaviors in aqueous solution saturated with different gases. To gain insight into the mechanism of the phototoxic reactions and to determine the role of the intermediates involved in the photoreactions in a biological environment, reactions of a number of representative amino acids, including TyrOH, TrpH, Gly and Cys with FQs were studied using LFP, and more direct evidences were obtained by pulse radiolysis.

2. Materials and methods

2.1. Materials

ENX, NFX and CPX were purchased from Sigma–Aldrich. Phosphate salts and amino acids were obtained from J&K Chemical Ltd. All solutions were prepared freshly with ultrapure water provided by a Millipore purification system.

2.2. Methods

Nd: YAG laser providing a 355 nm pulse with duration of 5 ns and the maximum energy of 240 mJ per pulse was used as the pump light source. The laser energy is 6 mJ in our experiments. A xenon lamp was employed as detecting light source. The laser and the analyzing light beam passed perpendicularly through a quartz cell. The transmitted light entered a monochromator equipped with an R955 photomultiplier. The output signal from the HP54510B digital oscillograph was transferred to a personal computer for further analysis. The LFP setup has been previously described [24]. All experiments were performed in water solution. Samples were bubbled with high-purity (99.99%) N$_2$, or O$_2$ (99.99%) for at least 20 min, before photolysis experiments were initiated.

Pulse radiolysis experiments were performed utilizing a 10 MeV linear accelerator which delivers an electron pulse with duration of 8 ns. The dosimetry of electron pulse was determined by thiocyanate dosimeter using G[(CNS)$_2$$^{-\bullet}$] = 5.8 in a 0.1 mM KCNS saturated with N$_2$O by taking ε_{480nm} = 7600 dm^3 m^{-1} cm^{-1} for (CNS)$_2$$^{-\bullet}$. The details of the setup and operation conditions were given in the previous paper [25]. The dose per electron pulse was 10 Gy.
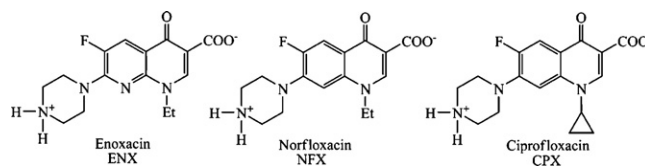

Chart 1. The structure of fluoroquinolones.

Table 1
Photophysical and photochemical properties of the FQ triplet states.

Compound	λ_{max}/nm[a]	$\tau_{1/2}$/µs	k_0/s^{-1}	k_d(self)/M^{-1}s^{-1}	k_d(O$_2$)/M^{-1}s^{-1}
ENX	520	0.7	7.3×10^5	2.7×10^9	2.7×10^9
NFX	620	3.0	2.1×10^5	4×10^8	2.8×10^9
CPX	610	2.1	2.8×10^5	1×10^9	2.7×10^9

[a] λ_{max} of ^{3}FQs*[10]. Halftime ($\tau_{1/2}$) of ^{3}FQs*, medium is net water, C(FQs) = 0.1 mM.

3. Results and discussion

3.1. Transient absorption spectra of the FQ triplet states

There are multiple proton binding sites in FQs that make the pattern of acid–base equilibria rather complex. The most significant proton binding sites, from the biological point of view, are the carboxylate group and the 4′-N of the piperazine ring [10]. At different pH in aqueous solution, the quantum yields for triplet formation of FQs (Φ_T) have apparent differences due to changes in the components [26]. In the neutral condition, FQs exist as zwitterions (see structure in Chart 1) in aqueous solution, with Φ_T that is higher than that of the other components [26].

In consideration of the reasonable lifetime (in the µs range), effective oxygen quenching (Table 1), and quenching by various triplet quenchers (such as 4-biphenylcarboxylic acid and naproxen [8]), the primary triplet state of FQs is characterized by the absorption maxima at 520 nm (ENX), 620 nm (NFX) and 610 nm (CPX), respectively [10] (Fig. 1).

The triplet states of FQs were quenched rapidly by ground states. Quenching-rate constants were in the range 10^8–10^9 M^{-1} s^{-1}, the decay of ^{3}FQ* is accelerated with rates proportional to the concentrations of the ground states. The pseudo-first-order decay kinetics of ^{3}FQs* were observed at 520 nm (ENX), 620 nm (NFX) and 610 nm (CPX) (Table 1) (Reaction (1)).
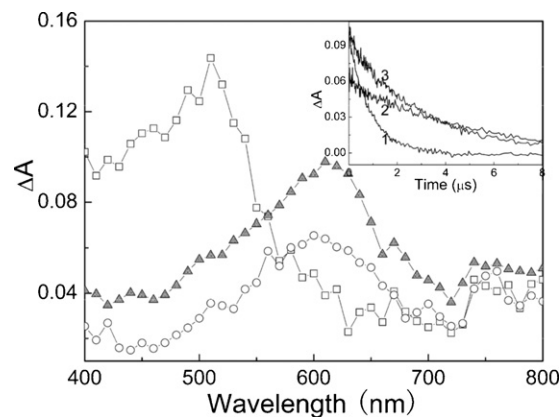
$$^3\text{FQs}^* + \text{FQs} \rightarrow 2\text{FQs} \tag{1}$$

Fig. 1. Transient absorption spectra observed at 0.2 µs after 355 nm laser flash excitation of 0.1 mM FQs (□: ENX; ○: NFX; ▲: CPX) in N$_2$-saturated aqueous solution. Inset: the decay of ^{3}ENX* at 520 nm (1), ^{3}NFX* at 620 nm (2) and ^{3}CPX* at 610 nm (3). Laser energy is 6 mJ.

$$^{3}FQs^{*} + O_{2} \rightarrow FQs + {}^{1}O_{2} \qquad (2)$$

Quenching of triplet states by ground state molecular oxygen (O_{2}) is characterized by rate constants in the range $2–3 \times 10^{9} \, M^{-1} \, s^{-1}$, which is typical for aromatic molecules. The major reactions are energy transfer with formation of singlet oxygen (Reaction (2)). Singlet oxygen was actually observed in light irradiated FQ solutions [10,16].

When pulse energy is lower than 7 mJ, the photoionization of FQs is negligible. However, under moderate laser energy conditions (laser energy >7 mJ), the photoejection of electron in aqueous solutions at $\lambda_{exc} = 355 \, nm$ is predominantly two-photon [27], and we cannot ignore the effect of photoionization. The FQ radical cations from photoionization are able to oxidize the amino acids directly. The hydrated electron or the FQ radical anions can react with O_{2} with formation of superoxide which can oxidize TrpH and TyrOH. We will discuss these photooxidation reactions in detail in another paper.

3.2. Reactions of FQs with hydrated electron

To determine whether or not electron transfer from amino acids to the triplet states of FQs, we investigated reactions of FQs with hydrated electron in a N_{2}-saturated phosphate buffer 2 mM (pH = 7.0) aqueous solutions of FQs containing t-butanol (0.1 M) by pulse radiolysis first.

$$FQs + e_{aq}^{-} \rightarrow FQs^{\bullet -} \qquad (3)$$

$$FQs^{\bullet -} + H^{+} \rightarrow FQsH^{\bullet} \qquad (4)$$

Under this condition, the $^{\bullet}OH$ radical reacts with t-butanol to form relatively unreactive free radicals. Considering that the signal-to-noise ratio at 640 nm is better than that at 720 nm, the reactivity of hydrated electron with FQs was studied by monitoring the decay of absorption at 640 nm (Reaction (3)). In the absence of FQs the hydrated electron decayed with a rate constant of $5.0 \times 10^{5} \, s^{-1}$, while with the increasing concentration of FQs, the decay at 640 nm proportionally became fast. In the presence of $1.0 \times 10^{-4} \, M$ FQs, the decay rate constant increased to $1.2 \times 10^{6} \, s^{-1}$, indicating that the hydrated electron reacts very efficiently with FQs. From the pseudo-first-order decay kinetics of hydrated electron observed at 640 nm with FQ concentrations, the reaction rate constants were determined, $6.3 \times 10^{9} \, M^{-1} \, s^{-1}$ (ENX), $5.2 \times 10^{9} \, M^{-1} \, s^{-1}$ (NFX), and $6.0 \times 10^{9} \, M^{-1} \, s^{-1}$ (CPX), respectively, which belong to diffusion controlled rate constants. The FQ radical anions were able to get a proton, forming protonated radicals whose absorption maxima were located at 320 nm (Reaction (4)).

After the hydrated electron decayed completely (after 3 μs), the transient absorption spectra exhibited absorption maxima at 370 nm and a weaker absorption around 440 nm ascribed to the FQ radical anions together with bleaching of ground state absorption below 350 nm (Fig. 2). In N_{2}-saturated solutions, the radical anions of FQs (monitored at 370 nm) decayed by a first-order process with a rate constant of $9.8 \times 10^{4} \, s^{-1}$ (ENX), $9.3 \times 10^{4} \, s^{-1}$ (NFX), $8.8 \times 10^{4} \, s^{-1}$ (CPX), with a halftime 7.0 μs (ENX), 7.5 μs (NFX), 8.0 μs (CPX), which are close to the value determined in LFP experiment.

Under moderate laser energy conditions (laser energy >7 mJ), the photoejection of electrons in aqueous solutions is predominantly two-photon [27]. So we can observe distinct absorption at 370 nm in a neutral aqueous solutions excited by $\lambda_{exc} = 355 \, nm$, when laser pulse energy is *ca.* 20 mJ. The decay rate constants and lifetimes were in agreement with the results obtained by pulse radiolysis. According to these results, we concluded that the absorption at 370 nm contained a significant contribution of the FQ radical anions in LFP experiments.
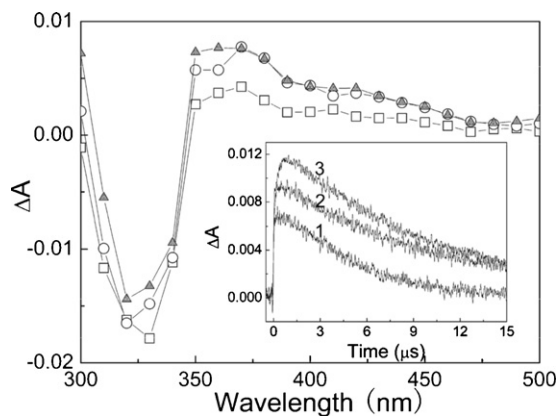

Fig. 2. Transient absorption spectra observed at 5 μs after pulsing (10 Gy; 1 cm path length) a N_{2}-saturated aqueous solution of $1 \times 10^{-4} \, M$ FQs (□: ENX; ○: NFX; ▲: CPX) in 2 mM phosphate buffer (pH = 7.0) and 0.1 M t-butanol. Inset: the time profile observed at 360 nm (1: ENX; 2: NFX; 3: CPX).

Table 2
Quenching-rate constants of the FQ triplet stats by amino acids, unit $M^{-1} \, s^{-1}$.

Compound	TrpH	TyrOH
ENX	1.2×10^{9}	8.7×10^{8}
NFX	9.5×10^{8}	$<10^{7}$
CPX	1.0×10^{9}	$<10^{7}$

3.3. Reactions of FQ triplet state with amino acids

In the presence of amino acids, the decay of $^{3}FQs^{*}$ was clearly accelerated, with rates proportional to the concentrations of the amino acids applied. Quenching-rate was in the range $10^{7}–10^{9} \, M^{-1} \, s^{-1}$ (Table 2).

The energy of ENX triplet state (E_{T}) is 273 kJ/mol, and 269 kJ/mol for NFX [8]. While the E_{T} of TrpH and TyrOH are 297 kJ/mol and 342 kJ/mol, respectively [28]. Consequently, energy transfer from the triplet states of FQs to TrpH and TyrOH is unlikely to occur. However, $^{3}FQs^{*}$ are able to oxidize TrpH, forming the FQ radical anions and oxidized radicals of TrpH (Reactions (5) and (6)). As shown in Fig. 3, the observed spectra at 6 μs in an ENX N_{2}-saturated solution containing TrpH showed considerably stronger absorption in the 480–560 nm region, which was ascribed to the contribution of the absorption of the deprotonated radical cation of TrpH [28,29],
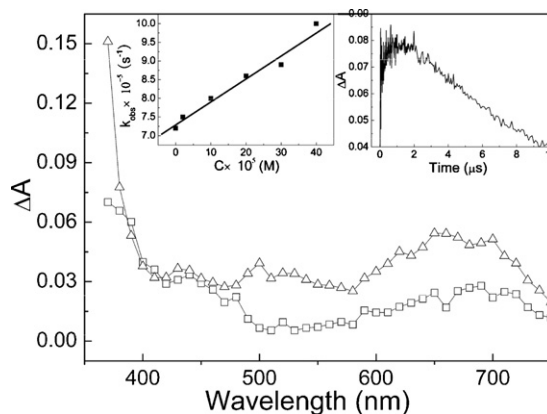

Fig. 3. Transient absorption spectra observed after 355 nm laser flash excitation of 0.1 mM ENX in N_{2}-saturated phosphate buffer 2 mM (pH = 7.0) aqueous solution containing different concentrations of TrpH at 6 μs. △: in the presence of TrpH $5 \times 10^{-4} \, M$; □: in the absence of TrpH. Inset: the time profile at 370 nm in N_{2}-saturated solutions of ENX in phosphate buffer 2 mM (pH = 7.0) containing TrpH. $5 \times 10^{-4} \, M$. Laser energy is 6 mJ.
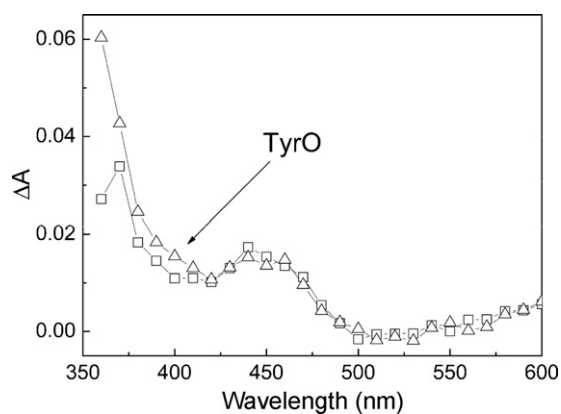
Fig. 4. Transient absorption spectra observed after 355 nm laser flash excitation of 0.1 mM ENX in N_2-saturated phosphate buffer 2 mM (pH = 7.0) aqueous solution containing TyrOH at 12 μs. □: in the absence of TyrOH; △: in the presence of TyrOH (0.12 mM). Laser energy is 6 mJ.

after the complete decay of ^{3}ENX*. At the same time, the absorption at 370 nm ascribed to the ENX radical anion was enhanced, and we can also observe an obvious generating process at 370 nm (inset in Fig. 2). The absorption at 370 nm decayed with a rate constant 1.0×10^5 s^{-1}, halftime 6.9 μs in agreement with the decay rate of ENX radical anion obtained by pulse radiolysis experiments. Thus, the absorption at 370 nm was attributed to the ENX radical anion. When TrpH was present at a higher concentration, the absorption at 370 nm and around 520 nm were enhanced much more.

$$^3FQs^* + TrpH \rightarrow FQs^{\bullet-} + TrpH^{\bullet+} \qquad (5)$$

$$TrpH^{\bullet+} \rightarrow Trp^\bullet + H^+ \qquad (6)$$

The same phenomenon could be observed in neutral solutions of NFX and CPX containing TrpH saturated by N_2. The obvious absorption of NFX/CPX radical anions at 370 nm and Trp$^\bullet$ around 520 nm was observed. Therefore, ENX, NFX and CPX were apparently able to get electrons from TrpH (Reactions (5) and (6)).

The quenching of ^{3}ENX* by TyrOH was much more rapid than that of NFX or CPX (Table 2). We observed that the obvious absorption with λ_{max} = 370 nm ascribed to the ENX radical anion and an absorption around 400 nm ascribed to TyrO$^\bullet$ [28,29] were enhanced in transient spectra of ENX solution containing TyrOH upon excitation by 355 nm laser (Fig. 4). For NFX and CPX, we did not observe that the triplet states were quenched by TyrOH distinctly, but also observed the absorption of the FQ radical anions and TyrO$^\bullet$. The absorption of TyrO$^\bullet$ can not be observed clearly, this is mainly due to the lower absorption coefficient of TyrO$^\bullet$ than that of FQ radical anions and their overlapped absorption. According to these results, we concluded that the reactions of ^{3}FQs* with TyrOH occurred through electron transfer (Reactions (7) and (8)).

$$^3FQs^* + TyrOH \rightarrow FQs^{\bullet-} + TyrOH^{\bullet+} \qquad (7)$$

$$TyrOH^{\bullet+} \rightarrow TyrO^\bullet + H^+ \qquad (8)$$
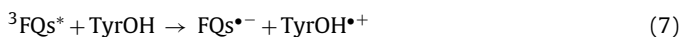
We did not observe any quenching reactions of the FQ triplet states by Gly and Cys, or absorption of the FQ radical anions. These results indicated that FQs may have reacted with Cys without electron transfer or energy transfer.

Under aerobic conditions, the FQ triplet states can be efficiently quenched by O_2 through energy transfer with a rate constant of $2–3 \times 10^9$ M^{-1} s^{-1}, forming singlet oxygen. The singlet oxygen quantum yields are 0.061 (ENX), 0.081 (NFX) and 0.092 (CPX), respectively [10,16]. As we know, TrpH and TyrOH can react with singlet oxygen at rates (3×10^7 and 8×10^6 M^{-1} s^{-1}, respectively) (Type II mechanism) [30]. Therefore, in the presence of O_2, Type I mechanism is competitive with singlet oxygen (Reaction (9)).
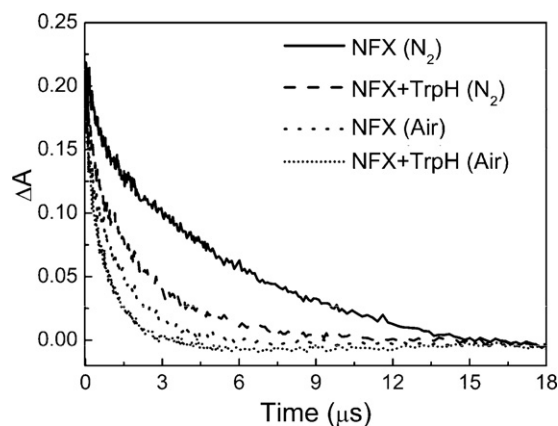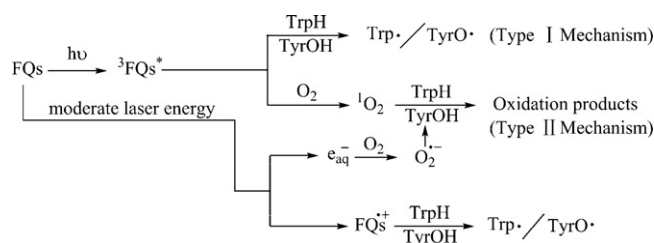

Fig. 5. The decay of ^{3}NFX* (0.1 mM) observed at 620 nm in different conditions (TrpH: 0.25 mM). Laser energy is 6 mJ.

Scheme 1. The possible interactions in the photosensitization of TrpH and TyrOH by FQs.

Which reaction is major pathway is dependent on the concentrations of O_2 and amino acids. As shown in Fig. 5, in the presence of O_2 and TrpH, the photooxidation TrpH mechanism of FQs involves both Type I and Type II processes. This is in agreement with the results obtained by gel electrophoresis experiments [6,16].

$$^1O_2 + TrpH/TyrOH \rightarrow \text{Oxidation products} \qquad (9)$$
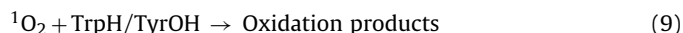
4. Conclusions

The LEP experiments described transient spectra and photochemical properties of ENX, NFX and CPX. The absorption maxima of triplet states of ENX, NFX and CPX were located at 520 nm, 620 nm and 610 nm, respectively. The radical anions of FQs were characterized by λ_{max} = 370 nm. All three FQs were able to oxidize TrpH and TyrOH, forming the reduced FQ radicals and the oxidized radicals of TrpH and TyrOH. No distinct quenching reactions of FQs with Gly and Cys were observed. Under aerobic conditions, the photooxidation of amino acids involves both Type I and Type II mechanisms. Photoinduced reactions of FQs with amino acids can occur through different pathways in different conditions (Scheme 1). Although it was unclear what the biological consequences of such oxidations of amino acids would be, our research provides some support for mechanisms of protein damage and for phototoxicity of FQs from a biological viewpoint.

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