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Photosensitized Oxidation of Methionine-Containing Dipeptides. From the Transients to the Final Products

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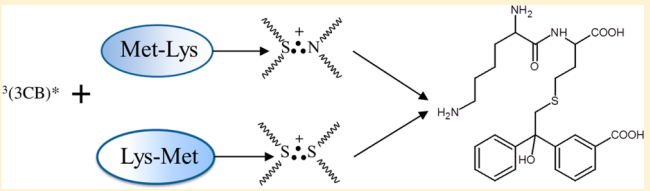
Supporting Information

ABSTRACT: The Met residue oxidation has been studied for decades. Although many efforts have been made on the identification of free radicals, some doubts remain about their final fates, i.e., the nature of stable oxidation products. The photosensitized oxidation processes of two peptides, methionyl lysine (Met-Lys) and lysyl methionine (Lys-Met), were investigated using 3-carboxybenzophenone (3CB) as a sensitizer. Therefore, not only the transients were characterized but also the final products (by high-performance liquid chromatography and mass spectrometry) together with the quantum yields. As for the transients, the sulfur radical cations stabilized by a two-center three electron bonds with a nitrogen $(S.\cdot.N)^+$ were identified in the case of Met-Lys. On the other hand, in Lys-Met, the intermolecular $(S.\cdot.S)^+$ radical cations were found. The peptide-3CB adduct was the only stable product detected and was accompanied neither by sulfoxide formation nor by decarboxylation. It shows that both $(S.\cdot.N)^+$ and $(S.\cdot.S)^+$ radicals are converted into the relatively long-lived α-(alkylthio)alkyl radicals, which add to the 3CB-derived radicals. This addition reaction prevented all other oxidation processes such as formation of sulfoxide. The lysine residue was totally protected, which may also be of importance in biological processes.



INTRODUCTION

The oxidation mechanisms of different amino acids, peptides, and proteins have been thoroughly studied over the past decades due to the biological importance of such processes and their roles in medicine, biology, and basic chemistry.[1,2] One of the sites frequently attacked by oxidative agents (excited states, free radicals, reactive oxygen species) is the thioether function in methionine (Met) residues.[3−6] The Met residue oxidation can cause deleterious consequences during oxidative stress (neurodegenerative diseases, aging, etc.).[7] However, despite the numerous studies concentrated on the one-electron oxidation processes of the methionine residue, some aspects of the processes still need to be further investigated (e.g., the fate of free radicals leading to stable products).

One-electron oxidation of Met-containing compounds in solution occurs easily [e.g., by using benzophenone derivatives as triplet sensitizers[8−11] or the strongly oxidizing hydroxyl radicals ($^\bullet$OH) (E° = 1.9 V vs NHE at pH 7)[12]] from water radiolysis[11,13−15] (through addition of the $^\bullet$OH radical followed by elimination of either OH^- or H_2O). The transients formed in the oxidation of Met-containing peptides and proteins by various one-electron oxidants have been well-characterized.[16−20] In the oxidation process, the sulfur radical cation initially formed can complex with any atom having a lone electron pair (O, N, or S), yielding a two-centered three-electron (2c-3e) bond.[19] It can also deprotonate, yielding a carbon-centered radical. Although many efforts have been made on the identification of free radicals [e.g., by means of pulse radiolysis, electron paramagnetic resonance,[21] and chemically induced dynamic nuclear polarization (CIDNP) techniques[22,23]], some doubts remain as for their final fates (i.e., the stable oxidation products). Surprisingly, there are only few reports about the combination of the complementary time-resolved and steady-state techniques in the photoinduced oxidation of Met-containing peptides.[24,25] Recently, we have investigated the nature of the final products of the oxidation of methionine residues by $^\bullet$OH radicals using an infrared multiphoton dissociation spectroscopy coupled with mass spectrometry (IRMPD-MS) technique. The major final product of the anaerobic one-electron oxidation of Met-containing peptides and related compounds in solution has been found to be methionine sulfoxide (MetSO). However, several other products were also found.[26−28]

In this work, we investigated the oxidation process starting from the transients formed by the photoinduced oxidation of methionine containing peptides, to the final oxidation products. The motivation to study the oxidation mechanism of methionyl-lysine (Met-Lys) and lysyl-methionine (Lys-Met)

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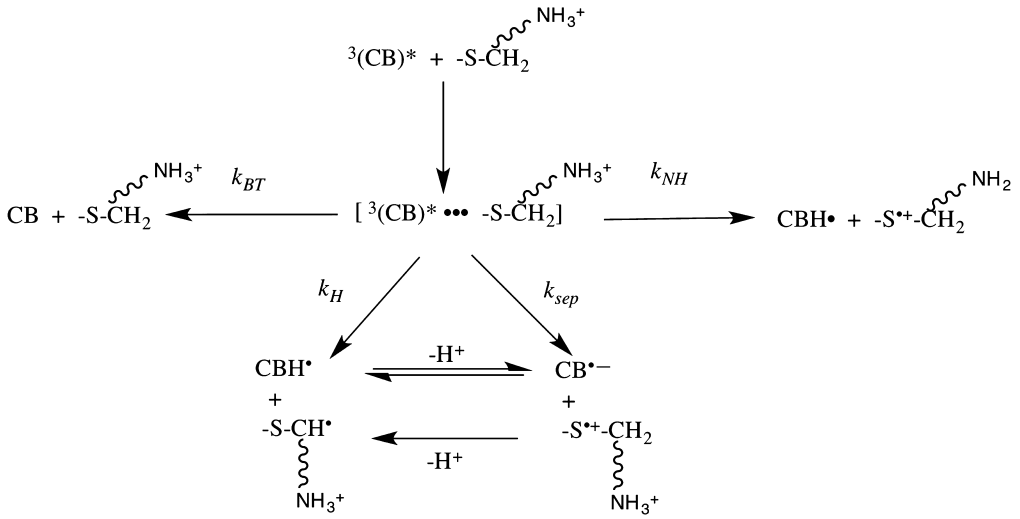
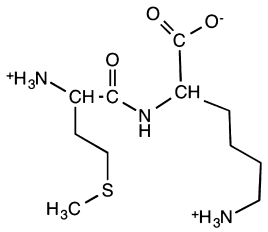
Scheme 1. First Steps of 3CB-Sensitized Oxidation of Met-Containing Peptides in Neutral Solutions[a]



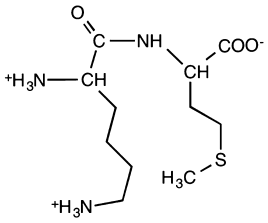
[a] k_{NH} route is for Met-X peptides, X being another residue.

Chart 1. Initial and Final Compounds Coming from Photo-Oxidation of, for example, Met-Lys[a]

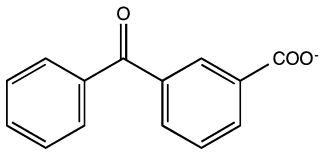
Initial compounds



Met-Lys

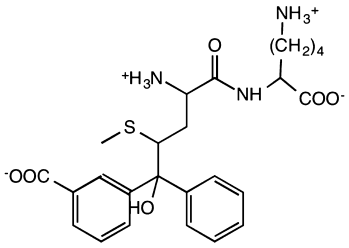


Lys-Met

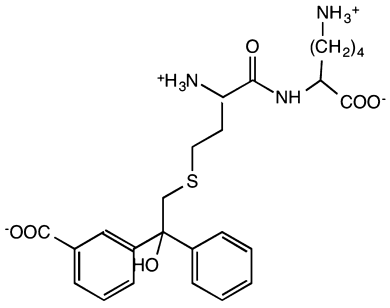


3-carboxy benzophenone

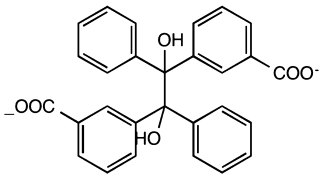
Final compounds



(i)



(ii)



(iii)

[a] Similar compounds were formed with Lys-Met.

dipeptides is related to the presence of such sequences in peptides and proteins like Met-Lys-bradykinin, an important neurotransmitter responsible for lowering of blood pressure.[29] In addition, in histones, many lysine residues are in the vicinity of methionines, as seen on the crystal structure. The oxidation was investigated by the combination of laser flash photolysis and steady-state UV irradiations combined with liquid chromatography−mass spectrometry (LC−MS) techniques. The use of both time-resolved and stationary techniques allowed us to perform the detailed analysis of initially formed transient radical species and of the final oxidation products.

The triplet 3-carboxybenzophenone (3CB) used in this work was shown to accept an electron from the sulfur moiety of methionine amino acid, thus yielding a charge transfer complex $[3CB^{\bullet-}\cdots>S^{\bullet+}]$[30] (Scheme 1). In the initial steps of the photoreaction, this complex can undergo different reaction pathways: back electron transfer (k_{BT}), separation of radical ions (k_{sep}), "in cage" proton transfer from the Met moiety to $CB^{\bullet-}$ (k_H), and the proton transfer from the protonated amino group of the peptide to the radical anion $CB^{\bullet-}$ (k_{NH}) (Scheme 1).

The progress of the process was monitored by resolving the transient spectra after selected delay times with respect to the

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laser pulse, and the concentration profiles for individual components were calculated from the multicomponent spectra.[31,32]

EXPERIMENTAL METHODS

Met-containing dipeptides Met-Lys, Lys-Met (BACHEM) and the 3-carboxybenzophenone (3CB) (Sigma-Aldrich) (CHART 1) were purchased with the best available grades and were used without further purification. Water was purified with a Millipore Milli-Q system and Elga maxima system (resistivity 18.2 MΩ cm).

Laser Flash Photolysis. Laser flash photolysis experiments were performed using 3CB as a photosensitizer. The setup has been described in detail elsewhere.[10] Briefly, this setup uses a Nd:YAG laser with a 355 nm excitation wavelength as a pump (Spectra-Physics) and a pulsed Xe lamp as a probe of the excited sample. The pulse duration was 6−8 ns. All flash photolysis experiments were carried out in rectangular quartz fluorescence cells (path length 10 mm). All solutions were prepared in purified water. pH was adjusted by adding potassium hydroxide and/or perchloric acid using a Mettler Toledo model Five Easy FE20 pH meter equipped with a semimicro InLab electrode from Mettler Toledo. Aqueous solutions (unbuffered) were prepared shortly prior to each experiment and were deoxygenated by bubbling high-purity argon through them for 20 min. The solutions were stirred during the experiments. Kinetic traces were taken between 360 and 700 at 10 nm intervals. The time-resolved absorption spectra were constructed from kinetic traces. Ten laser pulses were averaged for each kinetic trace in recording spectra and 30 in quenching experiments. The concentrations of Met-containing peptides in the laser flash photolysis experiments were in the range from 1×10^{-5} to 6×10^{-3} M (in the quenching experiments) and from 6×10^{-3} M to 2×10^{-2} M (for recording spectra in the time range following at least 99% quenching of the triplet state of 3CB. The concentration of 3CB was kept constant at 2×10^{-3} M in the quenching experiments and 4×10^{-3} M for recording spectra. All experiments were performed at room temperature (21 ± 1 °C).

Steady-State Photolysis. All experiments were performed in a 1 × 1 cm rectangular cell on an optical bench irradiation system using a Genesis CX355STM OPSL laser from Coherent, with 355 nm emission wavelength (the output power used was set at 50 mW). A solution of the Hatchard-Parker actinometer ($K_3[Fe(C_2O_4)_3]$) was used to measure the intensity of UV light.[33] The duration of the irradiation time was chosen to cause about 10−30% of 3CB conversion. The changes in the dipeptides and 3CB concentration during irradiation were determined by UV−vis spectroscopy using a Beckman Coulter DU 800 and Varian Cary 300 Bio spectrophotometers and by RP HPLC (Waters 600 setup with Waters 2996 photodiode array detector and Agilent 1260 Infinity with an Agilent 1260 photodiode array detector). Chromatographic analyses were done using a Waters column XBridge BEH136 C18, 3.5 μm, 4.6 × 150 mm. The concentration of Met-containing peptides and 3CB in steady-state photolysis was kept constant at 1×10^{-3} M. Solutions were purged with Ar for 30 min and were stirred during irradiations. The pH of the samples was adjusted by adding KOH or $HClO_4$.

Analysis of raw transient absorption spectra is difficult due to possible formations of transients with similar absorption features. For this reason spectra were resolved into component transients by a linear regression technique, absorption additive law, and reference spectra of possible transients using the following equation:

$$\Delta A(\lambda_j) = \sum_{i=1}^{n} \epsilon_i(\lambda_j) a_i \quad (1)$$

where $\Delta A(\lambda_j)$ is the sum of the absorbances of all species at λ_j, $\varepsilon_i(\lambda_j)$ is the molar absorption coefficient of the i^{th} species at the j^{th} wavelength of observation, and a_i is equal to the concentration c_i multiplied by the path length. This method has been described elsewhere.[6] Here, the transients considered were the excited triplet state from the sensitizer ^{3}(3CB)*, the ketyl radical 3CBH$^{\bullet}$, the radical anion 3CB$^{\bullet-}$, and the radical cations coming from the peptides symbolized by the 2c-3e bond between sulfur and O, N, or S atoms, respectively (S.·.O), $(S.\cdot.N)^+$, or $(S.\cdot.S)^+$. For the latter species, we assume that their absorption spectra are identical to those of simple sulfur-containing compounds.[34]

Mass Spectrometry. In order to be sure that all final compounds were detected, in a first step, the solution was always injected without separation by high-performance liquid chromatography (HPLC). In a second step, we used HPLC-MS. Two eluents in the HPLC analysis were used for the separation and isolation of substrates and stable products after irradiation: eluent A [water with 0.05% of trifluoroacetic acid (TFA)] and eluent B [95% of acetonitrile (AcN) with 5% of water and 0.03% of TFA]. After photolytic oxidation, samples were analyzed with the gradient mode starting from 100% of eluent A to 100% of eluent B in 30 or 45 min (depending on the compound). The mass spectra were recorded using a Bruker Esquire 3000$^+$ with a Paul ion trap. Solutions were diluted before recording mass spectra using water:methanol or water:acetonitrile and a small amount of formic acid (100/200 μL of the sample in 1 mL of solution). Compounds were ionized by electrospray ionization (ESI) under the following conditions: a flow rate of 180 μL/min, a spray voltage of 4500 V, a dry gas flow of 5.5 L/min, and a drying gas temperature of 250 °C. Mass spectra were analyzed using standard Bruker Esquire Control. Tandem mass spectrometry experiments were carried out using collision-induced dissociation with Helium atoms inside the Paul ion trap (radiofrequency 0.18−0.25). CO_2 analysis was performed using liquid chromatography by means of a Dionex ISC-900 with Ion Pack ICE-ASI 4 × 250 mm analytical column. Mass spectra interpretations were performed with help of massXpert.[35]

RESULTS AND DISCUSSION

Laser Flash Photolysis. *A. Triplet-Quenching Rate Constants.* The rate constants (k_q) for the ^{3}(3CB)* (excited triplet state) quenching by the dipeptides Met-Lys and Lys-Met were measured by monitoring the decay of the triplet−triplet absorption of the ^{3}(3CB)* at 520 nm (maximum of 3CB triplet−triplet absorption) (Figure S1 of the Supporting Information). The concentration of 3CB was set to 2×10^{-3} M, while the concentration of quencher was varied from 1×10^{-4} M up to 6×10^{-3} M. The pH was set to 6 or 11 (depending on the required experimental conditions). Linear least-squares fits of k_{obs} vs [Q] plots (Stern−Volmer plot) were used to calculate k_q with pseudo-first-order rate constant equations:

$$k_{obs} = (\tau_T)^{-1} + k_q[Q] \quad (2)$$

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where τ_T is the lifetime of ^{3}(3CB*) in the absence of quencher, and [Q] is the concentration of quencher (here the dipeptides). The rate constants are given in Table 1. They do not differ

Table 1. Rate Constants for Quenching of 3CB Triplet State by Met-Lys and Lys-Met in Aqueous Solution

	$k_q \times 10^{-9}$ (M^{-1} s^{-1})	
quencher	pH 6	pH 11
Met-Lys	1.81 ± 0.06[a]	1.32 ± 0.06[a]
Lys-Met	2.47 ± 0.08[a]	1.78 ± 0.04[a]

[a]Errors taken from double standard deviation; initial concentration of 3CB was 2 mM.

from those obtained for 4CB quenching by different Met-derivatives and Met-containing peptides.[18,34] This indicates the participation of the Met residue in the quenching event. The quenching rate constant by Met-Lys was a bit smaller than that of Lys-Met. Increasing the pH from 6 to 11 led to a lowering of the k_q value for both peptides. The global charge of 3CB in aqueous solution is negative, while the charge of the peptide changes with pH, mainly from 1 to −1. This small effect of pH on k_q can be explained by the Coulombic repulsion between two negatively charged molecules.

B. Resolution of Transient Spectra from Laser Flash Photolysis. Absorption spectra of aqueous solutions containing 3CB and the dipeptides were examined before laser flash photolysis to eliminate possible ground-state association. No evidence for association reactions involving the ground state of 3CB was found under the experimental conditions.

Met-Lys. The transient spectra recorded in Met-Lys and 3CB aqueous solutions are presented in Figure 1. The spectrum obtained 60 ns after the laser flash exhibited a maximum at 520 nm. It was assigned to the excited triplet state of 3CB.[30] Within 220 ns, the maximum shifted to 550 nm (pH 6), while the increase of pH caused a further red shift of the maximum to about 600 nm (Figure 1b). The band at 550 nm was assigned to the ketyl radical 3CBH$^\bullet$. The ketyl radical can easily deprotonate at higher pH (pK_a = 9.5) yielding the ketyl radical anion, 3CB$^{\bullet-}$ peaking at 600 nm.[10,30]

The resolution of spectra coming from the reaction of Met-Lys with 3CB at pH 6 after 100 ns and 7 μs is presented in Figure S2 of the Supporting Information. The triplet state from 3CB was still present in the solution 100 ns after the laser pulse; however, the ketyl radical 3CBH$^\bullet$ was already in equilibrium with the radical anion 3CB$^{\bullet-}$. As for the peptide, the resolution indicated the formation of the two-centered three-electron intramolecular radical cation that may be $(S.\cdot.N)^+$. It is difficult to distinguish between $(S.\cdot.O)$ and $(S.\cdot.N)^+$ due to their similar absorption spectra. However, the fitting of transient spectra using $(S.\cdot.N)^+$ as a component gave satisfactory results. A similar situation took place in basic solutions, meaning that four species were present 100 ns after the laser pulse: $(S.\cdot.N)^+$, ^{3}(3CB)*, 3CBH$^\bullet$, and 3CB$^{\bullet-}$ (Figure S3 of the Supporting Information). The resolution of spectra taken after 7 μs contained only two species: $(S.\cdot.N)^+$ and 3CBH$^\bullet$ at pH 6 or 3CB$^{\bullet-}$ in the basic medium.

The spectral resolution allows the extraction of the time-dependent concentration of the transient. Examples of concentration profiles at neutral and high pH for Met-Lys are presented in Figure 2. Clearly, the pH influences the decay of $(S.\cdot.N)^+$. It seems that the $(S.\cdot.N)^+$ radical cation of Met-Lys has a longer lifetime in neutral solutions than in basic medium. To assess the role of the pH on the $(S.\cdot.N)^+$ lifetime, the quenching of ^{3}(3CB)* by Met-Lys was examined at different pH values in the 6−11 range. Indeed, in basic solutions (pH > 9), the decay of the $(S.\cdot.N)^+$ radical cation becomes biexponential. This result is similar to the behavior of Met-Gly at high pH already discussed in the work of Hug et al.[36] (Scheme 2 a). The acceleration of the $(S.\cdot.N)^+$ decay with increasing pH was explained by the reaction between $(S.\cdot.N)^+$ and OH^- and formation of some unspecified intermediate called SNOH. The appropriate rate constants for Met-Lys of this equilibrium were similar to those calculated for Met-Gly (Table 2). In addition, it was proposed that, instead of the adduct SNOH, the basic form of $(S.\cdot.N)^+ - (S.\cdot.N)$ could be formed via OH^--assisted water elimination (fast component, k_1), while the slow component was assigned to the second-order decay of the basic form $(S.\cdot.N)$ (Scheme 2 b).[37] The k_3 value comes from the decay of the $(S.\cdot.N)^+$ radical cation at low OH^- concentration (in case of Met-Lys at pH 6.5). The decay rate constant of $(S.\cdot.N)^+$ at pH close to neutral was ten times higher than for Met-Gly. It may be explained by the influence of lysine, that has an additional positive charge.

Lys-Met. The transient absorption spectra for the reverse sequence peptide were slightly different from those observed with Met-Lys (Figure 3), especially at pH 6 and in the UV region. The band with a maximum at 520 nm, assigned to ^{3}(3CB)* was present in both cases at short delay times. However, this maximum did not shift to longer wavelengths

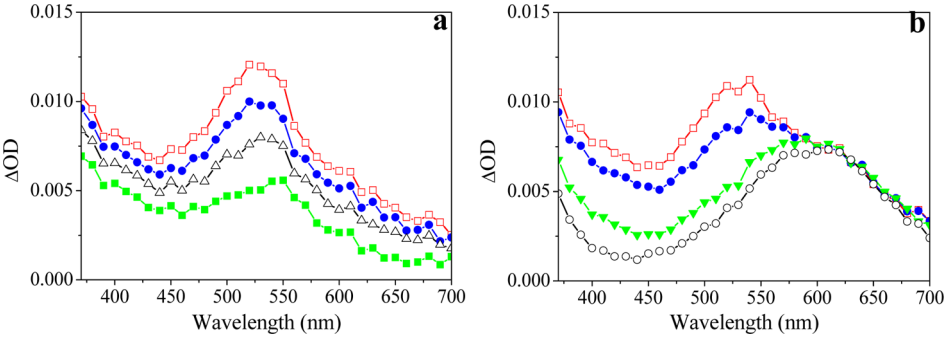

Figure 1. Evolution of transient absorption spectra from 3CB-sensitized oxidation of Met-Lys (6 mM) at pHs (a) 6 and (b) 11. The concentration of 3CB was 2 mM. The time-resolved laser flash photolysis was performed under the following conditions: energy of laser pulse ∼5 mJ, excitation wavelength 355 nm. Spectra were recorded at different delays following the laser pulse. (a) red □, 60 ns; blue ●, 80 ns; Δ, 120 ns; green ■, 220 ns. (b) red □, 70 ns; blue ●, 120 ns; green ▼, 300 ns; and ○, 6 μs.

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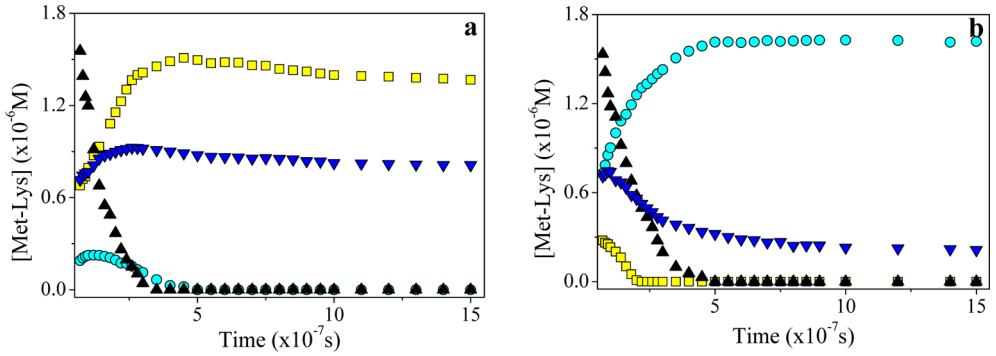
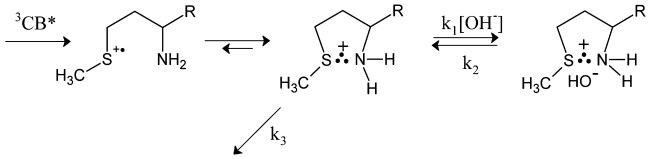


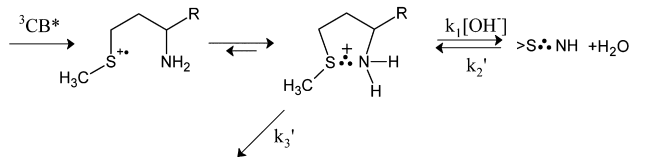
Figure 2. Concentration profiles of transients (▲) 3CB triplet state, (turquoise ●) $3CB^{\bullet-}$ radical anion, (yellow ■) $3CBH^{\bullet}$ ketyl radical, and (blue ▼) $(S.\cdot.N)^+$ radical cation in the laser flash photolysis of an aqueous solution of Met-Lys (6 mM) and 3CB (2 mM) at (a) neutral pH (≈ 6) and (b) pH = 11.1.

Scheme 2. Two Possible (Not Concurrent) Rearrangements of the Sulfur Radical Cation of Met-Lys in Basic Solution[a]

a



b



[a]The procedure of extracting the rate constants was published in refs 36 and 37.

Table 2. Rate Constants of Reactions Defined in Scheme 2, Calculated for Met-Lys and Compared with Literature Values Obtained for Met-Gly

	Met-Gly[36]	Met-Lys
k_1 (M^{-1} s^{-1})	(6.0 ± 1.0) × 10^{9a}	(2.3 ± 0.9) × 10^{9a}
k_2 (s^{-1})	(1.3 ± 0.7) × 10^{6a}	(1.2 ± 0.8) × 10^{6a}
k_3 (s^{-1})	2.0 × 10^{4a}	1.4 × 10^{5a}

[a]Errors taken from double standard deviation. k_3 is the $(S.\cdot.N)^+$ decay rate constant in neutral pH.

with the time after the triplet decayed in neutral solution (see Figure 3a). Instead a broad band was observed between 500 and 550 nm resulting of the overlap of two bands belonging to $CBH^{\bullet}$ and to the dimeric peptide radical cation stabilized by an intermolecular $(S.\cdot.S)^+$ 2c-3e bond whose maximum is located around 480 nm[14,38]). Increasing the concentration of peptide (10 and 20 mM), the intensity of the band was higher, and it was more visible. In the basic medium, the absorption band at 520 nm assigned to triplet state was observed at short delay times, and it was shifted to 600 nm (see Figure 3b). This latter band belonged to the $3CB^{\bullet-}$ radical anion.

The spectral resolution for Lys-Met 7 μs after the pulse is presented in Figure 3 (panels c and d). The $(S.\cdot.N)^+$ radical cation was short-lived ($\tau \sim 100$ ns), and its decay led to the formation of the dimeric peptide radical cation $(S.\cdot.S)^+$. This radical cation was present in basic and neutral solutions and remained visible for 6−7 μs after the laser pulse in neutral solutions. In accordance with Figure 4, the concentration profiles indicate that the yield and the decay of the $(S.\cdot.S)^+$ radical cation are dependent on the pH of the solution. Its lifetime was longer in neutral solutions ($\tau_{SS+} = 2.1 \times 10^{-5}$ s) than in basic medium ($\tau_{SS+} = 1.3 \times 10^{-5}$ s). The evolution of the spectra and their resolution into components for 3CB triplet quenching (2 mM of 3CB) with 20 mM of Lys-Met at pH 6 with appropriate kinetics are presented in Figures S4 and S5 of the Supporting Information. The decay rate constant of the $(S.\cdot.N)^+$ radical cation formed in Lys-Met at pH ∼6 was proportional to the peptide concentration (rate constant 8.5 × 10^8 M^{-1} s^{-1} with an estimated ±30% error) (Figure 5 a), while the rate constant for the growth of $(S.\cdot.S)^+$ was calculated (as in the case of $(S.\cdot.N)^+$ decay from concentration profiles) to be 1.1 × 10^9 M^{-1} s^{-1}. The $(S.\cdot.N)^+$ radical cation decay was followed by the formation of $(S.\cdot.S)^+$, as seen in Figure 5b. Thus, it can be proposed, that the dimeric $(S.\cdot.S)^+$ observed in the case of Lys-Met oxidation is formed in some extent with the participation of $(S.\cdot.N)^+$.

C. Quantum Yields. The quantum yields could be calculated by performing experiments under conditions whereby the quenching of the triplet state was almost complete (∼99%). They are presented in Table 3. The photochemical yields of $3CB^{\bullet-}$, $3CBH^{\bullet}$, and other transients were calculated at 250 ns, from concentration profiles and actinometry (see materials and methods). The yields of $3CB^{\bullet-}$ and of $3CBH^{\bullet}$ were the same within uncertainty with both peptides. In the case of Met-Lys in neutral solutions, where the peptide amino group was protonated, the $\Phi_{S.\cdot N^+}$ corresponds to the yield of k_{NH} process.

D. Stable Products Analysis. Stable products were formed using either a UV lamp (excitation wavelength 365 nm) or a continuous laser (excitation wavelength 355 nm). HPLC with UV−vis detection was used to determine the quantum yields and to separate and analyze the stable oxidation products. The chromatograms corresponding to the mixture containing Met-Lys and 3CB before and after laser irradiation at pH 6 are presented in Figure 6. The detection wavelength was adjusted to 210 nm for the peptide products and to 330 nm for the quantification of the 3CB disappearance. The peak with retention time 24.8 min was observed before irradiations and was assigned to 3CB. The peptide was eluted with the front of the phase. The laser irradiation caused the appearance of a group of peaks between 17 and 20.2 min and two well-separated products at 25.5 and 26 min retention times.

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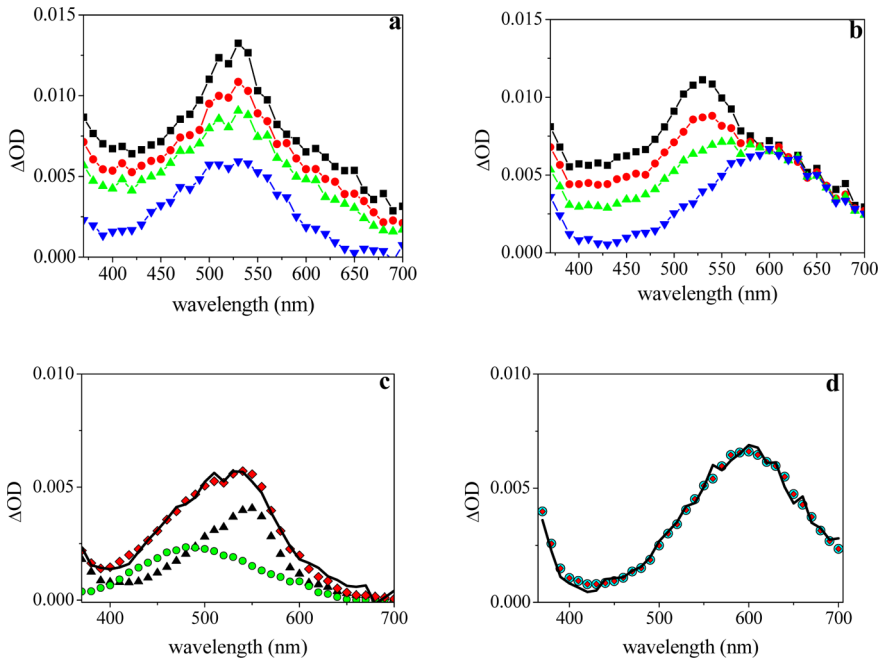

Figure 3. Evolution of the transient absorption spectra from the 3CB-sensitized oxidation of Lys-Met (6 mM) at pHs (a) 6 and (b) 11. The concentration of 3CB was 2 mM. The time-resolved laser flash photolysis was performed under following conditions: energy of laser pulse ∼5 mJ, wavelength of excitation 355 nm. Spectra recorded after different time delays from the laser flash. (a) ■, 70 ns; red ●, 100 ns; green ▲, 140 ns; blue ▼, 6 μs. (b) ■, 70 ns; red ●, 120 ns; green ▲, 200 ns; and blue ▼, 8 μs. Resolution of the spectral components in the transient absorption spectrum 7 μs after laser pulse following quenching of the 3CB triplet state by 6 mM Lys-Met at pHs (c) 6 and (d) 11 in an aqueous solution, [3CB] = 2 mM. Black line, experimental spectrum, red ◊, sum; green ●, $3CB^{\bullet-}$ radical anion; ▲, $3CBH^{\bullet}$ ketyl radical; and turquoise ●, $(S.\cdot.S)^{+}$ radical cation.

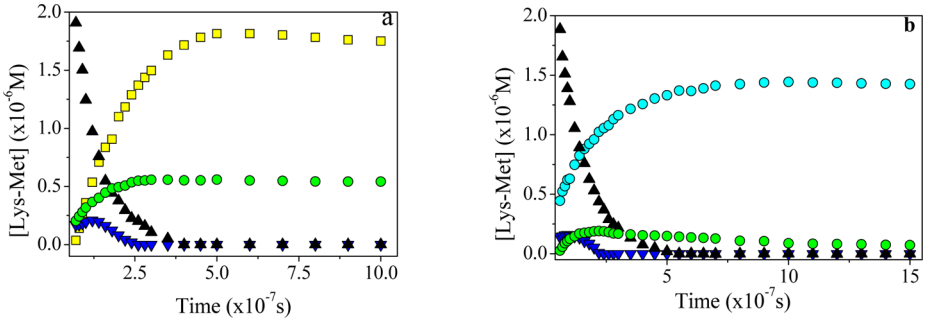

Figure 4. Concentration profiles of transients: ▲, 3CB triplet state; ●, $3CB^{\bullet-}$ radical anion; ■, $3CBH^{\bullet}$ ketyl radical; ▼, $(S.\cdot.N)^{+}$ radical cation; and ●, $(S.\cdot.S)^{+}$ radical cation in the laser flash photolysis of an aqueous solution of Lys-Met (6 mM) and 3CB (2 mM) at (a) neutral pH (≈ 6) and (b) pH = 11.2.

In the mass spectra of a nonirradiated mixture (reference samples) (Met-Lys 1 mM with 3CB 1 mM, pH 6) two peaks at *m*/*z* 278 and 227 (Figure 7 top) were present. The peak at *m*/*z* = 278 (z = 1) was assigned to the protonated dipeptide [Met-Lys + H^+] and was the main peak. The peak at *m*/*z* = 227 had a much lower intensity due to the difficult protonation of the 3CB molecule. After irradiation of samples containing 1 mM 3CB and 1 mM Met-Lys, a new peak appeared at *m*/*z* = 504 (*z* = 1) at both pH values 6 and 11 (Figure 7 middle for pH ∼ 6). Besides this peak, other minor products with *m*/*z* = 437 and 455 were identified as products formed after the 3CB-sensitized oxidation. The fragmentation of the ions with *m*/*z* of 504, showed the presence of an ion with *m*/*z* = 278, which belongs to the unchanged protonated dipeptide. This suggests that this product was an adduct of Met-Lys and 3CB. The exact monoisotopic *m*/*z* of this peak (504.217) is in agreement with the calculated elemental composition of such an adduct. Separation of the compounds by HPLC followed by MS analysis showed that all of the compounds eluted between 17 and 20.2 min exhibited the same *m*/*z* = 504. This means that those products were isomers of the adduct. An example of an MS analysis of the fraction with retention time 18 min is presented in Figure S6 of the Supporting Information. The pH change from 6 to 11 did not influence the formation of the photoadduct [peptide + 3CB] significantly.

The change of sequence from Met-Lys to Lys-Met and the change of pH from 6 to 11 led to similar addition products. The fragmentation spectra using collision-induced dissociation ($CID\text{-}MS^2$) spectra of the ion with *m*/*z* = 504 from Lys-Met and 3CB irradiations had the same pattern as for Met-Lys [i.e., the loss of a water molecule (−18 Da) and the peak from dipeptides (*m*/*z* = 278 Da)]. The quantum yields for the disappearance of substrates and the formation of adduct are presented in Table 4. Possible structures for the photoadducts are presented in Chart 1, panels i and ii.

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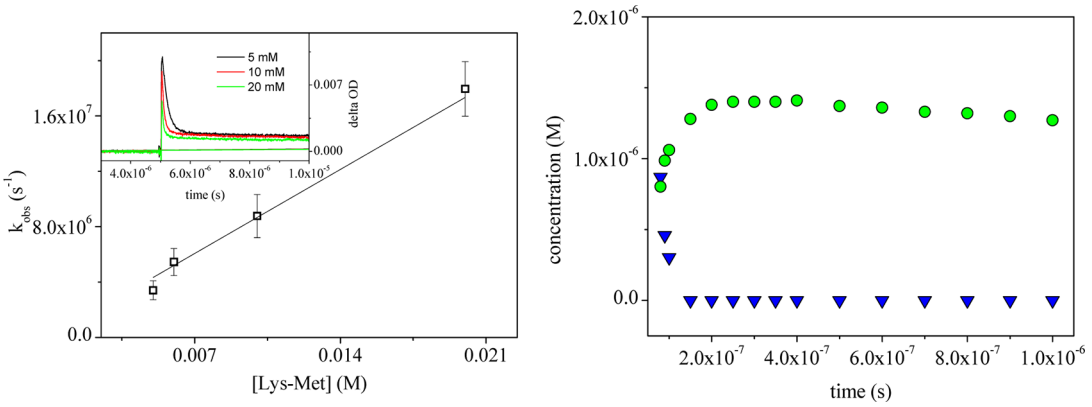

Figure 5. Left: the dependence of the $(S.\cdot.N)^+$ decay rate constant on the concentration of Lys-Met at pH ~6. Inset: the transient absorption traces recorded at 390 nm for 3CB (2 mM) sensitized oxidation of Lys-Met 5 (green), 10 (red), and 20 (black) mM. Right: concentration profiles of transients: blue ▼, $(S.\cdot.N)^+$ radical cation and green ●, $(S.\cdot.S)^+$ radical cation in the laser flash photolysis of an aqueous solution of Lys-Met (20 mM) and 3CB (2 mM) at neutral pH (≈6).

Table 3. Quantum Yields of Transients for 3CB-Sensitized Photo-Oxidation of Met-Containing Dipeptides in Aqueous Solution*

peptide	pH	$\Phi_{CB^{\bullet-}}$	$\Phi_{CBH^{\bullet}}$	$\Phi_{S.\cdot S^+}$	$\Phi_{S.\cdot N^+}$
Met-Lys (6 mM)	6.5	~0.03	0.55	–	0.33
	9.2	0.35	0	–	0.32
	10.1	0.45	0	–	0.27
	11.0	0.62	0	–	~. 0.12
Lys-Met (6 mM)	6.1	0	0.63	0.19	0
	11.2	0.58	0	0.06	0
Lys-Met (10 mM)	6	–	0.73	0.27	0.05
Lys-Met (20 mM)	6	–	0.83	0.42	0.05

*[3CB] = 2 mM.

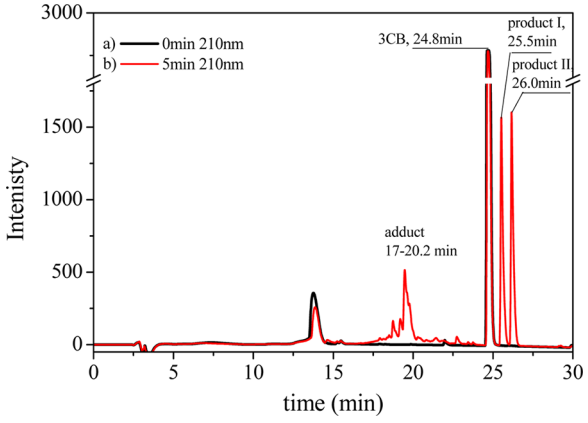

Figure 6. HPLC chromatogram of solution containing 100 μL of Met-Lys (4 mM) and 3CB (2 mM) in 900 μL of water at pH 6 (a) before irradiation, (b) after irradiation of a deoxygenated solution by continuous laser, irradiation time 5 min, laser energy 60 mW, excitation wavelength 355 nm.

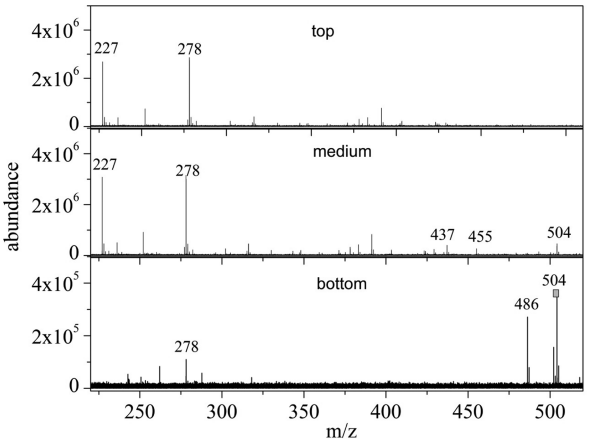

Figure 7. ESI-FT-ICR-MS of solution containing 1 mM of Met-Lys and 1 mM 3CB at pH 6. Top: before irradiation; middle: after irradiation of deoxygenated solution by laser, irradiation time 5 min, laser energy 50 mM, excitation wavelength 355 nm; bottom: CID spectra of ion with m/z 504 from sample irradiated 60 min with lamp (365 nm). MS analysis of 200 μL in ACN:water 1:1 with 1% of formic acid.

Table 4. Quantum Yields of 3CB Disappearance and Peptide-3CB Adduct Formation during Irradiation by Laser (λ_{ex} = 355 nm)*

	pH	$\Phi_{\text{disapp. 3CB}}$	$\Phi_{(\text{product (i)adduct}}$
Met-Lys + 3CB	6	0.34	~0.30
	10	0.34	~0.30
Lys-Met + 3CB	6	0.19	0.12
	10	0.24	0.17

*The peptide concentration was 1 mM.

The MS analysis of the products eluting at 25.5 and 26 min showed that they came from 3CB radical recombinations such as the benzopinacol-like products[39] with two additional carboxylic groups (in Chart 1, iii).

Moreover, for none of these peptides the sulfoxide was found. The permutation going from Met-Lys to Lys-Met causes only a lowering of the quantum yield of the major product (i.e., the adduct with 3CB). It is consistent with the lower yield of sulfur free radicals (Table 3).

The decarboxylation reaction was also searched in both peptides. CO_2 was not detected by gas chromatography, showing that there was no decarboxylation in natural or in basic medium irrespective of the irradiation time and the laser power used. In addition the MS analysis did not show any product that could be formed after the decarboxylation of Lys-Met.

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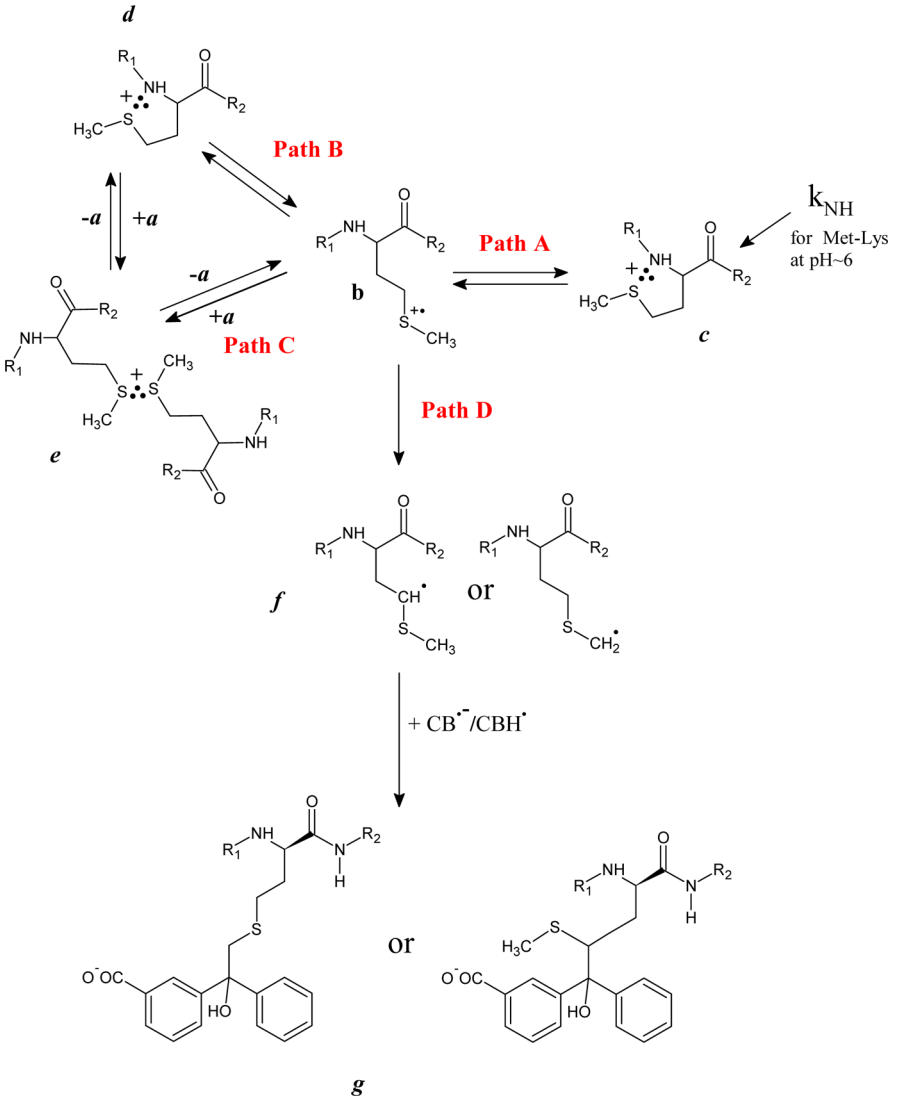
Scheme 3. Illustrative Reaction Scheme of Oxidation of Met-Containing Peptides[a]



[a]Met-Lys, species **b**, **f**: R_1 = −2 H (pH 6) or H (pH 11), R_2 = −Lys; species c and d: R_1 = H, R_2 = Lys Lys-Met, species **b**, **c**, **d**, **e**, **f**, **g**, R_1 = Lys, R_2 = −O−, **a**″ is a nonoxidized peptide.

CONCLUSION

The mechanisms of the 3CB sensitized photo-oxidation of Met-Lys and Lys-Met have the same primary steps as in the case of Met and its derivatives published previously (Scheme 1).[10,40,41] The global mechanism for the oxidation of these peptides is summarized in Scheme 3.

The sequence of the peptide was found to have some influence on the nature of the transients, however it had no influence on the final products. In both peptides, sulfur-centered radical cations **b** were initially formed (Scheme 3). In Met-Lys peptide (N-terminal Met), the **b** radical cation was stabilized by an intramolecular $(S.\cdot.N)^+$ species, that decayed with lifetimes that differed depending on the pH. This pH-dependent behavior was rationalized in Scheme 2.[36,37] At neutral pH, proton transfer from the protonated amino group in the peptides to the $CB^{\bullet-}$ radical anion was followed by the formation of the ketyl radical $CBH^{\bullet}$ and the ***c*** radical cation.[42] However, the quantum yield for the formation of ketyl radical was higher than that for $(S.\cdot.N)^+$. This can be explained by the reaction pathway of k_H (Scheme 1). A similar **d** radical cation $(S.\cdot.N)^+$ (path B) was formed with Lys-Met, but its decay rate constant was found to depend on the concentration of the peptide possibly leading to **e**.

The irreversible reaction path would be a deprotonation of **b** or **e** in the case of Lys-Met, leading to the formation of carbon-centered α-S radicals (**f**) (Scheme 3). This radical is known to be quite stable in the absence of scavengers and is well-characterized in the literature.[14] However, in the reaction investigated here, it is scavenged by the 3CB derived ketyl radicals or radical anions. Another source of *f* radicals is the proton transfer reaction from $>S^{\bullet+}$ to $CB^{\bullet-}$ (Scheme 1). Path d was also observed in pulse radiolysis experiments for these peptides (unpublished), where the relatively long-lived α-(alkylthio)alkyl radical could be directly observed (λ_{max} = 290 nm).[20]

The peptide-3CB adduct was the only stable product detected coming from the addition of 3CB ketyl radicals ($3CB^{\bullet-}$ or $3CBH^{\bullet}$, depending on the pH) to the α-S radical (m/z = 504). A very small amount of peptide dimer could be detected but the corresponding peak was in the noise.

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A similar experiment was performed in the presence of oxygen. As expected, a substantial amount of the triplet state was quenched. However, the final products (i.e., 3 CB dimer and adduct 3 CB-peptide) were the same with much lower yields. Finally, in MS, we have never measured a mass corresponding to a hydroperoxide (+32).

It was found that methionine offered protection against oxidation to the lysine residue. Recently, Ronsein et al. found the formation of a sulfilimine bond between oxidized methionine and lysine,[43] which would result in the loss of 2 Da from the mass of the peptide. Such a mass was found after oxidation by $^{\bullet}$OH radicals for Lys Met only.[27] However, the infrared spectra recorded by IRMPD still exhibited the umbrella band of the amine, showing that there was no S=N double bond (not shown). Thus, the formation of such a compound was ruled out.

In conclusion, as far as the final stable products are concerned, the major event is the transformations that led to carbon-centered radicals, α-(alkylthio)alkyl radicals, produced mostly from the deprotonation of the S-centered radical cation. Despite the similar mechanisms of $^{\bullet}$OH and ^{3}(3CB*)-induced oxidation of Met-containing peptides, different products were identified (e.g., sulfoxide or photoadduct). However, all of the products were formed with the participation of the same precursor-α-S radicals. For the first time, we show that the addition products of methionine and sensitizer replace the well-known oxidation product: methionine sulfoxide. Similar reactions could take place in biological media in the presence of natural photosensitizers.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1: The Stern−Volmer plot for the quenching of excited triplet state of 3CB by Lys-Met in aqueous solution at pHs 6 and 10. Figure S2: resolution of the spectral components in the transient absorption spectrum following quenching of 3CB triplet state by Met-Lys. Figure S3: resolution of the spectral components in the transient absorption spectrum after laser pulse following quenching of the 3CB triplet state by Met-Lys. Figure S4: evolution of transient absorption spectra and kinetic traces from 3CB-sensitized oxidation of Lys-Met at pH 6. Figure S5: resolution of the spectral components in the transient absorption spectrum following quenching of 3CB triplet state by Lys-Met. Figure S6: ESI-MS of the fraction coming from the HPLC analysis of irradiated Lys-Met and 3CB at pH 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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