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HYDROXYL RADICAL-INDUCED CROSS-LINKING OF THYMINE AND LYSINE: IDENTIFICATION OF THE PRIMARY STRUCTURE AND MECHANISM

Syota Morimoto,[a] Hiroshi Hatta,[a] Shin-ichi Fujita,[b] Tomochika Matsuyama,[c] Tôru Ueno[a] and Sei-ichi Nishimoto*[†a]

[a]*Department of Energy and Hydrocarbon Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-8501,*
[b]*Research Institute of Advanced Technology, University of Osaka Prefecture, Osaka 599-8231, and* [c]*Research Reactor Institute, Kyoto University, Osaka 590-0400, Japan*

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Abstract: Hydroxyl radical-induced formation of a cross-link of thymine (Thy) and lysine (Lys) in the γ-radiolysis of N_2O-saturated aqueous solution was studied. A Thy-Lys cross-link (**I**) of the formal structure that OH radical and 4-carbon-centered Lys radical added respectively to C(5) and C(6) positions of Thy was isolated by a preparative HPLC and identified by a FAB-HRMS. The primary cross-link **I** was dehydrated by treatment with HCl at 120 °C to yield the secondary structure (**II**) possessing a C(5)-C(6) double bond in the Thy moiety: the latter structure **II** was reported previously (Dizdaroglu, M.; Gajewski, E. *Cancer Res.* **1989**, *49*, 3463-3467). A pulse radiolysis study with a redox titration method indicated that 4-carbon centered Lys radical intermediate was of neutral redox reactivity in contrast to reducing reactivity of 5-hydroxy-5,6-dihydrothymin-6-yl radical intermediate. The cross-link **I** could be formed by a conventional radical recombination mechanism, but not by an ionic recombination mechanism involving a redox reaction between the radical intermediates.

Generation of excess free radicals in cells by exogeneous sources (e.g., UV and ionizing radiations, carcinogens) or endogeneous sources (e.g., normal cellular metabolism) causes potentially a variety of human diseases.[1] The carcinogenic, mutagenic, and lethal effects of ionizing radiation on living cells[2,3] are believed to be a consequence of various types of damages to cellular DNA by free radicals, especially by highly reactive hydroxyl (OH) radicals.[4-6] Formation of DNA-protein cross-links in nucleoprotein is among such radiation-induced damages[7-9] and has been studied intensively as well as modifications in base- and sugar-constitutes of DNA. A gas chromatography-mass spectrometry (GC-MS) has been employed to characterize trace amount of chemical modifications induced by free radicals in cellular DNA and chromatin.[10] The GC-MS characterization of cross-link structures which are composed of pyrimidine bases and various amino acids have been carried out in aqueous model systems[11-16] to get chemical insight into OH radical-induced DNA-protein cross-linking in cells.[17-19]

Previously,[14] using a GC-MS with a selected-ion monitoring (SIM) technique, Dizdaroglu and Gajewski detected a cross-link structure (**II**) or (**III**) of thymine (Thy) and lysine (Lys) among trimethylsilylated HCl-

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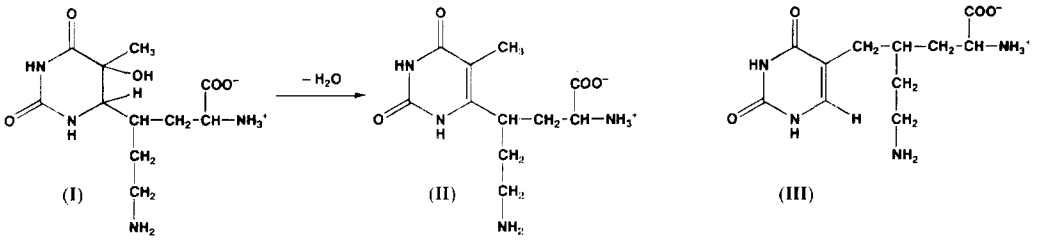


hydrolysates of calf thymus nucleohistone that was γ-irradiated in N_2O-saturated aqueous solution (OH radicals are generated under these conditions[5]). For selecting characteristic fragment ions to be monitored in the GC-MS/SIM analysis, a reference mass spectrum to the cross-link structure was first obtained from a model reaction system of γ-irradiated aqueous mixture of Thy and Lys after HCl-treatment and trimethylsilylation. The site of cross-linking on the Thy moiety producing **II** or **III** could not be identified from the previous GC-MS of trimethylsilylated cross-linked products. It was implicated that the formation of cross-link **II** might involve dehydration step of a primary product with the structure (**I**) *spontaneously or by acid hydrolysis*, while the cross-link **III** possessing C(5)-C(6) double bond in the Thy moiety could be a primary product.

We report herein identification by FAB-HRMS of the primary cross-link structure **I** that was isolated from a model γ-radiolysis system consisting of Thy and Lys. For better understanding of mechanism by which OH radicals induce cross-linking between Thy and Lys into the structure **I**, we characterized reactivity of radical intermediates by a redox titration method in the pulse radiolysis.[20]

Aqueous solutions of Thy (1 mM) and Lys (3-10 mM) were saturated with N_2O and irradiated with ^{60}Co γ-ray source at a dose rate of 141.6 Gy min^{-1} up to 17.0 kGy. Under these conditions of irradiation, 91% OH radicals and 9% H atoms are generated in the reaction system.[5] Figure 1 illustrates a representative HPLC chromatogram observed for aqueous mixture of Thy (1 mM) and Lys (10 mM) after 12.7-kGy γ-irradiation. The elution peaks 1 and 8 are assigned to Lys (positive ion FAB-LC-MS using glycerol (Gly) matrix provided characteristic ions at m/z 147 $[(M + H)^+]$ and 239 $[(M + H + Gly)^+]$) and Thy (m/z 127 $[(M + H)^+]$; 219 $[(M + H + Gly)^+]$), respectively. The peaks of 2, 3, 5, and 6 correspond to several oxidation products characteristic of OH radical reaction of Thy, as identified by reference to authentic samples.[21] The peak 4 is attributable

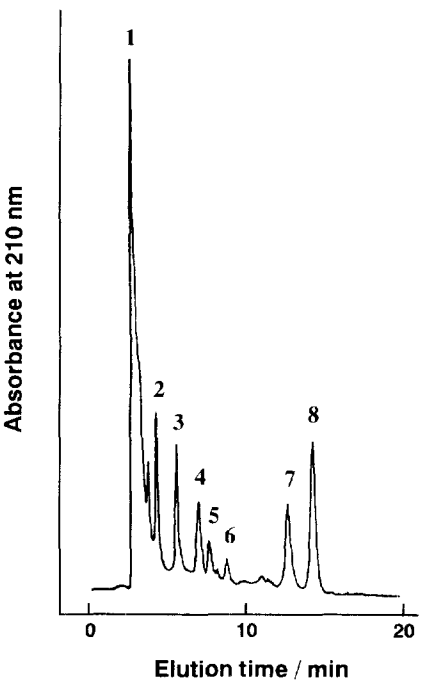


Figure 1. Reversed-phase HPLC analysis of aqueous mixture of Thy (1 mM) and Lys (10 mM) γ-irradiated to 12.7 kGy under N_2O at a dose rate of 141.6 Gy min^{-1}. The analysis was carried out on an ODS-type column (4.6 mm i.d.x150 mm) and phosphate buffer solution (pH 3.0) containing 2 vol% methanol was delivered at a flow rate of 0.6 ml min^{-1}: **1**, Lys; **2**, *cis*-thymine glycol; **3**, 5-(hydroxymethyl)uracil; **4**, unidentified product of OH-radical reaction of Lys; **5**, 6-hydroxy-5,6-dihydrothymine; **6**, N^1-formyl-N^2-pyruvylurea; **7**, Thy-Lys cross-link + 5,6-dihydrothymine; **8**, Thy.

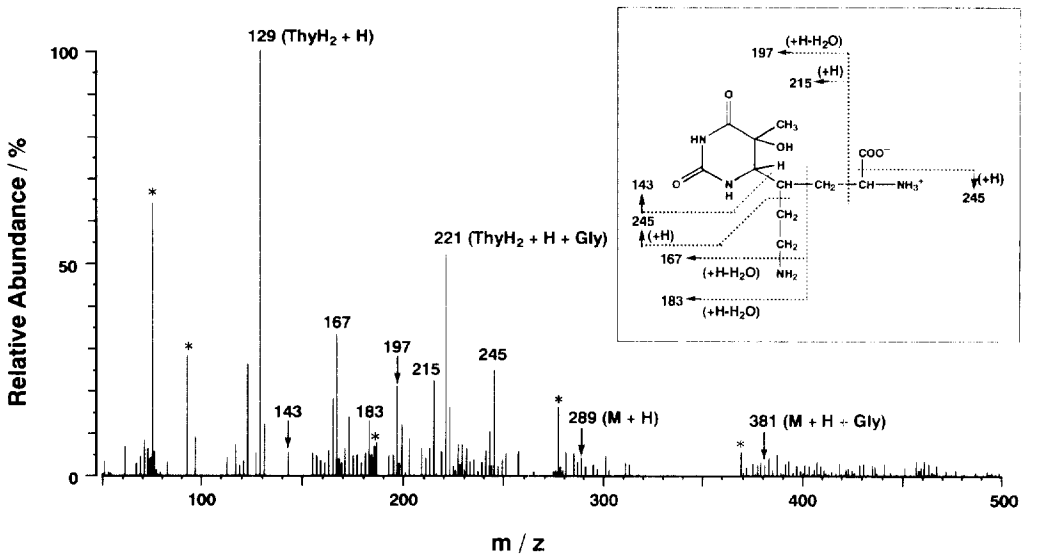


Figure 2. Positive ion FAB-LC/MS taken from the peak 7 in Figure 1 using xenon for FAB and glycerol (Gly) matrix. The ions marked by * originate from Gly.

to a product of OH radical reaction of Lys, since it was also obtained by a control γ-irradiation of Lys in N_2O-saturated aqueous solution. Although a FAB-LC-MS failed to identify the corresponding structure, a GC-EIMS of reaction mixture after trimethylsilylation demonstrated the formation of 4-hydroxylysine (see also Figure 3).

As confirmed by a positive ion FAB-LC-MS analysis (Figure 2), the peak 7 in Figure 1 contained a mixture of Thy-Lys cross-link **I** (m/z 289 [(M + H)$^+$], 4% relative abundance; 381 [(M + H + Gly)$^+$], 3%) and 5,6-dihydrotymine ($ThyH_2$: m/z 129 [(M + H)$^+$], 100%; 221 [(M + H + Gly)$^+$], 53%). Considerable yield of $ThyH_2$ in the γ-radiolysis of Thy in N_2O-saturated aqueous solution has been reported previously.[21] The fragment ions in the mass spectrum shown in Figure 2 are consistent with the Thy-Lys cross-link structure **I**: m/z 245 [(M + H - CO_2)$^+$ and/or (M + H - $CH_2CH_2NH_2$)$^+$], 25%; 215 [(M + H - $CH(COO^-)NH_3^+$)$^+$], 22%; 197 [(M + H - $CH(COO^-)NH_3^+$ - H_2O)$^+$], 22%; 183 [[(M + H - $CH_2CH(COO^-)NH_3^+$ - H_2O)$^+$], 13%; 167 [(M + H - NH_2 - $CH_2CH(COO^-)NH_3^+$ - H_2O)$^+$], 34%; 143 [(M - $CH(CH_2CH_2NH_2)CH_2CH(COO^-)$-$NH_3^+$)$^+$], 5%. The ions at m/z 197, 183 and 167 are accounted for by the dehydration of Thy moiety to form C(5)-C(6) double bond in the fragmentation processes. In light of a separate HPLC observation that the components at the peak 7 showed no absorption at 254 nm in contrast to Thy at the peak 8, we concluded that neither cross-link structure **II** nor **III** possessing a C(5)-C(6) double bond is contained in the peak 7. For further characterization of the cross-link structure, the elution peak 7 was also isolated by repeated preparative HPLC, evaporated to dryness, and then subjected to a direct positive FAB-HRMS (Gly matrix): calcd for $C_{11}H_{21}O_5N_4$ [(M + H)$^+$ of Thy-Lys cross-link **I**] 289.1512, found 289.1535 (15.7% relative abundance); calcd for $C_{11}H_{25}O_8N_2$ [(M + H + 2Gly)$^+$ of $ThyH_2$] 313.1611, found 313.1628 (100%).

The 12.5-kGy γ-irradiated aqueous reaction mixture that showed the HPLC profile in Figure 1 was evaporated to dryness. Aliquot (0.5 mg) of the residual solid was treated with 6 M HCl (1 ml) in an evacuated

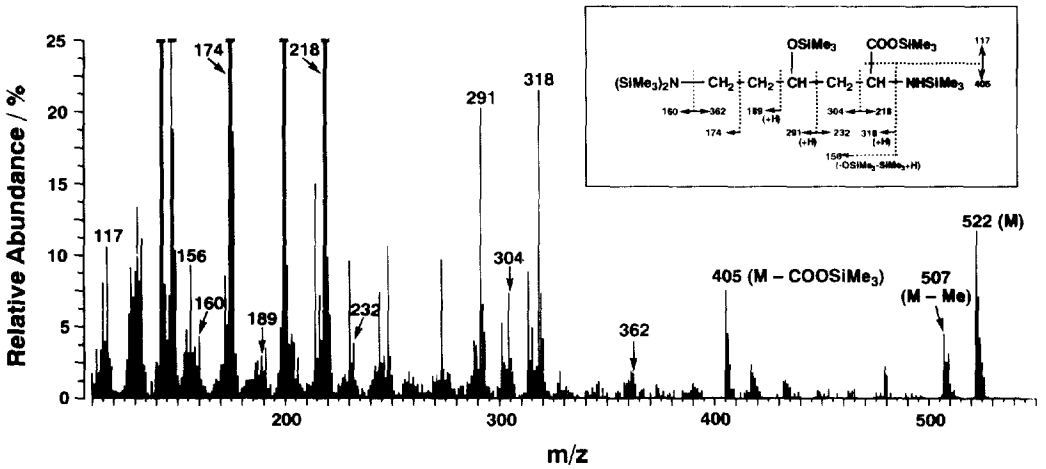


Figure 3. GC/EIMS of trimethylsilylated 4-hydroxylysine produced in the radiolysis of lysine in N_2O-saturated aqueous solution. The injection port, the ion source and the interface were maintained at 250°C. Separations were carried out using a fused silica capillary column (0.25 mm i.d.×50 m). Helium was used as the carrier gas and the mass spectra were obtained at 70 eV.

and sealed vial for 6h at 120 °C, evaporated to dryness. The solid mixture thus obtained was resolved in water and analyzed by HPLC. By reference to the profile in Figure 1, we found that all the major radiolysis products, but not $ThyH_2$ and unreacted residual Thy, were quantitatively hydrolyzed and thereby the corresponding elution peaks disappeared. It is noted that 43% of the component eluted at the peak 7 was decreased by the HCl treatment. Since $ThyH_2$ underwent no reaction by the HCl treatment, as confirmed by a separate experiment, the observed decrement is most likely due to dehydration of the Thy-Lys cross-link **I** into the structure **II** followed by further decompositions.

The hydrolysates obtained by the HCl-treatment and the evaporation were trimethylsilylated in a polytetrafluoroethylene-capped vial with 0.15 ml of an *N*-methyl-*N*-trimethylsilyl trifluoroacetamide/acetonitrile (1:2 v/v) mixture by heating for 30 min at 130 °C. The GC-EIMS analyses of the resulting OH radical-induced reaction products demonstrated the formation of trimethylsilylated derivatives of Thy-Lys cross-link structures **II** (and/or **III**), showing the characteristic MS fragmentation pattern that was essentially in accord with the previous report by Dizdaroglu and Gajewski.[14] It is therefore most likely that the HCl treatment converted the primary cross-link structure **I**, as identified from the FAB-LC-MS data in Figure 1, to the secondary structure **II** via dehydration. The cross-link structure **III** possessing C(5)-C(6) double bond in the Thy moiety seems to be less important in the OH radical-induced cross-linking between Thy and Lys, although its possible formation as a primary product could not be ruled out.

In a separate experiment, N_2O-saturated aqueous solution of Lys (10 mM) was γ-irradiated up to 6 kGy. The γ-radiolysis products were trimethylsilylated in a similar procedure as described above, then being subjected to a GC-EIMS analysis. Among the trimethylsilyl derivatives of OH radical reaction products of Lys, we could identify the formation of 4-hydroxylysine (Figure 3): m/z 522 [M^+], 12% relative abundance; 507 [$(M - CH_3)^+$], 4%; 405 [$(M - COOSi(CH_3)_3)^+$], 7%; 362 [$[(M - N(Si(CH_3)_3)_2)^+$], 2%; 318 [$(M - COOSi(CH_3)_3 - NHSi(CH_3)_3)^+ + H$], 21%; 304 [$(M - CH(COOSi(CH_3)_3)\text{-}NHSi(CH_3)_3)^+$], 7%; 291 [$(M - CH_2CH(COOSi(CH_3)_3)\text{-}NHSi(CH_3)_3 + H)^+$], 20%; 232 [$CH_2CH(COOSi(CH_3)_3)\text{-}NHSi(CH_3)_3^+$], 4%; 218

[CH(COOSi(CH$_3$)$_3$)-NHSi(CH$_3$)$_3$$^+$], 26%; 189 [(CH$_2CH_2$N(Si(CH$_3$)$_3$)$_2$ + H)$^+$], 3%; 174 [CH$_2$N(Si(CH$_3$)$_3$)$_2$$^+$], 100%; 160 [N(Si(CH$_3$)$_3$)$_2$$^+$], 4%; 156 [(M - COOSi(CH$_3$)$_3$ - NHSi(CH$_3$)$_3$ - OSi(CH$_3$)$_3$ - Si(CH$_3$)$_3$ + H)$^+$], 9%; 117 [COOSi(CH$_3$)$_3$$^+$], 10%. This hydroxylated product could be derived from radical recombination between OH radical and C(4)-centered radical of Lys (Lys C(4)•, see Scheme) produced by hydrogen abstraction. In the radiolysis of N_2O-saturated aqueous solution of Thy and Lys, the Lys C(4)• would in turn react with 5-hydroxy-5,6-dihydrothymin-6-yl radical (Thy-5-OH C(6)•) that is a known intermediate[20] in the OH radical reaction of Thy, thus producing the cross-link structure **I**.

For better understanding of a mechanism by which Lys C(4) cross-links to Thy-5-OH C(6), a pulse radiolysis study was also performed to characterize redox reactivity of the possible intermediates involved in the cross-linking, Thy-5-OH C(6)• and Lys C(4)•. As demonstrated previously,[20] the Thy-5-OH C(6)• can reduce tetranitromethane (TNM) to yield nitroform anion C(NO$_2$)$_3$$^-$. Actually, we reconfirmed such a reducing reactivity of the Thy-5-OH C(6)• (Figure 4). This evidence may lead to a hypothesis that Thy-5-OH C(6)• will reduce Lys C(4)• efficiently to produce Thy-5-OH C(6)$^+$ and Lys C(4)$^-$ followed by their ionic recombination into the cross-link structure **I** (a redox reaction path in Scheme). However, in the pulse radiolysis of Lys (5 mM) in N_2O-saturated phosphate buffer solution containing *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD, 0.1 mM) as a reductant,[20] we could not observe the formation of characteristic TMPD cation radicals (TMPD$^{+\bullet}$). This indicates that the Lys C(4)• is a neutral radical but not an oxidizing radical so long as it reacts with TMPD. Therefore, the cross-linking between Thy-5-OH C(6)• and Lys C(4)• into the structure **I** would favor a direct radical recombination mechanism rather than a redox reaction path (Scheme). The formation of the cross-link

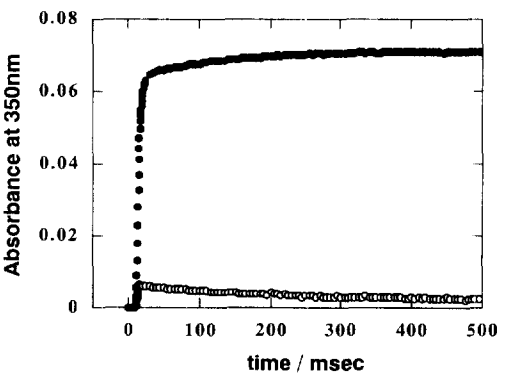
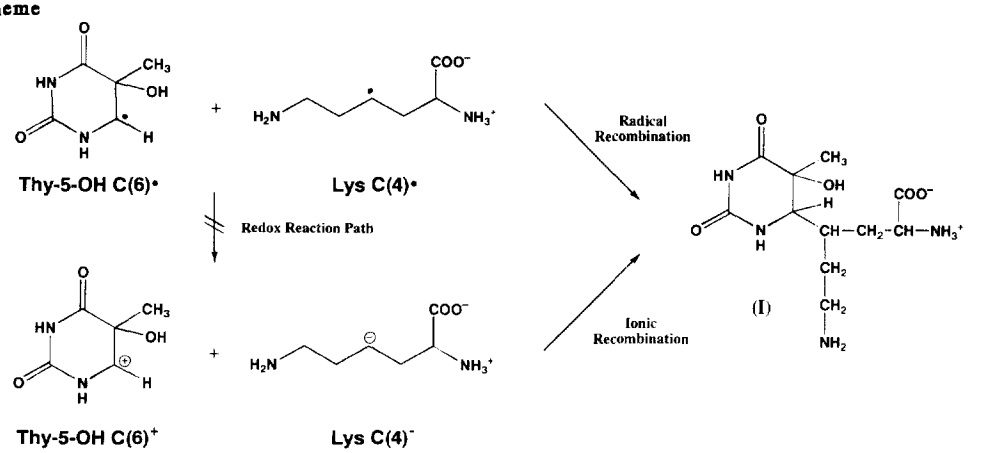


Figure 4. Buildup of C(NO$_2$)$_3$$^-$ as measured by absorbance at 350 nm in the pulse radiolysis of Thy (1 mM) in N_2O-saturated phosphate buffer solution containing TNM (0.35 mM) at pH 7.0.

Scheme



structure **II** seems to be relatively minor, in view of the previous pulse radiolysis study[20] that in the OH radical reaction of Thy the Thy-5-OH C(6)• is generated in about 6-times higher yield than a radical intermediate by hydrogen abstraction from methyl group.

References and Notes

† Corresponding e-mail address: nishimot@scl.kyoto-u.ac.jp

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