3-Hydroxylysine, a Potential Marker for Studying Radical-Induced Protein Oxidation†

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γ-Irradiation of several amino acids (Val, Leu, Ile, Lys, Pro, and Glu) in the presence of O_2 generates hydroperoxides. We have previously isolated and characterized valine and leucine hydroperoxides, and hydroxides, and have detected these products in both isolated systems [e.g., bovine serum albumin (BSA) and human low-density lipoprotein (LDL)] and diseased human tissues (atherosclerotic plaques and lens cataractous proteins). This work was aimed at investigating oxidized lysine as a sensitive marker for protein oxidation, as such residues are present on protein surfaces, and are therefore likely to be particularly susceptible to oxidation by radicals in bulk solution. $HO^{\bullet}$ attack on lysine in the presence of oxygen, followed by $NaBH_4$ reduction, is shown to give rise to (2*S*)-3-hydroxylysine [(2*S*)-2,6-diamino-3-hydroxyhexanoic acid], (2*S*)-4-hydroxylysine [(2*S*)-2,6-diamino-4-hydroxyhexanoic acid], (2*S*,5*R*)-5-hydroxylysine [(2*S*,5*R*)-2,6-diamino-5-hydroxyhexanoic acid], and (2*S*,5*S*)-5-hydroxylysine [(2*S*,5*S*)-2,6-diamino-5-hydroxyhexanoic acid]. 5-Hydroxylysines are natural products formed by lysyl oxidase and are therefore not good markers of radical-mediated oxidation. The other hydroxylysines are however useful markers, with HPLC analysis of 9-fluorenylmethyl chloroformate (FMOC) derivatives providing a sensitive and accurate method for quantitative measurement. Hydroxylysines have been detected in the hydrolysates of peptides (Gly-Lys-Gly and Lys-Val-Ile-Leu-Phe) and proteins (BSA and histone H1) exposed to $HO^{\bullet}/O_2$, and subsequently treated with $NaBH_4$. Quantification of the hydroxylysines yields, and comparison with hydroxyvalines and hydroxyleucines, supports the hypothesis that surface residues give higher yields of oxidized products than the hydrophobic leucines and valines, at least with globular proteins such as BSA. Hydroxylysines, and particularly 3-hydroxylysine, may therefore be sensitive and useful markers of radical-mediated protein oxidation in biological systems.

Introduction

Free radicals are known to be generated either by normal metabolic processes during electron transport chains and redox reactions of enzymes or as a result of exposure to exogenous factors such as UV light, radiation, and various chemicals. Until recently, it had been thought that radical damage on proteins was essentially a chain-terminating process and that the damage produced (cross-linking, fragmentation, and chemical modification of the amino acids) was mainly the result of inactive products. However, it has been shown recently that two products of protein oxidation, i.e., protein-bound reducing species, believed to be mainly 3,4-dihydroxyphenylalanine and protein hydroperoxides, are able to initiate further chemical reaction. Studies on free amino acids have shown that six amino acids (Val, Leu, Ile, Pro, Glu, and Lys) give significantly higher yields of hydroperoxides than the others (*1*, *2*). Such selectivity is in agreement with the hypothesis that most of the hydroperoxide groups occur on the side chains of free aliphatic amino acids.

Carbonyl formation on proteins has been used extensively to determine the oxidative state of proteins (*3*, *4*); however, this method is not specific for carbonyl groups on proteins associated with oxygen radical attack, and it can be confounded by carbonyls resulting from other processes such as glycoxidation and lipid peroxidation. As a result, it is often difficult to use in biological samples. Therefore, more specific methods for analyzing the oxidative state of proteins are needed. In the course of searching for a suitable marker for studying protein oxidation, we have previously focused on the oxidation products of valine and leucine. We have extended earlier work of Garrison and Kopoldova (reviewed in ref *5*) by characterizing the structures of three hydroxides of valine and five oxidation products of leucine produced upon γ-radiolysis in the presence of oxygen. These oxidation products have been detected with both isolated proteins and a number of biological samples, including human

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atherosclerotic plaques, normal human plasma and low-density lipoprotein (LDL[1]), cataract crystallins, and glycated rat-tail collagen (*6*−*11*) (S.-L. Fu and R. T. Dean, submitted for publication). Hydroxyleucines, like hydroxyvalines, have been proposed as useful markers to assess the extent of damage occurring on proteins during various pathological processes. Another novel hydroxylated product (5-hydroxy-2-aminovaleric acid) has been identified in radical-damaged proteins (*12*). This product is thought to arise from the oxidation of proline and arginine residues and has been postulated to be a specific marker of radical attack on proteins.

In this study, we focused on hydroxyl radical attack on lysine residues in proteins. Although valine and leucine as free amino acids are among the principal targets of hydroxyl radicals as assessed by the high yield of hydroperoxides, lysine is of potential interest because, as revealed by structural databases, the majority of these residues are preferentially located at the protein surface. EPR studies have shown that charged amino acids such as lysine and glutamate are important sites of formation of hydroperoxides (*13*, *14*). Furthermore, protein surfaces play an important role in cellular mechanisms through their interactions with other molecules. 5-Hydroxylysine has been identified previously with both free lysine and lysine-containing peptides, as a product of metal ion-catalyzed oxidation of lysine (*15*). We report here the identification of four oxidation products of lysine, (2*S*,5*R*)-5-hydroxylysine, (2*S*,5*S*)-5-hydroxylysine, 4-hydroxylysine, and 3-hydroxylysine, from γ-radiolysed lysine in the presence of oxygen. Among these, 3-hydroxylysine has been isolated by high-performance liquid chromatography with 9-fluorenylmethyl chloroformate (FMOC) precolumn derivatization. The characterization of this product was carried out on the basis of extensive spectroscopic measurements, including electrospray mass spectrometry (ES-MS) and ^{1}H and ^{13}C NMR. 3-Hydroxylysine has been detected on oxidized peptides and proteins, and a comparison of 3-hydroxylysine formation to that of other markers of protein oxidation (valine and leucine hydroxides) has been made in various peptides and proteins.

Materials and Methods

Materials. *o*-Phthalaldehyde (OPA) crystals and OPA diluent [3% KOH and 3% boric acid (pH 10.4)] were from Pickering Laboratories (Mountain View, CA). 2-Mercaptoethanol, fatty acid-free BSA, histone H1 (calf thymus), 5-hydroxylysine (mixed DL and DL-allo), 2,4-dinitrophenylhydrazine, and 9-fluorenylmethyl chloroformate were from Sigma (St. Louis, MO). L-Lysine and sodium borohydride were provided by Aldrich (Milwaukee, WI). DL-*trans*-4,5-Dehydrolysine and the tripeptide Gly-Lys-Gly (GKG) were obtained from Bachem (Budendorf, Switzerland). (2*S*,5*R*)-5-Hydroxylysine [5(*R*)OHLys] was purchased from Fluka (Bushs, Switzerland). Sodium borate and sodium acetate (analytical grade) were from Merck (Darmstadt, Germany). Water was purified by passage though a four-stage Milli-Q system (Millipore-Waters) equipped with a 0.2 μm pore size final filter. HPLC-grade methanol and acetonitrile were from EMScience (Gibbstown, NY). Tetrahydrofuran was from BDH (Poole, England).

γ-Irradiation of Lysine, Peptides, and Proteins. Irradiations of lysine (4 mM), tripeptide GKG (2 mM), histone H1 (4 mg/mL), and BSA (5 mg/mL) solutions were carried out using a ^{60}Co source (dose rate of ca. 21 Gy/min), and all samples were gassed throughout the irradiation with oxygen. After irradiation, the solutions were stored at −20 °C until they were used. The absolute quantity of hydroperoxides generated from γ-radiolysis was determined using an iodometric assay after adding a small volume of catalase (5 μg/mL) to the samples to remove radiation-generated hydrogen peroxide (*6*, *16*).

Gas-Phase Amino Acid Hydrolysis. The gas-phase hydrolysis method developed to obtain the best recovery for DOPA from protein hydrolysis was adopted (*6*). With this method, we utilized 1 mL of 6 M HCl containing 1% (w/v) phenol and 50 μL of mercaptoacetic acid as reductants. The hydrolysate was subsequently analyzed by HPLC.

FMOC Derivatization of Amino Acids. To 10 μL of sample were added 90 μL of borate buffer (0.1 M, pH 9.4) and 100 μL of FMOC (15 mM in acetone). The mixture was vortex mixed and after 1 min was extracted with 1 mL of pentane. The extraction was repeated twice to remove FMOC-OH, produced by reaction of FMOC-Cl with water. The aqueous solution was then diluted 10-fold for HPLC analysis.

Separation of FMOC-Derivatized Lysine Hydroxides for Structural Analysis. To obtain enough material for structural work, 500 mL of a 2 mM lysine solution was γ-irradiated (1000 Gy). After reduction with $NaBH_4$, the solution was concentrated 100-fold under reduced pressure and derivatized with FMOC by addition to 45 mL of sodium borate buffer (0.1 M, pH 9.4) and 50 mL of FMOC in acetone (50 mM). The solution was vortexed for 5 min, and the lysine derivatives were extracted twice with 50 mL of pentane. The mixture was separated by HPLC on a semipreparative Supelco C_{18} column (25 cm × 10 mm, 5 μm particle size) with UV detection at 300 nm, isocratic elution with 55% solvent A [acetonitrile/tetrahydrofuran/20 mM sodium acetate at pH 4.2 (19.5:0.5:80 v/v)] and 45% solvent B [acetonitrile/20 mM sodium acetate at pH 4.2 (80:20 v/v)] with a flow rate of 4 mL/min. After concentration, the four fractions collected from 30 injections were repurified on the same column. An extra HPLC purification step was performed using 55% solvent A [acetonitrile/20 mM ammonium acetate at pH 4.2 (20:80 v/v)] and 45% solvent B [acetonitrile/20 mM ammonium acetate at pH 4.2 (80:20 v/v)]. The samples were then lyophilized a minimum of three times to free the samples of ammonium acetate for MS and NMR studies.

HPLC Analysis of FMOC and OPA Derivatives of Amino Acids. The HPLC system consisted of a SIL-10A auto injector (Shimadzu, Kyoto, Japan), two LC-10AT pumps (Shimadzu), a F-1080 fluorescence detector (Hitachi, Tokyo, Japan), a SPD-10A UV detector (Shimadzu), and a column oven (30 °C, Waters, Millipore, Milford, MA). Data were digitized using a CBM-10A interface (Shimadzu) and processed on an IBM PC 123 computer.

(1) FMOC Derivatization of Lysine. The FMOC derivatives of amino acids were separated by HPLC using a Supelco C_{18} column (4.6 mm × 25 cm, 5μm particle size) with a Pelliguard column (2 cm, Supelco) at a flow rate of 1 mL/min, eluted with a gradient of solvent A [acetonitrile/tetrahydrofuran/20 mM sodium acetate at pH 4.05 (20:5:75 v/v)] and solvent B [same solvents (80:5:15 v/v)]. The gradient was generated as follows: isocratic elution for 30 min at 32% B, then a gradient to 70% B in 10 min, isocratic elution for 8 min, and then re-equilibration at 32% B for 7 min for the next analysis. Oxidized lysine products were monitored by fluorescence with an excitation wavelength of 265 nm and an emission wavelength of 310 nm. A calibration curve was carried out with authentic pure 5(*R*)OHLys. The parent amino acids were monitored by UV detection at 260 nm. The amount of protein analyzed was calculated from the lysine peak area after appropriate calibra-

[1] Abbreviations: BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; DQF-COSY, double-quantum filtered correlated spectroscopy; ES-MS, electrospray mass spectrometry; FMOC, 9-fluorenylmethyl chloroformate; HMQC, heteronuclear multiple-quantum coherence; LDL, human low-density lipoprotein; 3OHLys, (2*S*)-3-hydroxylysine; 4OHLys, (2*S*)-4-hydroxylysine; 5(*R*)OHLys, (5*R*,2*S*)-5-hydroxylysine; 5(*S*)OHLys, (5*S*,2*S*)-5-hydroxylysine; OPA, *o*-phthalaldehyde.

tion. All the data are expressed as the amount of oxidized product per lysine parent.

(2) OPA Derivatization of Valine and Leucine. The method, which has been previously described (*9*), involves a HPLC purification step on a LC-NH_2 column (4.6 mm × 25 cm, 5μm particle size, Supelco) prior to the OPA-HPLC assay. The eluting window (13.7−15.3 min) of both 3-hydroxyvaline (13.8 min) and 4-hydroxyleucines (14.4 and 15.0 min) was collected using an isocratic elution with 83% acetonitrile in 10 mM phosphate buffer (pH 4.3), eluting at 1.5 mL/min. The eluent was monitored at 210 nm. After evaporation to dryness, this fraction was derivatized with freshly made OPA reagent in the presence of 2-mercaptoethanol (0.5% v/v) using an SIL-10A autosampler (Shimadzu, South Rydalmere, Australia) as described previously (*6*). 3-Hydroxyvaline and 4-hydroxyleucines were analyzed using HPLC on an ODS column (*9*) and monitored by fluorescence (λ_{ex} = 340 nm and λ_{em} = 440 nm). The second HPLC step was also used to measure the lysine parent in the hydrolysate after appropriate dilution (usually ca. 1000-fold).

Spectroscopic Measurements. Electrospray (ES) MS was performed on a VG Platform mass spectrometer (Fisons, Homebush, Australia). The samples were dissolved in 50% aqueous acetonitrile. Formic acid (1%) was added to the solutions to assist with protonation of the sample. The solvent was delivered by a Phoenix (Fisons) syringe pump at a flow rate of 5 μL/min; 10 μL of each solution was injected. A dry nitrogen bath gas at atmospheric pressure was employed to assist evaporation of the electrospray droplets. The electrospray probe tip potential was 3.5 kV with 0.5 kV on the chicane counter electrode. A sample cone potential of 15 V was used. The ^{1}H spectra, double-quantum filtered COSY (DQF-COSY) spectra, and heteronuclear multiple-quantum coherence (HMQC) correlated NMR spectra were recorded in DMSO-d_6 (99.9%, Cambridge Isotopes, Andover, MA) in the Fourier transform mode on a Bruker AMX-600 NMR spectrometer. The chemical shifts are expressed in parts per million with respect to the DMSO-d_6 signal at δ 2.50 for the ^{1}H spectra and δ 39.50 for the ^{13}C spectra. Two-dimensional spectra were acquired, using standard Bruker software, over 2K data points and typically 512 t_1 increments with phase discrimination achieved using time-proportional phase incrementation (TPPI). The spectra were routinely processed with zero-filling in F_1 and a π/2 sine-bell function in each dimension.

Synthesis of 4-Hydroxylysine and 5-Hydroxylysine by Hydration of 4,5-Dehydrolysine. 4,5-Dehydrolysine (7 mg, 10 μM) was dissolved in 3.8 mL of water. Perchloric acid (70%, 185 μL, 0.55 M final concentration) was added and the mixture stirred at reflux for 24 h. An aliquot was then removed, neutralized with NaOH, and derivatized by the FMOC method described above for HPLC analysis. 5OHLys was identified by comparison with pure standard 5(*R*)OHLys and the mixture of DL- and DL-allo-5-hydroxylysine.

Derivatization of Aldehydes by 2,4-Dinitrophenylhydrazine (DNPH). The purpose of this experiment was to determine whether carbonyls were a significant source of hydroxides upon borohydride reduction; this was addressed by derivatizing carbonyls prior to borohydride reduction. Thus, γ-irradiated lysine was mixed with a solution of 1 mM DNPH in 1 M HCl (1:1) or with 1 M HCl alone (1:1) as a control, to derivatize carbonyls. After incubation for 1 h at ambient temperature, $NaBH_4$ was added to the sample. Lysine hydroxides were then derivatized using FMOC and analyzed by HPLC.

Results

FMOC Derivatization of the Samples. Initial attempts to detect and separate the oxidized products of lysine on an analytical LC-NH_2 column with an acetonitrile/aqueous 10 mM sodium phosphate solvent system gave broad overlapping peaks; this is probably due to the high hydrophilicity and the charged nature of lysine. Derivatization with 9-fluorenylmethyl chloroformate (FMOC) was therefore employed as this gives highly fluorescent species which are stable (*17*, *18*) in contrast to those with *o*-phthalaldehyde (*19*). This derivatization method was optimized by examining the effects of different extraction and FMOC solvents, and different reaction times, and the method tested for linearity and reproducibility.

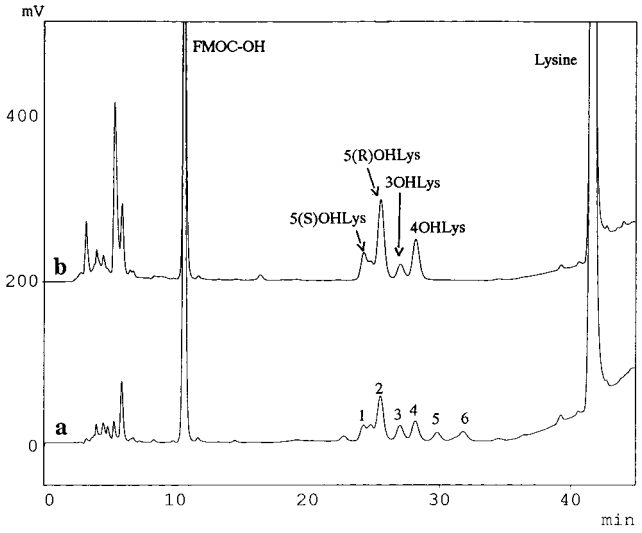


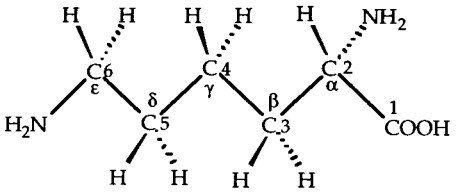
Figure 1. HPLC detection of FMOC derivatives of γ-irradiated lysine: (a) a γ-irradiated lysine solution and (b) a γ-irradiated and reduced lysine solution. For further details, see Materials and Methods.

(1) Effects of Extraction Solvent and FMOC Solvent. Two pH values (7.7 and 9.4) for the borate buffer (0.1 M) and two FMOC solvents (acetone and acetonitrile) were used to determine the best reaction conditions. FMOC in acetone and borate buffer at pH 9.4 provided the best recovery for lysine and 5(*R*)OHLys as well as a minimal contamination with hydrolyzed FMOC after appropriate extraction (data not shown). Two extraction solvents, ethyl acetate and pentane, were evaluated; pentane was employed as ethyl acetate, though efficient at removing hydrolyzed FMOC, caused loss of the lysine derivatives as previously described (*17*).

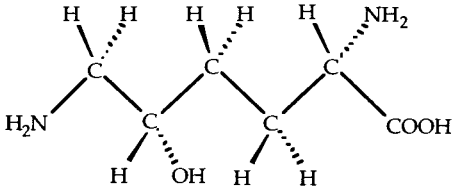
(2) Effects of Reaction Time. A reaction time of 30 s to 1 min gave the maximum fluorescence yield; longer times gave lower values.

(3) Linearity and Reproducibility. The linearity of the method was tested with lysine standards (1, 2.5, 5, 7.5, and 10 mM) with UV detection (260 nm) as well as with (2*S*,5*R*)-5-hydroxylysine (10, 25, and 50 μM) with fluorescence detection (λ_{ex} = 265 nm and λ_{em} = 310 nm). Five separate derivatizations were performed at each concentration level. Correlation coefficients for lysine and 5(*R*)OHLys were 0.997 and 0.998, respectively.

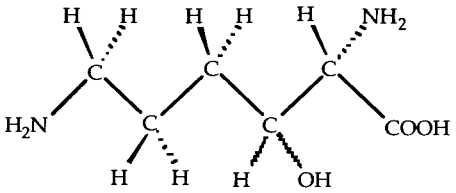
Isolation and Characterization of the Main FMOC Derivative Products of γ-Irradiated Lysine Solutions. The oxidation products of lysine, derivatized by FMOC, were analyzed by HPLC before and after reduction by $NaBH_4$. The HPLC chromatograms showed similar traces for the reduced and nonreduced samples (Figure 1) with the exception of the two latest eluting peaks (**5** and **6**) in the nonreduced samples. At least four different products (**1**−**4**) can be detected from the γ-radiolysed and reduced lysine. These products have been identified as FMOC derivatives of (2*S*,5*R*)-5-hydroxy-



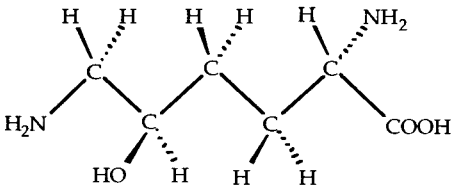
L-lysine



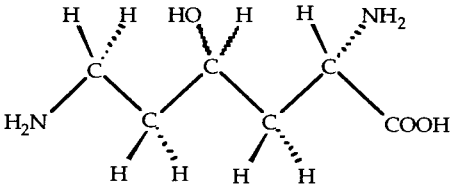
(2S, 5S)-5-hydroxylysine (**1**)



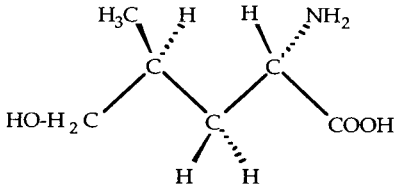
(2S)-3-hydroxylysine (**3**) [a]



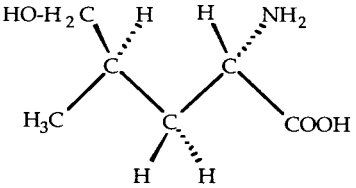
(2S, 5R)-5-hydroxylysine (**2**)



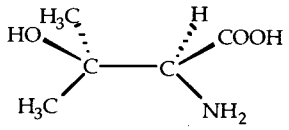
(2S)-4-hydroxylysine (**4**) [a]



(2S, 4S)-5-hydroxyleucine (**LeuOH2**)



(2S, 4R)-5-hydroxyleucine (**LeuOH3**)



(2S)-3-hydroxyvaline (**ValOH1**)

Figure 2. Structures of lysine and its oxidation products together with some of the corresponding valine and leucine materials. For those marked with a superscript a, the absolute configuration was not determined.

lysine [(2*S*,5*R*)-2,6-diamino-5-hydroxyhexanoic acid] (**1**), its diastereoisomer (2*S*,5*S*)-5-hydroxylysine [(2*S*,5*S*)-2,6-diamino-5-hydroxyhexanoic acid] (**2**), (2*S*)-3-hydroxylysine [(2*S*)-2,6-diamino-3-hydroxyhexanoic acid] (**3**), and (2*S*)-4-hydroxylysine [(2*S*)-2,6-diamino-4-hydroxyhexanoic acid] (**4**), on the basis of spectroscopic measurements and chemical evidence (vide infra) (Figure 2). None of these products were present in the native lysine (Figure 3).

ES-MS. The molecular weight of the four oxidized lysine derivatives **1**−**4** purified by HPLC was determined to be 607 by mass spectrometry (data not shown). The ES mass spectra exhibit a pseudomolecular ion at *m/z* 607.8 [M + H^+], which differs by 16 mass units from the lysine parent at *m/z* 591.7. This is indicative of the addition of a hydroxy group to the molecule and the fact that they differ from each other only by the position of the hydroxy group in their structures. The ion at *m/z* 369.5 in L-lysine corresponds to a molecular ion minus one FMOC group (mass of 223) presumably arising from the cleavage of one FMOC group and a concomitant transfer of two hydrogens to the residual fragment containing one FMOC group. This was not observed in the oxidized products.

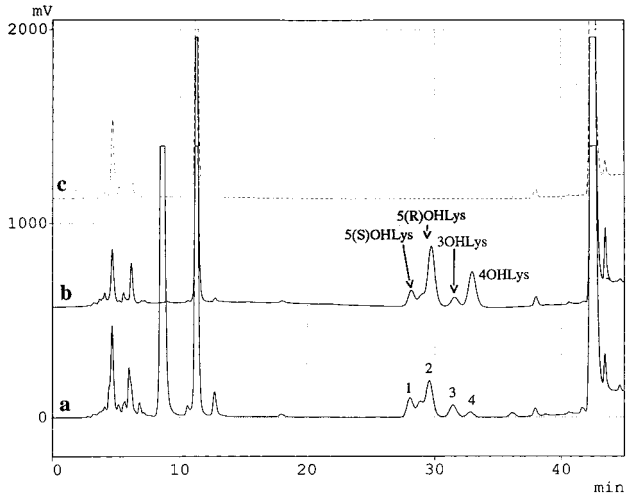


Figure 3. HPLC detection of FMOC derivatives of lysine hydroxides: (a) γ-irradiated and reduced lysine solution after protein hydrolysis treatment (see Materials and Methods), (b) γ-irradiated and reduced lysine solution, and (c) unirradiated lysine.

Characterization of (2*S*,5*S*)-5-Hydroxylysine (1) and (2*S*,5*R*)-5-Hydroxylysine (2). Formation of the two diastereoisomers of 5-hydroxylysine in γ-irradiated lysine solutions was confirmed by injection in the HPLC system of a pure (2*S*,5*R*)-5-hydroxylysine standard and a mixture of (2*S*,5*R*)- and (2*S*,5*S*)-5-hydroxylysine standards after derivatization by FMOC.

Characterization of (2*S*)-4-Hydroxylysine (4). Chemical evidence supporting the identification of 4OHLys came from the synthesis of a mixture of 4-hydroxylysine and 5-hydroxylysine by hydration of 4,5-dehydrolysine using perchloric acid. The mixture was derivatized by FMOC and analyzed by HPLC. The HPLC trace was compared to those of γ-irradiated lysine solutions and a mixture of 5*R* and 5*S* diastereoisomers of the 5OHLys standard. Four products were formed during this synthesis corresponding to the two diastereoisomers of 5OHLys and presumably the two diastereoisomers of 4OHLys. It is interesting to note that only one diastereoisomer of 4OHLys was detected with irradiated lysine as no HPLC peak was observed to elute after the 4OHLys diastereoisomer formed in irradiated lysine (Figure 1b).

Stability of Lysine Hydroxides under Acid Hydrolysis Conditions. Previous studies have shown that acid-catalyzed protein hydrolysis can result in loss of amino acids which contain a hydroxy group in their structures (*9*, *20*). Therefore, we studied the stability of the various lysine hydroxides under acidic conditions. The recoveries of the hydroxylysines were ca. 40 and 80% for 5(*S*)OHLys (**1**) and 5(*R*)OHLys (**2**), respectively, 80% for compound **3**, and <10% for 4OHLys (**4**) (Figure 3a). This is in agreement with previous studies where recoveries of 80 and 20% were reported for 3-hydroxyvaline and 4-hydroxyvaline, respectively (*20*). Similarly, protein hydrolysis of a γ-radiolysed leucine solution gave 70% and <5% recoveries for 5-hydroxyleucines and 4-hydroxyleucine, respectively (*9*). The low recoveries observed for 4-hydroxyamino acids are probably due to the formation of γ-lactones under acidic conditions. Lysine was almost fully recovered under similar conditions.

Since 5-hydroxylysine is a natural amino acid (and hence probably not a useful marker for oxidative damage), and 4-hydroxylysine is poorly recovered from protein hydrolysis, the structure of the FMOC derivative of compound **3** was investigated as a potential marker for oxidative damage.

Characterization of 3-Hydroxylysine (3). Further support for the assignment of peak **3** to 3-hydroxylysine was provided by the detailed NMR data of the di-FMOC derivative (Tables 1 and 2). ^{1}H assignments were determined from a ^{1}H−^{1}H COSY spectrum (Figure 4) by sequential correlation with the NH resonances at each end of the molecule. The corresponding ^{13}C resonance frequencies were established from an HMQC experiment.

First, we observed the loss of two protons in the upfield region of the ^{1}H spectrum with respect to the six protons corresponding to the three methylenic groups at C(3), C(4), and C(5) in lysine. This is in agreement with the addition of an OH group to one of the methylenic groups of the lysine side chain. The methinic proton in the resultant CHOH group resonates at low field (δ 3.58 ppm). The ^{1}H chemical shifts (Table 1) are evidently strongly influenced by conformational effects and, except for the proton H(3) directly attached to the hydroxylated carbon, cannot be reliably used for confirmation of the structure (*21*). We can nevertheless note the magnetic nonequivalence of the two protons at C(4) due to the formation of a new chiral center at C(3).

The ^{13}C data (Table 2) are entirely consistent with incorporation of a hydroxy group at C(3), with chemical shifts to higher frequency for the directly attached and adjacent C(2) and C(4), and to lower frequency for C(5), compared with the parent lysine derivative (*22*). Similar effects are observed for 5(*R*)OHLys.

The assignments for the 3OHLys FMOC derivative are in accordance with previous literature data for 3OHLys where the ^{13}C resonances were obtained in D_2O (*23*).

Formation of Lysine Hydroxides, Hydroperoxides, and Aldehydes in γ-Irradiated Lysine Solutions. ^{60}Co radiolysis (1000 Gy) of L-lysine (4 mM) in the presence of O_2 gave ca. 80 μM lysine hydroperoxides (i.e., ca. 2000 hydroperoxides/10^5 lysines, mean of three determinations) as determined by iodometric assay (*16*, *24*). No hydroperoxides were detected after reduction by $NaBH_4$. The lysine hydroperoxides present in the γ-radiolysed lysine solution were not detected with HPLC either after FMOC derivatization and reversed-phase HPLC or by injection of the nonderivatized solution onto an amino-phase HPLC column coupled with chemiluminescence detection. The hydroperoxides are presumably degraded during FMOC derivatization or HPLC analysis.

(1) Production of Lysine Hydroxides by Hydroxyl Radicals. FMOC measurement of the γ-radiolysed lysine solution gave a total yield of 2000 lysine hydroxides/10^5 lysines, and the γ-radiolysed and reduced lysine solution gave a yield of 4700 lysine hydroxides/10^5 lysines [5(*R*)OHLys, 2545/10^5 Lys; 5(*S*)OHLys, 774/10^5 Lys; 4OHLys, 1149/10^5 Lys; and 3OHLys, 336/10^5 Lys]. This means that 2700 lysine hydroxides/10^5 lysines were formed by $NaBH_4$ reduction. Almost half of the lysine hydroxides were therefore generated during γ-radiolysis as discussed below.

(2) Production of Lysine Aldehydes during γ-Radiolysis. The high yield of lysine hydroxides formed by $NaBH_4$ reduction (2700/10^5 lysines) which is greater than the total content of lysine hydroperoxides (2000/10^5 lysines, see above) may indicate that other oxidation

Table 1. ^{1}H Chemical Shifts for FMOC−Lysine Derivatives[a]

		L-lysine	5(*R*)OHLys	3OHLys
lysine moiety	H(2) (α)	3.84	3.90	3.60
	H(3_a) (β)	1.59	1.55	3.58
	H(3_b) (β)	1.70	1.91	−
	H(4_a) (γ)	1.30	1.25	1.01
	H(4_b) (γ)	1.30	1.51	1.31
	H(5_a) (δ)	1.39	3.44	1.32
	H(5_b) (δ)	1.39	−	1.60
	2H(6) (ε)	2.96	2.96	2.95
	NH(α)	7.37	7.59	6.23
	NH(ε)	7.26	7.22	7.25
	OH	−	4.68	nd[b]
FMOC moiety	CH_2	4.20	4.20	4.20
	CH	4.27	4.26	4.25
	aromatic ring	7.31, 7.40, 7.67, 7.88	7.32, 7.40, 7.70, 7.88	7.32, 7.39, 7.66, 7.87

[a] Reference CD_3SOCD_2H of δ 2.50. The NH chemical shifts were obtained from one-dimensional spectra. The ^{1}H data represent the midpoints of multiplets rather than true chemical shifts. [b] Not determined.

Table 2. ^{13}C Chemical Shifts for FMOC−Lysine Derivatives[a]

		L-lysine	5(*R*)OHLys	3OHLys
lysine moiety	C(2) (α)	53.9	53.9	57.1
	C(3) (β)	30.6	27.1	69.8
	C(4) (γ)	22.5	30.9	29.0
	C(5) (δ)	28.8	68.7	26.5
	C(6) (ε)	39.8	46.5	40.6
FMOC moiety	CH_2	65.0	65.2	65.1
	CH	46.4	46.4	46.5
	aromatic ring	119.8, 124.9, 126.7, 127.3	119.8, 124.9, 126.7, 127.3	119.8, 124.9, 126.9, 127.3

[a] Reference CD_3SOCD_3 of δ 39.5. The ^{13}C chemical shifts were obtained from HMQC spectra.

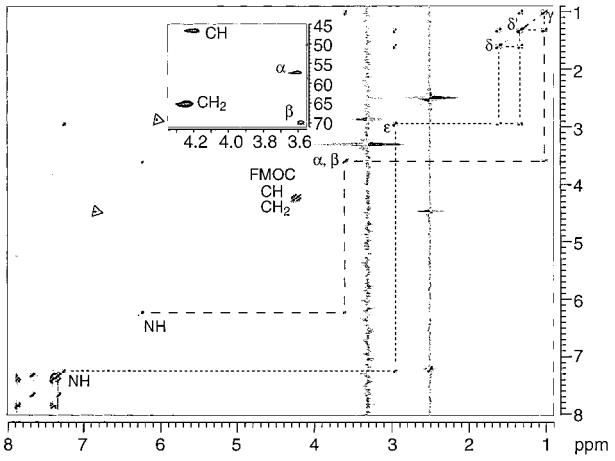


Figure 4. Double-quantum filtered COSY spectrum of the 3-hydroxylysine derivative. The spectrum was processed with zero-filling in F_1 and a π/2 shifted sine-bell function in each dimension, and ridges of t_1 noise due to the solvent and water resonances diminished by subtraction of a partial projection. Negative contours are shown with lighter shading; correlations from each of the NH protons are indicated by dashed lines. Artifacts due to quadrature images of the solvent resonances are enclosed in triangles. For clarification of chemical shifts not readily distinguished in the COSY spectrum, the relevant section of a HMQC spectrum is shown in the inset.

products are also produced during γ-radiolysis. Carbonyl formation during γ-radiolysis has been detected previously (*4*, *20*, *25*, *26*). To investigate this, we examined the reactivity of these non-hydroperoxide precursors with 2,4-dinitrophenylhydrazine, a compound which derivatizes carbonyl groups such as aldehydes (*27*). When a γ-radiolysed lysine solution was reacted with DNPH before reduction by $NaBH_4$, the level of hydroxylysines was slightly lower than that from direct $NaBH_4$ reduction. By this analysis, the amount of lysine aldehydes correspond to 200/10^5 lysines. This value does not account for all the non-hydroperoxide precursors of the hydroxides; other sources have not been investigated further.

Detection of 3-Hydroxylysine on Oxidized Peptides and Proteins. ^{60}Co radiolysis of Gly-Lys-Gly (2 mM), Lys-Val-Ile-Leu-Phe (1 mM), BSA (5 mg/mL), and calf thymus histone H1 (4 mg/mL) was carried out with various doses in the presence of O_2. Significant amounts of peptide-bound hydroperoxides and protein-bound hydroperoxides were measured by iodometric assay. For instance, BSA or histone H1 exposed to 2000 Gy of radiation gave 158 or 335 μM protein-bound hydroperoxides, respectively. After reaction with $NaBH_4$, which removed all the hydroperoxides, the samples were acid-hydrolyzed and analyzed using both the OPA- and FMOC-HPLC method.

The yields of 3-hydroxylysine in γ-irradiated (1500 Gy) and $NaBH_4$-reduced Gly-Lys-Gly and Lys-Val-Ile-Leu-Phe were determined to be, respectively, 2170/10^5 Lys and 230/10^5 Lys after taking into account their recovery. The extent of formation of 3-hydroxyvaline and 4-hydroxyleucines was also determined in γ-radiolysed and reduced Lys-Val-Ile-Leu-Phe (Table 3).

3-Hydroxylysine formation during γ-radiolysis of histone H1 was readily detected (Figure 5). An irradiation dose of 2000 Gy yielded a production of 269 3OHLys/10^5 Lys (Table 3). Species co-migrating with the other hydroxylysines were also formed (Figure 5), but were not investigated further for reasons already given.

Generation of 3OHLys during irradiation of BSA (Figure 6) showed a dose-dependent response increase from 500 to 2000 Gy (the yield at the lowest dose was below the detection limit). About 0.1% of the total lysine present in 5 mg/mL BSA was converted to 3OHLys. The formation of 3OHLys in γ-radiolysed BSA and histone

Table 3. Formation of Oxidized Products in γ-Irradiated Peptide, H1, and BSA[a]

	dose (Gy)	3OHLys/10^5 Lys	ValOH1[b]/10^5 Val	LeuOH2[b]/10^5 Leu	LeuOH3[b]/10^5 Leu
Lys-Val-Iso-Leu-Phe	1500	232 ± 44	220 ± 52	1421 ± 486	1086 ± 47
H1	2000	269 ± 19	112 ± 15	499 ± 103	326 ± 86
BSA	500	nd[c]	15 ± 1	34 ± 7	31 ± 8
	1000	37 ± 1	23 ± 6	64 ± 4	46 ± 1
	1500	80 ± 5	38 ± 4	150 ± 25	101 ± 19
	2000	90 ± 5	52 ± 3	223 ± 11	154 ± 29

[a] Data represent mean ± SD of three separate experiments conducted as described in Materials and Methods. [b] Structures of ValOH1, LeuOH2, and LeuOH3 are given in Figure 2. [c] Value below the HPLC detection limit.

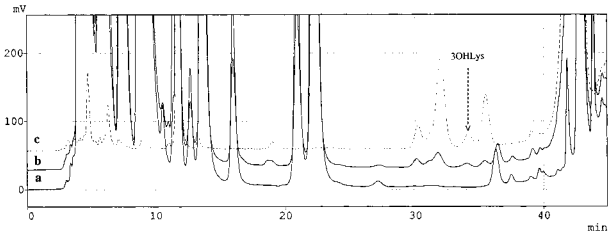


Figure 5. HPLC detection of the FMOC derivative of 3-hydroxylysine in oxidized lysine and histone H1 solutions. (a) Native and (b) γ-irradiated histone H1 (4 mg/mL) were reduced by $NaBH_4$, hydrolyzed, and derivatized using FMOC (see Materials and Methods). (c) A γ-irradiated lysine solution (4 mM) was treated with $NaBH_4$ and subsequently derivatized with FMOC (cf. Figure 2).

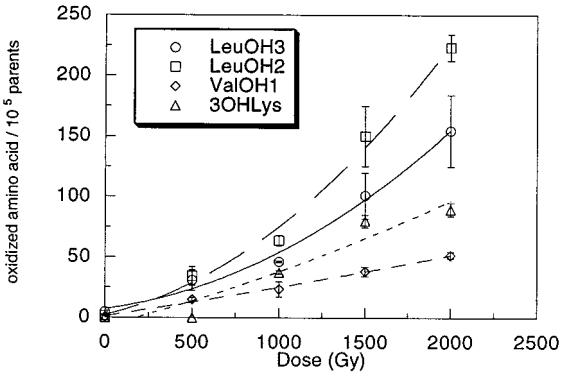


Figure 6. Detection of 3-hydroxylysine, 3-hydroxyvaline, and 5-hydroxyleucines on γ-radiolysed BSA. BSA (5 mg/mL in water) was exposed to the stated doses of radiation in the presence of O_2. After treatment with $NaBH_4$, the samples were hydrolyzed, derivatized by FMOC (for 3-hydroxylysine) or OPA (for 3-hydroxyvaline and 5-hydroxyleucines), and analyzed by HPLC as described in Materials and Methods. Results are means ± SD from two different experiments.

H1 was confirmed by spiking experiments using a mixture of the four lysine hydroxides.

Discussion

On the basis of chemical and spectroscopic evidence, we have identified the formation of four lysine hydroxides generated during γ-radiolysis of lysine in the presence of O_2. 3-Hydroxylysine derivatized by FMOC has been fully characterized by detailed structural studies using electrospray MS and ^1H and ^{13}C NMR.

The γ-radiolysed lysine solution, before reduction with $NaBH_4$, contains a mixture of lysine hydroperoxides (as inferred from iodometric assay), lysine hydroxides (as determined by HPLC), and some carbonyl species (as shown by DNPH derivatization). Carbonyl groups have been previously observed in irradiated valine and leucine solutions, although in the latter case the carbonyls undergo further rearrangement to generate cyclic products via Schiff base formation (*9*). A possible mechanism

Table 4. Ratio of 3OHLys to Hydroxylated Valine (ValOH1[a]) and Leucine (LeuOH2[a] or LeuOH3[a]) Derivatives in γ-Irradiated Peptide, H1, and BSA

	Lys-Val-Iso-Leu-Phe at 1500 Gy	H1 at 2000 Gy	BSA at 1500 Gy
3OHLys/ValOH1	1.05	2.42	2.09
3OHLys/LeuOH2	0.16	0.54	0.53
3OHLys/LeuOH3	0.21	0.82	0.79
ValOH1/LeuOH2	0.15	0.22	0.21
ValOH1/LeuOH3	0.20	0.34	0.37

[a] Structures of ValOH1, LeuOH2, and LeuOH3 are given in Figure 2.

for the generation of lysine hydroperoxides similar to that proposed previously for formation of valine and leucine hydroperoxides is shown in Figure 7. The initial step involves hydrogen abstraction by hydroxyl radicals to generate a carbon-centered radical and subsequent formation of a peroxyl radical species via O_2 addition. The formation of lysine hydroxides during γ-radiolysis can be explained by several pathways which have been described in some detail (*7*, *8*). The first route is the reduction of the preformed lysine hydroperoxides by electrons generated during water radiolysis (*8*). Another possibility is via a transient tetroxide formed by dimerization of two peroxyl radicals. This species can decompose to form oxygen and alkoxyl radicals (which can be expected to undergo hydrogen atom abstraction reaction with suitable R−H bonds) or decompose directly to form oxygen, an aldehyde, and a hydroxide (the Russell mechanism) (*28*, *29*).

The predominant formation of 5OHLys (3300/10^5 Lys) compared to those of 4OHLys (1100/10^5 Lys) and 3OHLys (330/10^5Lys) is consistent with EPR spin trapping studies which have demonstrated that hydrogen abstraction occurs mainly at C(4) and C(5) on the side chain when lysine is treated with $HO^•$ (*30*). As 5OHLys is a naturally occuring amino acid (present in collagen as a result of lysine oxidation by the enzyme lysyl oxidase) and 4OHLys is not fully recovered after protein hydrolysis treatment, these species are probably not useful markers for protein oxidation. However, 3OHLys which is stable during our procedure and apparently a specific product of oxidative conditions may be suitable as a marker for studying protein oxidation. Hydroxylation at C(2) and C(6) is not observed as protonated α-amino groups in free amino acids deactivate the carbon nearby with respect to hydrogen atom abstraction by $HO^•$ (*29*, *31*, *32*).

This study was undertaken in the light of the hypothesis that lysine residues on proteins might be especially susceptible to $HO^•$ attack because of their presence on the surface of globular proteins. We have therefore compared the formation of lysine hydroxides to that of valine and leucine hydroxides in γ-radiolysed peptides and proteins BSA and histone H1 (Table 3); the latter in particular has a very high lysine content. The extent of

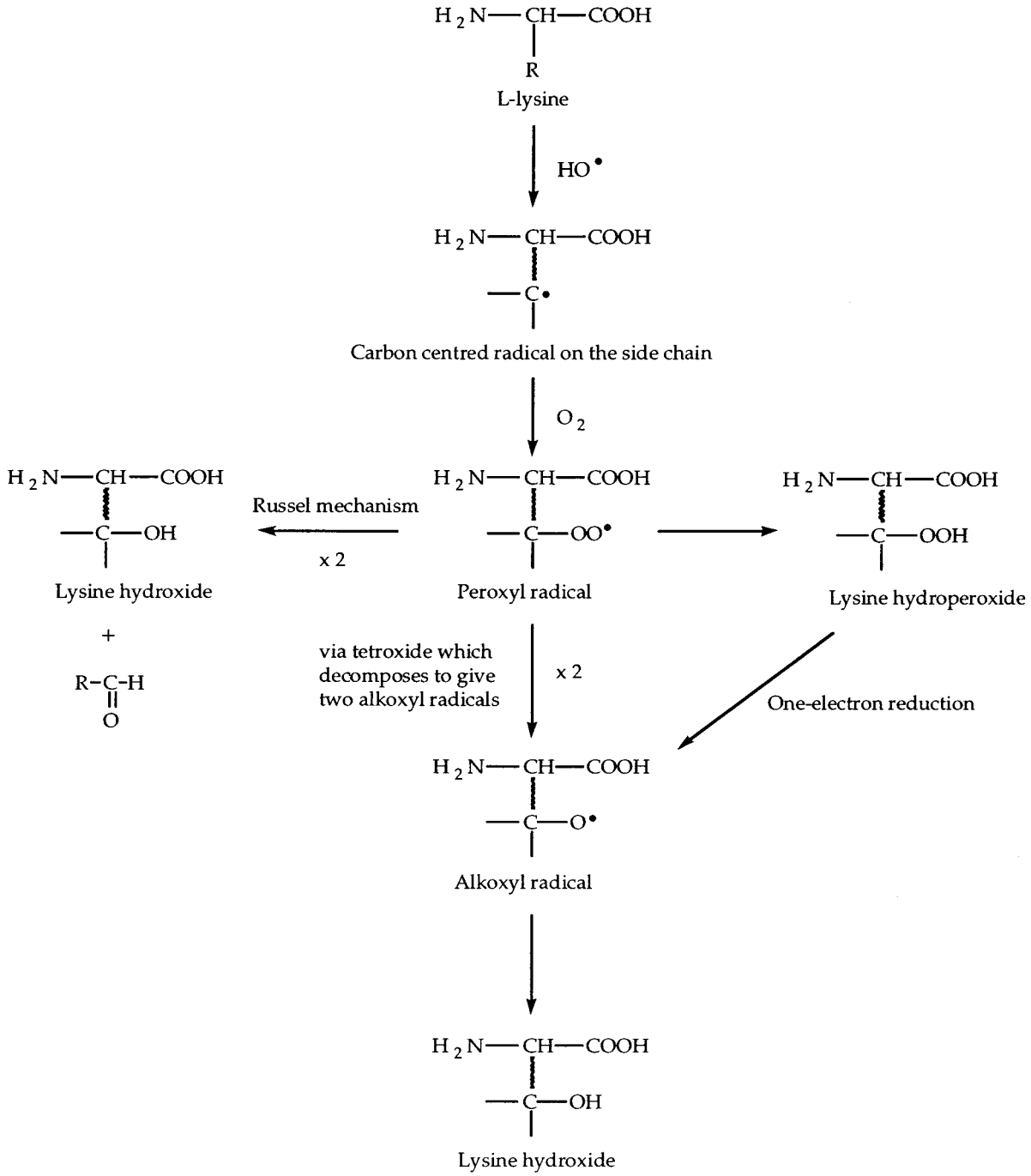


Figure 7. Proposed reaction mechanisms for the generation of lysine hydroperoxides and hydroxides.

hydroperoxide formation on free lysine (70 μM in a 1 mM lysine solution) was lower compared to that of valine (112 μM in a 1 mM valine solution) and leucine (88 μM in a 1 mM leucine solution) (*6*). However, we have demonstrated the formation of high yields of lysine hydroxides during the irradiation. In contrast, γ-radiolysis of valine and leucine did not directly produce hydroxide compounds.

Table 4 shows an analysis of our data in terms of ratios between 3OHLys and the leucine and valine hydroxides, and between the leucine and valine hydoxides themselves. The indices for the number of 3OHLys per leucine or valine derivative are approximately 2−4-fold higher in the two proteins (histone H1 and BSA) than in the peptide Lys-Val-Ile-Leu-Phe. In contrast, the indices for ValOH1/LeuOH2 and ValOH1/LeuOH3 are similar in all three cases. Thus, it seems that protein-bound lysine is preferred as target for radical attack compared to peptide lysine, whereas this is not true for Val (or Leu). This can be most readily explained as a consequence of the preferential location of lysine residues at protein surfaces, and the contrasting more internal disposition of the valine and leucine residues.

We conclude that 3-hydroxylysine may be a useful marker of radical-mediated protein oxidation and, in particular, that it may help in the further analysis of radical reactions localized on the surfaces of proteins.

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