Structure of a Fluorescent Compound Formed from 4-Hydroxy-2-nonenal and N^{α}-Hippuryllysine: A Model for Fluorophores Derived from Protein Modifications by Lipid Peroxidation

Koichi Itakura,*,† Toshihiko Osawa,‡ and Koji Uchida‡

Faculty of Education, Aichi University of Education, Kariya 448, Japan, and Laboratory of Food and Biodynamics, School of Agricultural Sciences, Nagoya University, Nagoya 464-01, Japan

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Introduction

It is well established that modification of proteins by lipid peroxidation produces fluorescence.[1] Because of the high sensitivity, this fluorescence is regarded as a good parameter for assessing oxidative damages in biological systems. Lipid peroxidation results in a variety of reactive aldehydes that are capable of covalently attaching to proteins.[2] There is increasing evidence that some of the aldehydes can produce fluorescent proteins.[3−10] 4-Hydroxy-2-nonenal (HNE, **1**) is a major lipid peroxidation-derived aldehyde that readily reacts with proteins under physiological conditions. Protein modifications by **1** have been implicated in various pathophysiologies such as atherosclerosis,[11,12] nephropathy,[13] Parkinson's disease,[14] and Alzheimer's disease.[15] It has been proposed that **1** contributes to the formation of fluorescent proteins during the lipid peroxidation of biological materials. For example, low-density lipoprotein (LDL) treated with **1** in vitro develops a protein fluorescence with an excitation maximum at 360 nm and an emission maximum at 430 nm, which agrees well with that observed in oxidized LDL.[16] Although it is generally accepted that the fluorescence in HNE-treated proteins involves the modification of lysine residues, the structure of the fluorophore has so far not been determined. Therefore, we studied the reaction of **1** with the lysine-containing dipeptide, N^{α}-hippuryllysine (N^{α}-benzoyl-glycyl-L-lysine, BGL, **2**), at neutral pH. We report here the first structural characterization of an HNE-derived fluorescent compound **3**, a proposed model for fluorophores formed in protein during lipid peroxidation.

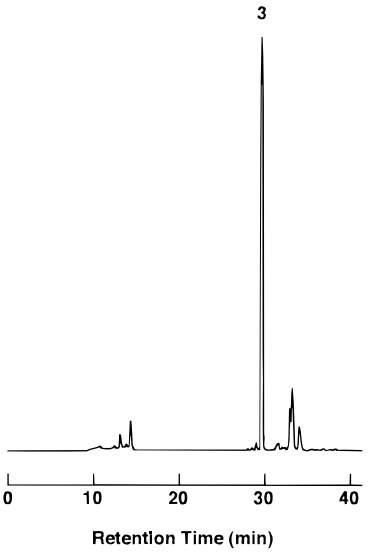

Figure 1. HPLC analysis of fluorescent products formed by the reaction of **1** with **2** after 7 days. Fluorescence detection was carried out at 360 nm (excitation) and 430 nm (emission). The reaction and HPLC conditions are described in the Experimental Section.

Results and Discussion

A reaction mixture (6.0 mL) of **1** (10 mM) and **2** (100 mM) in 0.1 M phosphate buffer (pH 7.4) was incubated at room temperature for 7 days. HPLC analysis of the mixture revealed a major peak eluting at 29.7 min (Figure 1). To permit its structural analysis, the same reaction was carried out on a large scale. Purifications by Amberlite XAD-2 and then by repeated reversed-phase HPLC afforded a fluorescent compound **3** that had an excitation maximum at 362 nm and an emission maximum at 436 nm in ca. 100:1 $H_2O-CH_3CO_2H$ (pH 2.7). It should be mentioned here that **3** is unstable in water. Fortunately, however, we found that it is quite stable in aqueous acetic acid. The mechanism by which acetic acid stabilizes **3** remains unclear. On the basis of NMR spectroscopy [^{1}H, ^{13}C, DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC (10 Hz)] and FAB mass spectrometry, the structure of **3** was confirmed as shown in Scheme 1. The FAB mass spectrum of **3** showed a pseudomolecular ion peak at *m*/*z* 749 $(M + H)^+$, suggesting that **3** was composed of one molecule of **1** and two molecules of **2**. Table 1 shows selected ^{13}C and ^{1}H NMR data for **3**. The ^{1}H NMR

* To whom correspondence should be addressed. Tel.: 0566-26-2596. Fax: 0566-26-2510. E-mail: kitakura@auecc.aichi-edu.ac.jp.
† Aichi University of Education.
‡ Nagoya University.

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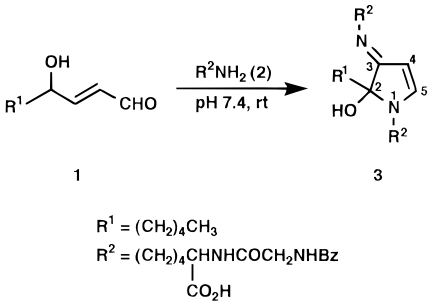
Table 1. Selected ^{13}C and ^{1}H NMR Data for 3[a]

C position[b]	δ_C	δ_H[c]	HMBC
2	95.89		
3	177.33		
4	88.54	5.37 ($^1/_2$H, d, 3.1), 5.38 ($^1/_2$H, d, 3.1)	C−2, C−5
5	167.20	8.08 ($^1/_2$H, d, 3.1), 8.10 ($^1/2$H, d, 3.1)	C−2, C−3, C−4
1-NCH_2	44.52, 44.57	3.46 (2H, m)	C−5
C=NCH_2	45.71	3.35 (2H, m)	

[a] A mixture of two diastereomers. [b] See Scheme 1 for numbering. [c] The *J* values are in parentheses (Hz).

Scheme 1



patterns of H-4 and H-5 and HPLC analysis of purified **3** (Figure 2) provide evidence for a mixture (ca. 1:1) of two diastereomers originating from the C-2 stereocenter. Support for modification of the ϵ-amino group of **2** were shifts downfield of the ϵ-CH_2 proton resonances (δ 2.99-δ 3.35 and 3.46). An HMBC correlation of δ_H 3.46 to C-5 suggested the connectivity of N-1 to C-5. On the other hand, an HMBC correlation of δ_H 3.35 to C-3 was not observed. The HMBC experiment also showed the correlations of H-4 to C-2 and C-5 and H-5 to C-2, C-3, and C-4. Among them, the most remarkable is the correlation of H-5 to C-2 because they correspond to H-1 and C-4 of **1**, respectively. This unexpected correlation suggested the connectivity of N-1 to C-2, leading to a five-membered ring. The coupling constants between H-4 and H-5 supported the presence of the cyclic structure.

A plausible pathway for the formation of **3** is outlined in Scheme 2. It was reported that an alkylamine readily reacts with C-1 and C-3 of **1** via Schiff base formation and Michael addition to produce the 1:2 HNE−amine adduct.[17] It is therefore reasonable to presume that the initial step involves the formation of the 1:2 HNE−BGL adduct **4**. The subsequent conversion of **4** into **3** obviously requires two oxidation steps and intramolecular cyclization, but its detailed mechanism is not yet clear.

It has long been considered that malondialdehyde (MDA), one of the lipid peroxidation-derived aldehydes, is responsible for the formation of fluorescent proteins during lipid peroxidation, and several models for MDA-derived fluorophores have been proposed.[3−7] However, their fluorescence properties are quite different from those observed in peroxidized systems.[18−20] Therefore, aldehydes other than MDA have become of interest in recent years.[8−10] Among them, **1** has received particular attention because of its quantitative importance and high reactivity with proteins.[2] Although several reports demonstrated that **1** may be responsible for the fluorescence of peroxidized biological materials, most of them only depend on the comparison of fluorescence properties.[16,21] In the present study, we identified the HNE−lysine adduct **3** possessing a fluorescence property similar to those observed in peroxidized systems. It is noteworthy that the formation of structures analogous to **3** in HNE-modified proteins would imply protein cross-linkings. We expect that this fluorophore would provide a chemical clue to investigate the involvement of **1** in the formation of fluorescent cross-linked proteins during lipid peroxidation.

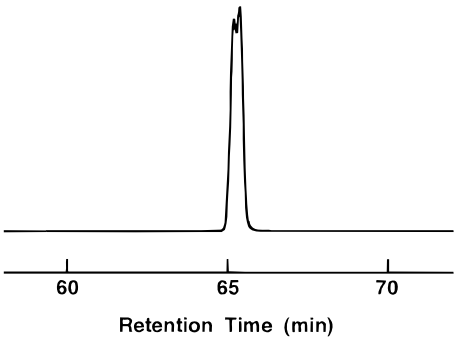
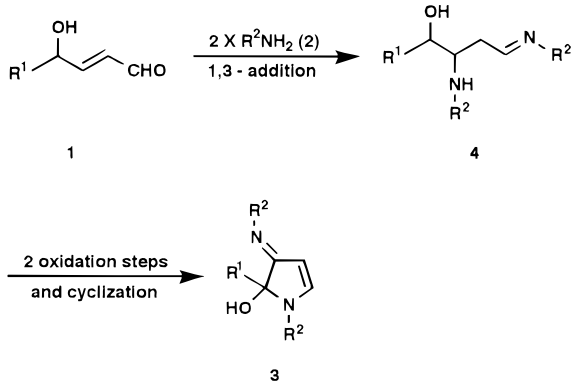


Figure 2. HPLC analysis of purified **3**. Elution was with H_2O−CH_3CO_2H (100:1) containing 0−50% CH_3CN−CH_3OH (1:1), linear gradient over 70 min. Fluorescence detection was carried out at 360 nm (excitation) and 430 nm (emission).

Scheme 2. Plausible Pathway for Formation of 3



Experimental Section

Materials and General Procedures. 4-Hydroxy-2-nonenal was prepared according to the procedure of Grée et al.[22] N^{α}-Hippuryllysine was obtained from the Peptide Institute, Inc. (Osaka, Japan) and Sigma Chemical Co. (Milwaukee, WI). All other chemicals were of the highest grade commercially avail-

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able. Analytical and preparative HPLC were carried out on a C18 reversed-phase column (5 μm particle size, 8.0 × 250 mm) at a flow rate of 2.0 mL/min. Products were monitored with a fluorescence detector at 360 nm (excitation) and 430 nm (emission). The concentration of Amberlite XAD-2 and HPLC eluents to dryness caused insolubilization of **3** in all solvents. Moreover, removal of acetic acid from the eluents by evaporation caused the decomposition of **3**. For these reasons, **3** was always treated as a water solution containing a small amount of acetic acid during its preparation and spectral measurements.

Reaction of 1 with 2. The reaction mixture (6.0 mL) containing **1** (10 mM) and **2** (100 mM) in 0.1 M phosphate buffer (pH 7.4) was allowed to stand at room temperature. After 7 days, the mixture was directly subjected to analytical HPLC; elution was with $H_2O-CH_3CO_2H$ (100:1) containing 0−50% CH_3CN, linear gradient over 40 min. Its HPLC profile is shown in Figure 1.

Preparation of 3. A solution (220 mL) of **1** (354 mg, 2.27 mmol) and **2** (6.76 g, 22.0 mmol) in 0.1 M phosphate buffer (pH 7.4) was allowed to stand at room temperature for 21 days. After the pH was adjusted to 5 with CH_3CO_2H, the solution was applied on an Amberlite XAD-2 column (2.5 × 25.5 cm) equilibrated with $H_2O-CH_3CO_2H$ (100:1). The column was eluted stepwise with $H_2O-CH_3CO_2H$ (100:1), 20%, 40%, 60%, and 80% $CH_3OH/H_2O-CH_3CO_2H$ (100:1), and CH_3OH (200 mL each). The fractions eluted with 60% and 80% $CH_3OH/H_2O-CH_3CO_2H$ (100:1) were combined and concentrated. The concentrated solution was subjected to preparative HPLC; elution was with $H_2O-CH_3CO_2H$ (100:1) containing 0−45% CH_3CN, linear gradient over 36 min. The HPLC fraction eluted at 29.2 min was collected and concentrated. The concentrated solution was again subjected to preparative HPLC; elution was with $H_2O-CH_3CO_2H$ (100:1) containing 0−70% CH_3OH, linear gradient over 50 min. The HPLC fraction eluted at 45.4 min was collected and concentrated. The concentrated solution was further subjected to HPLC purification; elution was with $H_2O-CH_3CO_2H$ (100:1) containing 0−49% CH_3CN-CH_3OH (1:1), linear gradient over 87 min. The HPLC fraction eluted at 78.4 min was collected, and concentration of the combined fraction afforded **3** as a mixture (ca. 1:1) of two diastereomers (1.1 mg, 0.063% yield based on starting **1**). No attempt was made to separate the diastereomers. The yield of **3** was determined by measuring the released glycine from the acid hydrolysis of **3** (6 N HCl, 105 °C, 24 h): UV (ca. 100:1 $H_2O-CH_3CO_2H$, pH 2.7) λ_{max} (ϵ) 361 nm (1.5×10^4); ^{13}C NMR (100 MHz; D_2O; CH_3CO_2D as δ 21.10) δ 13.69, 21.49, 22.14, 22.77, 22.81, 22.96, 23.06, 27.72, 27.80, 28.12, 28.15, 30.88, 31.23, 31.29, 31.32, 35.89, 43.72, 43.77, 44.52, 44.57, 45.71, 54.20, 88.54, 95.89, 127.84, 129.47, 133.15, 133.39, 167.20, 171.63, 171.74, 171.77, 171.82, 177.33, 177.84, 177.93 (although each diastereomer has 39 carbons, the ^{13}C NMR spectrum showed only 36 signals probably because of signal overlapping); ^{1}H NMR (400 MHz; D_2O; HOD as δ 4.80) δ 0.83 (3H, t, J = 6.7 Hz), 0.77−0.94 (2H, m), 1.17−1.29 (4H, m), 1.34−1.48 (4H, m), 1.59−1.99 (9H, m), 2.01−2.09 (1H, m), 3.29−3.41 (2H, m), 3.42−3.50 (2H, m), 4.08−4.22 (4H, m), 4.32−4.40 (2H, m), 5.37 ($^1/_2$H, d, J = 3.1 Hz), 5.38 ($^1/_2$H, d, J = 3.1 Hz), 7.55−7.89 (10H, m), 8.08 ($^1/_2$H, d, J = 3.1 Hz), 8.10 ($^1/_2$H, d, J = 3.1 Hz); FABMS (glycerol) m/z 749 $(M + H)^+$, 733 $(M + H - 16)^+$; HRFABMS (3-nitrobenzyl alcohol + poly(ethylene glycol)) calcd for $C_{39}H_{53}O_9N_6$ 749.3874, found m/z 749.3879 $(M + H)^+$.

Supporting Information Available: ^{1}H NMR, ^{13}C NMR, DEPT (90° and 135°), ^{1}H−^{1}H COSY, HMQC, HMBC, and FAB mass spectra of **3** (8 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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