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A novel fluorescent malondialdehyde-lysine adduct

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Abstract

We report a novel type of fluorescent product derived from the reaction of the lysine residue with malondialdehyde (MDA). When the lysine-containing peptide (*N*-acetyl-glycyl-L-lysine methyl ester) was treated with MDA prepared by the acid hydrolysis of 1,1,3,3-tetramethoxypropane, the main fluorescent product, which corresponded neither to the 1-amino-3-iminopropene derivative (**2**) nor to the 4-methyl-1,4-dihydro-3,5-dicarbaldehyde derivative (**3**), was detected by reverse-phase HPLC. By analysis of its UV, NMR, and high-resolution FAB mass spectra, it was confirmed to be 1-[5-carboxymethyl-5-(*N*-acetylglycylamino)pentyl]-3-[1-(5-carboxymethyl-5-(*N*-acetylglycylamino)-pentyl)-3,5-diformyl-1,4-dihydropyridin-4-yl]pyridinium (**1**). This finding may provide a new clue to the formation mechanisms of fluorescent lipofuscin-like pigment.

Keywords: Fluorescence; Malondialdehyde; Lysine; Protein; Cross-link; Lipofuscin

1. Introduction

It is generally believed that lipofuscin is a good parameter of in vivo lipid peroxidation. Malondialdehyde (MDA), one of the major secondary products of lipid peroxidation, produces fluorescence in proteins (Kikugawa and Beppu, 1987; Tsuchida et al., 1987; Esterbauer et al., 1991). This has been the subject of much interest because of its possible implication in the formation of fluorescent lipofuscin-like pigment. In MDA-modified proteins, the fluorophores have been considered to involve the ε-amino group of lysine residues (Chio and Tappel, 1969a; Kikugawa et al., 1985; Beppu et al., 1988). Several studies on the chemical nature of the fluorophores have been undertaken, and two models have been proposed. In early studies, Chio and Tappel (1969b) have obtained fluorescent 1-amino-3-iminopropenes from the reaction of amino acids with MDA, and have suggested that the 1-amino-3-iminopropenes are responsible for the fluorescence of lipofuscin-

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like pigment. Later, Kikugawa and co-workers (Kikugawa et al., 1981; Kikugawa and Ido, 1984) investigated the reaction of primary amines with MDA and have obtained highly fluorescent compounds, 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes. They have also suggested that the fluorophores that may be formed from MDA in biological systems seem to be these 1,4-dihydropyridine-3,5-dicarbaldehydes, since they can be easily produced under physiological conditions. Nair et al. (1986, 1988) have also isolated similar compounds and have discussed their formation mechanisms in detail.

In this paper, we report the isolation and structural elucidation of a novel fluorescent product derived from the reaction of *N*-acetyl-glycyl-L-lysine methyl ester, an analog of the lysine residues in proteins, with MDA.

2. Experimental procedures

2.1. Materials

N-Acetyl-glycyl-L-lysine methyl ester acetate salt (AGLME) was obtained from Peptide Institute, Inc. (Osaka, Japan). 1,1,3,3-Tetramethoxypropane (TMP) was obtained from Aldrich (Milwaukee, WI). All other reagents were of the highest grade commercially available.

2.2. Equipment

Ultraviolet spectra were recorded on a Shimadzu UV-160 UV-visible recording spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-3000 fluorescence spectrophotometer. High-resolution fast atom bombardment (HR-FAB) mass spectra were obtained on a JEOL JMS-DX 705 mass spectrometer with glycerol as the matrix. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker ARX-400 spectrometer with tetramethylsilane as the internal standard. High-performance liquid chromatography (HPLC) was performed with an intelligent HPLC system (Japan Spectroscopic Co., Ltd.) equipped with a FP-920 intelligent fluorescence detector (Japan Spectroscopic Co., Ltd.). A Develosil ODS-UG-5 column (8.0 × 250 mm; Nomura Chemical Co. Ltd., Japan) was used for analytical and preparative purposes.

2.3. Preparation of TMP hydrolysate

TMP (165 μl, 1.0 mmol) was added to 1 N HCl (835 μl). After a homogeneous solution was obtained, a portion (0.1 ml) of the solution was diluted with 1 N HCl (0.9 ml). The diluted solution was allowed to stand at room temperature for 1 h to prepare 100 mM TMP hydrolysate for use.

2.4. Reaction of AGLME with TMP hydrolysate

AGLME (16.1 mg) was dissolved in 450 μl of 0.1 M sodium acetate buffer (pH 4.5), and then 50 μl of freshly prepared 100 mM TMP hydrolysate was added. The reaction mixture containing 100 mM AGLME and 10 mM TMP hydrolysate (pH 4.0) was incubated at room temperature. After 4, 8, 24, and 48 h, the reaction mixture was directly subjected to HPLC analysis (100:1 H_2O/CH_3CO_2H containing 0–20% acetonitrile, linear gradient (50 min) at a flow rate of 2.0 ml/min). The HPLC profile of the mixture after 48 h is shown in Fig. 1.

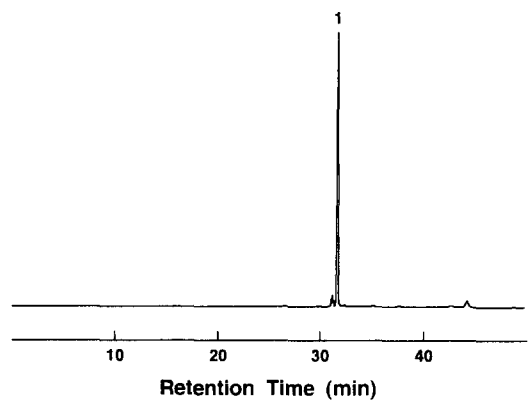


Fig. 1. HPLC analysis of fluorescent products formed by the reaction of AGLME with TMP hydrolysate after 48 h. Fluorescence detection was carried out at 370 nm (excitation) and 450 nm (emission). The reaction and analysis were carried out as described in Experimental procedure (Section 2).

2.5. Isolation of fluorescent product (1)

AGLME (64.2 mg) was dissolved in 1.8 ml of 0.1 M sodium acetate buffer (pH 4.5), and then 0.2 ml of freshly prepared 100 mM TMP hydrolysate was added. The reaction mixture containing 100 mM AGLME and 10 mM TMP hydrolysate (pH 4.0) was incubated at room temperature. After 2 days, the reaction mixture was directly subjected to preparative HPLC (100:1 H_2O/CH_3CO_2H containing 0–14% acetonitrile, linear gradient (55 min) at a flow rate of 2.0 ml/min). The HPLC fraction of **1** eluted at 49.5 min was collected and immediately neutralized with pyridine to avoid the decomposition of **1**. The combined fraction was evaporated to dryness. The resulting residue was further purified by HPLC (100:1 H_2O/CH_3CO_2H containing 0–24% CH_3OH, linear gradient (60 min) at a flow rate of 2.0 ml/min). The HPLC fraction eluted at 55.9 min was collected and immediately neutralized with pyridine. Evaporation of the combined fraction afforded **1** (yield 0.18 mg). The yield of **1** was determined by measuring the released glycine from the acid hydrolysis of **1** (6 N HCl, 105°C, 24 h). UV(H_2O) λ_{max} (ϵ) 235 (24 600), 260 (11 000), and 398 nm (8600). ^{13}C-NMR (CD_3OD) δ 22.5, 22.5, 23.2, 23.6, 30.1, 31.7, 31.9, 32.2, 34.3, 43.6, 43.6, 52.9, 52.9, 53.1, 53.2, 56.0 (ϵ-CH_2 adjacent to the dihydropyridine ring), 62.6 (ϵ-CH_2 adjacent to the pyridinium ring), 120.4, 129.4, 143.5, 145.6, 145.6, 147.4, 150.1, 171.8, 171.8, 173.7, 173.7, 173.9, 173.9, 190.8. ^{1}H-NMR (CD_3OD) δ 1.20–1.56 (4H, m, γ-CH_2), 1.67–2.11 (8H, m, β- and δ-CH_2), 1.99 (3H, S, $COCH_3$), 2.00 (3H, s, $COCH_3$), 3.70 (3H, s, CO_2CH_3), 3.72 (3H, s, CO_2CH_3), 3.80 (2H, dd, $J = 16.6$ and 4.8 Hz, NHC$\underline{H}_2$CO), 3.89 (2H, dd, $J = 16.6$ and 3.1 Hz, NHC$\underline{H}_2$CO), 4.45–4.56 (2H, m, NHC$\underline{H}$CO), 4.64 (2H, t, $J = 7.2$ Hz, ϵ-CH_2 adjacent to the pyridinium ring), 5.17 (1H, s), 7.56 (2H, s), 8.00 (1H, dd, $J = 8.0$ and 6.2 Hz), 8.44 (1H, d, $J = 8.1$ Hz), 8.74 (1H, d, $J = 6.1$ Hz), 9.05 (1H, s), 9.23 (2H, s). The ϵ-CH_2 protons adjacent to the dihydropyridine ring were obscured by the overlapping signals at δ 3.70 and 3.72. HR-FABMS m/z 699.3322 calc. for $C_{34}H_{47}O_{10}N_6$ 699.3354.

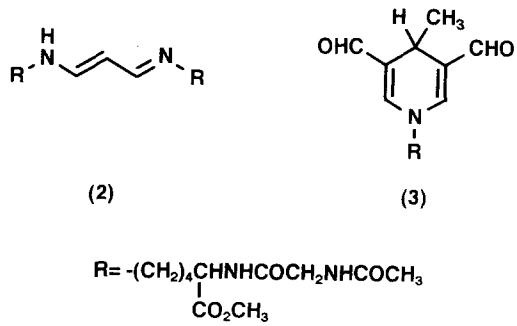


Fig. 2. 1-Amino-3-iminopropene derivative (**2**) and 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative (**3**).

3. Results

A reaction mixture of 100 mM *N*-acetyl-glycyl-lysine methyl ester (AGLME) and 10 mM 1,1,3,3-tetramethoxypropane (TMP) hydrolysate (pH 4.0) was incubated at room temperature for 48 h. HPLC analysis of the mixture revealed a major peak eluting at 31.6 min as illustrated in Fig. 1. Purifications by reverse-phase HPLC gave a fluorescent product (**1**) which had a fluorescence in H_2O at 400 nm (excitation) and 454 nm (emission). Product **1** also exhibited UV absorption maxima at 235, 260, and 398 nm. The HR-FABMS spectrum of **1** revealed a molecular ion peak at m/z 699.3322 indicating its molecular formula to be $C_{34}H_{47}O_{10}N_6$. This data definitely indicates that **1** was neither the 1-amino-3-iminopropene derivative (**2**) nor the 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative (**3**) (Fig. 2). Both **2** and **3** have been already reported as major fluorescent products derived from the reaction of primary amines with MDA (Chio and Tappel, 1969b; Kikugawa et al., 1981a,b; Kikugawa and Ido, 1984). Table 1 shows selected ^{13}C- and ^{1}H-NMR data of **1**. The ^{1}H-NMR spectrum showed a singlet (2H) for two equivalent uncoupled aldehyde protons δ 9.23, a singlet (2H) for two equivalent uncoupled olefinic protons at δ 7.56, and a singlet (1H) for an uncoupled methine proton at δ 5.17. These data suggested that a 4-substituted 1,4-dihydro-3,5-dicarbaldehyde moiety was present within **1**. Further support for this partial structure came from the UV and fluorescence spectra of **1**. Besides the dihydropyridine

Table 1
Selected ^{13}C- and ^{1}H-NMR data of **1** in CD_3OD[a]

Carbon number[b]	^{13}C (ppm)	^{1}H (ppm)
2	145.6	9.05 (1H, s)
3	147.4	
4	145.6	8.44 (1H, d)
5	129.4	8.00 (1H, dd)
6	143.5	8.74 (1H, d)
2′,6′	150.1	7.56 (2H, s)
3′,5′	120.4	
4′	34.3	5.17 (1H, s)
7′,8′	190.8	9.23 (2H, s)

[a]Assignments were established by ^{1}H-^{1}H COSY, HMQC, HMBC, and DEPT experiments.
[b]See Fig. 3 for numbering.

ring signals, four proton signals at δ 8.00, 8.44, 8.74, and 9.05 were also observed. The singlet (1H) at δ 9.05 was not an aldehyde proton but an aromatic proton because of its HMQC correlation to a carbon signal at δ 145.6. The doublets (1H, each) at δ 8.44 and 8.74 were coupled with the doubled doublet (1H) at δ 8.00 as is confirmed by a ^{1}H-^{1}H COSY experiment. These data suggested the presence of four aromatic protons in a pyridinium ring with a substituent at the 3-position. Collectively, the above data suggested the structure of **1** as shown in Fig. 3. The connectivity of C-3 of the pyridinium ring to C-4′ of the dihydropyridine ring was confirmed by the HMBC correlation of δ_H 5.17 (H-4′) to δ_c 145.6 (C-2 and/or C-4). Fig. 4 shows the time course of the formation of **1**. The concentration of **1** in the reaction mixture after 48 h was 0.31 mM.

Next we examined whether or not **1** could be produced under neutral conditions. When the reaction of AGLME with TMP hydrolysate was

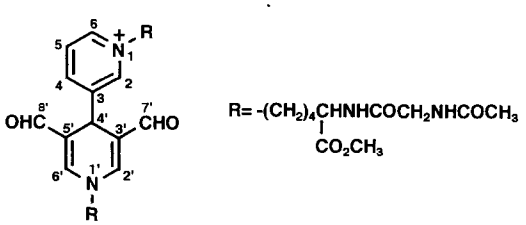


Fig. 3. Chemical structure of the fluorescent MDA-lysine adduct (**1**).

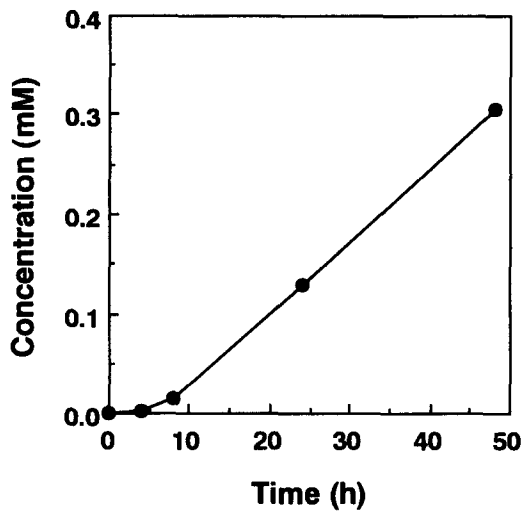


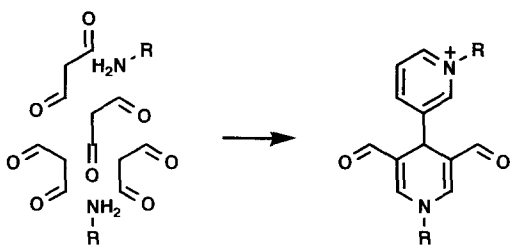
Fig. 4. Time course of the formation of **1** in the reaction mixture of AGLME and TMP hydrolysate. The reaction was carried out as described in Experimental procedure (Section 2). The concentrations of **1** were determined by HPLC with fluorescence detection at 370 nm (excitation) and 450 nm (emission).

conducted at pH 7.2, HPLC analysis of the mixture revealed a fluorescent peak at the expected retention time of **1** (data not shown). However, the peak was too weak to provide its complete confirmation.

4. Discussion

It is well-known that proteins are readily modified by MDA to form fluorescence. From a chemical point of view, considerable interest has been focused on the structure of the fluorophores. Chio and Tappel (1969b) have demonstrated that MDA reacts with amino compounds under strongly acidic conditions far from physiological to afford 1-amino-3-iminopropenes. Since the 1-amino-3-iminopropenes exhibited fluorescence spectra (excitation maxima at 370–400 nm; emission maxima at 450–470 nm) similar to those of lipofuscin-like pigment (excitation maxima at 340–390 nm; emission maxima at 430–490 nm), they have speculated that the fluorophores in lipofuscin-like pigment are these 1-amino-3-iminopropenes. Contrary to this, Kikugawa and

co-workers (Kikugawa and Ido, 1984; Kikugawa et al., 1981a,b, 1984) have demonstrated that the reaction of amino compounds with MDA under mild conditions close to physiological does not afford 1-amino-3-iminopropenes but affords strongly fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes. This observation was somewhat surprising because 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes cannot form cross-linkings. Proteins are readily cross-linked by MDA under mild conditions, and it has been widely accepted that the cross-linkings occur through the formation of 1-amino-3-iminopropene linkages. In order to investigate this discrepancy, we first attempted to obtain 1-amino-3-iminopropene-type product (**2**) by using an excess of AGLME over MDA at pH 4.0 (Nair et al., 1981; Ohya, 1993), but it could not be obtained. Instead we isolated and identified a new type of fluorescent product (**1**) which has dihydropyridine and pyridinium rings. Although **1** may be formed by the condensation of 2 mol AGLME with 4 mol MDA (Scheme 1),



Scheme 1. Formation of **1** from 2 mol AGLME and 4 mol MDA.

the detailed mechanism is not yet clear. The amount of MDA employed in our experiment was much less than that of AGLME, suggesting that even a small amount of MDA relative to the ϵ-amino group of the lysine residues can produce **1**. Furthermore, it is worthy of remark that cross-linkings in MDA-modified proteins can be explained by the formation of **1**. Although the cross-linkings are considered to be due to 1-amino-3-iminopropenes, we assume that **1** is also one of the possible candidates for the protein cross-linkages. With respect to this, further investigation will be needed.

In conclusion, we found a novel fluorescent MDA-lysine adduct (**1**). Further studies on the formation of this fluorophore in MDA-modified proteins are now in progress.

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