Formation of a New 1,1,1 Adduct in the Reaction of Malondialdehyde, n-Hexylamine and Alkanal under Neutral Conditions

Takeshi OHYA

Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-02, Japan.

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The reactions of malondialdehyde (MDA) with n-hexylamine (HA) in the presence of alkanals at a neutral pH were investigated. Two new compounds, 1,1,1 adduct (4a) and fluorescent compound (3a), were isolated from the reaction of MDA, HA and acetaldehyde. Compounds 4a and 3a were identified as 2-formyl-3-hexylamino-3-methylpropionaldehyde and 1-hexyl-3-hexyliminomethylene-4-methyl-1,4-dihydropyridine-3-carbaldehyde, respectively. Similar compounds (4b and 3b) were obtained from the reaction of MDA, HA and propionaldehyde. Compound 4 was obtained in a high yield. In addition, the reactivity of MDA towards phenylethylamine (PEA) in the presence or absence of alkanals was investigated. The results indicated that MDA was of low reactivity in the absence of alkanals at neutral pH. However, when alkanals coexisted, MDA showed high reactivity towards PEA.

Keywords malondialdehyde; alkanal; n-hexylamine; phenylethylamine; 2-formyl-3-hexylamino-3-methylpropionaldehyde; 1-hexyl-3-hexyliminomethylene-4-methyl-1,4-dihydropyridine-3-carbaldehyde

Introduction

Malondialdehyde (MDA) is formed from the peroxidation of polyunsaturated fatty acids and from the oxidative degradation of deoxyribose by a hydroxyl radical. MDA is also produced in mammalian tissues as a side product of prostaglandin and thromboxane biosynthesis.

Since MDA is toxic and mutagenic to the modification of lysine residue, the reactions of MDA with nucleic acid bases and primary amines have been investigated. Chio and Tapper isolated conjugated Schiff bases (1-amino-3-iminopropanes) from the reaction of MDA with n-hexylamine and leucine ethyl ester under strongly acidic conditions. Nair et al. demonstrated that the reaction of MDA with amino acid methyl esters gave 1:1 Schiff bases under mildly acidic conditions.

On the other hand, alkanals such as acetaldehyde, propanal and hexanal are produced along with MDA as the end products of unsaturated lipid peroxidation. Kikugawa et al. showed that highly fluorescent 1,4-dihydropyridine-3,5-dicarbaldehydes were formed when MDA reacted with methylethanolamine, ethylamine or glycine ethyl ester in the presence of alkanals. Nair et al. also isolated similar compounds from the reactions of MDA with amino acids in the presence of alkanals.

The author investigated the reactions of MDA with primary amines (n-hexylamine (HA) and phenylethylamine (PEA)) in the presence of alkanals (acetaldehyde and propanal) at a neutral pH in detail in order to elucidate the action of MDA on a primary amino group when alkanals coexisted under close to physiological conditions. It was found that the reaction of MDA, HA and alkanal afforded a new 1:1:1 adduct and fluorescent compound in addition to 1,4-dihydropyridine-3,5-dicarbaldehyde.

This paper describes the structures of new compounds and the reactivity of MDA towards HA and PEA in the presence or absence of alkanals.

Results

Structures of the Reaction Products

The reaction of MDA with HA in the presence of acetaldehyde produced three compounds at pH 7.0 and four compounds at pH 4.0. TLC of the reaction mixture revealed two fluorescent spots (Rf 0.90, compound 1a and Rf 0.44, compound 3a) and two UV-absorbing spots (Rf 0.56, compound 2 and Rf 0.27, compound 4a). These products (1a, 2, 3a, and 4a) were isolated from the reaction mixtures by use of a silica gel column.

Compounds 1a and 2 were identified as 1-hexyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde and 1:1 Schiff base of MDA with HA (3-hexylamino-2-propenal), respectively, by comparison with authentic samples prepared according to the literature.

The fast atom bombardment mass spectrum (FAB-MS) of 4a, the main product in the reaction at pH 7.0, showed an [M + H] + ion at 200, indicating its molecular weight to be 199. The FAB-MS spectral data and elemental analysis indicated a molecular formula of C11H23NO2, which suggested that 4a was a 1:1:1 adduct of MDA, acetaldehyde and HA. It is known that the reaction of MDA with alkylamine produces the Schiff base compound. However, 4a seemed to differ from the Schiff base derivative, since the UV-absorption spectrum, showing a maximum at 265 nm in water, was not similar to that of the Schiff base which exhibited a maximum at 280 nm in water. In addition, the reaction of the Schiff base (2) with acetaldehyde did not give 4a. The 'H-NMR spectrum showed signals due to an aldehyde (δ 8.61, s), CH-CH3 (δ 4.15, q, J = 7.0 Hz and δ 1.43, d, J = 7.0 Hz) and n-hexyl group (δ 0.91, t; δ 1.28—1.39, br, and δ 1.55—1.70, m). The spectrum of 4a was analogous in part to that of 2,4-dihyrdroxyethylene-3-methylglutaraldehyde (5, Chart 1) which was the 2:1 adduct of MDA and acetaldehyde (Table I). Accordingly, the structure of 4a was determined to be an enol form of...
2-formyl-3-hexylamino-3-methylpropanal (Chart 2). Further support for the structure came from the $^{13}$C-NMR spectral data together with the distortionless enhancement by polarization transfer (DEPT) experiment, which showed 10 carbon signals; the signal at $\delta 189.02$ (methylene carbon) due to an aldehyde group, the signal at $\delta 114.61$ (quaternary carbon) due to C-2, the signal at $\delta 52.25$ (methylene carbon) due to C-3, the signal at $\delta 18.20$ (methyl carbon) due to CH$_3$-3 and the signals at $\delta 46.61$, $32.37$, $27.30$, $27.26$, $23.40$ (each methylene carbon) and at $\delta 14.27$ (methyl carbon) due to an n-hexyl group.

The reaction of MDA with HA in the presence of propanal also gave an adduct (4B) similar to 4a. Compound 3a was obtained as a yellow oil, which was fluorescent, emitting at 480 nm upon excitation at 415 nm. The high-resolution mass spectrum (HR-MS) showed an M$^+$ ion at $m/z$ 318, indicating its molecular formula to be C$_{26}$H$_{34}$N$_2$O. Compound 3a was prepared by refluxing a mixture of 1a and HA in MeOH. In addition, the hydrolysis of 3a gave 1a (Chart 2). Therefore, it was assumed that 3a was a Schiff base of 1a with HA. The $^1$H-NMR spectrum of 3a was assigned by comparison with that of 1-hexyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde and identified as follows: the signals at $\delta 2.90$ (s), 6.67 (s), 6.22 (s), 4.05 (q, $J=6.6$ Hz) and 1.12 (d, $J=6.6$ Hz) were due to a 4-methyl-1,4-dihydropyridine-3-carbaldehyde moiety; the signal at $\delta 7.69$ (s) was due to $-\text{CH}==\text{N}$- and the signals at $\delta 0.86-0.92$ (m), 1.29-1.37 (m), 1.57-1.69 (m), 3.36 (t) and 3.43 (t) were due to two n-hexyl groups. Accordingly, the structure of 3a was determined to be 1-hexyl-5-hexyliminomethylene-4-methyl-1,4-dihydropyridine-3-carbaldehyde.

The reaction of MDA with HA in the presence of propanal also gave an adduct (3b) similar to 3a.

**Time Course Experiment**

During lipid peroxidation, alkanals are produced in larger amounts than MDA, and a number of biomolecules containing primary amino groups such as polyamines, proteins and amino phospholipids are present in a biological environment. Therefore, the time course experiments on the formation of 1a, 2, 3a and 4a were carried out in excess amounts of HA and acetaldehyde. A mixture of MDA (10 mM), HA (40 mM) and acetaldehyde (20 mM) in phosphate buffer (pH 7.0) or citrate buffer (pH 4.0) was incubated at 37 °C. The yields of the reaction products were determined by HPLC. The results are shown in Fig. 1. In the reaction at pH 7.0, the formation of 4a and 3a reached a maximum after 30 min and 4 h, respectively, while the yield of 1a gradually increased as the reaction time increased. No formation of 2 was observed, even after 7 h. In the reaction at pH 4.0, the formation of 2 and 4a reached a maximum after 1 h and then the yield

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**Table I. $^1$H-NMR Data for 2,4-Dihydroxymethylene-3-methylglutalaldehyde (5)$^a$ and 4a**

<table>
<thead>
<tr>
<th></th>
<th>5$^a$</th>
<th>4a$^b$</th>
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<tbody>
<tr>
<td>8.24 (H-1,1,1' ,5)</td>
<td>8.61 s</td>
<td></td>
</tr>
<tr>
<td>4.15 (H-3)</td>
<td>4.15 q</td>
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<tr>
<td>1.25 (C,H$_3$-3)</td>
<td>1.43 d</td>
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<td></td>
<td>2.82 t</td>
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<td></td>
<td>1.55-1.71 m</td>
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<tr>
<td></td>
<td>1.28-1.39 br</td>
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<tr>
<td></td>
<td>0.91 t</td>
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$^a$ In D$_2$O. $^b$ In CD$_3$OD.
of both compounds gradually decreased with increasing reaction time. The yield of 1a increased with the increase in reaction time in both cases, pH 4.0 and 7.0.

Effect of the HA Concentration The relation between the HA concentration and the yields of the reaction products (1a, 2, 3a and 4a) was examined. A mixture of MDA (10 mM), acetaldehyde (20 or 60 mM) and HA (0–80 mM) in a phosphate buffer (pH 7.0) or citrate buffer (pH 4.0) was incubated at 37°C for 4 h. The results are shown in Figs. 2A, B, C and D. The yields of 3a and 4a increased as the HA concentration increased, while the yield of 1a increased as the HA concentration increased up to 20 mM, then gradually decreased with further increases in the HA concentration in the reactions at both pH 7.0 and 4.0. The formation of 2, which was observed in the reaction at pH 4.0 and at a low concentration of acetaldehyde (20 mM), reached a maximum at an HA concentration of 60 mM. The reaction of MDA with excess amounts of HA (80 mM) and acetaldehyde (60 mM) at pH 7.0 gave 4a in a high yield (86%).

In the absence of acetaldehyde, only 2 was produced (Figs. 2E and F). The yield of 2 increased as the HA concentration increased. The yield of 2 in the reaction at pH 7.0 was much lower than that in the reaction at pH 4.0.

Influence of pH Figure 3 shows the influence of pH on the formation of 1a, 2, 3a and 4a. A mixture of MDA (10 mM), HA (40 mM) and acetaldehyde (20 mM) in citrate buffer (pH 4.0) or phosphate buffer (pH 5.5–8.0) was incubated at 37°C for 4 h. The yield of 2 increased as the pH decreased from 7.0 to 4.0.
incubated at 37°C for 4 h. The yield of 4a increased with increasing pH, while the yields of 1a and 2 decreased with increasing pH. The formation of 3a reached a maximum yield at pH 6.5.

**Reactivity of MDA towards PEA** The reactivity of MDA towards primary amine in the presence or absence of alkanals was investigated. In order to facilitate the detection by HPLC, PEA was used instead of HA as a primary amine. A mixture of MDA (10 mm), alkanals (10 mm) and PEA (10 mm) in a phosphate buffer (pH 7.0) was incubated at 37°C for 4 h, then the PEA that remained was determined by HPLC. The results are shown in Table II. In the absence of alkanal, the loss of PEA was only 6%, while in the presence of alkanals, 33−53% of the PEA was modified. This result indicated that MDA had a low reactivity towards a primary amine under physiological conditions. However, when alkanals coexisted, MDA was found to react smoothly with a primary amine.

**Discussion**

It has been shown that MDA produced during lipid peroxidation leads to the formation of an adduct with a lysine residue (ε-amino group) of proteins and that MDA reacts with a primary amine to give a 1:1 Schiff base. On the other hand, it is known that alkanals such as acetaldehyde, propanal and hexanal are formed together with MDA during lipid peroxidation. Therefore, it is of interest to investigate the reactivity of MDA towards a primary amine in the presence of alkanals.

Two new compounds, 3-alkyl-2-formyl-3-hexylamino-propanal (4) and 4-alkyl-1-hexyl-5-hexylaminomethylene-1,4-dihydropyridine-3-carbaldehyde (fluorescent compound, 3) were obtained along with 1,4-dihydropyridine-3,5-carbaldehyde from the reaction of MDA with HA in the presence of acetaldehyde or propanal at a neutral pH. Compound 4 was formed in a high yield when MDA reacted with excess amounts of HA and alkanals.

Although MDA is highly reactive towards primary amines under acidic or mildly acidic conditions as demonstrated by Nair et al., the experimental results showed that MDA was of low reactivity under neutral conditions. For example, the reaction of MDA (10 mm) with HA (40 mm) at pH 4.0 gave a 70% yield of Schiff base (2), while the reaction at pH 7.0 produced only a 10% yield (Figs. 2E and F), and in the reaction of MDA (10 mm) with PEA (10 mm) at pH 7.0, the PEA modified was only 6% (Table II). However, when alkanals coexisted, it was found that MDA was highly reactive towards a primary amine such as HA under neutral conditions. For example, the reaction of MDA (10 mm) with HA (40 mm) in the presence of acetaldehyde (20 mm) at pH 7.0 gave 4a in a 63% yield (Fig. 2A). These results suggested that the active site of MDA seemed to be the methylene group rather than the aldehyde group in the reaction at pH 7.0.

Carbonneau et al. demonstrated that more than 50% of the total MDA produced during lipid peroxidation in serum and plasma was bound to biomolecules such as proteins. It is probable that the bound MDA can exist in part in the form of an MDA-alkanamino group of biomolecule adduct.

Further studies are in progress on the interactions of MDA with biomolecules or their model compounds in the presence of alkanals.

**Materials**

Acetaldehyde (99.5%) was purchased from Merck Co., Ltd. Sodium malondialdehyde was prepared by the method in the literature.

**Preparation of 1-Hexyl-4-formyl-1,4-dihydropyridine-3-carbaldehyde** Compound 1a was prepared by a modification of the method of Kikugawa et al. A mixture of sodium malondialdehyde (150 mg), n-hexylamine hydrochloride (270 mg) and acetaldehyde (120 μl) in 50 ml of 0.1 M citrate buffer solution (pH 4.0) was incubated at 37°C for 24 h. The solid (80 mg) separated in the reaction mixture was collected by filtration and recrystallized from benzene-n-hexane. mp 95−96°C. MS m/z: 235 (M+). Anal. Caled for C9H14NO: C, 72.25; H, 9.09; N, 5.62. Found: C, 71.47; H, 8.91; N, 5.75.

**Preparation of 3-Hexylamino-2-propanal** Compound 2 was prepared by a modification of the method of Nair et al. A mixture of sodium malondialdehyde (150 mg) and n-hexylamine hydrochloride in 35 ml of 0.1 M citrate buffer solution (pH 4.0) was incubated at 37°C for 5 h. The solution was neutralized with 1 N NaOH, and the reaction mixture was evaporated to dryness in vacuo. Benzene (50 ml) was added to the residue and filtered to separate sodium citrate. The filtrate was evaporated to dryness in vacuo and the residue was subjected to column chromatography on silica gel (60 g) with benzene-MeOH (10:1) to give 2 as a pale yellow oil (65 mg). MS m/z: 155 (M+). HRMS Caled for C7H12N: 155.1310. Found: 155.1293.

**Formation of 2-Formyl-3-methyl-3-hexylamino-2-propanal** (4a) A mixture of sodium malondialdehyde (150 mg), n-hexylamine hydrochloride (540 mg) and acetaldehyde (120 μl) in 50 ml of 0.1 M phosphate buffer solution (pH 7.0) was incubated at 37°C for 40 min. The reaction mixture was evaporated to dryness in vacuo. Chloroform (50 ml) was added to the residue and filtered to separate sodium phosphate. The filtrate was evaporated to about 10 ml in vacuo, and extracted with water (3×15 ml). The aqueous layer was evaporated to dryness in vacuo. The residue was purified twice by column chromatography on silica gel (50 g) with benzene-MeOH (10:2), and 4a (120 mg) was obtained on recrystallization from MeOH-AcOEt-n-hexane as a colorless powder. mp 110°C (dec.) FAB-MS m/z: 200 (M+H+). UV λmax (H2O): 265 nm. Anal. Caled for C10H14NO2: C, 66.29; H, 10.62; N, 7.03. Found: C, 66.40; H, 10.59; N, 7.02. \( \text{HNMR (CD}_{3}\text{OD}) \): \( \delta \) 8.61 (2H, s), 4.15 (1H, q, J = 7.0 Hz), 2.82 (2H, t), 1.70−1.55 (2H, m), 1.43 (3H, d, J = 7.0 Hz), 1.39−1.28 (6H, br), 0.91 (3H, t). \( \text{13CNMR (CD}_{3}\text{OD}) \): \( \delta \) 189.02, 114.61, 52.25, 46.31, 32.37, 27.30, 27.26, 27.30, 18.20, 18.40, 14.27. 2-Formyl-3-ethyl-3-hexylamino-2-propanal (4b) was obtained from the reaction of sodium malondialdehyde (150 mg),...
n-hexylamine hydrochloride (540 mg) and propanol (160 μl) in the same manner described above. mp 106°C (dec). FAB-MS m/z: 214 (M + H)⁺. 
Anal. Caled for C₁₃H₂₂NO₂: C, 67.57; H, 10.87; N, 6.57. Found: C, 67.47; H, 10.78; N, 6.30. ¹H-NMR (CD₃OD) δ: 8.67 (2H, s), 3.98 (1H, dd), 2.93—2.75 (2H, m), 2.02—1.85 (1H, m), 1.85—1.70 (1H, m), 1.70—1.50 (2H, m) ppm.

Formation of 1-Hexyl-5-hexylimidazoline-4-methyl-1,4-dihydropyridine-3-carboxaldehyde (3a) - A mixture of sodium malondialdehyde (150 mg), n-hexylamine hydrochloride (825 mg) and acetaldehyde (120 μl) in 50 ml of 0.1 M phosphate buffer solution (pH 7.0) was incubated at 37°C for 5 h. The reaction mixture was extracted with benzene (30 ml × 2). The benzene layer was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The residue was subjected to column chromatography on silica gel (50 g) with CHCl₃-MeOH:AcOH (10:1:0.5) then with CHCl₃-MeOH (10:1). The eluate was evaporated to dryness in vacuo and the residue was rechromatographed on silica gel (50 g) with CHCl₃-MeOH (10:1) to give 3a as a yellow oil (10 mg). MS m/z: 318 (M⁺). HRMS Caled for C₂₃H₃₄N₂O₃: 318.2671. Found: 318.2666. ¹H-NMR (CDCl₃) δ: 9.20 (1H, s), 7.69 (1H, s), 6.67 (1H, s), 6.22 (1H, s), 4.05 (1H, q, J = 6.6 Hz), 3.43 (2H, t), 3.36 (2H, t), 1.69—1.57 (4H, m), 1.37—1.29 (12H, m), 1.12 (3H, d, J = 6.6 Hz), 0.92—0.86 (6H, m). 1-Hexyl-5-hexylimidazoline-4-ethyl-1,4-dihydropyridine-3-carboxaldehyde (3b) was obtained as a yellow oil from the reaction of sodium malondialdehyde (150 mg), n-hexylamine hydrochloride (825 mg) and propanol (160 μl) in the same manner described above. MS m/z: 332 (M⁺). HRMS Caled for C₂₅H₃₆N₂O₃: 332.2827. Found: 332.2841. ¹H-NMR (CDCl₃) δ: 9.24 (1H, s), 7.70 (1H, s), 6.80 (1H, s), 6.35 (1H, s), 4.17 (1H, t), 3.43 (2H, t), 3.35 (2H, t), 1.70—1.46 (6H, m), 1.26—1.20 (2H, m), 0.94—0.85 (6H, m), 0.72 (3H, t). Compound 3a was prepared from the reaction of 1a with HA. A mixture of 1a (50 mg) and n-hexylamine hydrochloride (50 mg) in MeOH (10 ml) was refluxed for 7 h. The reaction mixture was evaporated to dryness in vacuo and the residue was subjected to column chromatography on silica gel (50 g) with CHCl₃-MeOH (10:0.7) to give 3a.

Time Course Experiment - A mixture of MDA (10 μmol), HA (40 μmol) and acetaldehyde (20 μmol) in 0.1 M phosphate buffer solution (pH 7.0) was incubated at 37°C. Aliquots (1 ml) of the solution were periodically removed, diluted with water to 10 ml, and 10 μl of the solution was injected into HPLC to determine the reaction products. HPLC analysis was performed on a 4.6 × 250 mm ODS column (Ultron C₁₈, Shinwakakou Co., Ltd.) with a mobile phase of 0.05 M phosphate buffer (pH 3.0)—MeOH (35:65, v/v) at a flow rate of 1.0 ml/min at ambient temperature. The UV absorbances at 245 nm (for 3a, 3b and 4a) and at 280 nm (for 2) were integrated and the concentrations of the products in the reaction mixture were determined by using standard curves.

Reactivity of MDA towards PEA - A mixture of MDA (10 μmol), PEA (10 μmol) and alkaline (10 μmol) in 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 4 h. Aliquots (1 ml) of the solution were removed, diluted with water to 10 ml, and 10 μl of the solution was injected into HPLC for the determination of PEA. HPLC analysis was performed on a 4.6 × 250 mm ODS column (Ultron C₁₈, with a mobile phase of 0.05 M phosphate buffer (pH 3.0)—MeOH (75:25, v/v) at a flow rate of 1.0 ml/min and a detector at 210 nm.

References
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