Formation of Fluorescent Products in the Reaction of Butyraldehyde and Methylamine as a Model of the Reaction of Oxidized Lipids and Proteins

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Formation of fluorescent compounds in the reaction of butyraldehyde and methylamine at pH 7 and 37 °C was investigated as a model of the reaction of oxidized lipids and proteins. The reaction mixture was treated with borohydride for isolation of the products. Some fluorescent products remained unchanged and the other changed into other fluorescent compounds. One of the fluorescent compounds produced on borohydride treatment was identified as 2,4-diethyl-2,5-dihydrofuran, which exhibited fluorescence with an excitation maximum at 345 nm and an emission maximum at 406 nm. The product may be derived by the condensation of 2 molecules of butyraldehyde in the presence of methylamine. Non-fluorescent products composed of 2:1, 3:1 and 4:1 ratios of butyraldehyde and methylamine were isolated. The fluorescent products may include self-condensation products of butyraldehyde formed in the presence of methylamine.

Keywords fluorescence; butyraldehyde; 2,5-dihydrofuran; primary amine; lipofuscin

Lipofuscin pigment formed in aging tissues and cells[1,2] exhibits fluorescence with excitation maxima at 345—395 nm and emission maxima at 420—490 nm.[3,4] Accumulation of the fluorescent lipofuscin pigment has been assumed to be due to lipid oxidation of tissues or cells.[5,6] Fatty acid hydroperoxides give rise to similar fluorescence by interaction with primary amines, phospholipids or proteins, and the fluorophores have been considered to be derived from their secondary degradation products.[7-10]

Secondary degradation products such as malonaldehyde and the other aldehydes[11] produce fluorescence similar to that of lipofuscin pigment.[7-10] While the formation of the fluorophores from aldehydes other than malonaldehyde has been demonstrated,[12-17] their structures have not yet been elucidated. Identification of the fluorophores derived from the reaction of the aldehydes and primary amines may be useful for elucidation of the mechanisms involved in the formation of lipofuscin pigment.

Hexanal is one of the major components in oxidized lipids,[11] and butyraldehyde is a closely related aldehyde with a shorter alkyl chain. We have shown that the reaction of butyraldehyde with methylamine gives 2-ethyl-2-hexenal as a consequence of aldol condensation, a pyridinium salt and several fluorescent products.[16] At that time, however, the fluorescent products could not be isolated in pure forms owing to their instability. This time, we attempted to isolate the fluorescent products and characterize them after treatment with borohydride.

Experimental

Butyraldehyde and 2-ethyl-2-hexenal were obtained from Wako Pure Chemical Industries, Osaka, and Tokyo Kasei Kogyo Company, Tokyo, respectively.

Analysis Ultraviolet (UV) absorption spectra were taken on a Hitachi 557 dual-wavelength double-beam spectrophotometer. Fluorescence spectra were measured on a Hitachi 650-40 fluorescence spectrophotometer. Proton and carbon-13 nuclear magnetic resonance (^{1}H-and ^{13}C-NMR) spectra were taken on a JEOL JNM-GX 500 FT-NMR (or a Bruker AM400 FT-NMR) spectrometer by use of $CDCl_3$ as a solvent and tetramethylsilane as an internal standard. Signals in the ^{13}C-NMR spectra were assigned by the complete decoupling technique and insensitive nucleic enhanced by polarization transfer (INEPT) experiments. Mass spectra (MS) were taken on a Hitachi M-80 double-focusing mass spectrometer by the electron impact ionization (EI) technique operating at an ionization energy of 70 eV and an ion source temperature of 200 °C, or by the chemical ionization (CI) technique using isobutane as a reagent gas.

High-pressure liquid chromatography (HPLC) was performed by use of a Hitachi 655 liquid chromatograph equipped with a column of Inertsil octadecyl silica (ODS) (4.6 × 250 mm for analytical purposes, and 20 × 250 mm for preparative purposes) (Gasukuro Kogyo Company, Tokyo). The column was eluted with methanol/0.01 M triethylamine bicarbonate buffer (pH 7.6) (1:1, v/v) at a flow rate of 0.5 ml/min for analytical purposes and 5.0 ml/min for preparative purposes. Fluorescent peaks were detected with excitation at 350 nm and emission at 420 nm by use of a Shimadzu RF-530 fluorescence spectromonitor. Ultraviolet absorbing peaks were detected at 250 nm by use of a Hitachi 638-41 variable wavelength UV monitor. Gas chromatography (GC) was performed by use of a Hitachi 263-30 gas chromatograph equipped with a column of Silicone OV-101 (3 × 2000 mm). The gas chromatographic conditions were: injection port temperature 150 °C; column oven temperature programmed from 130 °C at 5 °C/min to 240 °C; carrier gas (nitrogen) flow rate set to 30 ml/min. Silica gel and silicic acid column chromatographies were carried out by use of silica gel for column chromatography (100 mesh) (Kanto Chemical Company, Tokyo) and silicic acid (100 mesh) (Mallinckrodt, Inc., Paris), respectively. Thin-layer chromatography (TLC) was carried out by use of Wakogel B-5F (Wako Pure Chemical Industries). The chromatogram was developed with chloroform–methanol (9:1, v/v) and the spots were visualized by exposure to iodine vapor or UV light at 254 nm, or by fluorescence measurement (excited at 365 nm).

Formation of Fluorescence by Reaction of Butyraldehyde or 2-Ethyl-2-hexenal with Methylamine A reaction mixture of butyraldehyde (or 2-ethyl-2-hexenal) and methylamine at indicated concentrations in 0.1 M phosphate buffer (pH 7.0) containing 70% methanol was incubated at 37 °C for 48 h. For the investigation of the effects of borohydride treatment, the reaction mixture was treated with 500 mM sodium borohydride at 0 °C for 1 h. The fluorescence spectrum of the mixture was measured after dilution into methanol and the intensity relative to that of 0.1 μM quinine sulfate in 0.1 N sulfuric acid was measured (Table I). For HPLC analysis, the reaction mixture was applied directly to an analytical Inertsil ODS column (Fig. 1).

Isolation of Fluorescent Products I, II and RI and Non-fluorescent Products a, b, c, e, g and l from the Borohydride-Treated Reaction Mixture of Butyraldehyde and Methylamine A 150-ml reaction mixture of 200 mM butyraldehyde and 100 mM methylamine in 0.1 M phosphate buffer (pH 7.0) containing 70% methanol was incubated at 37 °C for 48 h (reaction B in Table I). Sodium borohydride (75 mmol) was added to the reaction mixture, and the mixture was kept at 0 °C for 1 h. The mixture was extracted with 4 volumes of chloroform, and the extract was evaporated to dryness (2.4 g).

For isolation of the fluorescent products, the residue was applied to a column (25 × 190 mm) of silica gel. The column was eluted successively with *n*-hexane–chloroform (2:8, v/v), chloroform, and chloroform–methanol (19:1, v/v). All the fluorescent products were eluted in the chloroform/methanol fraction. The fraction was evaporated to dryness (about 0.4 g), and the residue was applied to a preparative Inertsil ODS column in two portions. The fractions containing products I, II, RI and RII were separately collected and evaporated to dryness. Each residue was rechromatographed through an analytical Inertsil ODS column. Fluorescent products I, II and RI were obtained as colorless oils which

showed single fluorescent and ultraviolet absorbing peaks on analytical HPLC. The retention times of I, II and RI were 10.5, 13.0 and 18.0 min, respectively. Products I and II were unstable and decomposed even in a refrigerator. About 0.4 mg of product RI was obtained from eight batches of the above reaction mixture. CI MS *m/z*: 127 [$M^+ + 1$]. The ^{13}C- and ^1H-NMR spectra of RI are shown in Table II, and the ultraviolet absorption and fluorescence spectra are shown in Fig. 2. Product RI was identified as 2,4-diethyl-2,5-dihydrofuran.

For isolation of the non-fluorescent products, the residue was subjected to GC and TLC. At least fourteen products (a—n) were detected on the gas chromatogram (Fig. 3), and many spots were detected on the thin-layer chromatogram (Fig. 4). The residue was then applied to a column (28 × 190 mm) of silica gel, and the column was eluted successively with *n*-hexane–chloroform (2:8, v/v), chloroform, and chloroform–methanol (49:1, v/v). All the products were eluted from the column. The fractions were subjected to GC and TLC, and the products detected in the GC corresponded to those in the TLC (Fig. 4). The fractions containing a, b, l, g, c, and e, which were eluted in that order, were rechromatographed by successive column chromatographies on a column (15 × 260 mm) of silicic acid. These products were obtained in pure oily forms showing single peaks or spots in the chromatographies.

Product a Retention time in GC: 1.6 min. *Rf* value in TLC: 0.70. Product b Retention time: 2.5 min. *Rf* value: 0.70. Product c was obtained in a yield of 30 mg. Retention time: 4.6 min. *Rf* value: 0.40. Product e was obtained in a yield of 3.5 mg. Retention time: 4.9 min. *Rf* value: 0.31. Product g was obtained in a yield of 20 mg. Retention time: 6.1 min. *Rf* value: 0.54. Product l was obtained in a yield of 0.3 mg. Retention time: 13.6 min. *Rf* value: 0.67.

Results

Formation of fluorescence in the reaction of butyraldehyde or 2-ethyl-2-hexenal, an aldol condensation product of butyraldehyde, with methylamine in 70% methanol at 37 °C for 48 h was investigated. Reactions A, B and C were conducted in the reactant ratios of 11:1, 2:1 and 1:11 of butyraldehyde and methylamine, respectively, and reaction D in the ratio of 1:1 of 2-ethyl-2-hexenal and methylamine. Fluorescence spectra and intensities of these reaction mixtures and those after treatment with borohydride are shown in Table I. All the reactions produced fluorescence with excitation maxima at 340—360 nm and emission maxima at 400—440 nm. Yields of the fluorescence were dependent on the ratios of butyraldehyde and methylamine, and increased with increasing ratios of butyraldehyde (reactions A, B and C). However, incubation of butyraldehyde alone did not produce any significant fluorescence. When butyraldehyde in reaction B was replaced by 2-ethyl-2-hexenal (reaction D), the yield of the fluorescence increased. On treatment with borohydride, excitation and emission maxima of each reaction mixture shifted slightly to shorter wavelength.

Reaction mixtures A, B and D were subjected to HPLC before and after treatment with borohydride (Fig. 1). Before borohydride treatment, different fluorescent compounds were produced in reactions A and B, indicating that the fluorescent products were dependent on the ratios of the reactants. In contrast, the fluorescent products of reactions B and D were similar; they produced the same four fluorescent compounds I—IV. The results indicated the fluorescent products I—IV were formed after self-condensation of butyraldehyde into 2-ethyl-2-hexenal. After borohydride treatment, the chromatograms of reaction mixtures A, B and D were similar, and these reactions gave the same products, I, II, RI and RII. When the fluorescent peak fractions due to I and II of reaction B were treated with borohydride they remained unchanged. When the fluorescent peak fractions due to III and IV of reaction B were treated with borohydride, they were transformed into the fluorescent peaks due to newly formed fluorescent products RII and RI, respectively.

Fluorescent products I, II, RI and RII in the borohydride-treated reaction mixture of reaction B were extracted with chloroform and purified by successive silica gel column chromatography and preparative HPLC. Products I, II and RI were obtained as chromatographically homogenous oils. Product I exhibited an excitation maximum at 365 nm and an emission maximum at 441 nm

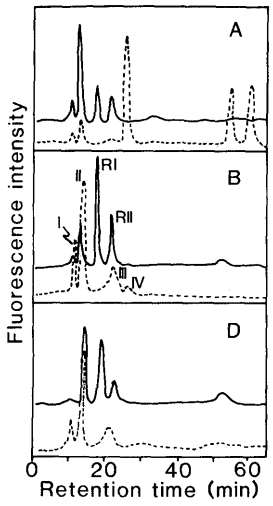

Fig. 1. Analytical HPLC of the Reaction Mixtures of Butyraldehyde (or 2-Ethyl-2-hexenal) and Methylamine (A, B and D shown in Table I) before (------) and after (——) Treatment with Borohydride

TABLE I. Fluorescence Spectra and Intensities of the Reaction Mixtures of Butyraldehyde or 2-Ethyl-2-hexenal with Methylamine

Reaction	Reactant	Treatment with borohydride after the reaction	Fluorescence		
			Excitation maximum (nm)	Emission maximum (nm)	Relative intensity
A	275 mM BAL+	−	348	419	87
	25 mM MA	+	345	410	35
B	200 mM BAL+	−	357	437	48
	100 mM MA	+	345	408	37
C	25 mM BAL+	−	351	398	22
	275 mM MA	+	348	400	16
D	100 mM EH+	−	347	418	96
	100 mM MA	+	340	402	119

BAL, butyraldehyde; EH, 2-ethyl-2-hexenal; MA, methylamine. The reaction conditions were described in Experimental. The control reaction mixtures without MA did not produce any significant fluorescence before or after borohydride treatment. Fluorescence intensity relative to that of 0.1 μM quinine sulfate in 0.1 N sulfuric acid is shown.

Table II. NMR Signals of Fluorescent Product RI

^{13}C-NMR			^{1}H-NMR				
ppm	INEPT	Assignment	ppm	Multiplicity	Coupling constant (Hz)	Number of protons	Assignment
144.5	C	4	5.36	dd	7.95, 0.90	1	3
129.6	CH	3	4.38	q	7.95	1	2
69.4	CH	2	4.28	dd	0.90, 12.0	1	5
61.2	CH_2	5	4.08	d	12.0	1	5
30.6	CH_2	a	2.16	q	7.30	2	c
28.4	CH_2	c	1.53	m		2	a
12.5	CH_3	d	1.05	t	7.30	3	d
9.8	CH_3	b	0.96	t	7.25	3	b

Multiplicity: d=doublet, t=triplet, q=quartet, m=multiplet.

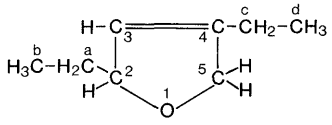

Chart 1

in methanol. Product II exhibited an excitation maximum at 349 nm and an emission maximum at 425 nm in methanol. Structural analysis of I and II was unsuccessful owing to their instability.

Product RI was identified as 2,4-diethyl-2,5-dihydrofuran (Chart 1) by analysis of its ^{13}C-and ^{1}H-NMR spectra. The ^{13}C-NMR spectrum with complete decoupling and INEPT experiments revealed that it had 8 carbon atoms: one quaternary, two tertiary, three secondary and two primary (Table II). The CI MS showed an ion peak at 127 m/z [$M^+ + 1$], indicating that this product was an isomer of 2-ethyl-2-hexenal and contained no nitrogen atoms. The compound may be produced from 2-ethyl-2-hexenal by intramolecular cyclization and subsequent reduction with borohydride. All the carbon signals in the ^{13}C-NMR spectrum (Table II) suggested the structure. The ^{1}H-NMR spectrum with decoupling also supported the structure (Table II). A multiplet at 1.53 ppm and a triplet at 0.96 ppm were assignable to the ethyl group attached to the 2-position. A quartet at 2.16 ppm and a triplet at 1.05 ppm were assignable to the ethyl group attached to the 4-position. A doublet at 4.08 ppm and a double doublet at 4.28 ppm were assigned to the geminal protons of the 5-position, the latter being long-range-coupled with the proton at the 3-position. A quartet at 4.38 ppm was assigned to the proton at the 2-position, which was coupled with the methylene protons of the attached ethyl group and the proton at the 3-position. A double doublet at 5.36 ppm could be assigned to the proton at the 3-position, which was coupled with the proton at the 2-position and long-range-coupled with one of the two protons at the 5-position.

UV absorption and fluorescence spectra of RI are shown in Fig. 2. It exhibited absorption maxima at 270 nm (ε: 107) and 331 nm (ε: 113), and fluorescence with an excitation maximum at 345 nm and an emission maximum at 406 nm. The molar fluorescence intensity relative to quinine sulfate was about 1.0%. While the compound was a product of borohydride treatment, it was derived from fluorescent product IV initially produced. Product IV may be a similarly cyclized compound containing no nitrogen atom.

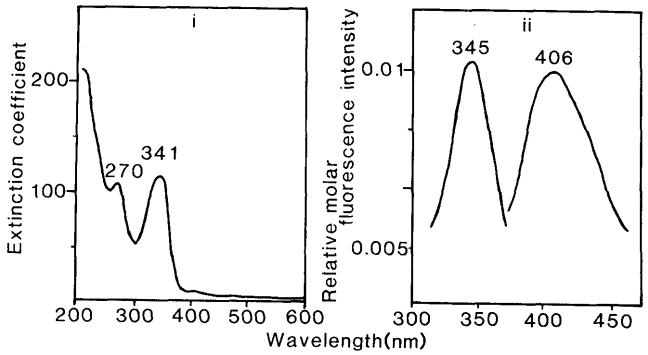

Fig. 2. Ultraviolet Absorption (i) and Fluorescence (ii) Spectra of Fluorescent Product RI in Methanol

Molar intensity relative to quinine sulfate in 0.1 N sulfuric acid is expressed.

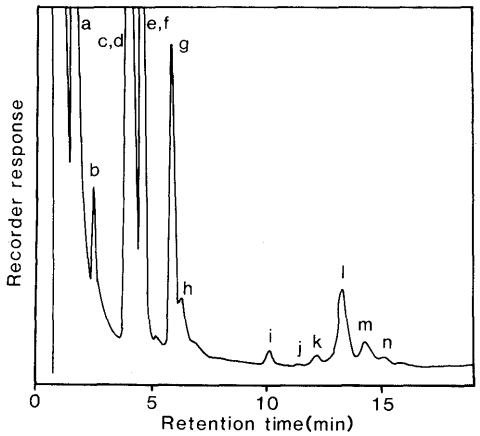

Fig. 3. Gas Chromatography of the Chloroform Extract of the Borohydride-treated Reaction Mixture of Reaction B in Table I

Methylamine may act only as a catalyst for the cyclization.

Besides the fluorescent products, many non-fluorescent products containing methylamine were produced in the reaction of butyraldehyde and methylamine. The chloroform extract of the borohydride-treated reaction mixture of reaction B was subjected to GC (Fig. 3) and TLC (Fig. 4). GC revealed at least fourteen products (a—n). TLC revealed many products other than fluorescent products. Products a, b, c, e, g and l were purified and obtained as pure oils by use of successive silica gel and silicic acid column chromatographies. Product a was identified as 2-ethyl-2-hexenol, a reduction product of 2-ethyl-2-hexenal,

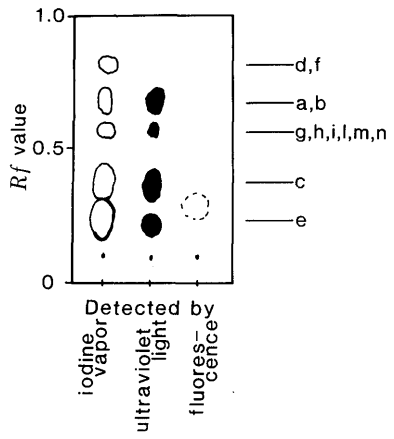

Fig. 4. Thin-Layer Chromatography of the Chloroform Extract of the Borohydride Reaction Mixture of Reaction B in Table I

The products seen in gas chromatography (a—n) corresponded to the indicated spots.

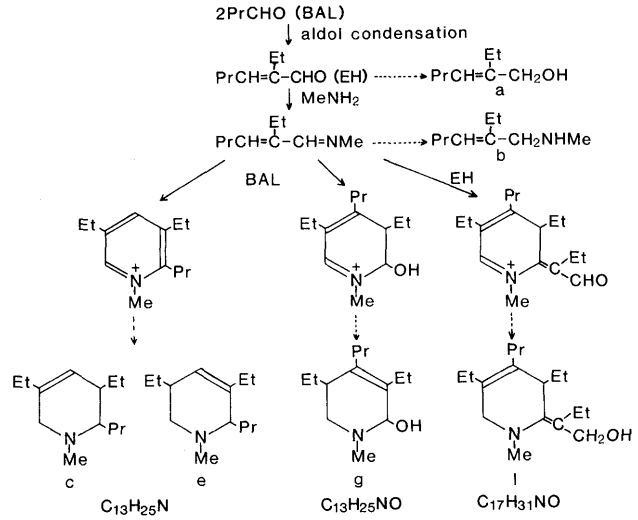

Chart 2

by its EI MS with ion peaks at 128 m/z [M^+] (relative intensity 50), 110 [$M^+ -18$ (H_2O)] (20), 99 [$M^+ -29$ ($C_2H_5\cdot$)] (40) and 85 [$M^+ -43$ ($C_3H_7\cdot$)] (100). Product b was identified as *N*-methyl(2-ethyl-2-hexenyl)amine, a reduction product of the Schiff base of 2-ethyl-2-hexenal, by analysis of its EI MS with ion peaks at 141 m/z [M^+] (2), 140 [$M^+ -1$] (50), 111 (60) and 83 (100).

Product c showed an EI MS with ion peaks at 195 m/z [M^+] (6), 166 [$M^+ -29$ ($C_2H_5\cdot$)] (16) and 152 [$M^+ -43$ ($C_3H_7\cdot$)] (100). The high resolution MS of the fragment ion peak of mass weight 195.1959 revealed the formula of $C_{13}H_{25}N$ (195.1984). ^{1}H-NMR spectrum ppm: 0.91 (6H, t, $J=7.5$ Hz, $2\times CH_3$), 1.00 (3H, t, $J=7.5$ Hz, CH_3), 1.25 (2H, m, CH_2), 1.36 (2H, m, CH_2), 1.43 (2H, m, CH_2), 1.88 (1H, m, CH), 1.95 (2H, q, $J=7.5$ Hz, CH_2), 2.26 (1H, m, CH), 2.33 (3H, m, NCH_3), 2.86 (2H, s, CH_2) and 5.34 (1H, d, $J=1.6$ Hz, =CH). It was identified as 3,5-diethyl-1,2,3,6-tetrahydro-*N*-methyl-2-propylpyridine.

Product e showed an EI MS with ion peaks at 195 m/z [M^+] (6), 166 [$M^+ -29$ ($C_2H_5\cdot$)] (8) and 152 [$M^+ -43$ ($C_3H_7\cdot$)] (100). The high resolution MS of the fragment ion peak of mass weight 195.1982 revealed the formula of $C_{13}H_{25}N$ (195.1985). ^{1}H-NMR spectrum ppm: 0.91—1.01 (9H, m, $3\times CH_3$), 1.25—1.36 (6H, m, $3\times CH_2$), 1.93 (2H, q, $J=7.5$ Hz, CH_2), 2.21 (1H, m, CH), 2.33 (3H, s, NCH_3), 2.52 (1H, m, CH), 2.79 (1H, d, $J=16.8$ Hz, gemH), 3.07 (1H, d, $J=16.8$ Hz, gemH) and 5.36 (1H, m, =CH). It was identified as 3,5-diethyl-1,2,5,6-tetrahydro-*N*-methyl-3,5-diethyl-2-propylpyridine. Products c and e were tetrahydro derivatives of the 3,5-diethyl-1-methyl-2-propylpyridinium salt[16] derived from the reaction of the Schiff base of 2-ethyl-2-hexenal and butyraldehyde. Formation of this type of pyridinium salt was well documented by Suyama and Adachi.[18]

Product g showed an EI MS with ion peaks at 211 m/z [M^+] (4), 182 [$M^+ -29$ ($C_2H_5\cdot$)] (40) and 168 [$M^+ -43$ ($C_3H_7\cdot$)] (100). The high resolution MS of the fragment ion peak of mass weight 211.1915 revealed the formula of $C_{13}H_{25}NO$ (211.1934). ^{1}H-NMR spectrum ppm: 0.86—1.00 (9H, m, $3\times CH_3$), 1.30—1.59 (7H, m, $3\times CH_2$ and CH), 1.80 (2H, q, CH_2), 2.24 (3H, s, NCH_3), 2.53 (1H, d, $J=13.6$ Hz, gemH), 2.84 (1H, s, CHOH) and 2.99 (1H, d, $J=13.6$ Hz, gemH). It was suggested to be 3,5-diethyl-1,2,5,6-tetrahydro-2-hydroxy-*N*-methyl-4-propylpyridine, and may be derived from the reaction of butyraldehyde with the Schiff base of 2-ethyl-2-hexenal in another way.

Product l showed an EI MS with ion peaks at 265 m/z [M^+] (10), 250 [$M^+ -15$ ($CH_3\cdot$)] (5), 236 [$M^+ -29$ ($C_2H_5\cdot$)] (10), 194 [$M^+ -71$] (18) and 152 (100). The high resolution MS of the fragment ion peak of mass weight 265.2441 revealed the formula of $C_{17}H_{31}NO$ (265.2404). It may be derived from the reaction of 2-ethyl-2-hexenal with the Schiff base of 2-ethyl-2-hexenal as shown in Chart 2. Analysis of these non-fluorescent products indicated that butyraldehyde readily undergoes condensation of two, three and four molecules in the presence of methylamine. The fluorescent products observed in the HPLC chromatograms (Fig. 1) may be derived from the condensed derivatives of butyraldehyde.

Discussion

Formation of fluorescence in the reaction of oxidized lipids and proteins is important with respect to the formation of fluorescent lipofuscin in tissues and cells.[1-6] It is well known that aldehydes other than malonaldehyde can produce fluorescence by reaction with primary amines or proteins.[12-17] In the present paper, the reaction of butyraldehyde and methylamine under mild conditions was investigated in order to obtain information on the mechanisms of the formation of fluorescent components in the reaction of aldehydes and proteins.

Suyama and Adachi[18] have demonstrated that the mild reaction of alkanals with primary amines (including amino acids) readily affords the dimerized aldehydes as a consequence of aldol condensation, and pyridinium salts composed of 3 molecules of alkanals and one molecule of primary amines. We have shown previously that the reaction of butyraldehyde with methylamine gave several fluorescent products together with these nonfluorescent products.[16] We extended the investigation and it was found that the fluorescent products formed were dependent on the reactant ratios, and various fluorescent products were formed. The 2:1 reaction of butyraldehyde and methyl-

amine (reaction B) afforded four fluorescent products, I—IV. Products I and II remained unchanged and products III and IV changed into fluorescent compounds RII and RI, respectively, on treatment with borohydride. Product RI derived from IV was identified as 2,4-diethyl-2,5-dihydrofuran. Product IV must have been produced without introduction of methylamine. As shown in Table I, the fluorescence yields in the reaction mixtures increased with increasing ratios of butyraldehyde to methylamine, suggesting that the fluorescence formation required selfcondensation of butyraldehyde. Identification of the non-fluorescent products composed of 2 : 1, 3 : 1 and 4 : 1 ratios of butyraldehyde and methylamine indicated that butyraldehyde readily condensed into high-molecular-weight substances under the reaction conditions used.

We have shown recently that the reaction of butyraldehyde and benzylamine afforded fluorescent and non-fluorescent products.[19] Among the non-fluorescent products, three products composed of 4 molecules of butyraldehyde and two molecules of benzylamine were isolated and their structures were suggested. Condensation of butyraldehyde occurred regardless of the kind of primary amine. It is interesting to note that a self-condensation product of butyraldehyde, 2,5-dihydrofuran, exhibited fluorescence whose spectrum is similar to that of lipofuscin pigment. Aldehydes in oxidized lipids can be self-condensed into fluorescent products in the presence of amino acids or proteins. This type of reaction may contribute in part to the formation of lipofuscin-like fluorescent components in the reaction of oxidized lipids and proteins.

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