[Chem. Pharm. Bull. 29(5)1423—1432(1981)]

Studies on Peroxidized Lipids. II.[1] Fluorescent Products Relevant to Aging Pigments derived from Malondialdehyde and Methylamine

Kiyomi Kikugawa,* Teppei Maruyama, Yasuko Machida, and Tsutao Kurechi

Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

(Received October 8, 1980)

Malondialdehyde (MDA) is a secondary product of *in vitro* and *in vivo* lipid peroxidation. Reactions of MDA, prepared by acid hydrolysis of malonaldehyde bis(dimethylacetal), with methylamine were performed at 37° under various pH conditions (pH 1—8). Two major fluorescent compounds, 1,4-dimethyl-1,4-dihydropyridine-3,5-dialdehyde (**1**) and 1-methyl-4-(dimethoxyethyl)-1,4-dihydropyridine-3,5-dialdehyde (**2**), and two non-fluorescent UV-absorbing by-products, β-methylaminoacrolein (**3**) and 2,6-dimethyl-2,6-diazabicyclo[3.3.1]3,7-nonadiene-4,8-dialdehyde (**4**), were isolated. The structures of these products were elucidated by analysis of their ^{13}C and ^{1}H NMR spectra, mass spectra and UV absorption spectra. Compounds **1** and **2** were produced in the best yields at pH 6—8, **3** was produced at every pH, and **4** was produced at pH 2—5. The 1,4-dihydropyridine-3,5-dialdehyde fluorophore in **1** and **2** may have been formed *via* a Hantzsch-type reaction of MDA and methylamine. The fluorescence spectrum of **1** with a maximum at 460 nm was the same as that of a conjugated Schiff base of MDA and amino acid or those of aging or lipofuscin pigments accumulated in the cells.

Keywords——malondialdehyde; 1,4-dimethyl-1,4-dihydropyridine-3,5-dialdehyde; 1-methyl-4-(dimethoxyethyl)-1,4-dihydropyridine-3,5-dialdehyde; β-methylaminoacrolein; 2,6-dimethyl-2,6-diazabicyclo[3.3.1]3,7-nonadiene-4,8-dialdehyde; Hantzsch reaction; lipofuscin pigments

Lipid peroxidation has been said to be of basic importance in aging, in damage to cells by air pollution, in some phases of atherosclerosis and in oxygen toxicity. It is generally regarded as being involved in the formation of aging or lipofuscin pigments that accumulate in the cells of certain organs with age, and these pigments exhibit a characteristic fluorescence spectrum with a maximum at around 460 nm.[2-9] Malondialdehyde (MDA) has been recognized as one of the secondary products of peroxidation of polyunsaturated fatty acids on the basis of its thiobarbituric acid coloration.[10,11] Fluorescent compounds are formed *in vitro* by interaction of MDA with amino acids,[12] and this has led to the suggestion that the fluorophore of lipofuscin pigments is a conjugated Schiff base of MDA.[5,6]

Reactions of MDA, prepared by acid hydrolysis of malonaldehyde bis(dialkylacetal),[13] with amino-containing compounds have generally been performed under strongly acidic or severe conditions. Thus, the reactions with amino acids giving Schiff bases[14,15] and fluorescent conjugated Schiff bases,[12] with aromatic amines giving fluorescent dianils,[16,17] with guanidines giving 2-aminopyrimidines,[18,19] with ureas giving 2-hydroxypyrimidines,[20-23] and with hydrazines giving pyrazoles,[24] have been described. In the previous paper,[1] we described the reactions of MDA with secondary amines to yield N-disubstituted aminoacroleins under mild pH conditions. This time, the reaction of MDA with methylamine under mild conditions was investigated, and it was found that the reaction afforded compounds bearing a new fluorophore and exhibiting a fluorescence spectrum similar to those of conjugated Schiff bases or lipofuscin pigments.

Results

A. Reaction Profiles of Malondialdehyde and Methylamine

Malondialdehyde (MDA) was treated with an excess of methylamine at 37° for 23 hr under various pH conditions (pH 1—8.3). Fluorescent compounds were gradually produced,

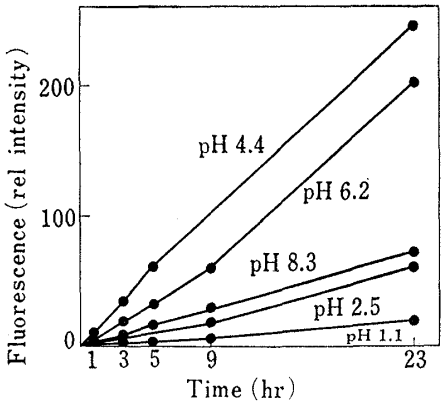

Fig. 1. Increases in Fluorescence of the Reaction Mixture of 43 mM MDA and 87 mM Methylamine at 37° under Various pH Conditions

At various times, an aliquot was withdrawn from the reaction mixture and diluted with water (1:125), then the fluorescence at 460 nm with excitation at 365 nm was measured. Relative intensity was expressed as a percentage of the intensity of 6 μM 1.

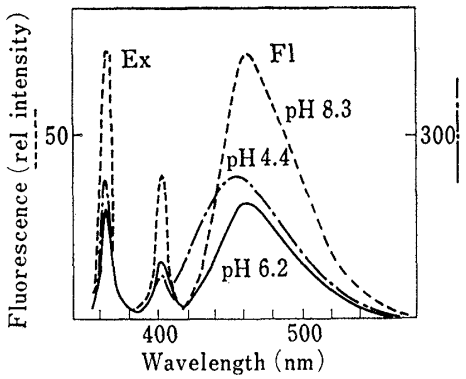

Fig. 2. Excitation and Fluorescence Spectra of a 1: 125 Dilution with Water of the Reaction Mixtures of MDA and Methylamine at 37° for 23 hr

and the rate of increase in fluorescence intensity at 460 nm decreased in the following order: pH 4.4>6.2>8.3>2.5>1.1 (Figure 1). The control experiments with MDA alone did not yield any fluorescent compounds under the conditions used. Excitation and fluorescence spectra of the reaction mixtures at pH 4.4, 6.2 and 8.3 after 23 hr are illustrated in Figure 2; identical excitation maxima at 365 and 403 nm were seen with all three reactions, though slightly different fluorescence maxima appeared at 453 nm with the pH 4.4 reaction and at 460 nm with the pH 6.2 and pH 8.3 reactions.

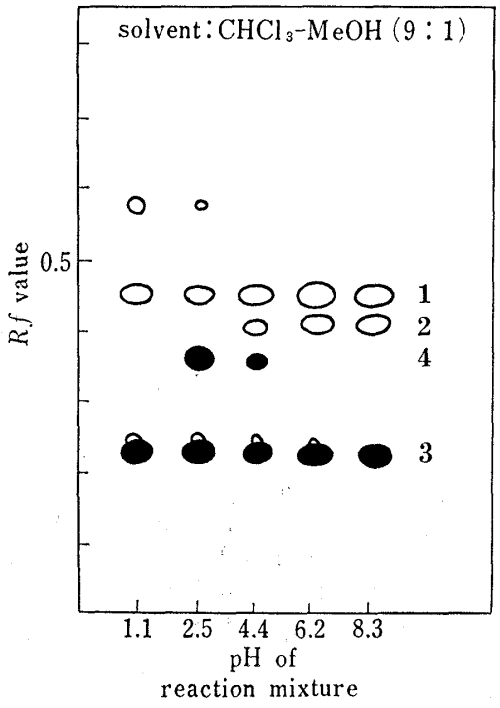

Fig. 3. TLC of the Chloroform Extracts of the Reaction Mixtures of MDA and Methylamine treated at 37° for 23 hr

UV-absorbing spots (irradiated at 254 nm) (●) and fluorescent spots (irradiated at 365 nm) (○) are indicated.

When the reaction mixtures were extracted with chloroform after alkalization, the recovery of the fluorescent products in chloroform was relatively high with the reactions at pH 6.2 and 8.3, but it was much lower with the reaction at pH 4.4 (Table I). The results indicate that the pH 4.4 reaction yielded water-soluble products, while the pH 6.2 and pH 8.3 reactions yielded chloroform-soluble products. Patterns of excitation and fluorescence spectra of the chloroform extracts were quite similar to those before extraction. When the reaction mixtures were extracted with *n*-butanol instead of chloroform, similar results were obtained. The UV-absorption spectra of the reaction mixtures, as well as the control mixture with MDA alone, showed complex spectra, but the chloroform extracts showed rather simple spectra. The extract of the pH 6.2 reaction exhibited a characteristic absorption maximum at around 400 nm with an absorbance value of 15 (see "Experimental" for the reaction conditions).

Table I. Recovery of the Fluorescent Products in Chloroform Extracts from the Reaction Mixtures of MDA and Methylamine at 37° for 23 hr

pH of the reaction mixture	Fluorescence (relative intensity)		
	Reaction mixture	$CHCl_3$-extract	Recovery (%)
1.1	21.8	20	92
2.5	65.0	20	31
4.4	249	50	20
6.2	203	179	88
8.3	72	57	79

TLC of the chloroform extracts (Figure 3) revealed two major blue fluorescent spots (*Rf* 0.45 and 0.40) corresponding to compounds **1** and **2** and two non-fluorescent UV-absorbing spots corresponding to compounds **3** and **4**. Compound **1** was produced at every pH value, the yield being the highest at around neutral pH values (6.2 and 8.3). Compound **2** was formed at pH values higher than 4.4. Compound **3** was produced at every pH value, the yield being the highest at pH 8.3. The formation of compound **4** was significant only at pH 2.5 and 4.4. Direct TLC of the reaction mixtures gave chromatograms similar to those of the chloroform extracts, except for the presence at the origins of a fluorescent spot in the case of the reaction at pH 4.4 and a UV-absorbing spot with all the reactions.

Since MDA was prepared by acid hydrolysis of malonaldehyde bis(dimethylacetal)[1] the preparation was not always homogenous MDA, but contained a small amount of the bis-

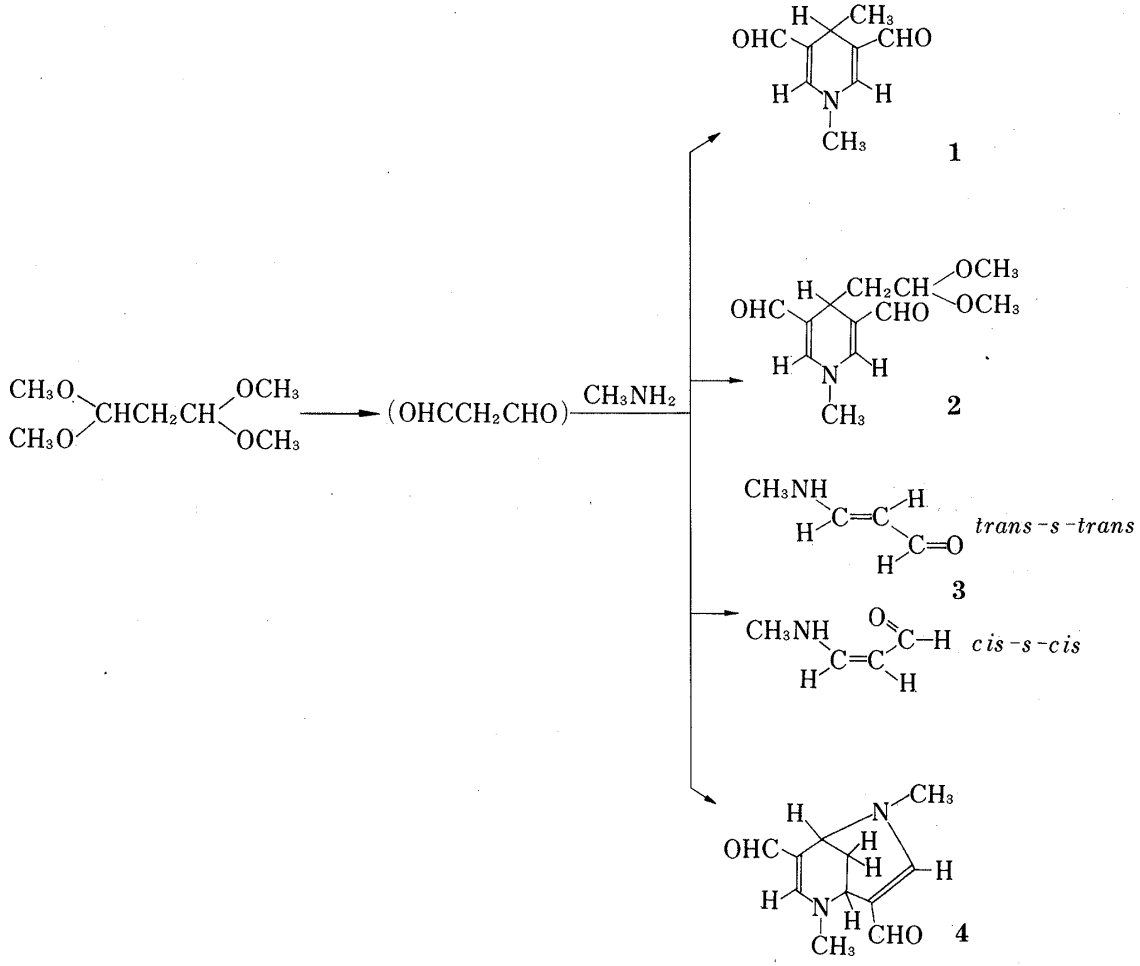

Chart 1

(dimethylacetal) and the partially hydrolyzed acetal. When the bis(dimethylacetal) and methylamine were treated at pH 6.2 and 8.3, neither fluorescent nor UV-absorbing compounds were produced. Thus, the formation of the fluorescent and the UV-absorbing products was attributed to MDA and in part to the partially hydrolyzed acetal.

B. Structure of the Products

The products (**1**, **2**, **3** and **4**) were isolated from the reaction mixtures by the use of silica gel columns. Compounds **1** and **2** were isolated as fluorescent yellow crystals in yields of 3% and 2.3%, respectively, after reaction at about pH 8.

Elemental analysis and high resolution mass spectroscopy showed the empirical formula of **1** to be $C_9H_{11}NO_2$ with a molecular weight of 165. The noise-decoupled ^{13}C NMR spectrum taken in $CDCl_3$ of **1** revealed six ^{13}C signals. From the ^{13}C chemical shifts, together with one-bond carbon-proton couplings observed by the off-resonance decoupling technique, the signals were identified as follows. The signal at 188.68 ppm, split into a doublet, was assinged to a conjugated aldehyde group (=C–CHO); the signals at 146.97 (doublet) and at 123.84 (singlet) ppm were at sp^2 regions (–CH= and =C<); the signal at 41.71 ppm, split into a quartet, was assigned to NCH_3; and the signals at 22.87 (doublet) and at 22.22 (quartet) ppm were at sp^3 regions (>CH and $-CH_3$). The ^1H NMR spectrum taken in $CDCl_3$ revealed 11 protons and these were identified as follows: a singlet at 9.27 ppm was due to two equivalent uncoupled protons of CHO; a singlet at 6.68 ppm was due to two equivalent uncoupled protons of –CH=; a singlet at 3.33 ppm was due to three protons of NCH_3; and signals at 3.90 and 1.09 ppm appeared as a quartet (1 proton) and a doublet (3 protons,) respectively, coupled with a coupling constant of 6.5 Hz and thus indicating the presence of a >CH–CH_3 group. The structure 1,4-dimethyl-1,4-dihydropyridine-3,5-dialdehyde accounts for the spectral data of **1**.

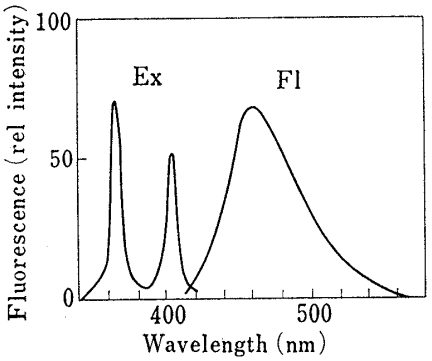

Fig. 4. Excitation and Fluorescence Spectrum of **1**

Mass spectral fragmentations of **1** indicate that the fragmentation processes involved the formation of aromatic pyridinium ions by loss of a hydrogen (*m/e* 164, M^+-1) or a methyl radical (*m/e* 150, M^+-15) at the 4-position and by successive loss of an aldehyde radical (*m/e* 121, 122) at the 3 or 5 position. These fragmentation processes are characteristic of 1,4-dihydropyridines.[25] An intense peak at *m/e* 150 corresponds to the stable pyridinium cation with loss of a methyl radical at the 4 position. The UV-absorption spectrum of **1** showing three maxima at 236, 264 and 399 nm resembled the spectrum of 1,4-dimethyl-3,5-diacetyl-1,4-dihydropyridine.[26] The IR spectrum indicated the presence of aldehyde. The results support the proposed structure of **1**.

Table II. Excitation and Fluorescence Intensities of Compounds **1** and **2**

Compound (1 μM)	Solvent	max, nm			Relative molar intensity		
		Ex_1	Ex_2	Fl	Ex_1 (at, nm)	Ex_2 (at, nm)	Fl (at, nm)
Quinine sulfate	0.1 M H_2SO_4	365		450	100 (450)		100 (365)
1	H_2O	365	403	460	71.5 (460)	51 (460)	68 (365)
2	H_2O	365	403	460	50.6 (460)	33 (460)	52.3 (365)

The excitation and fluorescence spectrum of **1** exhibited excitation maxima at 365 nm and 403 nm and a fluorescence maximum at 460 nm (Figure 4). The relative molar intensity of fluorescence with respect to quinine sulfate was about 70% (Table II), and it was higher than that of a conjugated Schiff base produced by the reaction of MDA and amino acid.[12] The excitation and fluorescence spectrum of **1** was identical with those of the reaction mixture of MDA and methylamine at pH 6.2 and 8.3, as well as with those of their chloroform extracts.

The elemental composition of **2** was found to be $C_{12}H_{17}NO_4$ with a molecular weight of 239. UV-absorption maxima, and excitation and fluorescence maxima of **2** were very close to those of **1**, suggesting that **1** and **2** have the same chromophore and fluorophore. Mass spectral fragmentations of **2** gave the same intense peak at *m/e* 150 (M^+-89) and the same peaks at *m/e* 121 and 122 as in the case of **1**, and indicated the presence of two methoxy groups (*m/e* 207 and 176). The ^{13}C and ^1H NMR spectra revealed the presence of a 1-methyl-1,4-dihydropyridine-3,5-dialdehyde moiety ($C_8H_8NO_2$) in **2**. The residual substituent ($C_4H_9O_2$) must be a dimethoxyethyl group ($-CH_2CH(OCH_3)_2$) attached to the 4-position of the chromophore. Thus, the signal at 52.5 ppm in the ^{13}C NMR spectrum and the signal at 3.22 ppm in the ^1H NMR spectrum revealed two CH_3O groups; the signals at 102.7 and 38.2 ppm in the ^{13}C NMR spectrum and at 4.39 and 1.72 ppm in the ^1H NMR spectrum corresponded to a $-CH_2CH<$ moiety; and ^1H NMR coupling (5.5 Hz) between the 4-proton of the chromophore and the adjacent CH_2 protons of the substituent indicated that the attachment was at the 4 position. The structure of **2** was thus elucidated as 1-methyl-4-(dimethoxyethyl)-1,4-dihydropyridine-3,5-dialdehyde.

Compound **3** was obtained as a non-fluorescent UV-absorbing yellow oil in a yield of 2.4% from the reaction at around pH 8. The mass spectrum showed the molecular ion peak at *m/e* 85, and the spectral fragmentations gave fragment ions at *m/e* 84 (M^+-1), 68 (M^+-17) and 56 (M^+-29), as were observed in the case of *β*-dimethylaminoacrolein.[1] The UV-absorption spectra showed maxima at 279 nm in water, at 271 nm in chloroform and at 302 nm in cyclohexane. The large differences in maximum wavelength depending on solvent are characteristic of *β*-alkylaminoacroleins in equilibrium between *trans-s-trans* and *cis-s-cis* conformations.[27,28] The ^1H NMR spectrum of **2** taken in dimethylsulfoxide-d_6 revealed AMX system signals of an aldehyde proton and two olefinic protons with coupling constants of $J_{\alpha\beta}=14$ Hz and $J_{CHO,\alpha}=9$ Hz, indicating that the conformation was *trans-s-trans*, while the spectrum taken in deuterochloroform showed two AMX system signals of *trans-s-trans* and *cis-s-cis* ($J_{\alpha\beta}=8$ Hz and $J_{CHO,\alpha}=3$ Hz) conformers in a ratio of 2:1.[29,30] Similar solvent-dependent equilibrium between the two conformers has been demonstrated by ^1H NMR spectroscopy of *β*-benzylaminoacrolein and *β*-anilinoacrolein.[30,31] The results supported the identification of **3** as *β*-methylaminoacrolein.

Compound **4** was isolated as non-fluorescent UV-absorbing colorless crystals in a yield of 0.7% from the reaction at pH 2.5. The elemental composition was found to be $C_{11}H_{14}N_2O_2$ with a molecular weight of 206. The UV-absorption spectrum, showing a maximum at 283 nm in water, was similar to that of **3**. The ^{13}C NMR and ^1H NMR spectra indicated the presence of two $CH_3\overset{|}{N}CH=\overset{|}{C}-CHO$ moieties ($C_3H_{10}N_2O_2$) in the molecule, and the residual composition (C_3H_4) could be readily ascribed to a $>CHCH_2CH<$ moiety with two equivalent $>CH-$ groups, since the signals at 46.8 and 25.1 ppm in the ^{13}C NMR spectrum, split into a doublet and a triplet, respectively, were in sp^3 regions, and the signals at 4.57 and 1.81 ppm in the ^1H NMR spectrum appeared as coupled triplets. Thus, the structure of **4** was assumed to be 2,6-dimethyl-2,6-diazabicyclo[3.3.1]3,7-noadiene-4,8-dialdehyde, in which two methylaminoacrolein moieties are symmetrically bridged through a three-carbon moiety without any conjugation between the acrolein moieties. Mass spectral fragmentations showing the loss of a methyl radical (*m/e* 191, M^+-15) and an aldehyde or a methylamino radical (*m/e* 177, M^+-29) and suggesting the formation of a stable aromatic pyridinium cation with an intense peak at *m/e* 122 and another pyridinium cation with a peak at *m/e* 146 supported the

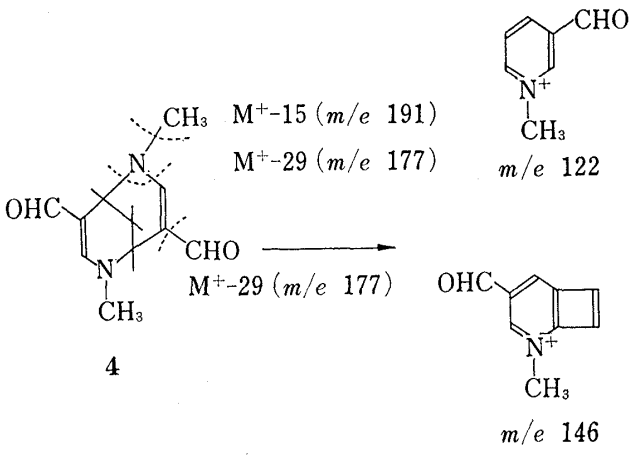

Chart 2

above structure.

C. Stability and TBA Reaction of the Products

Compounds **1** and **4** were stable at pH 1 and pH 7 at 37° overnight. While **2** was stable at pH 7, it was readily transformed into another fluorescent compound and a UV-absorbing neither of which was identified. Compound **3** was rather stable at pH 7, but was gradually converted at pH 1 into a strongly UV-absorbing compound and two fluorescent compounds, one of which corresponded to **1**. Compound **3** was more readily changed into the same two fluorescent compounds when it was adsorbed on a silica gel plate and exposed to the air. When **3** was treated with MDA in the neutral range, two fluorescent compounds corresponding to **1** and were produced.

Color formation of compounds **1**, **2** and **4** in the thiobarbituric acid (TBA) test was studied, as it has been demonstrated that, as well as MDA, MDA-adducts such as Schiff bases of dianils[19] and N-disubstituted aminoacroleins[1] give a pink coloration in the test. While the raction of MDA with TBA showed an absorption maximum at 532 nm with a shoulder at 502 nm, those of compounds **1** and **2** with TBA showed slightly different spectra; **1** showed a maximum at 526 nm with a shoulder at 505 nm, and **2** showed maxima at 500 and 530 nm. Compound **4** remained uncoupled, as did the blank. The ratios of the color yields at 532 nm of **1**, **2** and **4** against that of MDA were very low (0.03, 0.004 and 0.00, respectively).

Discussion

MDA, a secondary product of lipid peroxidation,[10,11] is labile *in vivo*, and reacts with biomaterials, disappearing from the serum.[32,33] The *in vitro* reaction of MDA with amino acid under acidic conditions gave a Schiff base (N-monosubstituted aminoacroleins)[14,15] and a conjugated Schiff base (N,N′-disubstituted 1-amino-3-iminopropenes) which give a fluorescence spectrum with a maximum at 460 nm.[12] The fluorophore of lipofuscin pigments which are formed *in vivo* and can be extracted with organic solvents is considered to be this type of conjugated Schiff base.[5,6] The conjugated Schiff base, however, has been produced in the strongly acidic region,[12,16] and the reaction under conditions close to physiological has not been investigated. It was shown in the present experiments that MDA reacted with methylamine to produce fluorescent compounds of a new type under mild conditions. Unidentified water-soluble fluorescent compounds (s) were formed in the acidic region, but no fluorescent and chloroform-soluble compounds other than **1** and **2** were produced in the neutral region. The fluorescent compounds **1** and **2** were identified as 1,4-dihydropyridine-3,5-dialdehyde derivatives.

Since Hantzsch[34] demonstrated the synthesis of a 1,4-dihydropyridine derivative by the reaction of ammonia, acetoacetate and acetaldehyde, various derivatives of 1,4-dihydropyridine has been prepared from amines, β-dicarbonyl compounds and aldehydes by the so-called "Hantzsch dihydropyridine synthesis".[25] MDA bearing β-dialdehyde functions may undergo this type of reaction in the presence of an appropriate amine. The reaction may, however, be rather complex because the MDA preparation used in the experiment was not homogeneous and contained a small amount of partially hydrolyzed acetals as active reactants. The mechanisms of formation of **1** and **2** might be as follows. Reaction of methylamine or the

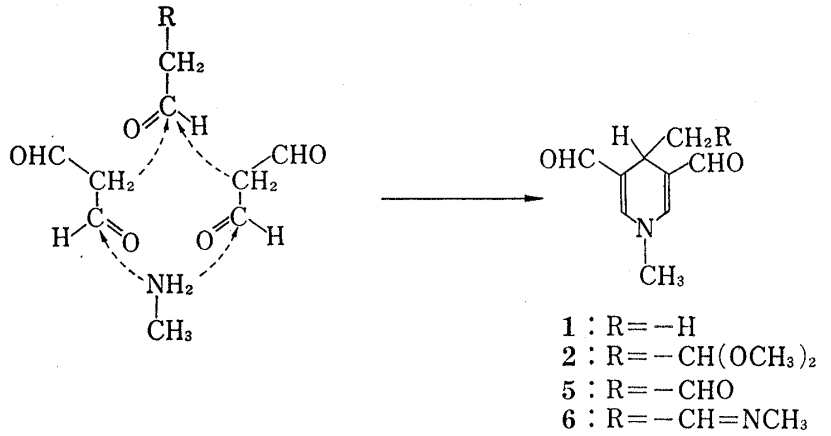

Chart 3

Schiff base **3**, initially produced in the reaction, with MDA may give an intermediate compound (**5** or **6**), which is sufficiently labile to be converted into a stable compound **1** by elimination of a one-carbon unit. Reaction of methylamine with 2 mole of MDA and 1 mole of malonaldehyde mono(dimethylacetal), a partial hydrolysis product of the starting bis (dimethylacetal), may give a relatively stable compound **2**. The fact that the formation of **2** was not significant in the acidic region may indicate that the mono (dimethylacetal) was absent in the acidic reaction mixtures due to its complete hydrolysis into MDA. It is unlilely that **1** was produced *via* **2** because treatments of **2** at pH 1 and 7 did not give **1**. Although **2** may be formed by a reaction involving MDA and the mono (dimethylacetal), **1** may be produced by the reaction of MDA alone or MDA and its reaction product with methylamine.

Non-fluorescent UV-absorbing compounds **3** and **4** were isolated as chloroform-soluble by-products in the reaction under acidic or neutral conditions. Compound **3**, a 1:1 Schiff base of MDA and methylamine, tended to transform into fluorescent compounds depending upon the conditions. Compound **4** was a unique adduct of MDA and methylamine in a ratio of 3:2. Three moles of MDA and 2 mol of methylamine may be condensed into a Schiff base to produce the symmetrical intermediate shown in Chart 4, which was in turn transformed into a stable symmetrical compound **4**.

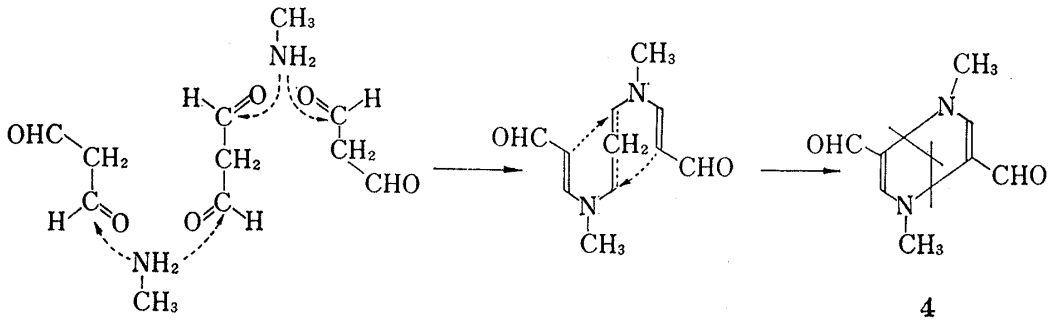

Chart 4

The reaction of MDA and methylamine under conditions close to physiological did not afford a conjugated Schiff base but afforded a fluorescent 1,4-dihydropyridine-3,5-dialdehyde derivative **1**. The derivative **1** exhibited the same fluorescence spectrum, with a maximum at 460 nm, as a conjugated Schiff base of MDA and amino acid. In contrast, **1** showed an excitation spectrum with two maxima at 365 and 403 nm, but the Schiff base exhibited a single maximum at 365 nm.[12] The relative molar intensity of fluorescence of **1** was higher than that of the Schiff base.[12] The fluorescence spectrum of **1** resembled those of lipofuscin

pigments, Showing a fluorescence maximum at 420—470 nm with an excitation maximum at 340—380 nm.[2,6] Compound **1** was almost inert to the TBA reaction, whereas the conjugated Schiff base was as active in the reaction as MDA.[19]

It is conceivable that fluorescent 1,4-dihydropyridine-3,5-dialdehyde derivatives may be produced by interaction of MDA with biogenic amines with primary amino functions under neutral conditions. Such derivatives are structurally related to nicotinamide adenine dinucleotide coenzymes and might possess some pharmacological activity, since certain derivatives of 1,4-dihydropyridine have been studied and developed as clinically useful vasodilators.[35,36]

Experimental

Methods——A solution of 1 M malondialdehyde (MDA) was prepared by acid hydrolysis of malonaldehyde bis(dimethylacetal) (Tokyo Kasei Kogyo Co., Ltd.) according to the method described previously.[1]

Melting points are uncorrected. TLC was performed on Wako gel B-5F (Wako Pure Chemical Industries, Ltd.) with $CHCl_3$–MeOH (9: 1) as a solvent. Silica gel column chromatography was performed by the use of silica gel for column chromatography (100 mesh, Kanto Chemical Company, Ltd.). UV-absorbing spots and fluorescent spots were located by UV irradiation at 254 and 365 nm, respectively. Absorption spectra were measured with a Shimadzu UV-200S double beam spectrometer. Excitation and fluorescnce spectra were measured with a Hitachi 204A fluorescence spectrophotometer. ^{13}C NMR spectra were taken in $CDCl_3$ on a JEOL JNM-FX-60 Fourier-Transform NMR spectrometer with Me_4Si as an internal standard, utilizing both noise decoupling and off-resonance decoupling techniques. ^1H NMR spectra were taken in $CDCl_3$ or Me_2SO-d_6 on a JEOL PS-100 machine with Me_4Si as an internal standard. Mass spectra were obtained with a Hitachi RMU-7L double focusing mass spectrometer. IR spectra were taken with a Hitachi 215 grating infrared spectrophotometer.

Reaction of MDA with Methylamine under Various pH Conditions——A mixture of 43 mM MDA and 87 mM methylamine was treated at 37° under various pH conditions. Thus, 0.90 ml of 1 M MDA solution and 1.80 ml of 1 M methylamine hydrochloride were added to 18.0 ml of each of the following solutions: 0.1 N HCl, 0.2 M sodium citrate–HCl buffer (pH 3.0), 0.1 M acetate buffer (pH 5.0), 0.1 M phosphate buffer (pH 7.0) and 0.05 M sodium borate (pH 9.5). Each of the mixtures was incubated at 37° for 23 hr. The pH values of the reaction mixtures were maintained at 1.1, 2.5, 4.4, 6.2 and 8.3, respectively, during the incubation. The control experiments, incubations of MDA alone and incubations of malonaldehyde bis(dimethylacetal) with methylamine, were similarly performed. A 0.20 ml aliquot of each reaction mixture was withdrawn at the indicated time and made up to 25.0 or 150 ml with water, and its fluorescence spectrum was recorded. At the end of the reaction TLC of the reaction mixtures was performed.

A 10 ml portion was withdrawn from each reaction mixture at the end of the reaction, mixed with 5.0 ml of 1 N NaOH and 2.0 g of NaCl, and extracted with 20 ml of $CHCl_3$. The chloroform extracts were evaporated to dryness and made up to 10.0 ml with EtOH. The ethanolic solutions (3 μl) were subjected to TLC. The UV-absorption spectrum of the ethanolic solutions between 200 and 500 nm was measured after 125-fold dilution with 0.1 N HCl. The fluorescence spectrum was recorded after 125- or 750-fold dilution with water. Extraction with *n*-BuOH instead of $CHCl_3$ was similarly performed and the extracts were examined in the same way.

1,4-Dimethyl-1,4-dihydropyridine-3,5-dialdehyde (1) and 1-Methyl-4-(dimethoxyethyl)-1,4-dihydropyridine-3,5-dialdehyde (2)——A solution of 100 ml of 1 M MDA (0.10 mol) and 90 ml of 1 M methylamine (0.09 mol) in 2.0 l of 0.05 M borate buffer (pH 9.5) was treated at 37° for 48 hr. The reaction mixture was saturated with NaCl and extracted with 1.5 l of $CHCl_3$ in several portions. The extracts were evaporated to dryness and applied to a column of silica gel (30 g). The column was eluted with $CHCl_3$–MeOH (19: 1); the fractions (75—150 ml) were evaporated to dryness to yield 650 mg (dry weight) of a mixture of **1** and **2** as yellow needles. The crude mixture was again applied to a column of silica gel (15 g) and eluted with benzene–MeOH (49: 1). The early fractions (50—230 ml) gave pure yellow leaflets of **1** (230 mg) the intermediate fractions (230—410 ml) gave a crystalline mixture of **1** and **2** (270 mg) and the later fractions (410—620 ml) gave pure yellow needles of **2** (169 mg).

Recrystallization of **1** from $CHCl_3$–*n*-hexane gave yellow leaflets melting at 144—147° (145 mg, 2.94% yield based on MDA). TLC: *Rf* 0.45. ^1H NMR (Me_2SO-d_6): ppm; 9.30 (2H, s), 7.30 (2H, s), 3.63 (1H, q), 3.39 (3H, s), and 0.93 (3H, d). UV: $\lambda_{max}^{H_2O}$ nm (ε): 236 (21000), 264 (6800), 399 (10900); $\lambda_{max}^{0.1N\ HCl}$ 236 (21000), 264 (7000), 399 (10600); $\lambda_{max}^{0.1N\ NaOH}$ 236 (18600), 264 (6800), 308 (3000), 399 (9800). IR ν_{max}^{KBr} cm^{-1}; 1640 (C=O), 2750 and 2850 (aldehyde C–H). MS *m/e* (rel intensity): 165 (37), 164 (10), 151 (34), 150 (100), 136 (6), 122 (12), 121 (11), 106 (6), 94 (13), 93 (12). High resolution mass spectroscopy showed the empirical formula of the molecular ion peak to be $C_9H_{11}NO_2$. *Anal.* Calcd for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.27; H, 6.86; N, 8.60.

Recrystallization of **2** from $CHCl_3$–*n*-hexane gave yellow needles melting at 121—123° (145 mg, 2.34% yield based on MDA). TLC: *Rf* 0.40. ^{13}C NMR ($CDCl_3$): ppm; 188.55 (d, 3,5-CHO), 147.36 (d, $C_{2,6}$), 121.63 (s, $C_{3,5}$), 102.66 (d, –CH<), 52.50 (q, 2O-CH_3), 41.71 (q, N-CH_3), 38.20 (t, $-CH_2-$), and 24.43 (d, C_4). ^1H NMR ($CDCl_3$): ppm; 9.29 (2H, s, 3,5-CHO), 6.77 (2H, s, $H_{2,6}$), 4.39 (1H, t, –CH<, $J=5.5$ Hz), 4.06 (1H, t, H_4, $J=5.5$ Hz), 3.36 (3H, s, N-CH_3), 3.22 (6H, s, 2O-CH_3), and 1.72 (2H, t, $-CH_2-$, $J=5.5$ Hz). ^1H NMR (Me_2SO-d_6): ppm; 9.27 (2H, s), 7.35 (2H, s), 4.20 (1H, t), 3.73 (1H, t), 3.37 (s), 3.10 (s), and 1.50 (2H, t). UV: $\lambda_{max}^{H_2O}$ nm (ε); 237 (18500), 263 (6000), 395 (10000); $\lambda_{max}^{0.1N\ HCl}$ 238 (21000), 262 (6400), 396 (8400); $\lambda_{max}^{0.1N\ NaOH}$ 238 (15000), 264 (8000), 302 (6300), 392 (7900). IR ν_{max}^{KBr} cm^{-1}: 1640 (C=O), 2810 and 2890 (aldehyde C–H). MS *m/e* (rel intensity): 239 (very low), 207 (13), 176 (10), 151 (16), 150 (100), 121 (5), 75 (8). *Anal.* Calcd for $C_{12}H_{17}NO_4$: C, 60.24; H, 7.16; N, 5.85. Found: C, 60.27; H, 7.21; N, 5.95.

β-Methylaminoacrolein (3)——A mixture of 90 ml of 1 M MDA and 180 ml of 5 M methylamine hydrochloride in 2.0 l of 0.05 M borate buffer (pH 9.5) was treated at 37° overnight. The reaction mixture was saturated with NaCl and extracted with 1.5 l of $CHCl_3$. The extracts were evaporated to dryness then the residue was applied to a column of 30 g of silica gel and eluted with $CHCl_3$–MeOH (19: 1). The fractions containing **3** were evaporated to dryness. The residue was rechromatographed in the same way, and **3** was isolated as a slightly yellow-colored oil, 187 mg (2.4% based on MDA). TLC: *Rf* 0.22. ^1H NMR ($CDCl_3$): ppm; 9.03 (2/3H, d, CHO, $J=9$ Hz), 9.01 (1/3H, d, CHO, $J=3$ Hz), 7.25 (1/3H, d, H_β, $J=8$ Hz), 7.17 (2/3H, d, H_β, $J=14$ Hz), 5.23 (2/3H, dd, H_α, $J=9$ Hz, $J=14$ Hz), 4.97 (1/3H, dd, H_α, $J=3$ Hz, $J=8$ Hz), 3.02 (3/3H, d, N-CH_3), 2.82 (6/3H, d, N-CH_3). ^1H NMR (Me_2SO-d_6): ppm; 8.90 (1H, d, CHO, $J=9$ Hz), 7.33 (1H, d, H_β, $J=14$ Hz), 4.97 (1H, dd, H_α, $J=9$ Hz, $J=14$ Hz), 2.65 (3H, d, N-CH_3, $J=5$ Hz). UV: $\lambda_{max}^{H_2O}$ nm; 279; $\lambda_{max}^{0.1N\ HCl}$ 268; $\lambda_{max}^{0.1N\ NaOH}$ 279; $\lambda_{max}^{CHCl_3}$ 271; $\lambda_{max}^{cyclohexane}$ 302. MS *m/e* (rel intensity): 85 (35), 84 (21), 70 (6), 68 (26), 56 (15), 55 (8), 54 (6), 43 (14), 42 (100).

2,6-Dimethyl-2,6-diazabicyclo[3.3.1]3,7-nonadiene-4,8-dialdehyde (4)——A mixture of 162 ml of 1 M MDA (0.162 mol) and 324 ml of 1 M methylamine hydrochloride (0.324 mol) in 3.24 l of 0.2 M citrate buffer (pH 3.0) was treated at 37° overnight. The mixture was saturated with NaCl, adjusted at pH 9 by addition of 5 N NaOH, and extracted with 6 l of $CHCl_3$ in several portions. The extracts were concentrated *in vacuo* to obtain a brown gummy residue. The residue was applied to a column of 90 g of silica gel, and the column was eluted stepwise with 1.0 l of $CHCl_3$ followed by $CHCl_3$–MeOH (39: 1). The fractions (1.0—1.15 l) were evaporated to dryness. The residue was further purified by passage through a column of 15 g of silica gel with elution by $CHCl_3$–MeOH (39: 1). The fractions containing **4** were evaporated to dryness. Crystallization and recrystallization from EtOH–*n*-hexane gave pure colorless prisms decomposing at 200° (72 mg, 0.72% yield based on MDA). TLC: *Rf* 0.35. ^{13}C NMR ($CDCl_3$): ppm; 185.17 (d, 4,8-CHO), 155.55 (d, $C_{3,7}$), 113.83 (s, $C_{4,8}$), 46.78 (d, $C_{1,5}$), 42.62 (q, 2,6-NCH_3), 25.08 (t, C_9). ^1H NMR ($CDCl_3$): ppm; 8.96 (2H, s, 4,8-CHO), 7.01 (2H, s, $H_{3,7}$), 4.57 (2H, t, $H_{1,5}$, $J=3$ Hz), 3.26 (6H, s, 2,6-NCH_3), 1.81 (2H, t, H_9, $J=3$ Hz). UV: $\lambda_{max}^{H_2O}$ nm (ε); 283 (57800); $\lambda_{max}^{0.1N\ HCl}$ 270 (50100), 287 shoulder; $\lambda_{max}^{0.1N\ NaOH}$ 283 (56200); $\lambda_{max}^{cyclohexane}$ 274. IR ν_{max}^{KBr} cm^{-1}: 2720 and 2790 (aldehyde C–H). MS *m/e* (rel intensity): 207 (23), 206 (84), 205 (11), 192 (5), 191 (32), 178 (9), 177 (52), 176 (14), 163 (9), 162 (8), 150 (10), 149 (9), 148 (16), 147 (11), 146 (21), 136 (12), 134 (7), 123 (15), 122 (100), 120 (8), 118 (8), 108 (14), 98 (10), 94 (15), 93 (5), 80 (5). *Anal.* Calcd for $C_{11}H_{14}N_2O_2$: C, 64.04; H, 6.84; N, 13.58. Found: C, 64.12; H, 6.84; N, 13.71.

Stability of Compounds 1, 2, 3 and 4——Each of the compounds **1**, **2**, **3** and **4** was treated in 0.1 N HCl and 0.1 M phosphate buffer (pH 7.0) at 37° overnight. Compounds **1** and **4** were unaffected by the treatments when checked by TLC. Treatment of **2** in the acid gave two spots, a fluorescent spot having a lower *Rf* value of 0.30 and a UV-absorbing spot (*Rf* 0.0). The maxima of the UV-absorption spectra of **2**, after treatment in the acid, were shifted to 264 and 400 nm (neutral and H^+) and to 300 and 364 nm (OH^-). Treatment of **3** in the acid gave three spots, *Rf* 0.45 (faint fluorescence), 0.42 (faint fluorescence) and 0.05 (strongly UV-absorbing), besides the spot corresponding to **3** (*Rf* 0.22). The UV-absorption spectra of **3** changed to show maxima at 245 nm (H^+) and 267 nm (OH^-).

Compound **3** became fluorescent when it was adsorbed on a silica gel plate and exposed to the air for up to 1 week or heated at 90° for 2—3 hr. TLC, after **3** had been treated on the plate at 90° for 3 hr, revealed two fluorescent spots having *Rf* 0.45 and 0.42, both of which exhibited the same absorption maxima as **1** after chloroform extraction.

Reaction of 3 with MDA——A mixture of 0.45 ml of 1 M MDA and 0.9 ml of 1 M **3** in 9.0 ml of 0.1 M phosphate buffer was treated at 37° for 42 hr, then 5.0 ml of 0.1 N NaOH and 2.0 g of NaCl were added, and the solution was extracted with 10 ml of $CHCl_3$. The extract was evaporated to dryness and made up to 10 ml with EtOH. The absorption spectrum of the 1: 250 dilution of the ethanolic solution with 0.1 N HCl showed the same characteristic absorption maximum at 400 nm as **1** (absorbance at this maximum: 0.12). TLC of the ethanolic solution revealed two fluorescent spots corresponding to **1** (*Rf* 0.45) and **2** (*Rf* 0.40) besides the spot corresponding to **3**.

Thiobarbituric Acid Test of Compounds 1, 2 and 4——To a mixture of 1.0 ml of glacial acetic acid and 1.0 ml of 20 mM 2-thiobarbituric acid was added 0.1 ml of one of the following solutions; water (blank), 20 mM malonaldehyde bis(dimethylacetal) (control), 20 mM **1**, 20 mM **2** and 20 mM **4**. The mixture were heated on a water bath for 30 min and then made up to 25 ml with water to record the absorption spectra between 400 and 600 nm.

Acknowledgement The authors are grateful to Dr. S. Yoshimura, Kanto Ishi Pharmaceutical Co., for measurement of ^{13}C NMR spectra and for valuable discussions on the structure of the compounds.

References and Notes

1) Preceding paper: K. Kikugawa, K. Tsukuda, and T. Kurechi, *Chem. Pharm. Bull.*, **28**, 3323 (1980).
2) H. Hydén and B. Lindström, *Discussions Faraday Soc.*, **9**, 436 (1950).
3) D.D. Hendley, A.S. Mildvan, M.C. Reporter, and B.L. Strehler, *J. Gerontol.*, **18**, 144 (1963).
4) A.L. Tappel, B. Fletcher, and D. Deamer, *J. Gerontol.*, **28**, 415 (1973).
5) A.L. Tappel, *Fed. Proc.*, **32**, 1870 (1973).
6) B.L. Fletcher, C.J. Dullard, and A.L. Tappel, *Anal. Biochem.*, **52**, 1 (1973).
7) A.L. Tappel, "Pathological Aspects of Cell Membranes," Vol. 1 ed. by B.F. Trumps and A. Arstilla, Academic Press, NY. 1975, p. 145.
8) R. Trombly, A.L. Tappel, J.G. Coniglio, W.M. Grogan, Jr., and R.K. Rhamy, *Lipids*, **10**, 591 (1975).
9) M.L. Seligman, E.S. Flamm, B.D. Goldstein, R.G. Poser, H.B. Demopoulas, and J. Ransohoff, *Lipids*, **12**, 945 (1977).
10) B.G. Tarladgis and B.M. Watts, *J. Am. Oil Chemists' Soc.*, **37**, 403 (1960).
11) R.O. Sinnhuber and T.C. Yu, *Food Technol.*, **12**, 9 (1958).
12) K.S. Chio and A.L. Tappel, *Biochemistry*, **8**, 2821 (1969).
13) R. Huttel, *Ber.*, **74**, 1825 (1941).
14) D.L. Crawford, T.C. Yu, and R.O. Sinnhuber, *J. Agric. Food. Chem.*, **14**, 182 (1966).
15) H. Buttkus, *J. Am. Oil Chemists' Soc.*, **46**, 88 (1969).
16) E. Sawicki, T.W. Stanley, and H. Johnson, *Anal. Chem.*, **35**, 199 (1963).
17) S. Tamura and M. Ono, *Chem. Pharm. Bull.*, **26**, 3167 (1978).
18) T.P. King, *Biochemistry*, **5**, 3454 (1966).
19) H. Buttkus and R.J. Bose, *J. Am. Chemists' Soc.*, **49**, 440 (1972).
20) T.V. Protopopova and A.P. Skoldinov, *J. Gen. Chem. USSR*, **28**, 360 (1968).
21) O.S. Tee and M. Endo, *J. Het. Chem.*, **11**, 441 (1974).
22) E. Kobayashi, *Yakugaku Zasshi*, **82**, 445 (1962).
23) K. Kaya, *Agric. Biol. Chem.*, **42**, 171 (1978).
24) H. Buttkus and R.J. Bose, *J. Org. Chem.*, **36**, 3895 (1971).
25) U. Eisner and J. Kuthan, *Chem. Rev.*, **72**, 1 (1972).
26) P.J. Brignell, U. Eisner, and P.G. Farrell, *J. Chem. Soc. B*, 1083 (1966); 1,4-dimethyl-3,5-diacetyl-1,4-dihydropyridine showed λ_{max} (EtOH) 236 (ε: 17600), 272 (8800), and 393 (9800) nm.
27) P.M. Vay, *Chem. Commun.*, **1969**, 861.
28) J. Dabrowski and K. Kamieńska-Trela, *J. Am. Chem. Soc.*, **98**, 2826 (1976); β-Alkylaminoacroleins exhibit absorption maxima at 266 nm (*trans-s-trans*) and at 306 nm (*cis-s-cis*).
29) M. Karplus, *J. Chem. Physics*, **30**, 11 (1959); J_{cis} and J_{trans} for olefinic protons are 6—11 Hz and 13—18 Hz, respectively.
30) S. Tamura and E. Yabe, *Chem. Pharm. Bull.*, **21**, 2105 (1973); J_{s-cis} and $J_{s-trans}$ for acroleins are 2 Hz and 8.5 Hz, respectively.
31) H.E.A. Kramer, *Ann.*, **696**, 15 (1966).
32) Z. Placer, A. Veselkova, and R. Rath, *Experientia*, **21**, 19 (1965).
33) R.O. Recknagal and A.K. Ghoshal, *Exp. Mol. Pathol.*, **5**, 108 (1966).
34) A. Hantzsch, *Ann.*, **215**, 1 (1882).
35) B. Loev, M.M. Goodman, K.M. Snader, R. Tedeschi, and E. Macko, *J. Med. Chem.*, **17**, 956 (1974).
36) M. Iwanami, T. Shibanuma, M. Fujimoto, R. Kawai, K. Tamazawa, T. Takenaka, K. Takahashi, and M. Murakami, *Chem. Pharm. Bull.*, **27**, 1426 (1979).