[Chem. Pharm. Bull. 29(10)3003—3011(1981)]

Studies on Peroxidized Lipids. III.[1] Fluorescent Pigments derived from the Reaction of Malonaldehyde and Amino Acids

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(Received March 24, 1981)

Reactions of malonaldehyde (MA), a secondary product of lipid peroxidation, with amino acids and related compounds were performed under mild conditions. Thus, mixtures of 200 mM MA–50 mM amino acid (or related compound) were treated at around pH 7 at 37°C. The major fluorescent products I_G, I_{GE} and I_H were isolated as crystals from the reaction mixtures of glycine, its ethyl ester and *n*-hexylamine, respectively, and their structures were established to be 1-substituted 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (I). The compounds showed fluorescence with λ_{max}^{ex} 365, 403 nm and λ_{max}^{em} 440—460 nm. The reaction mixtures of MA and amino acids except for L-tryptophan, crysteamine and L-cysteine exhibited the same fluorescence spectra. The reactions of MA with cysteamine and L-cysteine produced a different type of fluorescent products. Under the reaction conditions employed here, formation of conjugated Schiff bases (III) as described by Chio and Tappel (*Biochemistry*, **8**, 2821 (1969)) could not be detected. The fluorescence spectrum of I resembled those of lipofuscin pigments extracted from the tissue homogenates.

Keywords——malonaldehyde; amino acids; 1-substituted 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes; fluorescence

It has been considered that lipids undergo peroxidation by oxygen to yield hydroperoxides, which are in turn converted into a complex mixture of secondary products. Malonaldehyde (MA) has been recognized as one of the secondary products of the peroxidation.[2] This sequence of reactions has been generally regarded as being involved in causing cell damage, in aging, and in the formation of lipofuscin or ceroid pigments with characteristic fluorescence.[1-5] These fluorescent aging pigments have been assumed to be conjugated Schiff bases between MA and amino acids and primary amines, on the basis of the experiments described by Sawicki *et al.*[6] and by Chio and Tappel.[7] They treated amino acids with MA, prepared by acid hydrolysis of malonaldehyde bis(dialkylacetal), under relatively strongly acidic conditions far from the physiological, and obtained amorphous conjugated Schiff bases (N,N′-disubstituted 1-amino-3-iminopropenes) with a fluorescence maximum similar to that of lipofuscin pigments.[7]

In our previous paper,[1] we showed that MA reacts with methylamine under mild conditions close to physiological, affording a major fluorescent compound, 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde, whose fluorescence maximum is also similar to that of lipofuscin pigments. This time, MA was reacted with several amino acids and related compounds under mild pH conditions and it appeared that most of these compounds produced the dihydropyridine derivatives but not the conjugated Schiff bases.

Results

Malonaldehyde (MA), prepared from malonaldehyde bis(dimethylacetal) by acid hydrolysis, was treated with glycine and glycine ethyl ester at reactant molar rations of 4: 1 and 1: 2. Thus, solutions of 200 mM MA–50 mM glycine (or its ester) and 50 mM MA–100 mM glycine (or its ester) were treated at pH 1 and pH 7 at 37°C for 24 h. All the reaction mixtures produced blue fluorescence when irradiated at 365 nm. The excitation and fluorescence spectra showed identical maxima at λ_{max}^{ex} 365 and 403 nm and λ_{max}^{em} 455 nm, respectively; a representative

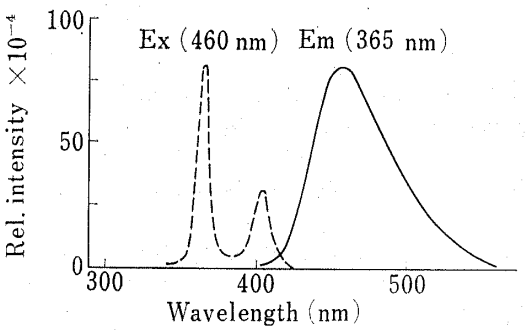

Fig. 1. Excitation and Fluorescence Spectra of the Reaction Mixture of 200 mM MA–50 mM Glycine treated at pH 7 and 37°C for 24 h

profile is illustrated in Fig. 1. This suggested that reaction of MA with glycine and its ester yielded the same type of fluorescent compound(s) regardless of the molar ratios of the reactants and of the pH of the reaction mixtures. The time courses of increase in the fluorescence intensity in the reaction mixtures were followed (Fig. 2). The increases in fluorescence intensity at pH 7 were much larger than those at pH 1 in all cases. Thin layer chromatography (TLC) revealed that the pH 7 reactions of both glycine and its ester yielded the major fluorescent spots, I_G and I_{GE}, respectively. The pH 1 reactions of both glycine and its ester seemed to afford ultraviolet (UV)-absorbing products more readily than the fluorescent products I_G and I_{GE} (Fig. 3).

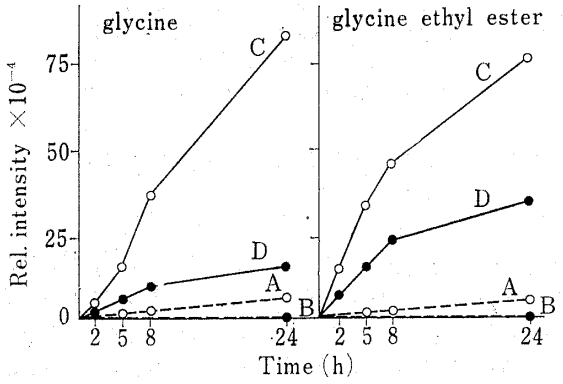

Fig. 2. Changes in Fluorescence Intensity of the Reaction mixture of MA and Glycine or Glycine Ethyl Ester, treated at 37°C

200 mM MA+50 mM amino acid at pH 1 (A) and at pH 7 (C); 50 mM MA+100 mM amino acid at pH 1 (B) and at pH 7 (D). All the reaction mixtures showed fluorescence spectra with λ^{ex}_{max} 365 and 403 nm and λ^{em}_{max} 460 nm.

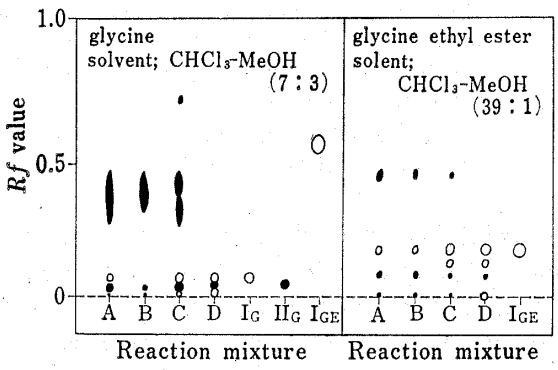

Fig. 3. TLC of the Reaction Mixture of MA and Glycine or Glycine Ethyl Ester, treated at 37°C for 24 h

The reaction mixtures were the same as described in the legend to Fig. 2. Spots were detected by UV-absorption (irradiated at 254 nm) (●) and fluorescence (irradiated at 365 nm) (○) measurements.

The major fluorescent product I_{GE} from glycine ethyl ester was isolated from the reaction of 400 mM MA–80 mM glycine ethyl ester at pH 5 by chloroform extraction and successive passages through a silica gel column. The elemental composition of I_{GE} was found to be $C_{12}H_{15}NO_4$ with a molecular weight of 237. The UV-absorption spectrum and fluorescence spectrum (Table I) of the product were very similar to those of the known 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde I_M derived from the reaction of MA and methylamine,[1] indicating that the product I_{GE} had the same chromophore and fluorophore. The nuclear magnetic resonance (NMR) spectrum revealed the characteristic signals of the 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde structure[1]; a singlet at 9.25 ppm (two equivalent uncoupled aldehyde protons), a singlet at 6.60 ppm (two equivalent uncoupled olefinic protons), signals at 3.92 (a quartet, 1 proton) and at 1.13 ppm (a doublet, 3 protons) coupled with a coupling constant of 6 Hz, and signals due to the ethyl acetate moiety. Mass spectral fragmentations gave an intense peak at *m/e* 222 ($M^+ - 15$) indicating the formation of the aromatic pyridinium ion by loss of a methyl radical at the 4-position.[1] The structure 3,5-diformyl-4-methyl-1,4-dihydropyridine-1-acetic acid ethyl ester accounts for these spectral data. The

Rf values (Fig. 3) and the fluorescence spectrum (Table I) were identical with those of the reaction mixture, indicating that I_{GE} was not a secondary product formed during the work-up.

The major fluorescent product I_G could hardly be purified from the reaction mixture of MA and glycine since it could not be extracted with organic solvents. The product I_G obtained as crystals in a fairly good yield from I_{GE} by mild alkaline hydrolysis. The structure of I_G was confirmed to be 3,5-diformyl-4-methyl-1,4-dihydropyridine-1-acetic acid by the physicochemical evidence given in Experimental. The compound I_G thus prepared showed the

TABLE I. Excitation and Fluorescence Intensities of the Products derived from the Reaction of MA and Amino-containing Compounds

Compound (1 μM)	Solvent	λ_{max}, nm			Rel. molar intensity			Ref.
		Ex(1)	Ex(2)	Em	Ex(1) at λ_{max}^{em}	Ex(2) at λ_{max}^{em}	Em at $\lambda_{max}^{ex\ (1)}$	
Quinine	0.1 N H_2SO_4	365		450	100		100	
I_M	H_2O	365	403	460	71.5	51	68	1
I_{GE}	H_2O	365	403	448	83	20	83	Present paper
I_G	H_2O	365	403	455	80	36	82	Present paper
I_H	H_2O	365	403	460	54	35	55	Present paper
III_G	H_2O	370		450	25		25	7
III_H	EtOH	396		462	20		20	7

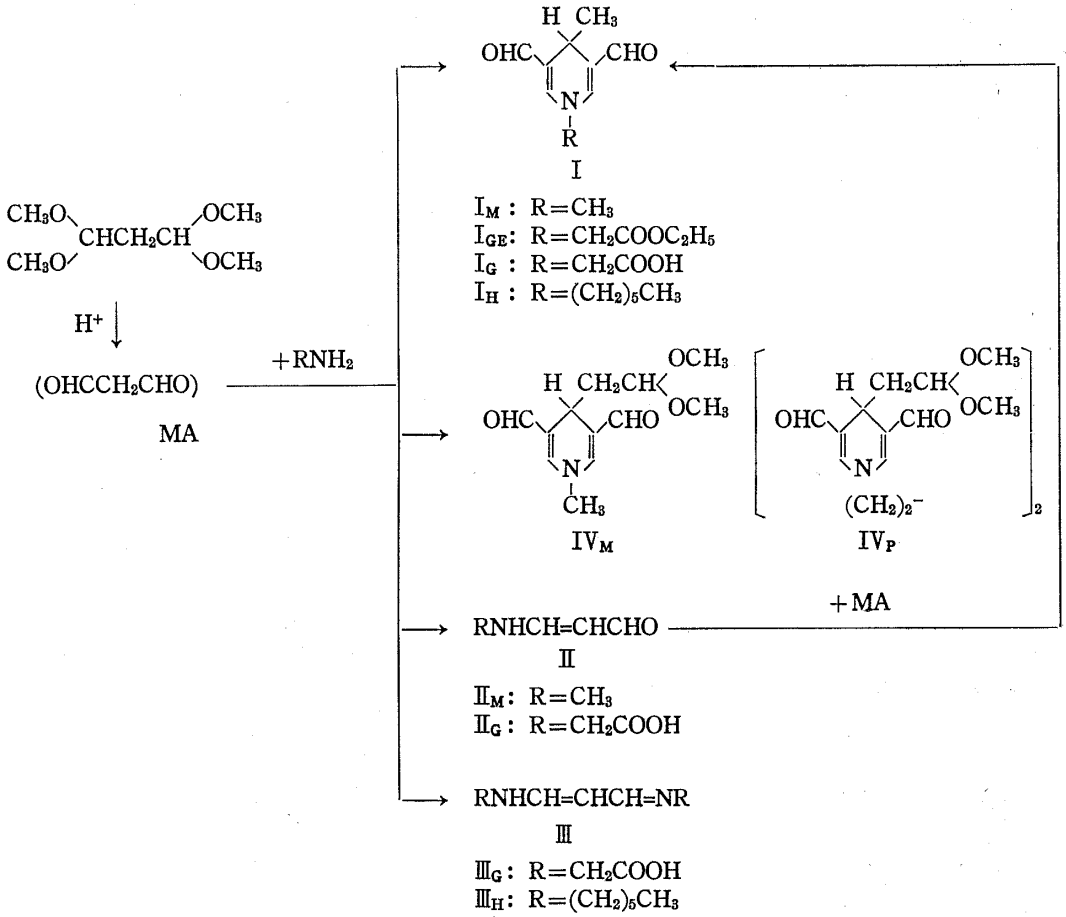

Chart 1

same *Rf* value as that of the major fluorescent product in the reaction mixture of MA and glycine at pH 7 (Fig. 3). The fluorescence spectrum of I_G, shown in Fig. 4 and Table I, was identical to that of the reaction mixture of MA and glycine (Fig. 1).

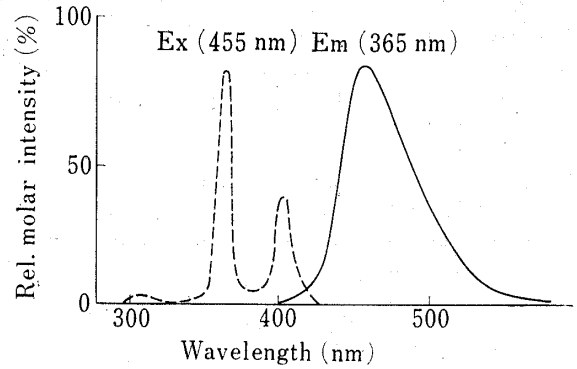

Fig. 4. Excitation and Fluorescence Spectra of I_G taken in H_2O

It has long been considered that the fluorescent aging pigments formed *in vivo* are conjugated Schiff bases between MA and amino acids or proteins, on the basis of the experiments of Chio and Tappel,[7] who reacted MA with glycine (as well as L-leucine, L-valine and *n*-hexylamine) and obtained the unstable yellow-colored fluorescent product III_G together with the non-fluorescent II_G. Chio and Tappel[7] reacted MA with glycine under rather severe conditions: the concentrations of both MA and glycine were higher than 3 M at a molar ratio of 3:2, and the pH of the reaction mixture may have been as low as below pH 1. The product II_G is a 1:1 Schiff base and the product III_G is a 1:2 Schiff base of MA and glycine as illustrated in the chart. The product III_G showed a fluorescence spectrum with λ^{ex}_{max} 370 nm and λ^{em}_{max} 450 nm (Table I) and had a complex UV-absorption spectrum.[7]

Although the product II_G was found in the reaction mixtures of MA and glycine treated under the conditions employed here, the fluorescent product III_G could not be recognized on the chromatogram (Fig. 3). It may be readily assumed that III_G is produced by the reaction of glycine and II_G. The product II_G, isolated and purified according to the method of Crawford

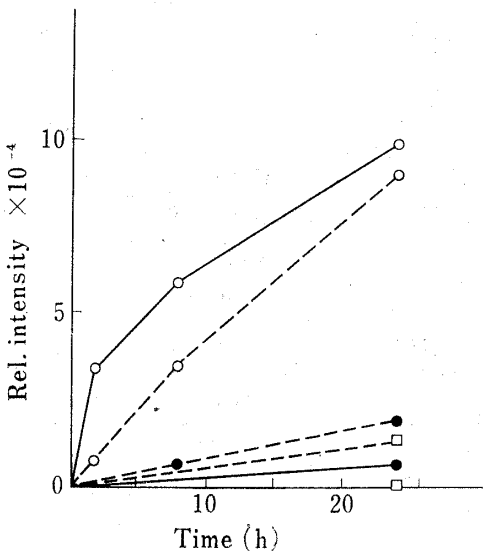

Fig. 5. Changes in Fluorescence of 50 mM II_G treated at 37°C

+None (□);+200 mM MA (○);+200 mM glycine (●). —— at pH 7; and ----, at pH 1. All the reaction mixtures showed fluorescence spectra with λ^{ex}_{max} 365 and 403 nm and λ^{em}_{max} 460 nm.

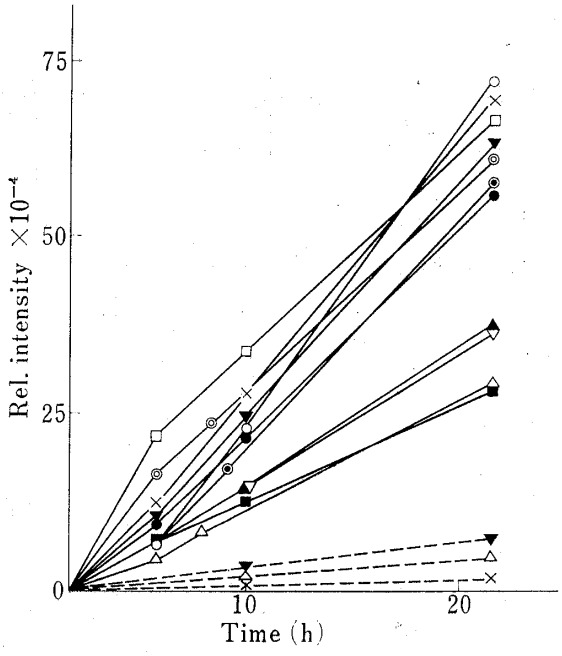

Fig. 6. Changes in Fluorescence Intensity of the Reaction Mixture of 200 mM MA–50 mM amino acid at pH 7 (——) and at pH 1 (----) at 37°C

L-Leu (○), L-phe (●), L-ser (□), L-lys (■), putrescine (△), L-arg (▲), L-glu (⊙), L-glu(NH_2) (◎), L-his (▽), histamine (▼) and L-met (×). All the reaction mixtures showed fluorescence spectra with λ^{ex}_{max} 365 and 403 nm and λ^{em}_{max} 460 nm.

et al.,[8] was reacted with glycine or MA at pH 1 and 7 over a period of 24 h. The mixtures of 200 mM glycine–50 mM II_G very gradually produced blue fluorescence, λ^{ex}_{max} 365, 403 nm and λ^{em}_{max} 450 nm, identical to that of I_G: the rate being the higher at pH 1 than at pH 7 (Fig. 5). However, the fluorescence intensity did not significantly exceed that of a solution of 50 mM II_G alone treated under the same conditions. TLC of these mixtures at pH 1 showed a single fluorescent spot having the same *Rf* value as I_G, and III_G could not be recognized. The formation of I_G from II_G in both the presence and absence of glycine in the acidic region may be conducted by the reaction of the liberated MA and glycine, since it has been noted that II_G and related compounds are unstable and degrade into MA.[8,9]

The mixtures of 200 mM MA–50 mM II_G produced blue fluorescence, the rate being higher at pH 7 than at pH 1 (Fig. 5). The rate at pH 7 was ,however, lower than that of the reaction of 200 mM MA–50 mM glycine at pH 7 shown in Fig. 2. TLC of the reaction mixture showed a single fluorescent spot corresponding to I_G. The results may indicate that I_G could be produced by the reaction of MA with II_G, but the rate was much lower than that of the direct reaction of MA and glycine.

The reaction of MA with *n*-hexylamine under mild conditions gave the same type of compound. Thus, mixtures of 43 mM MA–87 mM *n*-hexylamine were treated at pH 1, 3, 5, 7 or 9.5 at 37°C over a period of 24 h. The chloroform extracts from the reaction mixtures above pH 3 showed identical major fluorescent spots (I_H) on a thin layer chromatogram. The product (I_H) was isolated as crystals from the reaction mixture, and it was identified as 1-*n*-hexyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (see Experimental).

MA was reacted with several amino acids and related compounds including L-leucine, L-phenylalanine, L-serine, L-lysine, putrescine (1,4-diaminobutane), L-arginine, L-glutamic acid, L-glutamine, L-histidine, histamine, L-tryptophan, DL-tryptophan ethyl ester, L-methionine, cysteamine (2-aminoethanethiol) and cysteine. Thus, 200 mM MA was reacted with 50 mM amino acid at pH 1 and 7 at 37°C for 24 h. Except for the reactions of L-tryptophan, DL-tryptophan ethyl ester, cysteamine and L-cysteine, all the reactions produced blue fluorescence similar to that of I_M, I_G, I_{GE} and I_H. Time courses of the increases in fluorescence intensity are shown in Fig. 6. The rates of increase in fluorescence intensity were much higher at pH 7 than at pH 1. The production of fluorescence was generally greater with the neutral or acidic amino acids than with the basic amino acids. The reaction mixtures at pH 7 revealed stable blue-fluorescent spots on a thin layer chromatogram developed with chloroform-methanol. The reaction mixture of MA and putrescine exhibited several fluorescent spots on the chromatogram, one of which was isolated as crystals and identified as 1,4-bis (3,5-diformyl-4-(2,2-dimethoxyethyl)-1,4-dihydro-1-pyridyl)butane (IV_P). The compound showed the same UV-absorption and fluorescence spectra as the series of compounds I and 1-methyl-4-(2,2-dimethyoxyethyl)-1,4-dihydropyridine-3,5-dicarbaldehyde (IV_M),[1] and the NMR spectrum gave the evidence of the presence of two methoxyl groups. It may be a product derived from the reaction of putrescine with a partially hydrolyzed product of malonaldehyde bis-(dimethylacetal) contained in the MA preparation. Other fluorescent products could not be isolated in a homogeneous state. These results may indicate that these amino acids and related compounds undergo the Hantzsch type reaction with MA to produce 1,4-dihydropyridine derivatives.

Treatment of MA with L-tryptophan or DL-tryptophan ethyl ester at both pH 1 and 7 did not produce marked fluorescence. The reasons are obscure, but the amino acids may undergo a reaction with MA similar to the well-known reactions with aldehyde leading to carbolines by formation of a bridge through the amino group and the 2-position of the indole ring.[10]

Reactions of MA with cysteamine or L-cysteine produced yellow fluorescence on irradiation at 365 nm, and the profiles of fluorescence spectra were different from those of the reaction products of other amino acids containing no free thiol (Fig. 7). The pH 7 reaction mixture of MA and cysteamine showed fluorescence with λ^{ex}_{max} 365 and 403 nm and λ^{em}_{max} 460 nm; the

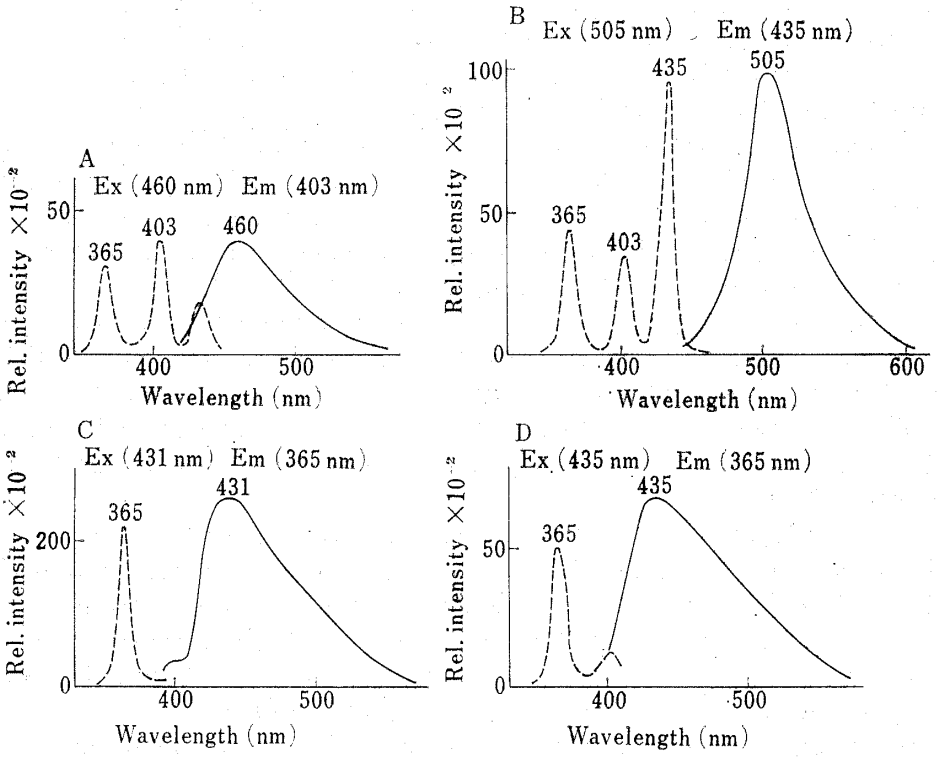

Fig. 7. Excitation and Fluorescence Spectra of the Reaction Mixtures of MA and Cysteamine or L-Cysteine, treated at 37°C

A: 200 mM MA+50 mM cysteamine at pH 7 for 24 h. B: 200 mM MA+50 mM cysteamine at pH 1 for 8 h. C: 200 mM MA+50 mM cysteamine at pH 1 for 24 h. D: 200 mM MA+50 mM L-cysteine at pH 1 for 24 h.

fluorescence intensity after 24 h was about 100 times less than those with other amino acids (Fig. 7A). The pH 1 reaction mixture showed complex fluorescence spectra: the mixture after 7.5 h exhibited fluorescence with λ^{ex}_{max} 365, 403 and 435 nm and λ^{em}_{max} 505 nm (Fig. 7B). and the mixture after 24 h exhibited fluorescence with λ^{ex}_{max} 365 nm and with λ^{em}_{max} 431 nm (Fig. 7C). The production of fluorescence at pH 1 was slightly higher than that at pH 7. The pH 7 reaction mixture of MA and L-cysteine exhibited faint fluorescence with λ^{ex}_{max} 365 nm and λ^{em}_{max} 550 nm (not shown). The pH 1 reaction was very similar to that of cysteamine; the spectra depended upon the reaction time. Fluorescence with λ^{ex}_{max} 403 nm and λ^{em}_{max} 505 nm observed after 5 h changed into fluorescence with λ^{ex}_{max} 365 nm and λ^{em}_{max} 435 nm after 24 h (Fig. 7D). Since mercaptans such as benzylmercaptan were found to be inert as regards the production of fluorescence, the production of fluorescence by reaction between MA and the thiol-containing amino acids must be due to complex interactions of both amino and thiol groups with MA. While Buttkus[11] reported the reaction of MA and L-cysteine at pH 5 to yield a UV-absorbing 3:2 adduct of MA and L-cysteine, they did not described the production of fluorescent compounds.

Discussion

The fluorophore of lipofuscin pigments which are formed *in vivo* in aged tissues is considered to be a conjugated Schiff base between MA and amino acids or proteins.[2-5] This view is based on experiments on the reaction of MA and amino acids and related compounds to yield N,N′-disubstituted 1-amino-3-imino-propenes such as III_G and III_H.[7] The fluorescence spectra of III_G and III_H (Table I) resemble those of lipofuscin pigments extracted from tissue

homogenates: λ_{max}^{ex} 340—380 nm and λ_{max}^{em} 420—470 nm.[5] However, the reaction conditions were far from the physiological. Furthermore, it has been pointed out by Buttkus and Bose[12] that more rigorous assignments of the structures of these compounds may be required, since the compounds were unstable and obtained in an amorphous state.

In our previous paper,[1] it was described that the mild reaction of MA with methylamine, one of the simplest primary amines, afforded two fluorescent dihydropyridines (I_M and IV_M) besides the minor UV-absorbing II_M and a diazabicyclononadiene derivative. In the present work, MA was reacted with glycine, its ester, *n*-hexylamine, other amino acids and related compounds under the mild reaction conditions. The major fluorescent products I_G, I_{GE} and I_H were isolated from the reaction mixtures of glycine, its ethyl ester and *n*-hexylamine, respectively, and were estabilished to be 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivatives by comparisons with the known I_M.[1] All of these fluorescent products exhibited fluorescence with λ_{max}^{ex} 365 and 403 nm and λ_{max}^{em} 440—460 nm with a strong relative molar intensity against quinine sulfate (Table I). The spectra were similar to those of lipofuscin pigments.[5] Under the conditions employed here, compounds III_G and III_H could not be detected in the reaction mixtures. Furthermore, compound III_G could not be produced in the reaction of II_G with excess glycine. Most of the amino acids except for L-tryptophan, its ester, cysteamine and L-cysteine produced the same fluorescence when reacted with MA, and it might be concluded that the major fluuorescent compounds were those bearing the 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde chromophore.

The mechanisms of the reaction have not yet been clearly elucidated although the products may be formed by the reaction of MA and amino acids (3: 1) *via* a Hantzsch type reaction,[13] as discussed in the previous paper.[1] While the yields of the fluorescent dihydropyridines were higher when a large amount of MA was present, they were formed to a significant extent even when amino acids were present in excess. Thus, it is possible that the fluorescent dihydropyridines are produced *in vivo* regardless to the amount of MA or the amount of amino acids or related compounds if MA is formed in a biological environment.

The MA preparation, which was prepared by acid hydrolysis of malonaldehyde bis(dimethylacetal), was not homogeneous but contained the partially hydrolyzed product, malonaldehyde mono(dimethylacetal), and thus compounds IV bearing two methoxyl groups may be produced by reaction of the monoacetal instead of MA. Since I_M could not be produced from IV_M under the conditions used, formation of IV was not essential for the production of compound I.[1]

In conclusion, it was elucidated that the reaction of MA with amino acids under mild physiological conditions gave fluorescent dihydropyridines regardless of the molar ratio of MA to the amino acids. These findings may provide a clue to the mechanisms of formation of lipofuscin pigments, since it has been considered that they might be formed by the reaction of MA with amino-containing compounds.[2]

Experimental

Materials and Methods——Glycine, L-leucine, L-phenylalanine, L-glutamic acid, L-glutamine, L-histidine, L-cysteine, L-tryptophan and L-methionine were kindky supplied by Ajinomoto Co., Inc. *n*-Hexylamine, glycine ethyl ester hydrochloride, histamine dihydrochloride, DL-tryptophan ethyl ester hydrochloride, putrescine (1,4-diaminobutane) and cysteamine (2-aminoethanethiol) were the products of Tokyo Kasei Kogyo Co., Ltd.

A solution of 1 M MA was prepared by acid hydrolysis of malonaldehyde bis (dimethylacetal) (Tokyo Kasei Kogyo Co., Ltd.) according to the method described previously.[9]

Melting points are uncorrected. TLC was performed on Wako gel B-5F (Wako Pure Chemical Industries, Ltd.). Silica gel column chromatography was performed on silica gel for column chromatography (100 mesh, Kanto Chemical Company, Ltd.). UV-absorbing spots and fluorescent spots were located by irradiation at 254 nm and 365 nm, respectively. Absorption spectra were measured with a Shimadzu UV-200S double beam spectrometer. Excitation and fluorescence spectra were measured with a Hitachi 204A fluorescence spectrophotometer. 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 (MS) were obtained with a Hitachi RMU-7L double-focusing mass spectrometer.

Reaction of MA with Amino Acids——Mixtures of 200 mM MA–50 mM amino acid (or its ester), or mixtures of 50 mM MA–100 mM amino acid (or its ester), were incubated at pH 1 or 7 at 37°C for 24 h. Thus, a mixture of 10 ml (or 2.5 ml) of 1 M MA and 2.5 ml (or 5.0 ml) of 1 M amino acid (or its ester) was adjusted to to pH 1 or 7 by addition of 1 N HCl or 1 N NaOH and then made up to 50 ml with water. After they had been incubated at 37°C for the indicated period, they were subjected to TLC and fluorometric assay after dilution with 1—10000 volumes of water. Excitation and fluorescence spectra of each reaction mixture were recorded. The relative intensity of the fluorescence was measured at λ_{max}^{ex} and λ_{max}^{em} and expressed as a percentage of the intensity of 1.12 μM quinine sulfate (λ_{max}^{ex} 365 nm and λ_{max}^{em} 450 nm).

When 200 mM MA alone was treated at pH 1 and 7 for 24 h, the solutions became fluorescent with λ_{max}^{ex} 365 nm and λ_{max}^{em} 485 nm (rel. intensity: 1.4×10^4) (pH 1) and λ_{max}^{ex} 365 nm and λ_{max}^{em} 555 nm (rel. intensity: 2.4×10^4) (pH 7), but the fluorescence faded rapidly.

Reaction of II_G with MA and Glycine——A solution of 50 mM II_G was incubated with 200 mM MA or 200 mM glycine at pH 1 or 7 at 37°C for 24 h, as described above. TLC and fluorometric assay were performed in the usual way.

3,5-Diformyl-4-methyl-1,4-dihydropyridine-1-acetic Acid Ethyl Ester (I_{GE})——A mixture of 2.8 g (20 mmol) of glycine ethyl ester hydrochloride and 100 ml (100 mmol) of 1 M MA was made up to 200 ml with water and adjusted to pH 5 by addition of about 50 ml of 1 N NaOH. The mixture was treated at 37°C overnight. It was then saturated with NaCl and extracted with 100 ml of $CHCl_3$ four times. TLC of the extracts and the aqueous layer showed that the major fluorescent compound (I_{GE}) was thoroughly transferred into the extracts. The extracts were evaporated to dryness and the residue was applied to a column (2.5×60 cm) of silica gel (70 g). The column was eluted stepwise with $CHCl_3$, $CHCl_3$–MeOH (99: 1), $CHCl_3$–MeOH (39: 1) and $CHCl_3$–MeOH (19: 1). The fractions containing the product (I_{GE}) were evaporated to dryness, and the residue was crystallized from $CHCl_3$–*n*-hexane to afford pale yellow columns of I_{GE} melting at 105—111°C, 350 mg (yield, 7.4%). Recrystallization from the same solvent gave pure pale yellow columns, mp 109 114°C. TLC: *Rf* 0.20 ($CHCl_3$–MeOH, 39: 1), 0.55 ($CHCl_3$–MeOH, 7: 3) single spot (Fig. 3). UV $\lambda_{max}^{H_2O}$ nm (ε): 231 (22600), 259 (9600), 381 (10800); $\lambda_{max}^{0.1N\ HCl}$: 231 (22400), 259 (9400), 381 (10700). NMR ($CDCl_3$) ppm: 9.25 (2H, s, 3,5-CHO), 6.60 (2H, s, $H_{2,6}$), 4.26 (2H, q, ester CH_2, $J_{CH_2,CH_3}=6$ Hz), 4.18 (2H, s, NCH_2), 3.92 (1H, q, H_4, $J_{H(4),CH_3}=6$ Hz), 1.33 (3H, t, ester CH_3, $J_{CH_2,CH_3}=6$ Hz), 1.13 (3H, d, 4-CH_3, $J_{H(4),CH_3}=6$ Hz). MS (rel. intensity) *m/e*: 237 (10), 223 (13), 222 (100), 195 (10), 194 (74), 164 (11), 149 (17), 136 (6), 92 (6). *Anal.* Calcd for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.82; H, 6.39; N, 5.64.

3,5-Diformyl-4-methyl-1,4-dihydropyridine-1-acetic Acid (I_G)——A solution of 98 mg of I_{GE} in 5.0 ml of water was adjusted to pH 10 with concentrated ammonium hydroxide and incubated at 37°C for 4 h. The mixture was evoporated to dryness *in vacuo* and redissolved in 5.0 ml of ethanol. The solution was passed through a column of 4.0 ml of Dowex W×4 (H^+ form, 100—200 mesh) and eluted with about 30 ml of ethanol. The eluate was evoporated to dryness and the residue was crystallized from EtOH-*n*-hexane to afford pale yellow columns of I_G decomposing at 211—212°C, 47 mg (yield 53%). Recrystallization from the same solvent gave pure columns, mp 211—213°C (dec.). TLC: *Rf* 0.05 ($CHCl_3$–MeOH, 7: 3) single spot (Fig. 3). UV $\lambda_{max}^{H_2O}$ nm (ε): 234 (23700), 262 (9200), 390 (11300); $\lambda_{max}^{0.1N\ HCl}$: 231 (22000), 259 (8800), 381 (10300); $\lambda_{max}^{0.1N\ NaOH}$: 234 (23700), 262 (10200), 390 (11300). NMR (Me_2SO-d_6) ppm: 9.18 (2H, s, 3,5-CHO), 7.24 (2H, s, $H_{2,6}$), 4.20 (2H, s, NCH_2), 3.62 (1H, q, H_4, $J_{H(4),CH_3}=6$ Hz), 0.96 (3H, d, 4-CH_3, $J_{H(4),CH_3}=6$ Hz). *Anal.* Calcd for $C_{10}H_{11}NO_4\cdot1/3H_2O$: C, 55.81; H, 5.46; N, 6.51%. Found: C, 55.64; H, 5.19; N, 6.65.

N-(2-Formylvinyl)glycine (II_G)——A mixture of 24.6 g of malonaldehyde bis (dimethylacetal) and 13.5 ml of 1 N HCl was heated at 40°C until the solution became homogeneous, and to this was added a solution of 7.5 g of glycine in 20 ml of water. The mixture was stirred at room temperature for 1 h and then stored in a refrigerator. The precipitate that separated was collected by filtration and purified as the sodium salt of II_G according to the method described by Crawford *et al.*[8] It was converted into white needles of the free form (II_G), mp 158—159°C (dec.). TLC: *Rf* 0.03 ($CHCl_3$–MeOH, 7: 3) single spot (Fig. 3). UV $\lambda_{max}^{H_2O}$ nm: 280; $\lambda_{max}^{H^+}$: 275. NMR (Me_2SO-d_6-D_2O) ppm: 8.93 (1H, d, CHO, $J_{\alpha,CHO}=8$ Hz), 7.40 (1H, d, H_β, $J_{\alpha,\beta}=13$ Hz), 5.04 (1H, dd, H_α, $J_{\alpha\beta}=13$ Hz, $J_{\alpha,CHO}=8$ Hz), 3.85 (2H, s, NCH_2). The coupling constants, $J_{\alpha\beta}$ and $J_{\alpha,CHO}$, indicate that the conformation of the product was *trans,s-trans* in the solvent mixture. [Lit.[8] mp 157—158°C (dec.), UV $\lambda_{max}^{H_2O}$: 271—272 nm].

1-*n*-Hexyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (I_H)——A suspension of 2.0 g (20 mmol) of *n*-hexylamine in 100 ml of water was added to 100 ml of 1 M MA solution. The mixture was adjusted to pH 5 by addition of about 30 ml of 1 N NaOH, then incubated at 37°C overnight to afford a gummy precipitate. The supernatant was extracted with 300 ml of $CHCl_3$ after addition of 70 g of NaCl. TLC ($CHCl_3$–MeOH, 9: 1) revealed that a major fluorescent product (*Rf* 0.6) besides several UV-absorbing products was present in the $CHCl_3$ extract and the gummy precipitate. The major fluorescent compound (I_H) was isolated by silica gel column chromatography. Thus, the gummy precipitate was applied to a column of silica gel (160 g) and eluted with $CHCl_3$. Fractions (2—2.2 l) containing the product were evoporated to dryness, and the residue was crystallized from *n*-hexane affording yellow leaflets of I_H, 106.5 mg (yield 2.27%), mp 99—101°C. Identical leaflets were similarly isolated by the use of the column from the $CHCl_3$ extract,

66.7 mg (yield 1.42%). Recrystallization from *n*-hexane afforded pure yellow leaflets melting at 98—100°C. TLC: 0.63 ($CHCl_3$–MeOH, 9: 1) single spot. UV $\lambda_{max}^{H_2O}$ nm (ε): 235 (23900), 263 (8600), 396 (11500); $\lambda_{max}^{0.1N\ HCl}$: 235 (23100), 263 (8000), 396 (11200); $\lambda_{max}^{0.1N\ NaOH}$: 235 (23000), 263 (9600), 396 (11000). NMR ($CDCl_3$) ppm: 9.22 (2H, s, 3,5-CHO), 6.65 (2H, s, $H_{2,6}$), 3.91 (1H, q, H_4, $J_{H(4),CH_3}$=6 Hz), 3.44 (2H, t, NCH_2, J_{NCH_2,CH_2}= 6 Hz), 1.7 (3H, m), 1.3 (5H, bs), 1.09 (3H, d, 4-CH_3, $J_{H(4),\ 4\text{-}CH_3}$=6 Hz), 0.90 (3H, t, 6′-CH_3, J_{CH_3,CH_2}=6 Hz). MS (rel. intensity): *m/e*: 235 (8), 221 (14), 220 (100), 166 (21), 165 (10), 136 (34), 95 (5). *Anal.* Calcd for $C_{14}H_{21}NO_2$: C, 71.45; H, 9.00; N, 5.95. Found: C, 71.45; H, 9.03; N, 5.72.

Reaction of MA with Putrescine——A mixture of 1.76 g (20 mmol) of putrescine and 100 ml (100 mmol) of 1 M MA was made up to 200 ml with water and adjusted to pH 5 by addition of 1 N NaOH. The mixture was treated at 37°C overnight. It was saturated with NaCl and extracted with $CHCl_3$. TLC ($CHCl_3$–MeOH, 9: 1) of the extracts showed two major fluorescent products (*Rf* 0.26 and 0.19) and two minor fluorescent products (*Rf* 0.62 and 0.35). The extracts were evaporated to dryness *in vacuo* to afford 1.44 g of residue, which was applied to a column of silica gel (30 g) and eluted stepwise with $CHCl_3$–MeOH (19: 1) and $CHCl_3$–MeOH (9: 1). The fractions containing the major product (*Rf* 0.26) and those containing the other major product (*Rf* 0.19) were each evaporated to dryness. The former fractions were successively chromatographed until the product became chromatogrophically homogeneous, and 136 mg of yellow oil was obtained. TLC: *Rf* 0.26 ($CHCl_3$–MeOH, 9: 1) single spot. Fluorescence spectrum (H_2O) λ_{max}^{ex} nm: 365, 403; λ_{max}^{em}: 460. Its NMR spectrum showed that the oil was not in a pure state.

The latter fractions were evaporated to dryness, and the residue was recrystallized from EtOH to afford 127 mg of yellow leaflets of IV_P decomposing at 175—176°C (dec.). TLC: *Rf* 0.19 ($CHCl_3$–MeOH, 9:1) single spot. UV $\lambda_{max}^{H_2O}$ nm (ε): 234 (42500), 261 (16800), 388 (21300); $\lambda_{max}^{0.1N\ HCl}$: 232 (42200), 259 (16300), 388 (21200); $\lambda_{max}^{0.1N\ NaOH}$: 234 (37100), 261 (29500), 290 shoulder, 388 (17300). Fluorescence spectrum (H_2O) λ_{max}^{ex} nm (rel. intensity): 365 (50), 403 (25); λ_{max}^{em}: 460 (50). NMR ($CDCl_3$) ppm: 9.23 (2H, s, 3′,5′-CHO), 6.72 (4H, s, $H_{2',6'}$), 4.33 (2H, t, –CH<, J_{CH,CH_2}=6 Hz), 4.03 (2H, t, H_4', $J_{H(4'),CH_2}$=6 Hz), 3.5 (bm, NCH_2), 3.20 (12H, s, O–CH_3), 1.7 (bm). Its NMR spectrum taken in Me_2SO-d_6 showed that the product was unstable in the solvent. *Anal.* Calcd for $C_{26}H_{36}N_2O_8$: C, 61.89; H, 7.19; N, 5.55. Found: C, 62.04; H, 7.08; N, 5.55.

References and Notes

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