Fluorescent Pigments by Covalent Binding of Lipid Peroxidation By-Products to Protein and Amino Acids

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ABSTRACT

The fluorescent products formed on reaction of 12-oxo-*cis*-9-octadecenoic acid (12-keto-oleic acid) with about 20 different amino acids, polylysine and bovine serum albumin (BSA) were studied. Besides glycine, only the basic amino acids histidine, lysine and arginine gave products with strong fluorescence. N-Acetylation of amino acids greatly reduced the fluorescence of their reaction products. The formation of fluorescent products was inhibited strongly by SH-amino acids such as N-acetyl-cysteine and glutathione. Polyacrylamide gel electrophoresis showed that BSA treated with 12-keto-oleic acid was more acidic than untreated or ricinoleic acid-treated BSA, indicating that basic amino acid residues in BSA were modified by reaction with the keto fatty acid. None of the structural analogs of 12-keto-oleic acid tested—12-oxo-*trans*-10-octadecenoic acid, 12-oxo-octadecanoic acid, 12-hydroxy-*cis*-9-octadecenoic acid (ricinoleic acid), *cis*-9-octadecenoic acid (oleic acid) and linoleic acid—reacted with glycine to give a fluorescent product. The fluorescent products formed on reaction of 12-keto-oleic acid methyl ester with benzyl amine and glycine methyl ester were shown to be 8-(N-substituted-4,5-dihydro-4-oxo-5-hexyl-5-hydroxy-2-pyrrolyl) octanoic acid methyl esters. The fluorescence properties of these compounds were attributed to the chromophobic system NC=CC=O which contains 6π electrons. This investigation contributes to insight of the mechanism of formation of fluorescent pigments, probably by a similar reaction of other compounds of the β,γ-unsaturated carbonyl type.

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INTRODUCTION

Lipofuscin and ceroid pigments are known to be associated with aging (1–6) or E hypovitaminosis (7–11) in animal tissues. These fluorescent substances generally are considered to consist of peroxidized lipid-protein complexes (12–14). Their characters and structures have been investigated by in vitro studies on the reactions of proteins with peroxidized lipids, but still are obscure. One of these compounds is thought to be a conjugated Schiff base product formed by the reaction of protein amino groups with α,β-unsaturated carbonyl groups of malondialdehyde, a decomposition product of peroxidized lipids (15).

Oral 12-oxo-*cis*-9-octadecenoic acid (12-keto-oleic acid: 12-KOA) is reported to enhance the incidence of encephalomalacia in chicks receiving a vitamin E-deficient diet (16,17). Moreover, accumulation of fluorescent pigments was observed in endotherial cells of cerebellar and cerebral vessels in encephalomalacic chicks (10). 12-KOA also was reported to have toxic effects, such as inactivation of enzymes (18) and acceleration of the formation of lipid peroxides (19.20) and fluorescent substances (21).

In the present study, we investigated the formation mechanism and the characterization of fluorescent pigments by covalent binding of 12-KOA, used as a model of secondary products of lipid peroxidation with β,γ-unsaturated carbonyl structure (22–24), to protein and amino acids.

MATERIALS AND METHODS

Materials

12-KOA and 12-oxo-*trans*-10-octadecenoic acid (conjugated 12-keto acid: 12-KCA) were prepared by the method of Nichols and Schipper (25). The methyl ester of 12-KOA was prepared by addition of diazomethane and purified by silicic acid column chromatography. 12-oxo-octadecanoic acid (12-keto stearic acid: 12-KSA) was prepared by hydrogenation of 12-KOA under a stream of hydrogen gas with 5% Pd on carbon as catalyst. BSA (fatty acid free), poly-L-lysine (M.W. 55000), amino acids and N-acetylated amino acids were purchased from Sigma Chemical Co. (St. Louis, Missouri). Benzylamine was obtained from Nakarai Chemical Co. (Tokyo, Japan).

Reaction of 12-KOA with BSA, Polylysine and Amino Acids

A solution of 12-KOA in chloroform in a test

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tube was evaporated under nitrogen. Then 1 ml of BSA in 0.1 M phosphate buffer, pH 7.4, was added to the dry lipid and shaken on a vortex mixer for one min. The final concentration of 12-KOA was 1 mM and that of BSA was 3 mg/ml. A ethanol solution of 12-KOA (0.4 ml) was added to 40 ml of polylysine in 0.1 M phosphate buffer, pH 7.4. The final concentrations of 12-KOA and polylysine were 0.2 mM and 0.5 mg/ml, respectively. The solution in the test tube was incubated at 37 C for 24 hr.

Mixtures of 1 mM 12-KOA and 50 mM amino acids in 0.1 M phosphate buffer, pH 7.4, containing 20% ethanol were incubated at 37 C for 24 hr. After incubation, pH of each sample was adjusted to 7.4. The fluorescence intensity of the solution then was measured directly in a Hitachi 650 fluorospectrometer and calculated relative to that of quinine sulfate standard (0.2 mg/l of 0.1 N H_2SO_4, Ex 351 nm, Em 448 nm), which was taken as 100.

Quantitative Determination of 12-KOA

12-KOA was determined by gas chromatography with 12-KSA as internal standard. Mixtures after reaction of 12-KOA with amino acids were extracted three times with an equal volume of hexane. The extracts were combined, the hexane was evaporated under nitrogen, and 12-KOA was methylated with diazomethane. The amount of methyl ester in the sample then was measured in a Shimadzu gas chromatograph GC-6A equipped with a column (3 mm × 2 m) packed with 10% EGSP on 60–80 mesh chromosorb WAW. The column temperature was 195 C.

Polyacrylamide Gel Electrophoresis

After incubation for five days, the reaction products of BSA and 12-KOA were analyzed by polyacrylamide gel electrophoresis. Electrophoresis was carried out in 7.5% polyacrylamide gel with Tris-glycine buffer, pH 8.3, for five hr at 6.4 mA/cm under cooling at 18 C. The gel was stained with 0.1% Coomassie Brilliant Blue.

Preparation and Purification of a Main Fluorescent Product of 12-KOA Methyl Ester with Benzylamine

The reaction mixture containing 2.0 g of 12-KOA methyl ester and 1.08 g of benzylamine was stirred at 50 C. After 36 hr, the fluorescent products were separated by silicic acid column chromatography with chloroform/acetone (4:1, v/v) as solvent and purified by silica gel thin layer chromatography (TLC) with chloroform/acetone (1:1, v/v). The main product (more than 80%) was 12-oxo-10-octadecenoic acid methyl ester. The main fluorescent product (Ia in Fig. 4, Rf = 0.71) was obtained in a yield of 64 mg. The methyl ether (Ib) of Ia was prepared by mixing 3 ml of solution of 40 mg of Ia in methanol with 0.1 ml of 1 M HCl-methanol for five hr at room temperature. The solvent was evaporated and Ib was separated by silicic acid column chromatography with chloroform/acetone (94:4, v/v) as solvent.

Preparation of a Fluorescent Product of 12-KOA Methyl Ester with Glycine Methyl Ester

The fluorescent product (II) was prepared by mixing a solution of 1.0 g of 12-KOA methyl ester with 0.42 g of glycine methyl ester HCl in 2.5 ml of triethylamine for 12 hr at 60 C. The fluorescent products were separated and purified by silicic acid column chromatography and TLC with chloroform/acetone (3:1, v/v) and chloroform/acetone/methanol (6:3:1, v/v/v), respectively, as solvents. The Rf value of the main fluorescent product (II) was 0.34.

Characterization of Ia and II

The fluorescent products were characterized by infrared (IR), ultraviolet (UV), fluorescence, ^1H- and ^{13}C-nuclear magnetic resonance (NMR) and high- and low-resolution mass (MS) spectrometries. Electron impact ionization mass (EI-MS) spectra were measured with a JEOL JMS-D300 double-focusing mass spectrometer. Ionization was complete at 70 eV. High resolution MS spectra were obtained with perfluorokerosene as a standard. ^1H- and ^{13}C-NMR spectra were recorded in a JEOL JNM-FX200 spectrophotometer at 200 MHz and 50 MHz. The samples were dissolved in $CDCl_3$ with 1% tetramethylsilane as an internal reference. Uncorrected fluorescence spectrum of Ia was recorded with a Hitachi 650 instrument. IR spectra were determined in a JASCO DS-701G instrument by the liquid film method.

RESULTS AND DISCUSSION

On incubation of 12-KOA with BSA at pH 7.4, the fluorescence with an excitation maximum of 350 nm and an emission maximum at 420 nm increased markedly (Table 1). The spectrum was similar to that reported for the reaction product of peroxidized linoleic acid with BSA (13).

To obtain further insight into the reaction, we examined the fluorescence of reaction mixtures of 12-KOA and various amino acids. As shown in Figure 1, except in the case of

TABLE 1

Fluorescent Formation by the Reaction of 12-KOA with Amino Acids and Protein and Its Inhibition by SH-Amino Acids

	Ex (nm)	Em (nm)	Fluorescent intensity
Histidine	339	413	206.6
Arginine	319	383	108.0
Lysine	349	419	167.5
Glycine	342	414	135.8
Glutathione	323	423	15.0
N-Acetyl-glycine	322	401	17.1
N-Acetyl-cysteine	313	351	<1.0
Glycine + N-acetyl-cysteine	327	369	1.2
Glycine + glutathione	335	425	26.1
Lysine + N-acetyl-cysteine	340	419	45.0
BSA	350	420	153.0
Polylysine	334	408	225.0

Concentration of each amino acid was 50 mM.

Concentrations of BSA and polylysine were 3 mg/ml and 0.5 mg/ml, respectively.

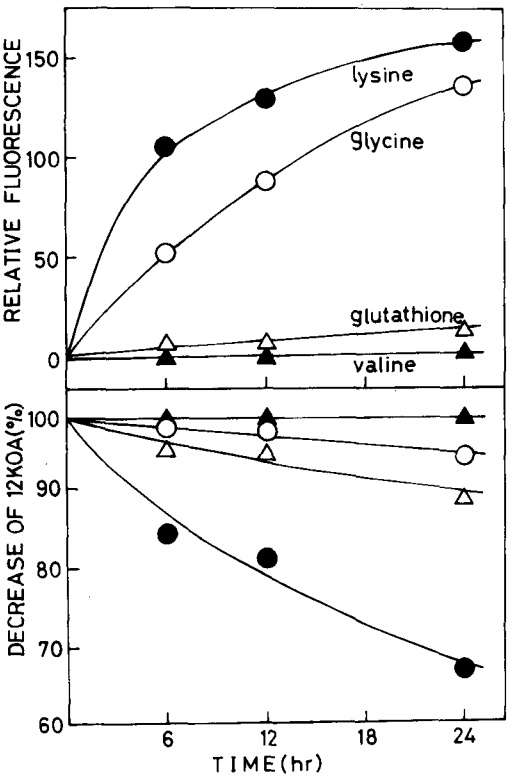

FIG. 1. Time courses of formation of fluorescence and 12-KOA consumption during its reacting with lysine (●), glycine (○), valine (▲) and glutathione (△). Reaction mixture containing 1 mM 12 KOA, 50 mM amino acids, 20% ethanol and 0.1 M phosphate buffer, pH 7.4, was incubated at 37 C. The fluorescent intensity of 0.2 mg of quinine sulfate/l of 0.1 N H_2SO_4 (Ex 351 nm, Em 448 nm) was set at 100.

glutathione, the increase in fluorescence intensity was proportional to the amount of 12-KOA consumed, in the order lysine > glycine > valine = 0. The mixture with glutathione showed little fluorescence, though significant 12-KOA was consumed during the reaction. Table 1 shows that besides glycine, only the basic amino acids histidine, lysine and arginine gave strongly fluorescent products. Acidic and neutral amino acids examined (asparatic acid, glutamic acid, glutamine, alanine, isoleusine, valine, serine, threonine, phenylalanine, proline and methionine) did not react with 12-KOA, resulting in little fluorescence (data not shown). In the reaction of peroxidized lipid also, only basic amino acids produce fluorescent products (13). Little fluorescence was observed with N-acetyl-glycine, but glycine gave strong fluorescence, indicating that a free amino group was essential for formation of a fluorescent product. The amino groups at the α-position in acidic and neutral amino acids other than glycine may be unable to react with 12-KOA due to steric hindrance of side chains. These results indicate that one of the reaction sites of BSA responsible for fluorescence is free amino groups of lysine and arginine moieties in the protein. This conclusion was supported by the result that polylysine, which contains only free amino groups in the molecule, gave strong fluorescence by reaction with 12-KOA (Table 1).

Figure 2 shows the results obtained on polyacrylamide gel electrophoresis of BSA with and without treatment with 12-KOA or ricinoleic acid. The higher mobilities of the two components of 12-KOA-treated BSA than those of untreated or ricinoleic acid-treated BSA indicate the increase in acidic properties of the components. This finding suggests that basic amino acid residues, such as lysine, arginine and histidine, in BSA were modified by 12-KOA.

Glutathione or N-acetyl-cysteine, which reacted with 12-KOA but produced no fluorescent products, strongly inhibited formation of fluorescent products on reaction of glycine or lysine with 12-KOA (Table 1). These results suggest that a thiol group is more reactive than an amino group with 12-KOA.

The formation of a fluorescent product on reaction of 12-KOA with glycine was investi-

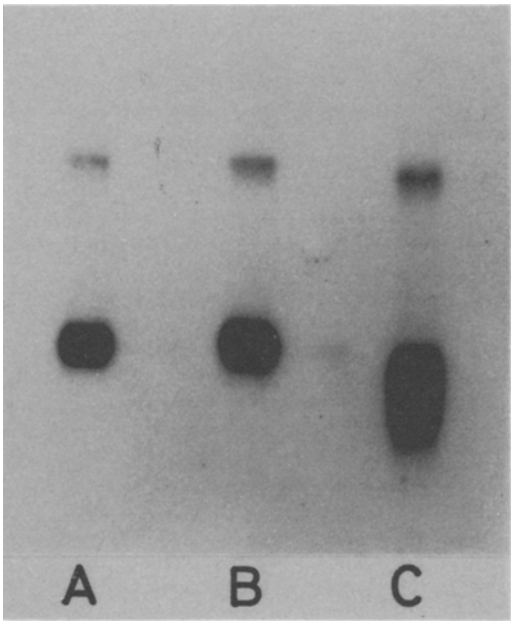

FIG. 2. Polyacrylamide disk gel electrophoresis of reaction products of BSA with 12-KOA. A, BSA; B, BSA + ricinoleic acid; C, BSA + 12-KOA. Fluorescent intensity of reaction products of 12-KOA with BSA was 328 (Ex 338 nm, Em 411 nm) when the intensity of 0.2 mg of quinine sulfate/l of 0.1 N H_2SO_4 was set at 100.

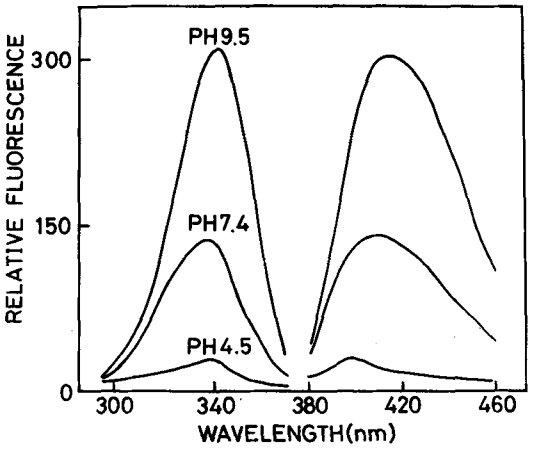

FIG. 3. pH-Dependent fluorescence formation during reaction of 12-KOA with glycine. Experimental conditions were the same as for Fig. 1, except that the incubation mixture at pH 4.5 contained 40% ethanol. Each pH was adjusted with NaOH or HCl. After incubation at the indicated pH values for 24 hr at 37 C, samples were adjusted to pH 7.4 and then fluorescence was measured.

gated at pH values of 4.5, 7.4 and 9.5. As shown in Figure 3, the fluorescence increased with increase of pH, suggesting that a NH_2, not NH_3^+, group reacted with 12-KOA.

We next examined the relationship between the structure of fatty acids and their ability to form a fluorescent product. As shown in Table 2, fatty acids with structures analogous to that of 12-KOA (β,γ-unsaturated keto acid), such as 12-KCA (α,β-unsaturated keto acid), 12-KSA (saturated keto acid), ricinoleic acid (β,γ-unsaturated hydroxy acid) and oleic acid, were tested, but none formed a fluorescent product when incubated with glycine. Thus, a β,γ-unsaturated carbonyl structure appeared essential for production of the fluorescent product. It is very interesting that 12-KOA had the highest activity to form a fluorescent product, since the potency of 12-KOA in induction of encephalomalacia in chicks has been reported also to be higher than those of 12-KCA and 12-KSA (16). Hexenal, another possible model of secondary products of peroxidized lipids (26,27), also produced a fluorescent product on reaction with glycine, but this had less fluorescence than the product of 12-KOA with glycine.

For elucidation of the structure of the fluorescent products, we prepared the Ia, a main fluorescent product, formed by a reaction of 12-KOA methyl ester with benzylamine, an aliphatic primary amine. As shown in Figure 5, Ia shows a fluorescence spectrum with an excitation maximum at 340 nm and an emission maximum at 410 nm, unlike the malondialdehyde-protein complex, which shows values of 395–405 nm and 460–465 nm, respectively (15). The spectrum of Ia was similar to that of the reaction product of oxidized lipid and protein (12).

The molecular weight of compound Ia was determined as 429.2881 ($C_{26}H_{39}N_1O_4$) by high

TABLE 2

Fluorescence Produced by Reactions of 12-KOA Analogs with Glycine

Fatty acid	Ex (nm)	Em (nm)	Fluorescent intensity
12-KOA	342	414	132.0
12-KCA[a]	340	408	47.7
12-KSA	308	368	<1.0
Ricinoleic acid	313	373	<1.0
Oleic acid	305	379	<1.0
Linoleic acid	314	373	<1.0
Hexenal	326	411	41.6
Hexanal	340	411	13.7

[a]Contaminated with 30% 12-KOA.

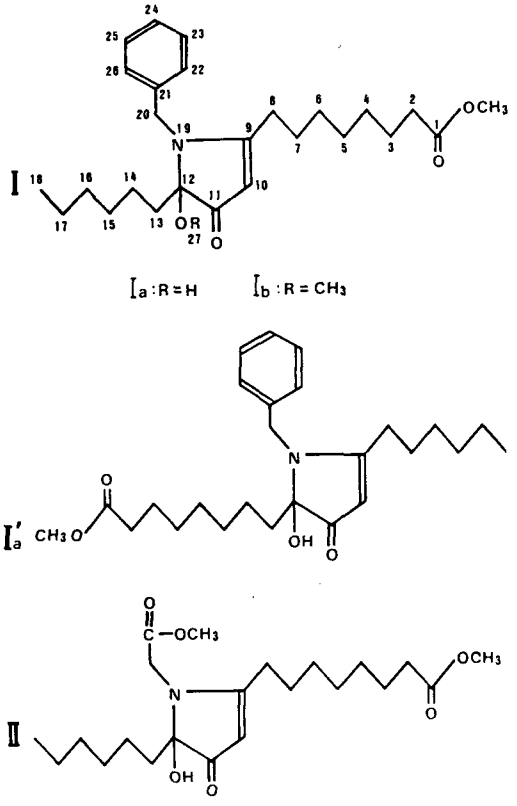

FIG. 4. Numerical key and structures of main fluorescent products and derivatives of the 12-KOA methyl ester and benzylamine and glycine methyl ester.

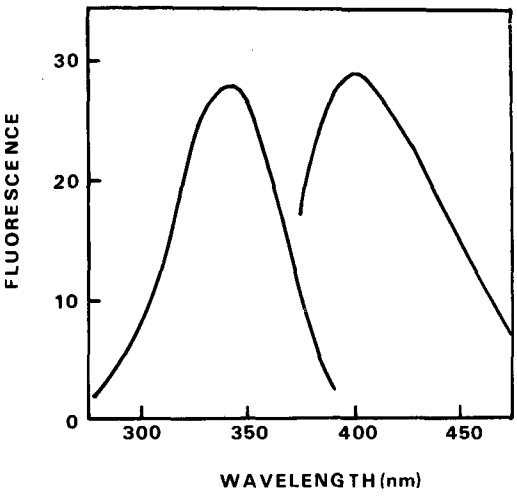

FIG. 5. Fluorescence spectrum of Ia in methanol. The concentration of Ia was 28.8 μM (0.295 absorbance at 340 nm). The fluorescent intensity of 0.5 mg of quinine sulfate/l of 0.1 N H_2SO_4 was set at 100 (Ex 340 nm, Em 408 nm).

resolution MS spectrometry. This value indicates the introduction of one oxygen atom as well as a benzyl amino group into the original β,γ-unsaturated ketone. Other fragment ions m/z 411 (M-H_2O), 398 (M-OCH_3), 338 (M-91), 316 (M-113) and 91 were also characteristic of Ia (Fig. 6).

Data on ^{1}H-NMR analysis shown in Table 3 indicate the presence of a methoxy, a terminal methyl and a benzylamino group and twelve methylene groups, of which two (corresponding to C_2 and C_8 of Ia, Fig. 4) are observed in a lower field than other methylene groups of Ia.

The absorptions at 1650 cm^{-1} in the IR spectrum and at 340 nm (ε = 10240) in the UV spectrum indicate the presence of an α,β-unsaturated ketone group. In the ^{1}H-NMR spectrum, the olefinic proton resonance is observed as a proton singlet at a higher field than that of normal olefinic proton conjugated with a ketone group. This position (δ4.95) is consistent with the conjugation of the enone group with some hetero atoms.

The other oxygen atom of the molecule is attributed to the tertiary hydroxyl group on the basis of the ^{1}H-NMR (δ4.48, singlet, exchangeable with D_2O) and the IR spectra (3240 cm^{-1}).

From these spectroscopic data and the molecular formula, in which one ring structure or one unsaturated bond other than that described above is necessary, the formula Ia or Ia′ is possible for the fluorescent compound. The latter formula (Ia), however, is not compatible with the fragmentation pattern of the MS spectrum in Figure 6. The ^{13}C-NMR data (Table 4) are fully consistent with formula Ia.

For confirmation of the aminol moiety (C_{12}-N_{19} in Fig. 4) of the molecules, compound Ia was treated with 1 M HCl in methanol at room temperature to give the methyl ester (Ib) in good yield as described in the Experimental section. The spectroscopic data for Ib were very similar to those for Ia except that a methoxy group (δ3.05, singlet) was observed in place of a hydroxyl group (δ4.48) in the ^{1}H-NMR spectrum. The EI-MS spectrum of Ib also showed the ion m/z 443 (M^+), 428 (M-CH_3), 412 (M-OCH_3), 358 (M-85), 352 (M-91) and 91 (Fig. 6). The IR spectrum of Ib showed less absorption at 3420 cm^{-1} than that of Ia, indicating loss of a hydroxyl group.

Addition of trifluoroacetic acid to the chloroform solution of Ia resulted in profound change of the UV spectrum. As shown in Figure 7, a new absorption peak appeared at 273 nm with decrease in absorption at 340 nm, suggesting ring opening of the aminol moiety (Fig. 8). The spectra had two isobestic points at 315 and 370 nm, and the change induced by

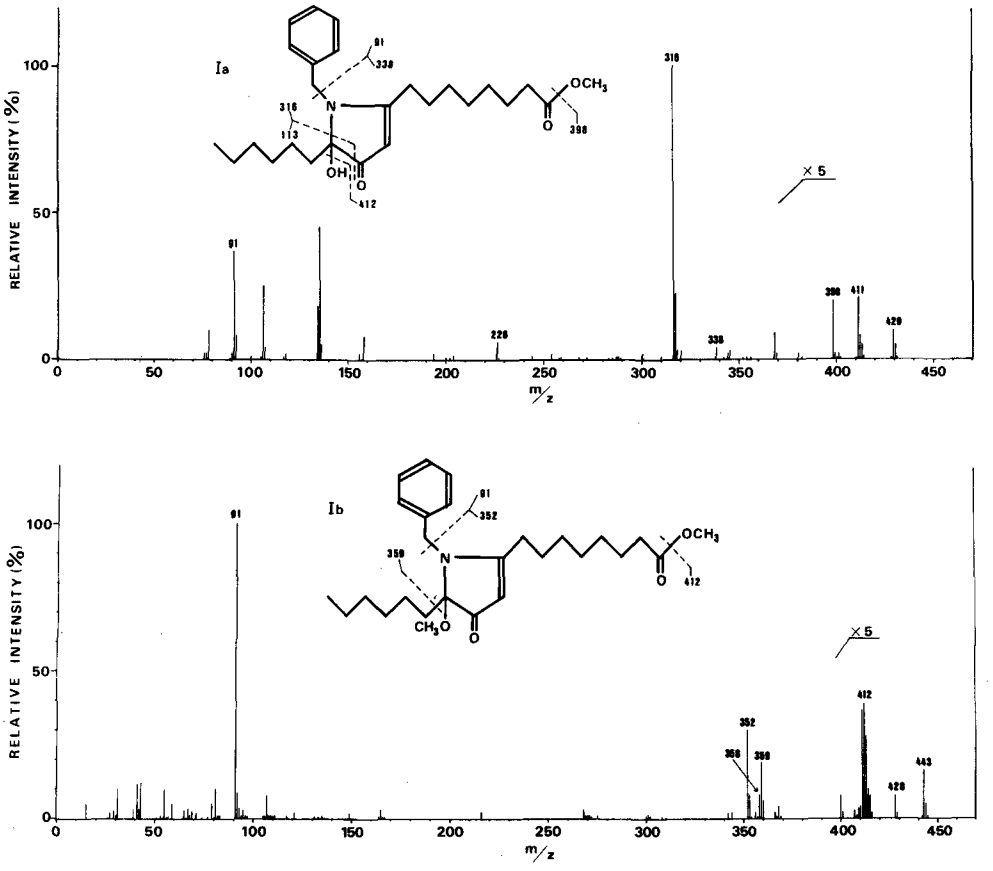

FIG. 6. Electron impact ionization mass spectra of Ia and Ib.

TABLE 3

^{1}H-NMR Spectra of the Fluorescent Product (Ia) and Its Methyl Ether (Ib)

ppm			Coupling constant	No. of	
Ia	Ib	Multiplicity[a]	(Hz)	protons	Assignment
7.32	7.27	s		5	Ar
4.95	5.05	s		1	H-10
4.62		d	J = 17	1	H-20
4.49		d	J = 17	1	H-20
	4.46	s		1	H-20
4.48		br, s		1	OH
3.66	3.67	s		3	OCH_3
	3.05	s		3	OCH_3 (H-27)
2.28		t	J = 7	4	H-2, H-8
	2.49	t	J = 7	2	H-8
	2.30	t	J = 7	2	H-2
1-2	1-2	br		18	H-3-7, H-14-17
0.84	0.81	t	J = 6	3	H-18

[a]Multiplicity: br = broad, s = singlet, d = doublet, t = triplet.

TABLE 4

^{13}C-NMR Spectra of the Fluorescent Product (Ia) and Its Methyl Ether (Ib)

ppm			
Ia	Ib	Multiplicity[a]	Assignment
200.72	198.00	s	C-11
182.29	182.35	s	C-9
174.09	174.06	s	C-1
138.02	137.44	s	C-21
128.68	128.77	d	C-23, C-25
127.43	127.72	d	C-24
127.31	127.57	d	C-22, C-26
94.96	97.35	d	C-10
91.39	96.15	s	C-12
	51.92[b]	q	C-27
51.42	51.45[b]	q	OCH_3
45.40	45.11	t	C-20
36.18	35.18	t	
33.99	33.99	t	
31.62	31.54	t	
29.17	29.17	t	
29.05	29.05	t	
28.96	28.99	t	
28.88	28.90	t	C-2-8, C-14-17
28.79		t	
26.60	26.92	t	
24.79	24.82	t	
22.71	22.48	t	
22.51	22.19	t	
14.02	14.05	q	C-18

[a]Multiplicity: s = singlet, d = doublet, t = triplet, q = quartet.

[b]May be reverse.

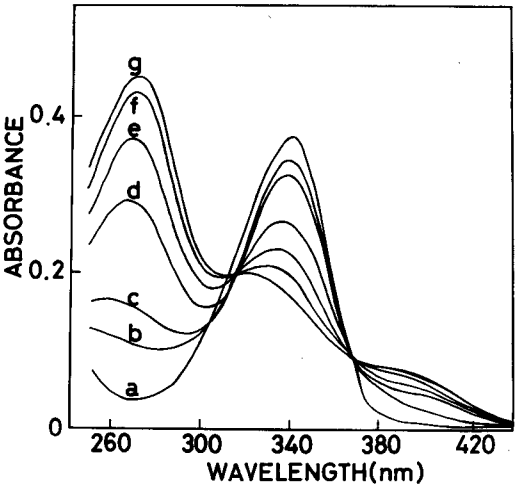

FIG. 7. Ultraviolet spectra of Ia in methanol with different concentrations of trifluoroacetic acid. Letters indicate concentrations (v/v) of trifluoroacetic acid of a, 0%; b, 0.3%; c, 0.6%; d, 0.9%; e, 2.7%; f, 5.4% and g, 8.1%.

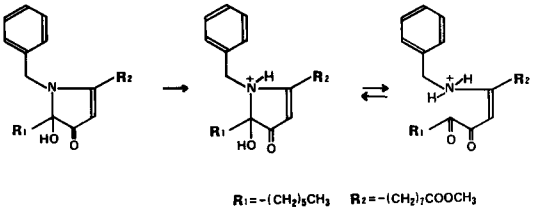

FIG. 8. Reversible structural change of Ia.

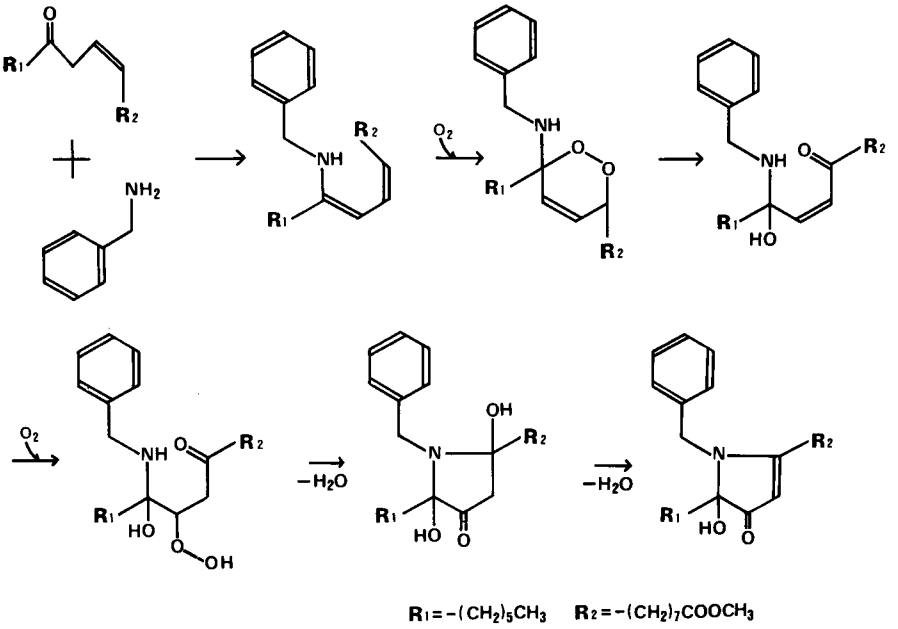

FIG. 9. Proposed pathway for formation of addition products. The fatty acid structure is abbreviated.

trifluoroacetic acid was reversed by addition of KOH. These findings support the proposed structure Ia.

The main fluorescent product (compound II, Fig. 4) of the glycine methyl ester with 12-KOA methyl ester was characterized by its EI-MS spectrum, having ion peaks m/z 411 (M^+, $C_{22}H_{37}N_1O_6$), 393 (M-H_2O, $C_{22}H_{35}N_1O_5$), 380 (M-OCH_3, $C_{21}H_{34}N_1O_5$), 352 (M-$COCH_3$, $C_{20}H_{34}N_1O_4$) and 298 (M-113, $C_{15}H_{24}N_1O_5$).

A possible pathway for formation of Ia is shown in Figure 9. The reaction of 12-KOA and benzylamine or glycine involves nucleophilic attack on the β,γ-unsaturated system of 12-KOA, which is followed by loss of water to yield the conjugated Shiff base. Oxygenation of the diene moiety followed by cleavage of the resulting peroxy ring gives the α,β-unsaturated ketone. Further oxygenation and dehydrations should give Ia. Other lipid peroxidation by-products having β,γ-unsaturated ketone, such as 12-oxo-9-dodecenoate (24), and proteins may undergo similar reactions.

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