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Properties of Conjugated Schiff Bases of Malonaldehyde

KIYOMI KIKUGAWA* and YUMIKO SUGIMURA

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

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Malonaldehyde is one of the end products of lipid oxidation, and because of its bifunctionality it can produce a conjugated Schiff base with a primary amine. The fluorescence properties, the reactivity to thiobarbituric acid (TBA) and the antioxidative activity of conjugated Schiff bases of malonaldehyde and aniline (I₁), *p*-toluidine (I₂), *p*-anisidine (I₃), *p*-chloroaniline (I₄) and *p*-aminoacetophenone (I₅) were investigated. Compounds I₁—I₅ showed no significant intrinsic fluorescence. The unstable compound I₅ produced highly fluorescent compound(s) in a mixture of dimethylformamide-tetra-*n*-propylammonium hydroxide. In the TBA reaction, compounds I₁—I₄ produced less than 30% as much a red color as malonaldehyde, while the unstable compound I₅ produced as intense a red color as malonaldehyde. Compounds I₁—I₄ were active as antioxidants with methyl oleate, lard and soybean oil.

Keywords—malonaldehyde; conjugated Schiff base; fluorescence; thiobarbituric acid; anti-oxidant

Malonaldehyde has been recognized as one of the most important secondary products of lipid oxidation.^{1,2)} Measurement of malonaldehyde with thiobarbituric acid (TBA) is a general method for evaluating the degree of oxidation of lipid and tissue samples in the fields of both medical and food science.^{2,3)} When malonaldehyde is formed it may coexist with protein or phospholipid containing free amino groups. This highly reactive aldehyde may react with these materials, and the reactions may cause deterioration or produce some signs of aging of tissues, and may affect the assay of malonaldehyde or the subsequent oxidation of tissues. Several studies on the reaction of malonaldehyde have appeared. Chio and Tappel⁴⁻⁶⁾ treated malonaldehyde with aliphatic primary amines under strongly acidic conditions to obtain amorphous conjugated Schiff bases with high fluorescence. Since then, it has been believed that the reaction of malonaldehyde and amino acids gave fluorescent conjugated Schiff bases which cross-link protein molecules. Nair *et al.*,⁷⁾ however, demonstrated that the reaction of malonaldehyde with amino acids under mildly acidic conditions failed to give conjugated Schiff bases but gave the non-fluorescent 1 : 1 Schiff bases, as had been reported.⁸⁾ We obtained crystalline 1,4-dihydropyridine-3,5-dicarbonyl aldehydes as the major fluorescent substances from the reactions of malonaldehyde and primary amines, and their structures were unambiguously established.⁹⁻¹¹⁾ Because of its bifunctionality, malonaldehyde can produce conjugated Schiff bases, but it is questionable whether they are highly fluorescent.

This time, we prepared conjugated Schiff bases of malonaldehyde and aromatic amines (malonaldehyde dianils) according to the reported methods, and examined their fluorescence properties and TBA-coloration, in addition to evaluating their antioxidant activities.

Experimental

Malonaldehyde bis(dimethylacetal), *p*-anisidine, *p*-chloroaniline and *p*-aminoacetophenone were the products of Tokyo Kasei Kogyo Company, Ltd. Aniline, *p*-toluidine, 10% tetra-*n*-propylammonium hydroxide and TBA were the products of Wako Pure Chemical Industries, Ltd. Butylated hydroxytoluene (BHT) was a product of Nikki

Universal Company, Ltd., and was recrystallized from ethyl alcohol for use. Methyl oleate with peroxide value (POV) 2.1 meq/kg was a product of Tokyo Kasei Kogyo Company, Ltd. Soybean oil with POV 0 meq/kg was a product of Showa-Sangyo Company, Ltd. Fresh lard was prepared for use by warming and squeezing and showed POV 34 meq/kg.

Absorption spectra were measured with a Shimadzu UV-200S double-beam spectrophotometer. Fluorescence spectra were measured with a Hitachi 650-40 fluorescence spectrophotometer.

Preparation of Conjugated Schiff Bases of Malonaldehyde (Malonaldehyde Dianils) (I)—Conjugated Schiff bases were prepared according to the method of Tamura *et al.*¹²⁾ Concentrated hydrochloric acid (2.0 ml) was added at room temperature to a mixture of an aromatic amine (20 mmol) and malonaldehyde bis(dimethylacetal) (10 mmol) in 30 ml of ethyl alcohol. The mixture was allowed to stand at room temperature overnight, and yellow crystals that precipitated were collected by filtration (I₁–I₅). The crystalline precipitate (I₁–I₄) was suspended in a solution of 7% sodium bicarbonate and extracted with ethyl acetate. The extract was evaporated to dryness to afford yellow crystals. The products (I₁–I₅) were purified by repeated recrystallization; mp 109–117°C for I₁ [113–114°C,¹²⁾ 118–120°C¹³⁾], 160–165°C for I₂ [165–166°C¹²⁾], 181–183°C for I₃ [184–185°C¹²⁾], 156–157°C for I₄ [158–159°C¹²⁾], and 205–210°C for I₅ [216–218°C¹⁴⁾].

TBA Test^{2,3)}—**Method A:** A mixture of 0.3 ml of 0.1–0.5 mM test sample in ethyl alcohol, 2.0 ml of 0.5% TBA in water and 6.0 ml of 10% trichloroacetic acid was heated at 60°C for 90 min, and the absorption spectrum was recorded between 400–600 nm as quickly as possible.

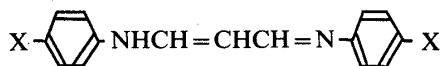
Method B: A mixture of 0.3 ml of 0.1–0.5 mM test sample in ethyl alcohol, 2.0 ml of 0.5% TBA in water and 6.0 ml of glacial acetic acid was heated at 100°C for 20 min.

Antioxidant Activity—Each sample was incorporated in methyl oleate, lard and soybean oil at a concentration of 0.01% with the aid of a small amount of ethyl alcohol. BHT at a concentration of 0.01% was similarly tested as a standard. An equivalent amount of ethyl alcohol was added to the control sample. Portions were placed in tubes designed as described,¹⁵⁾ and aerated with purified air at the rate of 4.0 ml/s, at 98°C [active oxygen method (AOM)].¹⁶⁾ At regular intervals, a 0.5–1.0 g sample was removed and the POV was determined according to the method of Wheeler.¹⁷⁾

Results

Conjugated Schiff bases (malonaldehyde dianils) (I₁–I₅) of malonaldehyde and aromatic amines such as aniline, *p*-toluidine, *p*-anisidine, *p*-chloroaniline and *p*-aminoacetophenone were prepared according to the reported methods.¹²⁾ Absorption maxima and molecular extinction coefficients of I₁–I₅ in ethyl alcohol, methyl alcohol, 0.1 M phosphate buffer (pH 7), 0.1 N HCl and 0.1 N NaOH are summarized in Table I. All the compounds exhibited absorption maxima at 350–400 nm with molecular extinction coefficients of 35000–55000 in ethyl alcohol and methyl alcohol. The absorption maxima of I₁–I₄ shifted batho- and hyperchromically in 0.1 M phosphate buffer and 0.1 N HCl. The absorption maxima of I₁–I₄ in 0.1 N NaOH were close to those in ethyl alcohol and methyl alcohol, but they gradually changed indicating that the compounds were degraded. While I₅

TABLE I. Absorption Maxima of Conjugated Schiff Bases I₁–I₅



Compd.	X	Absorption maximum nm (mε)				
		Ethyl alcohol	Methyl alcohol	0.1 M phosphate (pH 7.0)	0.1 N HCl	0.1 N NaOH
I ₁	H	358 (36.5)	365 (38.3)	381 (48.7)	381 (53.0)	357 (unstable)
I ₂	CH ₃	367 (40.1)	378 (43.0)	389 (50.2)	389 (51.6)	361 (unstable)
I ₃	OCH ₃	375 (38.0)	392 (41.5)	395 (47.8)	393 (50.3)	368 (unstable)
I ₄	Cl	364 (47.4)	364 (47.0)	389 (57.9)	387 (68.8)	364 (unstable)
I ₅	COCH ₃	394 (53.8)	393 (55.1)	410 (unstable)	410 (unstable)	398 (unstable)

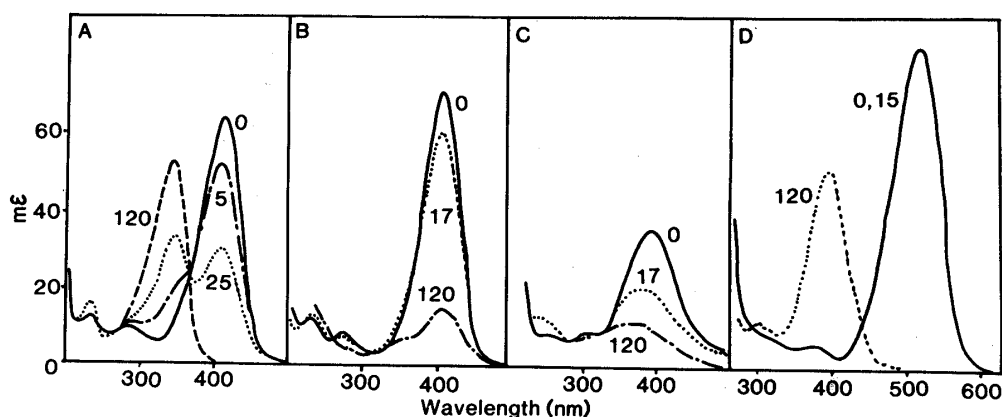


Fig. 1. Absorption Spectra of I_5

A, in 0.1 M phosphate buffer (pH 7.0); B, in 0.1 N HCl; C, in 0.1 N NaOH; and D, in dimethylformamide-tetra-*n*-propylammonium hydroxide (9 : 1). The numerals indicate time (min) of standing.

TABLE II. Fluorescence Spectra of Conjugated Schiff Bases I_1 — I_5

Compd.	In 0.1 M phosphate (pH 7.0)			In dimethylformamide-10% tetra- <i>n</i> -propylammonium hydroxide (9 : 1)		
	Ex^{max} (nm)	Em^{max} (nm)	RMI	Ex^{max} (nm)	Em^{max} (nm)	RMI
I_1	402	466	0.01	436	489	0.01
I_2	405	466	0.01	452	494	0.01
I_3	403	466	0.01	450	495	0.01
I_4	402	465	0.02	442	507	0.01
I_5	404	466	0.02	524	588	1.14

RMI, relative molar intensity with respect to quinine sulfate in 0.1 N sulfuric acid (Ex 352 nm and Em 448 nm).

was stable in ethyl alcohol and methyl alcohol, it was readily degraded in 0.1 M phosphate buffer, 0.1 N HCl and 0.1 N NaOH. Thus, the absorption maximum at 410 nm in the phosphate buffer gradually shifted to 340 nm after 2 h at room temperature (Fig. 1A). The absorbance at 410 nm in 0.1 N HCl and that at 398 nm in 0.1 N NaOH were greatly decreased after 2 h (Fig. 1B and C). Thus, the stability of I_5 in the solvents was very different from those of I_1 — I_4 . When I_5 was dissolved in a mixture of dimethylformamide-tetra-*n*-propylammonium hydroxide,¹⁴⁾ the solution turned reddish and showed an absorption maximum at 520 nm, which was maintained during 15 min at room temperature but changed into a maximum at 395 nm within 2 h (Fig. 1D). Compounds I_1 — I_4 showed slightly different spectra in dimethylformamide-tetra-*n*-propylammonium hydroxide from those in an aqueous alkali, but did not show such a large bathochromic shift as was observed with I_5 .

The fluorescence spectra of I_1 — I_5 exhibited excitation maxima at 400—405 nm and emission maxima at 465—470 nm (Table II). However, the relative molar intensity of the fluorescence was lower than 2% of that of quinine sulfate in 0.1 N sulfuric acid. Thus, the conjugated Schiff bases I_1 — I_5 had practically no fluorescence. The fluorescence spectra of I_1 — I_5 in dimethylformamide-tetra-*n*-propylammonium hydroxide¹⁴⁾ were measured (Table II). While the excitation and emission maxima of I_1 — I_4 shifted to higher wavelength, the relative molar intensity of the fluorescence was as low as 1% of that of quinine sulfate. It is interesting that compound I_5 showed a significant fluorescence with an excitation maximum

TABLE III. TBA Test Results of Conjugated Schiff Bases I₁—I₅

Compd.	Relative molar ratio of absorbance at 532 nm	
	Method A ^{a)}	Method B ^{a)}
Malonaldehyde bis (dimethylacetal)	1.00 ^{b)}	1.00 ^{c)}
I ₁	0.07	0.10
I ₂	0.03	0.06
I ₃	0.06	0.03
I ₄	0.29	0.26
I ₅	0.98	0.88

a) See Experimental. b) The absorbance at 532 nm of 0.2 mm reagent was 1.19. c) The absorbance at 532 nm of 0.2 mm reagent was 0.25.

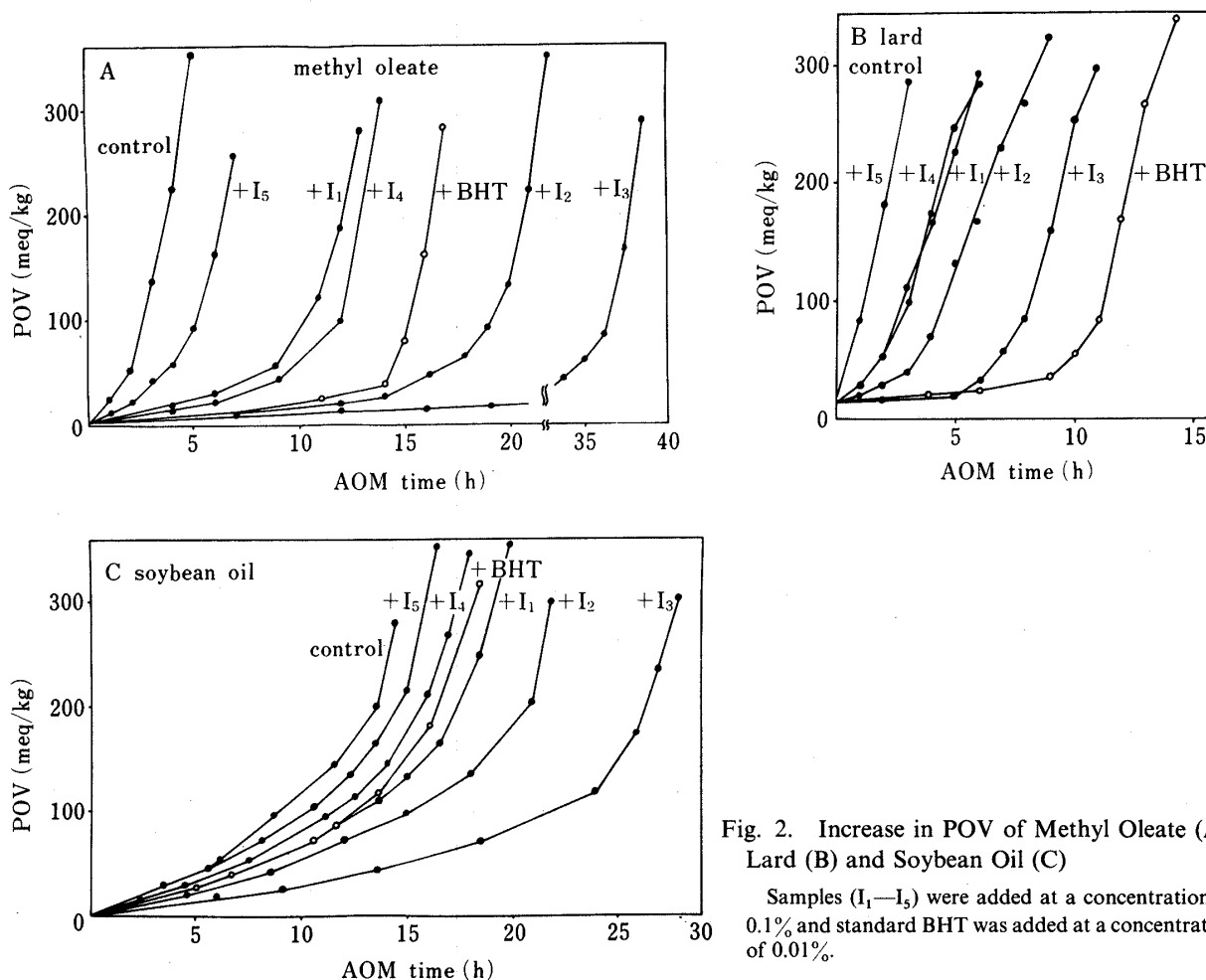


Fig. 2. Increase in POV of Methyl Oleate (A), Lard (B) and Soybean Oil (C)

Samples (I₁—I₅) were added at a concentration of 0.1% and standard BHT was added at a concentration of 0.01%.

at 524 nm and an emission maximum at 588 nm, and the relative molar intensity was as high as 1.14. The fluorescence was stable for 15 min, but was lost completely within 2 h. This fluorescence was also produced in dimethylformamide–aqueous sodium hydroxide, but not in dimethylformamide alone. The fluorescence was not formed in dimethylformamide–tetra-*n*-propylammonium hydroxide which had been kept for more than 1 h. Thus, the fluorescence of I₅ in dimethylformamide–tetra-*n*-propylammonium hydroxide must be due to the reaction product(s) of an unstable compound in the solvent mixtures.

In order to obtain information on the TBA-coloration of conjugated Schiff bases, I_1 — I_5 were subjected to the TBA test. The TBA test was performed in two ways; in trichloroacetic acid (method A) and in acetic acid (method B) (Table III). Standard malonaldehyde bis-(dimethylacetal) produced a red color with an absorption maximum at 532 nm, and all the test compounds produced a red color with the same absorption spectrum. Compounds I_1 — I_4 produced a red color amounting to less than 30% of that of the standard. Compound I_5 produced as intense a red color as the standard in method A and 88% as intense a red color in method B. Therefore, TBA-coloration of the conjugated Schiff bases must depend on their stability.

The time courses of the increase in POV of methyl oleate, lard and soybean oil with 0.1% of the conjugated Schiff bases were plotted against time according to the AOM (Fig. 2). As a reference standard, 0.01% BHT was similarly tested. Most compounds showed antioxidant activity with these substrate oils, and the antioxidative potency of the compounds was different depending on the substituents on the benzene ring of the compounds. The antioxidative potency of the compounds was in the order of $I_3 > I_2 > I_1$, $I_4 > I_5$ with every substrate oil. The AOM time at which the POV value reached 100 meq/kg is a measure of the antioxidative potency of the compounds. The most potent compound I_3 at 0.1% showed AOM times of 36 h (methyl oleate), 8 h (lard) and 22 h (soybean oil). Control AOM times were 2.5 h (methyl oleate), 1 h (lard) and 9 h (soybean oil), and BHT (0.01%) showed AOM times of 15 h (methyl oleate), 11 h (lard) and 13 h (soybean oil). The weakest compound was the unstable I_5 , which exhibited only slight antioxidative activity with methyl oleate and soybean oil and practically no activity with lard. It was found that the antioxidative potency of the conjugated Schiff bases I_1 — I_5 depended upon the substituents on the benzene ring of the compounds.

Discussion

It has been demonstrated that fluorescent lipofuscin is produced in aging tissues, and the formation has been regarded as a consequence of lipid oxidation of tissues.¹⁸⁾ Several studies have appeared with regard to the formation of fluorescent substances from malonaldehyde. Chio and Tappel^{4,5)} obtained conjugated Schiff bases of amino acids and *n*-hexylamine in an amorphous state from the reaction mixtures of malonaldehyde and the corresponding amino compounds under strongly acidic conditions. The conjugated Schiff bases⁴⁾ exhibited fluorescence with excitation maxima at 370—395 nm and emission maxima at 450—470 nm, with relative molar intensity of less than 0.5 with respect to quinine sulfate. Buttkus and Bose¹³⁾ demonstrated that while amorphous conjugated Schiff bases of amino acids fluoresced with excitation and emission maxima at 395 and 470 nm, respectively, the crystalline conjugated Schiff base of aniline I_1 had no fluorescence, and they suggested that more highly purified conjugated Schiff bases of amino acids were needed for fluorescence studies. Our recent studies⁹⁻¹¹⁾ demonstrated that highly fluorescent 1,4-dihydropyridine-3,5-dicarbaldehydes were formed instead of the fluorescent conjugated Schiff bases in the reaction of malonaldehyde and aliphatic primary amines under neutral conditions. The 1,4-dihydropyridine-3,5-dicarbaldehydes were obtained in a crystalline state and their structures were unambiguously established. The compounds showed excitation and emission maxima at 390—405 and 450—470 nm, respectively, with relative molar intensity of 0.9—1.5 with respect to quinine sulfate. Some of the amorphous conjugated Schiff bases of amino acids obtained by Chio and Tappel^{4,5)} may have contained these compounds as impurities.¹⁹⁾

Malonaldehyde produces cross-links in protein molecules,^{5,6,19-22)} and these may be due to the formation of conjugated Schiff bases or to the reaction products of malonaldehyde polymers. It is therefore important to clarify the properties of the purified conjugated Schiff

bases. It is difficult to obtain highly purified conjugated Schiff bases of aliphatic primary amines, but conjugated Schiff bases of aromatic amines (malonaldehyde dianils) can be prepared by reaction of malonaldehyde bis(dimethylacetal) and aromatic amines under strongly acidic conditions.¹²⁻¹⁴⁾ Although spectral data on the conjugated Schiff bases of aromatic amines have been reported,^{14,23-25)} there are few data on their properties, including fluorescence characteristics. Sawicki *et al.*¹⁴⁾ demonstrated that conjugated Schiff bases of 4,4'-sulfonyldianiline, ethyl *p*-aminobenzoate, *p*-aminobenzoic acid and *p*-aminoacetophenone I₅ fluoresced with excitation maxima at 475–520 nm and emission maxima at 520–580 nm in dimethylformamide–tetra-*n*-propylammonium hydroxide. The results gave the impression that conjugated Schiff bases were fluorescent. However, the present investigation on the fluorescence properties of the conjugated Schiff bases of aromatic amines showed that their intrinsic fluorescence was very weak, which is in accord with the results of Buttkus and Bose.¹³⁾ The bases with an electron-withdrawing function at the *para* position of the benzene ring fluoresced strongly in dimethylformamide–tetra-*n*-propylammonium hydroxide. This fluorescence may be due to the reaction product(s) of the conjugated Schiff bases in the solvent. The formation of fluorescent product(s) from the conjugated Schiff bases in dimethylformamide–tetra-*n*-propylammonium hydroxide may be useful for detection and determination of malonaldehyde.²⁶⁾

As regards the TBA-coloration of the conjugated Schiff bases, Buttkus and Bose¹³⁾ showed that I₁ produced the same ratio of coloration in the TBA test as malonaldehyde. However, the present experiments showed that compounds I₁–I₄ were much less colored than malonaldehyde, according to two methods. As an exception, unstable I₅ produced as intense a color as malonaldehyde in both methods. Therefore it is apparent that the degree of TBA-coloration of the conjugated Schiff bases depends on the substituents of the compounds.

Conjugated Schiff bases I₁–I₄ showed some antioxidative activity against oxidation of methyl oleate, lard and soybean oil. This result implies that if the conjugated Schiff bases were produced as end products of lipid oxidation, they might serve as antioxidants against subsequent lipid oxidation. The properties are in contrast to those of 1,4-dihydropyridine-3,5-dicarbaldehydes, which were quite inactive as antioxidants.²⁷⁾

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