Antioxidant Effect of Naturally Occurring Furan Fatty Acids on Oxidation of Linoleic Acid in Aqueous Dispersion

Youji Okada, Haruo Okajima, Hirokazu Konishi, Misaka Terauchi, Kazuo Ishii, I-Min Liu and **Hiroshi Watanabe***

School of Health Sciences, Kyorin University, Miyashita-cho, Hachioji 192, Japan

Naturally occurring furan fatty acids were synthesized and their antioxidant activity has been studied during the oxidation of linoleic acid in the phosphate buffer, pH 6.9, in the dark. The extent of the oxidation was followed both by the accumulation of conjugated diene and by the measurement of the residual amounts of linoleic acid. The tetra-alkylsubstituted furan fatty acids were found to suppress the oxidation. The tri-alkylsubstituted compound also showed antioxidant activity, being about 50% as effective as the tetra-alkylsubstituted ones. The di-alkylsubstituted one revealed no significant activity. The antioxidant activity of furan fatty acids depended on the number of substituents on the furan ring. Therefore, a tetra-alkylsubstituted furan ring may be necessary for the antioxidant action of furan fatty acids. The tetra-alkylsubstituted furan fatty acids reduced 1,1-diphenyl-2-picrylhydrazyl, reacted with the peroxyl radical generated from the thermal decomposition of a radical initiator, 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH), and also suppressed the AAPH-induced oxidation of linoleic acid, indicating that, by scavenging, the peroxyl radical furan fatty acids inhibit the oxidation.

KEY WORDS: Antioxidant, furan fatty acid, linoleic acid, peroxyl radical.

Furan fatty acids (F acids) have been widely distributed in fish lipids (1-7). However, F acids are not limited to fish and have been found in soft coral (8), rubber latex (9) and *Exocarpus* seed oil (10). We also detected F acids including new ones in crayfish (11-13), amphibian and reptile (12). Recently Hannemann *et al.* (14) showed that F acids were common constituents of plants.

The biosynthetic study Sand *et al.* (15) in fish demonstrated that the fish cannot synthesize from acetate the terminal alkyl chain including the carbons in the ring of the furan fatty acid. On the other hand, the biological role of naturally occurring F acids is still not clear in spite of active studies.

There is much evidence that the furan derivative is an effective scavenger of singlet oxygen (16,17), but little is known about its free radical-trapping ability (18-20). In the preceding paper (21), we reported that phosphatidylcholines (PCs) containing F_3 showed antioxidant activity on the oxidation in multilamellar soybean PC liposomes. As a continuation of our work on the biological role of naturally occurring F acids, we investigated their antioxidant activity on the oxidation of linoleic acid in aqueous dispersion in the dark.

There has been some discussion of the toxicity of synthetic antioxidants such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) (22,23). Recently much attention has been paid to naturally occurring antioxidants (24-28) for their safety.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. Linoleic acid (>99%) supplied by Sigma Chemical Co. (St. Louis, MO) was found to be free of hydroperoxides by thin-layer chromatography (TLC) and by potassium iodide spray. α-Tocopherol was purchased from Eastman Kodak Co., Rochester, NY. 2,2′-Azobis(2-amidinopropane)hydrochloride (AAPH) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and used as received. Linoleic acid hydroperoxide (LOOH) was prepared by the method of Gamage *et al.* (29) and purified by silica gel column chromatography, with benzene-methanol (98:2, v/v).

Synthesis of furan fatty acids. The naturally occurring F acids, F_2, F_3, and F_6, were synthesized by the methods of Rahn *et al.* (30) and Schödel and Spiteller (31), and 9,12-epoxyoctadeca-9,11-dienoic acid (NMF) by the method of Lie Ken Jie *et al.* (32).

Oxidation of linoleic acid. The system under study is similar to that described by Koskas *et al.* (33). Linoleic acid (10 mM) was dispersed in 25 mM sodium phosphate buffer with 0.5% Tween 20, pH 9.0. The prepared stock dispersion was stored at 4°C. One mL of the chloroform solution of each F acid (F_2, F_3, F_6 and NMF) was placed in a glass tube (67 mm i.d.), and the solvent was removed by flushing with N_2 until completely dried. The residue was dispersed in a mixture of 25 mL of the stock dispersion and 65 mL of 25 mM sodium phosphate buffer with 0.5% Tween 20, pH 6.9 (P.buffer). The dispersion was shaken with a vortex mixer for 2 min, and adjusted to pH 6.9 with 5% HCl. Then total vol was adjusted to 100 mL by P.buffer, and the final concentration of linoleic acid was 2.5 mM. The sample was left in the dark under air at 25-26°C, and the control without F acid was placed in similar conditions.

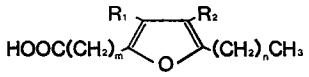
For the AAPH-induced oxidation of linoleic acid, the chloroform solution of linoleic acid (13 μmol), and F_3 (1.5 μmol), when necessary, were taken into a glass test tube (100 × 13 mm i.d.), and the solvent was removed under N_2. The residue was dispersed in 4.9 mL of P.buffer and shaken well with a vortex mixer for 2 min. The oxidation was initiated by the addition of 0.1 mL of 200 mM AAPH in P.buffer. The final concentrations of linoleic acid, F_3 and AAPH were 2.6, 0.3 and 4 mM, respectively. The samples were incubated at 37°C under continuous shaking.

Analytical procedure. The residual amounts of linoleic acid and F acids were determined by gas chromatographic (GC) analysis using methyl palmitate as an internal standard (7) after extraction from the sample with a chloroform-methanol mixture (1:1, v/v) (33) and esterification of free acids with trimethylsilyldiazomethane.

Accumulation of conjugated diene was estimated by measuring the absorbance at 234 nm (34).

TABLE 1

Structures of Furan Fatty Acids



Furan fatty acid	R_1	R_2	m	n
F_2[a]	CH_3	H	8	4
F_3[a]	CH_3	CH_3	8	4
F_6[a]	CH_3	CH_3	10	4
NMF[b]	H	H	7	5

[a]Named as in reference 3.

[b]9,12-Epoxyoctadeca-9,11-dienoic acid.

Decomposition of LOOH by furan fatty acid. A mixture of LOOH (12 μmol) (1.2 mM) and additives such as F acid (F_2, F_3 and NMF) (12 μmol) (1.2 mM) and α-tocopherol (0.12 μmol) (0.012 mM) in 10 mL of P.buffer was shaken well with a vortex mixer for 2 min. The resulting dispersion was left at 25–26°C in the dark. The peroxide value of each dispersion was measured by the ferric thiocyanate method (35) with a slight modification (36). To 0.2 mL of the test solution, were added 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid in this order. The resulting solution was left at 30°C exactly for 3 min and the absorbance at 500 nm was measured.

Reduction of DPPH by furan fatty acid. To a mixture of 1 mL of 200 mM acetate buffer (pH 5.5), 1 mL of deionized water and 2 mL of 0.25 mM DPPH ethanol solution was added 1 mL of 1.0 mM F acid (F_2, F_3 and NMF) ethanol solution, and then the sample was incubated at 37°C under continuous shaking. The final concentrations of DPPH and F acid were 0.1 and 0.2 mM, respectively. The control without F acid was placed in similar conditions. The absorbance of DPPH at 517 nm was read every 30 min.

Reaction of furan fatty acid with AAPH. To a dispersion of F acid (F_2, F_3 and NMF) (13 μmol) (final concentration, 2.6 mM) in 4.9 mL of P.buffer was added 0.1 mL of 200 mM AAPH aqueous solution (final concentration, 4 mM). The sample was incubated at 37°C under continuous shaking.

RESULTS AND DISCUSSION

The naturally occurring furan fatty acids (F acids) (Table 1) were synthesized, and their antioxidant activity had been studied during the oxidation of linoleic acid in the phosphate buffer, pH 6.9, containing 0.5% Tween 20 in the dark. The extent of oxidation was examined both by the accumulation of conjugated dienes and by the measurement of residual amounts of linoleic acid.

Antioxidant activity of tetra-alkylsubstituted furan fatty acids, F_3 and F_6. Figure 1A shows the time courses of the accumulation of the conjugated dienes during the oxidation of linoleic acid with various concentrations of tetra-alkylsubstituted F acid, F_3. In the absence of F acid the oxidation proceeded smoothly, but in its presence the oxidation was found to be suppressed significantly and in

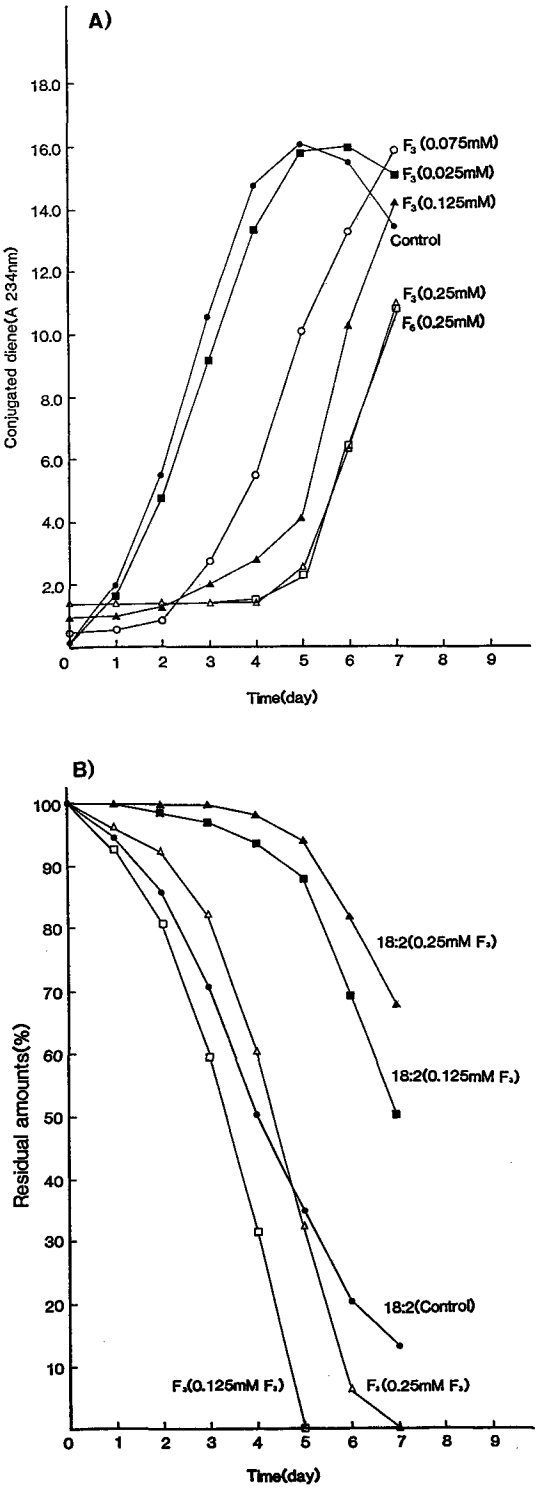


FIG. 1. Effects of F_3 and F_6 on linoleic acid (2.5 mM) autoxidation in 25 mM phosphate buffer (pH 6.9) with 0.5% Tween 20 at 25–26°C. A) Measurement of accumulation of conjugated diene. (●), Linoleic acid alone (control); (△), linoleic acid + F_3 (0.250 mM); (▲), linoleic acid + F_3 (0.125 mM); (○), linoleic acid + F_3 (0.075 mM); (■), linoleic acid + F_3 (0.025 mM); (□), linoleic acid + F_6 (0.250 mM). B) Measurement of residual amounts of linoleic acid and F_3. Linoleic acid (●) alone (control); linoleic acid (▲) + F_3 (0.250 mM) (△); linoleic acid (□) + F_3 (0.125 mM) (■).

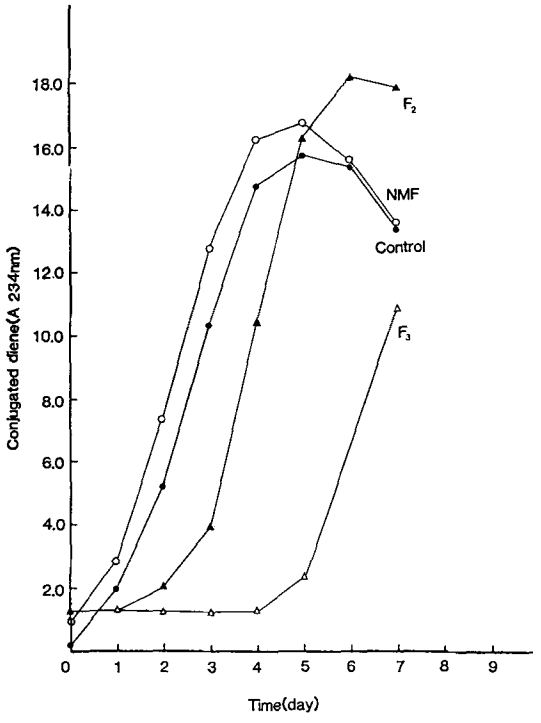


FIG. 2. Effects of F_2 and NMF on linoleic acid (2.5 mM) autoxidation 25 mM phosphate buffer (pH 6.9) with 0.5% Tween 20 at 25-26°C; A comparison with those of F_3 and F_6. Measurement of accumulation of conjugated diene. (•), linoleic acid alone (control); (△), linoleic acid + F_3 (0.250 mM), cited from Figure 1A for a comparison: (▲), linoleic acid + F_2 (0.250 mM); (○), linoleic acid + NMF (0.250 mM).

a concentration-dependent way. The additions of F_3, at levels of 0.250, 0.125 and 0.075 mM, retarded the oxidation clearly and at 0.025 mM only slightly. F_6, which is generally the major F acid in nature, revealed essentially the same activity as F_3. For clarification of Figure 1A, only the time course of F_6 at 0.250 mM was shown.

In order to confirm the results obtained by the accumulation of conjugated dienes, residual amounts of F_3 and linoleic acid were determined by GC analysis (Fig.1B). Linoleic acid with F_3 decreased more slowly, compared to that without F_3. Added F_3 was consumed gradually during the oxidation, and when the residual amount of F_3 reached about 30% of initial amount, the rapid oxidation of linoleic acid took place. Thus, the results obtained by two different methods agreed well with each other.

Antioxidant activity of tri-alkylsubstituted furan fatty acid, F_2 and di-alkylsubstituted furan fatty acid, NMF. In order to see detailed structural requirements of antioxidant activity of F acids in the oxidation of linoleic acid, those of tri- (F_2) and di-alkylsubstituted F acid (NMF) were examined. As shown in Figure 2, F_2 also has antioxidant activity, being about 50% as effective as F_3. In contrast, NMF revealed no significant activity. Dimethylfuran, which is known to be the effective singlet oxygen scavenger, did not suppress the oxidation as well. The results described above demonstrated that the activity of naturally occurring F acids decreased in the order of F_3>F_2>NMF. The tetra-alkylsubstituted F acids, F_3 and F_6, which are the major components among naturally occurring F acids, show the strongest antioxidant activi-

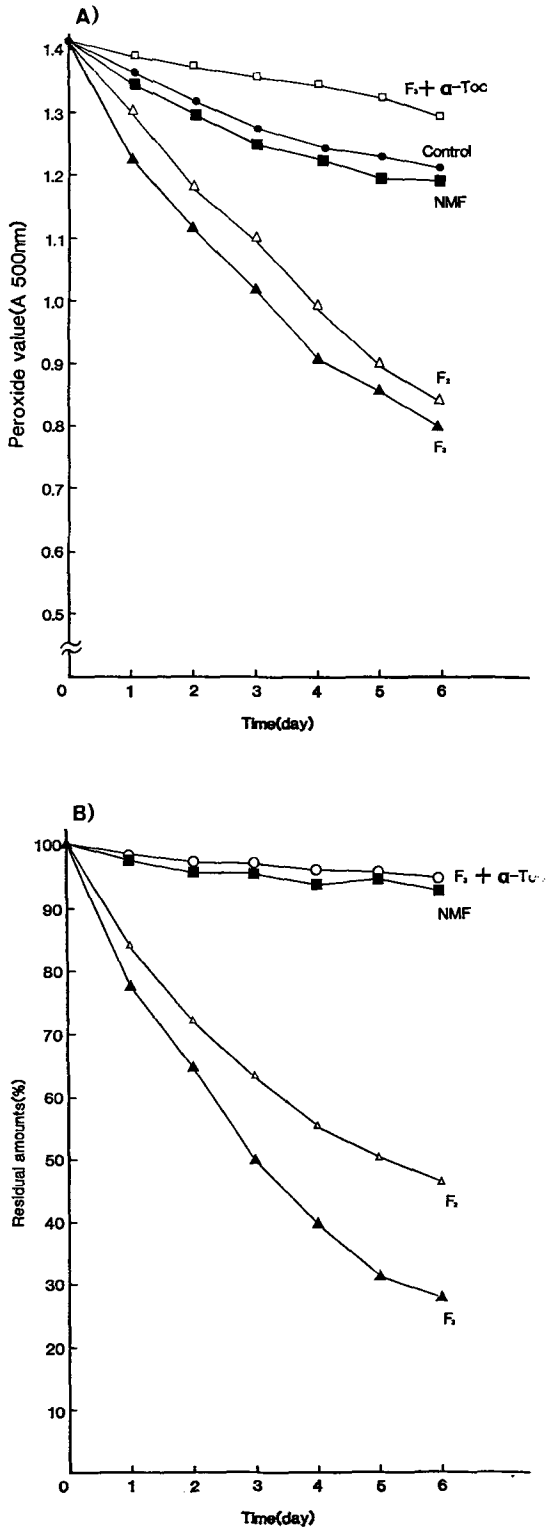


FIG. 3. Decomposition of linoleic acid hydroperoxide (LOOH) (1.2 mM) by F acids (F_3, F_2 and NMF) (1.2 mM) in 25 mM phosphate buffer with 0.5% Tween 20 at 25-26°C. A) Time courses of decreasing of peroxide value. (•), LOOH alone (control); (▲), LOOH + F_3; (□), LOOH + F_3 + α-tocopherol (0.012 mM); (△), LOOH + F_2; (■), LOOH + NMF. B) Residual amounts of F acids. LOOH + F_3 (▲); LOOH + F_3 (○) + α-tocopherol (0.012 mM); LOOH + F_2 (△); LOOH + NMF (■).

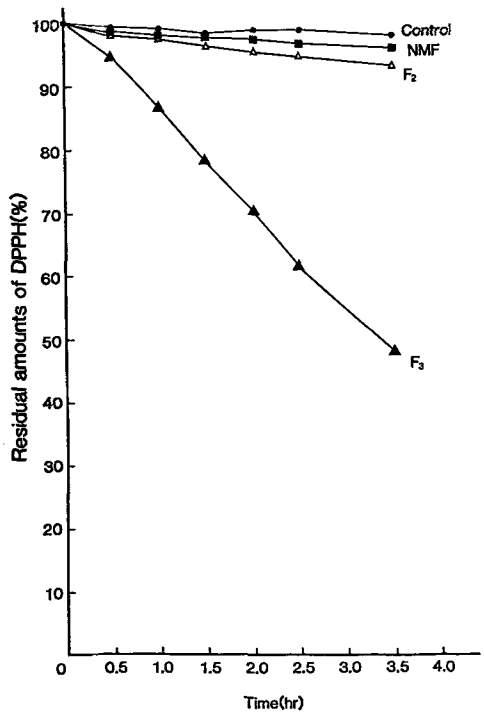


FIG. 4. Reduction of 1,1-diphenyl1-2-picrylhydrazyl (DPPH) (0.1 mM) by F acids (F_3, F_2 and NMF) (0.2 mM) at 37°C. (●), DPPH alone (control); (▲), DPPH + F_3; (△), DPPH + F_2; (■), DPPH + NMF.

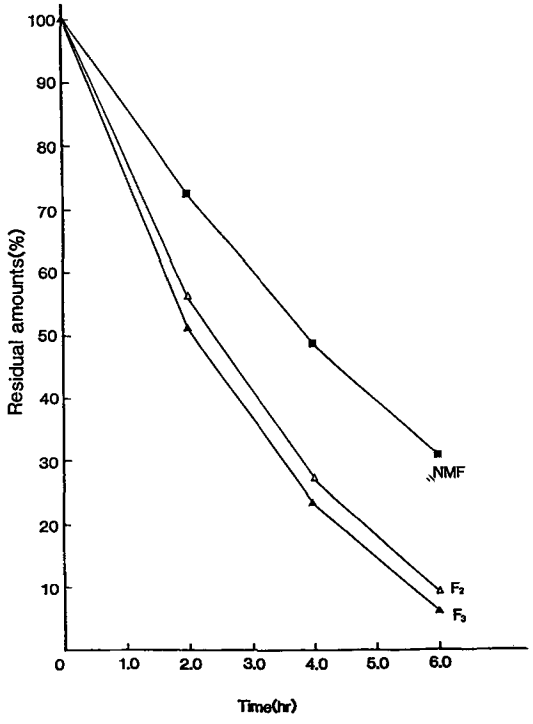


FIG. 5. Reaction of F acids (F_3, F_2 and NMF) (2.6 mM) with AAPH (4 mM) in 25 mM phosphate buffer (pH 6.9) with 0.5% Tween 20 at 37°C. F_3 (▲), F_2 (△) and NMF (■).

ty. Accordingly, the tetra-alkylsubstituted furan ring may be necessary for the antioxidant action of F acids.

Mechanism of antioxidant action. As shown in Figure 1B, F_3 which was added in the aqueous dispersion of linoleic acid was gradually consumed. On the other hand, in the dispersion of myristic acid which was inert to oxidation under the experimental conditions, F_3 was very stable and even after seven days was recovered quantitatively. This implied that F_3 reacted with oxidation products (including free radicals) of linoleic acid and revealed antioxidant activity. Thus F_3 did not undergo autoxidation spontaneously.

Generally antioxidants are divided into two categories: i) a hydroperoxide-decomposer, and ii) a chain-breaking antioxidant. Figure 3 shows that F acids decomposed linoleic hydroperoxide (LOOH) slightly. However, α-tocopherol, a radical scavenger, inhibited the decrease of peroxide value (Fig.3A) and the consumption of F acid (Fig.3B) completely. These observations indicated that F acids decomposed LOOH involving the generation of free radicals which was presumed to induce new chain reactions. Therefore, we conclude that the antioxidant activity of F acid was not ascribed to its ability to decompose hydroperoxides.

In order to examine whether F acid could act as a chain-breaking antioxidant, we carried out the following two experiments. First, the reduction of DPPH by F acid was examined (Fig.4). It is known that the reduction of the stable radical DPPH can be an indication of the presence of the abstractable hydrogen atom, which can interrupt the radical chain reaction (37). F_3 decreased the absorbance of DPPH at 517 nm. On the other hand, F_2 and NMF decreased the absorbance only slightly. This result suggests that F acid, especially F_3, may trap free radicals generated during the oxidation. Secondly, the reactivity of F acids toward the peroxyl radical (ROO·) generated from the thermal decomposition of a radical initiator, AAPH (38), was examined. As shown in Figure 5, F acids were consumed gradually with the same order as that of their antioxidant activity toward the oxidation of linoleic acid, indicating that F acids could react with peroxyl radical. The results obtained from two experiments described above suggest that F acids may scavenge the peroxyl radical generated during the oxidation of linoleic acid and break the radical chain reaction. This is supported by the fact that F_3 suppressed the AAPH-induced oxidation of linoleic acid significantly (Fig. 6). In general, the chain-breaking antioxidants such as α-tocopherol and BHT have an abstractable hydrogen atom to be donated to peroxyl radical and break the radical chain reaction. However, in the F acid such a hydrogen atom is not found. As examples of the chain-breaking antioxidant without the abstractable hydrogen, β-carotene (24,25), chlorophyll (CHL) (26) and others (39-41) have been reported. β-Carotene, which is known as an effective quencher of singlet oxygen, also functions as a radical trapping antioxidant. For the mechanism of its antioxidant activity, an addition of the peroxyl radical to the conjugated diene system of β-carotene was proposed. On the other hand, CHL reduced free radicals such as DPPH. The presence of π-cation radical was recorded during the oxidation of CHL in methyl linoleate solution by electron spin resonance (ESR). The following mechanism involving an electron transfer for the antioxidant activity was reported:

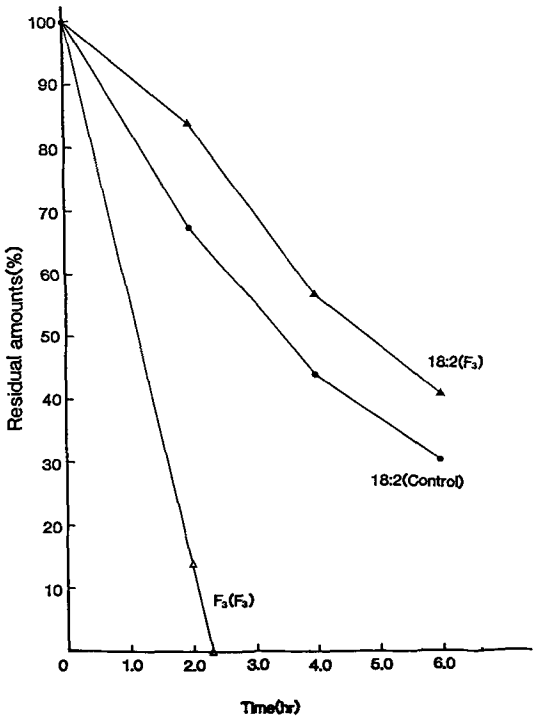


FIG. 6. Effect of F_3 (0.3 mM) on AAPH (4 mM) induced oxidation of linoleic acid (2.6 mM) in 25 mM phosphate buffer (pH 6.9) with 0.5% Tween 20 at 37°C. Measurement of residual amounts of linoleic acid and F_3. Linoleic acid alone (control) (●); linoleic acid (▲) + F_3 (△).

$$ROO\cdot + CHL \rightarrow ROO^{(-)}: CHL^{(+)\cdot}$$

$$ROO^{(-)}: CHL^{(+)\cdot} + ROO\cdot \rightarrow \text{Inactive product (ROO}\cdot\text{ ;peroxyl radical)}$$

As described above, F acids have no abstractable hydrogen atom, can reduce DPPH and react with the peroxyl radical generated from AAPH. From these findings we presumed that the mechanism for antioxidant activity of F acids involves the electron transfer from the furan ring to peroxyl radical or the addition of peroxyl radical to the furan ring. Now study on the detailed mechanism is in progress.

The results in the present paper show that furan fatty acids may serve as antioxidants in biological systems and be of importance as safe food additives to prevent the fat rancidity in the place of synthetic antioxidants such as BHT and BHA. In addition, the furan compounds, especially tetra-alkylsubstituted ones, were found to be capable of inhibiting the free-radical oxidation as well as the singlet oxygen-induced oxidation.

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