Hydroxyl Radical Scavenging Activity of Naturally Occurring Furan Fatty Acids

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As a part of our work on the antioxidant properties of naturally occurring furan fatty acids (F acids), we evaluated their hydroxyl radical (HO•) scavenging activity by an electron spin resonance (ESR) spin trapping technique with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The additions of F acids to the incubation mixture of Fe2+—diethylenetriaminepentaacetic acid complex, H2O2, and DMPO decreased the intensity of the DMPO-OH adduct signal in a dose-dependent way. This decrease was not attributed to the destruction of DMPO-OH adduct by F acids. Kinetic competition studies indicated that the decrease in DMPO-OH signal intensity was mainly due to the competition of F acids with DMPO for HO•, and not to the inhibition of the HO• generation system itself. F acids were found to react rapidly with HO• at approximately a diffusion-controlled rate (1.7 × 1014 M−1 s−1). Comparison with the common HO• scavengers indicated that the rate constant of F acids is higher than those of mannitol and ethanol, and is compatible with those of histidine and dimethylsulfoxide, demonstrating that F acids are a potent HO• scavenger. It is suggested that F acids may serve as antioxidants in biological systems through their ability to scavenge HO•.

Key words furan fatty acid; hydroxyl radical; antioxidant; ESR spin trapping

Furan fatty acids (F acids) are widely distributed in the lipid extracts of fish,1−4) and have also been found in Exocarups seed oil,5) soft coral6) and rubber latex.7) We also detected F acids including new ones in crustaceans (crayfish), amphibians (bullfrog), and reptiles (tortoise).8,9) Hannemann et al. reported that F acids are found in comparatively high amounts in the green parts of some plants.10) F acids have also been found in some vegetable oils by Guth and Grosch.11) Very recently, Shirasaka et al. detected an F acid in the cellular lipids of marine bacteria.12) The biosynthesis of F acids has been studied intensively by Spitteller et al. Labelling experiments demonstrated that the methyl groups on the furan ring originate from S-adenosylmethionine,13) and the oxygen atom in the furan ring is from molecular oxygen in the air.14) Two types of dibasic-substituted F acids were reported by Spitteller and his colleagues,15,16) which were extracted from the blood and urine of men and rats and thought to be degradation products of F acids. The biological roles of naturally occurring F acids remain unknown, however, in spite of active studies.2,17)

As a part of our work on the biological roles of naturally occurring F acids, we have investigated their antioxidant activities. There is much evidence that the furan derivatives such as dimethylfuran and diphenylfuran are effective scavengers of singlet oxygen,18) but little is known about their scavenging activity against oxygen-derived free radicals such as hydroxyl radicals (HO•),19,20) peroxyl radicals21) and superoxide anion. In previous papers we reported that F acids inhibited the peroxidation of linoleic acid in aqueous dispersion,22) and that phosphatidylycerines (PCs) containing F acid showed antioxidant activity on the oxidation in multimammalian soybean PC liposomes.23) In the present work, we report the ability of these acids to scavenge an extremely potent oxidant, HO•.

MATERIALS AND METHODS

Materials Diethylenetriaminepentaacetic acid (DE-APAC), hydrogen peroxide (H2O2), ironous iron (FeSO4) and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical Co. (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Labo Tech (Tokyo, Japan) in the highest grade available and used without further purification. Other reagents were of analytical grade.

Synthesis of F Acids The structures of F acids are shown in Fig. 1. F2 and F3 were synthesized by slightly modified methods of Rahn et al.24) and Schödel and Spitteller,25) and 9,12-epoxyoctadeca-9,11-dienoic acid (NMF) according to the method of Lie Ken Jie et al.26) Scavenging of HO• The experimental procedure used was that described by Kohn et al.27) To a mixture of 34 mm potassium phosphate buffer, pH 7.8, 340 μM DETAPAC, 340 μM FeSO4, 840 μM DMPO, and additives such as F3, F2 and NMF at various concentrations, H2O2 was added at a final concentration of 340 μM. An aliquot of the reaction mixture was transferred into a quartz ESR flat cell, and the ESR signal was measured exactly 40 s thereafter. Measurements were carried out under the following conditions: magnetic field, 335.6 ± 5 mT; modulation frequency, 100 kHz; microwave power, 8.0 mW; response, 0.1 s; and scanning time, 2.0 min. The intensity of the DMPO-OH adduct signal was calculated from the height of the first signal of the DMPO-OH spin adduct

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relative to the Mn$^{2+}$ signal as an internal standard.

**Treatment of DMPO-Adduct with F$_3$** The experiment was carried out using the method of Hiramoto et al.$^{29}$ In brief, a mixture of 34 mM potassium phosphate buffer, 340 $\mu$M DETAPAC, 340 $\mu$M FeSO$_4$, 840 $\mu$M DMPO and 340 $\mu$M H$_2$O$_2$ was stirred for 5 min (the DMPO-OH adduct solution). To 213.4 $\mu$l of the DMPO-OH adduct solution, 6.6 $\mu$l of a solution of F$_3$ in the phosphate buffer was added at a final concentration of 300 $\mu$M. As a control experiment, the phosphate buffer (6.6 $\mu$l) in place of a solution of F$_3$ was added to the DMPO-OH adduct solution. ESR spectrum of the reaction mixture was recorded exactly 40 s after the addition of a solution of F$_3$ or the phosphate buffer alone (control).

**Kinetic Competition Studies** The second order rate constants for F acids and the common HO· scavengers with HO· were calculated from the following Eq. 1.$^{29}$

$$F = \frac{k_s}{k_{DMPO} + [DMPO]} [S]$$

Here, $k_s$ and $k_{DMPO}$ are the second order rate constants for the reaction of HO· scavengers and DMPO, respectively, and [S] and [DMPO] refer to the scavenger and DMPO concentrations (mol/l). F is the inhibition rate of the DMPO-OH signal. The rate constant $k_s$ is determined from the slope ($k_s/k_{DMPO}$) of the straight line obtained by plotting $F/(1-F)$ against [S]/[DMPO] and the previously reported rate constant $k_{DMPO}$ (2.1 $\times$ 10$^9$ m$^{-1}$ s$^{-1}$).$^{29}$

**The Oxidation Product of F Acid** A mixture of 34 mM potassium phosphate buffer, pH 7.8, 136 $\mu$M NMF methyl ester, 340 $\mu$M DETAPAC, and 340 $\mu$M H$_2$O$_2$, in a total volume of 10 ml, was incubated at 25°C for 1 min. The mixture was extracted with CHCl$_3$ and 10 $\mu$l of the extract was subjected to HPLC analysis. HPLC was carried out with a JUSCO PU-980 and a JUSCO UV-970 variable wavelength UV detector. A Shim-packed CLC-ODS (4.6 mm × 150 mm, Shimazu) was used with elution by a 15-min linear gradient of CH$_3$CN/H$_2$O (80:20 to 100:0, v/v) at a flow rate of 1.5 ml/min. The effluent was monitored at 234 nm.

The cis-diketone derivative (Fig. 6) of NMF methyl ester was synthesized by the method reported previously.$^{9,30}$

**Residual Amounts of F Acid during the Reaction with HO·** A mixture of 34 mM potassium phosphate buffer, pH 7.8, 136 $\mu$M NMF, 340 $\mu$M DETAPAC, and 340 $\mu$M H$_2$O$_2$, in a total volume of 10 ml, was incubated at 25°C for 1 min. The residual amounts of NMF were determined by gas chromatography analysis using methyl palmitate as an internal standard after extraction from the reaction mixture with CHCl$_3$ and esterification of free acid with trimethylsilyldiazomethane.

**RESULTS AND DISCUSSION**

Incubation of Fe$^{2+}$-DETAPAC complex (340 $\mu$M) with H$_2$O$_2$ (340 $\mu$M) in the presence of DMPO (840 $\mu$M) gave a characteristic ESR spectrum which consisted of a quartet signal (intensity ratio of 1:2:2:1) (Fig. 2). The g value (2.006 G) and hyperfine coupling constant ($a_{H} = a_{N} = 1.49$ mT) are consistent with the values for the DMPO-OH adduct.$^{31}$ Additions of a tetraalkylsubstituted F acid, F$_3$, to Fe$^{2+}$-DETAPAC complex (340 $\mu$M) with H$_2$O$_2$ (340 $\mu$M) in the presence of DMPO decreased the intensity of the DMPO-OH signal in a dose-dependent way.

**The Possibility of Destruction of DMPO-OH Adduct by F Acids** Very recently, it was reported that DMPO-OH adduct is destroyed by most of the plant phenolics.$^{28,32}$ We considered the possibility that the decrease in the DMPO-OH adduct signal intensity might be due to the destruction of DMPO-OH adduct by F acids. As shown in Fig. 3, when F$_3$ at a final concentration of 300 $\mu$M was added to DMPO-OH adduct, its signal intensity was essentially equal to that of DMPO-OH adduct without the addition of F$_3$ (control), demonstrating that the decrease in this signal intensity is not due to the destruction of DMPO-OH adduct by F acids.

**Competition of F$_3$ with DMPO for HO·** To examine whether the decrease in DMPO-OH signal intensity is due to the competition of F$_3$ with DMPO for HO· or the inhibition of the HO· generation system itself, we used two DMPO concentrations, 1,672 and 0.836 mM. According to Eq. 1, we plotted $F/(1-F)$ versus [F$_3$]/[DMPO] at each concentration. If the decrease in the DMPO-OH signal intensity is due to the competition of F$_3$ with DMPO for HO·, the two plots should show straight lines and their slopes should be consistent. If the decrease is due to the inhibition of the HO· generation system, however, the plots should deviate from linearity.$^{33}$ As shown in Fig. 4, the two plots showed straight lines and their slopes were consistent. Thus, the decrease in DMPO-OH signal intensity with the addition of F$_3$ appears due mainly to the competition of F$_3$ with DMPO for HO·, but not to the inhibition of the HO· generation system. Furthermore, added F$_3$ was found to be decreased to 45% of the initial amount within 2 min after the initiation of reaction, supporting the assumption that F$_3$ scavenged HO· (Fig. 5).
Fig. 3. Effects of Treatment with F₃ on the Intensity of ESR Signal of DMPO-OH Adduct

DMPO-OH adduct was prepared by stirring a mixture of 34 mM potassium phosphate buffer, pH 7.8, 340 μM DETAPAC, 340 μM FeSO₄, 340 μM DMPO and 340 μM H₂O₂ for exactly 5.0 min (the DMPO-OH adduct solution). To 213.4 μl of the DMPO-OH adduct solution, 6.6 μl of a solution of F₃ in the phosphate buffer was added at a final concentration of 300 μM. As a control experiment, the phosphate buffer (6.6 μl) in place of a solution of F₃ was added to the DMPO-OH adduct solution. ESR of the mixture was recorded exactly 40 s after the addition of a solution of F₃ or the phosphate buffer alone (control). ESR settings were as follows: magnetic field, 335.6±5 mT; modulation frequency, 100 kHz; microwave power, 8.0 mW; response, 0.1 s; and scanning time, 2.0 min.

Fig. 4. Competition between F₃ and DMPO for HO· Generated by the Fenton Reaction System

A mixture of 34 mM potassium phosphate buffer, pH 7.8, 340 μM FeSO₄, 340 μM DETAPAC, 846 or 1672 μM DMPO, and 340 μM H₂O₂, in a total volume of 220 μl, was kept at room temperature for 40 s with various concentrations of F₃. F (0 < F < 1) is the inhibition rate of the DMPO-OH signal. R is a correlation coefficient.

Second Order Rate Constants between F Acids and HO·

From the slope (kₑ/k_DMPO) of the straight line plot (correlation coefficient: 0.999) of F/1 - F versus [F₃]/[DMPO] and the previously reported rate constant k_DMPO (2.1 × 10⁹ M⁻¹ s⁻¹) between DMPO and HO·, F₃ was found to react rapidly with HO· at approximately a diffusion controlled rate (kₑ = 1.7 × 10¹⁰ M⁻¹ s⁻¹) (Table 1). Di- and trialkylsubstituted F acids, NMF and F₂, have second order rate constants almost identical with that of F₃, though the three acids have different numbers of alkyl substituents on their furan rings. This can presumably be explained by the lack of selectivity due to the high reactivity of HO·. We previously reported²²² that the reactivity of F acids toward the peroxyl radical, which is less reactive than HO·,²⁴⁴ depends on the number of alkyl substituents on the furan rings.

Comparison of F Acids with Those of the Common HO· Scavengers

The second order rate constants of common HO· scavengers, mannitol, DMSO, histidine and ethanol were determined for comparison with those of F acids. As shown in Table 1, F acid rate constants were found to be slightly higher than those of mannitol and ethanol, and were comparable to those of DMSO and histidine.

An Oxidation Product of F Acid

We examined the oxidation products of F acids by HO·. Diphenylfuranyl and dimethylfuranyl, which is known as effective singlet oxygen scavengers, react with HO· to give the diketene derivatives, cis-dibenzyloxyethylene¹⁹¹ and cis-diacetoxyethylene,²⁴⁰ respectively. Therefore, F acids are also expected to undergo oxidation by HO· to afford the corresponding cis-diketenes. A mixture of NMF methyl ester, FeSO₄, DETAPAC and H₂O₂ in the phosphate buffer was incubated for 1 min, and the CHCl₃ extract of the reaction mixture was then analyzed by HPLC (Fig. 6). The mass spectrum and retention time of the peak “a” in Fig. 6
Table 1. Second Order Rate Constants in the Reaction of the F Acids and Other HO· Scavengers with HO·

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rate constant (m⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>2.64 × 10⁹</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.80 × 10⁹</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.14 × 10¹⁰</td>
</tr>
<tr>
<td>NMF</td>
<td>1.65 × 10⁹</td>
</tr>
<tr>
<td>F₂</td>
<td>1.68 × 10⁹</td>
</tr>
<tr>
<td>F₃</td>
<td>1.71 × 10⁹</td>
</tr>
<tr>
<td>DMSO</td>
<td>2.66 × 10⁹</td>
</tr>
</tbody>
</table>

A mixture of 34 mM potassium phosphate buffer, pH 7.8, 340 μM FeSO₄, 340 μM DETAPAC, 840 μM DMPO, and 340 μM H₂O₂, and substances at various concentrations in a total volume of 220 μl was kept at room temperature for 40s. ESR settings were as follows: magnetic field, 335.6 ± 5 mT; modulation frequency, 100 kHz; microwave power, 8.0 mW; response, 0.1 s; and scanning time, 2.0 min. The rate constant was determined from the equations as

\[
\frac{\dot{F}}{1 - \dot{F}} = \frac{k_0}{k_{\text{DMPO}}} [S]
\]

\[k_0\] and \(k_{\text{DMPO}}\) are the second order rate constants for reaction of HO· with substances and HO· with DMPO, respectively. \(F(0 < F < 1)\) is the inhibition rate of the DMPO-OH signal.

REFERENCES


In conclusion, this study demonstrates that naturally occurring F acids are a potent HO· scavenger. Their HO· scavenging abilities are characterized by second order rate constants higher than \(1 \times 10^{10} \text{m}^{-1}\text{s}^{-1}\), as measured by the ESR spin trapping technique. Our results suggest that F acids may serve as antioxidants in biological systems through their ability to scavenge HO·.

Fig. 6. HPLC Analysis of cis-Diketone Produced by the Reaction of NMF with HO·

A mixture of 34 mM potassium phosphate buffer, pH 7.8, 136 μM NMF methyl ester, 340 μM FeSO₄, 340 μM DETAPAC, and 340 μM H₂O₂ in a total volume of 10 ml, was incubated for 1 min at 25 °C. The reaction mixture was extracted with CHCl₃ and an aliquot of the extract was injected into the HPLC. Details are given in Materials and Methods.

The results were fully consistent with those of cis-diketone which was synthesized from the oxidation of NMF by m-chloroperbenzoic acid.\(^{30}\)

In conclusion, this study demonstrates that naturally occurring F acids are a potent HO· scavenger. Their HO· scavenging abilities are characterized by second order rate constants higher than \(1 \times 10^{10} \text{m}^{-1}\text{s}^{-1}\), as measured by the ESR spin trapping technique. Our results suggest that F acids may serve as antioxidants in biological systems through their ability to scavenge HO·.