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Antioxidant Mechanism Studies on Ferulic Acid: Identification of Oxidative Coupling Products from Methyl Ferulate and Linoleate

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In our studies of the chain-breaking antioxidant mechanism of natural phenols in food components, ferulic acid, a phenolic acid widely distributed in edible plants, especially grain, was investigated. The radical oxidation reaction of a large amount of ethyl linoleate in the presence of the methyl ester of ferulic acid produced four types of peroxides as radical termination products. The isolation and structure determination of the peroxides revealed that they had tricyclic structures which consisted of ethyl linoleate, methyl ferulate, and molecular oxygen. Based on the formation pathway of the products, a radical scavenging reaction occurred at the 3′-position of the ferulate radical with the four types of peroxyl radicals of ethyl linoleate. The produced peroxides subsequently underwent intramolecular Diels−Alder reaction to afford stable tricyclic peroxides.

KEYWORDS: Antioxidant mechanism; ferulic acid; methyl ferulate; peroxide; ethyl linoleate

INTRODUCTION

Some phenolic compounds in edible plants have received much attention as powerful antioxidants to protect against oxidative deterioration of food. Ferulic acid is one of the antioxidatively active phenolic acids (*1*), which is widely distributed in the plant kingdom. Especially, the content of ferulic acid in grains is very high; for example, in wheat its level is 50−500 μg/g of plant material (*2*). Ferulic acid occurs mainly as various ester forms with polar compounds such as sugars (*3*) and nonpolar ones such as sterols in plants (*4*). These ester forms of ferulic acid should work as potent antioxidants in plants and in plant-derived foods. The main mechanism for a phenolic antioxidant in food is the trapping and stabilizing of radical species, such as the lipid peroxyl radical, which is generated from the radical chain oxidation of food components. The antioxidation mechanism of ferulic acid and its derivatives has attracted much attention. Recent antioxidation mechanism studies of ferulic acid have been carried out by a kinetic approach (*5*) or a structure−activity relationship approach (*6*). The antioxidation process of the phenolic compounds is thought to be divided into two stages (*7*):

(1) radical trapping stage

$$\text{S-OO}\cdot + \text{AH} \rightarrow \text{S-OOH} + \text{A}\cdot$$

(2) radical termination stage

$$\text{A}\cdot + \text{X}\cdot \rightarrow \text{nonradical materials}$$

where S is the substance for oxidation, the S-OO· is the peroxyl radical of S, AH is the antioxidant, the A· is the antioxidant radical, and the X· is another radical species or the same species as the A·. Although the first stage is a reversible process, the second stage is irreversible and must produce stable radical termination products. Structural information about these nonradical products would afford important contributions to the antioxidation mechanism studies (*8*−*10*). During the course of an antioxidant mechanism study of ferulic acid, we recently showed that the dimerization and subsequent construction of a dihydrobenzofuran ring was main termination process of its antioxidation reaction, using methyl ferulate as the model compound of the ester derivatives of ferulic acid (*11*). It is well-known that the nonpolar esters of ferulic acid distribute in the lipidic part of foods or food stuffs (*12*, *13*) and show a protective effect against their oxidative deterioration (*14*, *15*). Therefore, analysis of the antioxidation process of the ferulic esters in the lipid media is also necessary. In lipid, radical species from the lipid may act as the X radical and react with the radical of the ferulic ester in the second stage of the above-mentioned antioxidation scheme. In this investigation, we sought such a radically coupling product in the antioxidation reaction media, which consisted of methyl ferulate and a large amount of ethyl linoleate, an oxidizable lipid model. In this paper, the isolation and structural identification of the radical coupling products from methyl ferulate and ethyl linoleate are reported.

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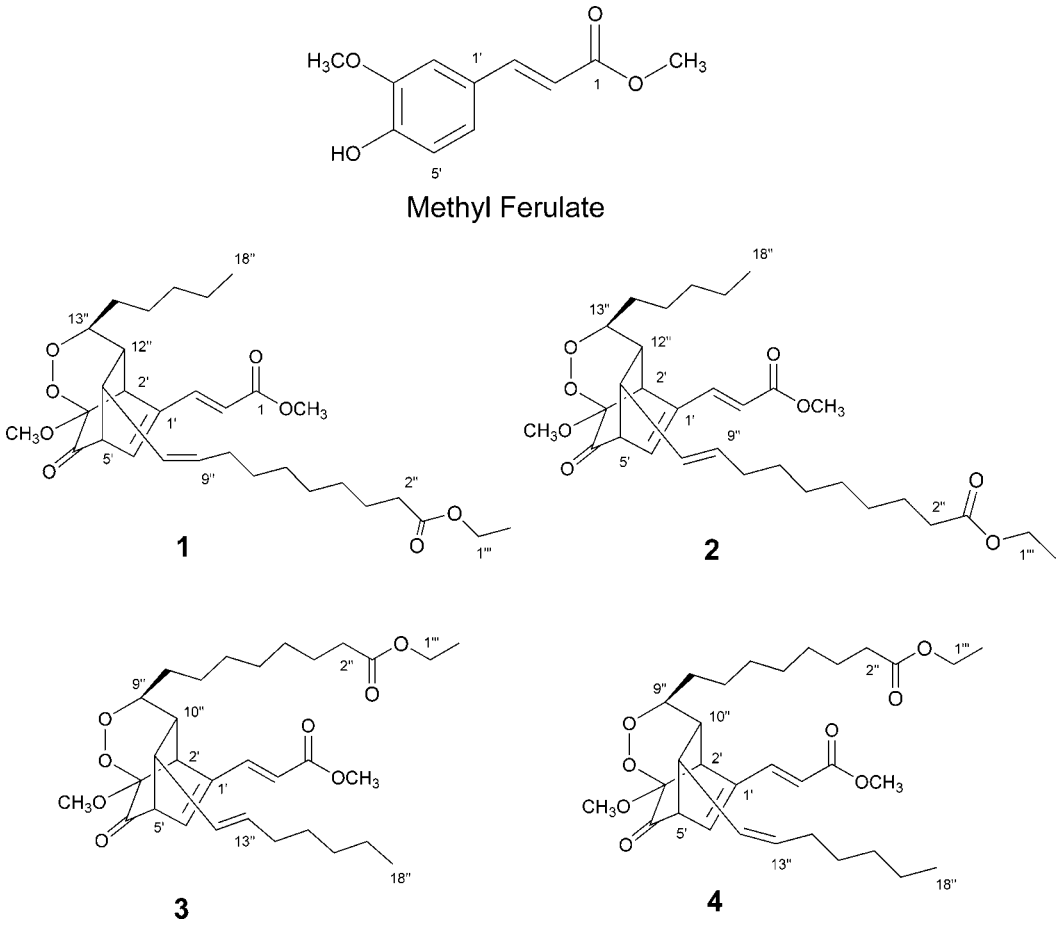

Figure 1. Chemical structures of methyl ferulate and coupling products **1**–**4**. (Tentative position numbering is given on the basis of the numbering system of the starting ferulate and linoleate.)

MATERIALS AND METHODS

Chemicals and Instruments. Methyl ferulate (**Figure 1**) was synthesized by a previously reported method. 2,2′-Azobis(isobutyronitrile) (AIBN) was obtained from Tokyo Kasei (Tokyo, Japan). Ethyl linoleate was purchased from Kanto Chemicals (Tokyo, Japan) and used after purification by silica gel (silica 60, Merck, Darmstat, Germany) chromatography developed with 2.5% ethyl acetate in hexane. All solvents and other reagents were obtained from Nacalai Tesque (Kyoto, Japan). The NMR spectra were measured on a Unity Plus 500 spectrometer (Varian, Palo Alto, CA) or an EX-400 spectrometer (JEOL, Tokyo, Japan) using the manufacturer-supplied pulse sequences [^{1}H, ^{13}C, correlated spectroscopy (HH-COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC)]. The mass spectra were measured with an SX-102A spectrometer (JEOL, Tokyo, Japan) in the positive fast atom bombardment (FAB) mode in the presence of *m*-nitrobenzyl alcohol as a matrix. A PU-980 high-pressure gradient system (Jasco, Tokyo, Japan) equipped with a photodiode array detector (SPD-M10AVP, Shimadzu, Kyoto, Japan) was employed for the analytical high-performance liquid chromatography (HPLC). A PU-980 pump equipped with a UV-975 detector (Jasco) was used for analysis of lipid hydroperoxides. A LC-6AD recycle system (Shimadzu) equipped with a UV-970 detector (Jasco) was used for preparative HPLC.

HPLC Detection of Antioxidation Products from Methyl Ferulate in Ethyl Linoleate. To 4.49 mL of ethyl linoleate in a 100 mL straight vial (40 mm, diameter) were added 0.4 mL of 50 mM methyl ferulate solution (CH_3CN), AIBN (788 mg), and CH_3CN (4.45 mL). The control vial was also prepared in a similar manner with addition of 0.4 mL of CH_3CN instead of the 50 mM methyl ferulate solution. Both solutions were well stirred and then incubated at 40 °C in the air for 3 or 4 h. An aliquot (100 μL) was removed and diluted with CH_3CN (200 μL). Ten microliters of the diluted solution was injected into the analytical HPLC system under the following conditions, column, Cosmosil 5SL-II (4.6 × 150 mm, Nacalai Tesque); solvent system, *n*-hexane (solvent A) and ethyl acetate (solvent B); elution, linear gradient from 2% to 20% solvent B for 25 min, and then isocratic mode of 100% solvent B for 10 min; flow rate, 1 mL/min; detection, 280 nm.

Isolation Procedure for Coupling Products 1−4. To 4.49 mL of ethyl linoleate in a 100 mL straight vial (40 mm diameter) were added 1 mL of 50 mM methyl ferulate solution (CH_3CN), AIBN (788 mg), and CH_3CN (3.85 mL). The vial was well stirred and then incubated at 40 °C in air for 15 h. The combined reaction solution from 40 vials was allowed to stand at −20 °C for 1 h. The precipitated AIBN was removed by filtration from the solution. The filtrate was evaporated in vacuo and then dissolved in 160 mL of CH_3CN. The CH_3CN solution was cooled at −30 °C to freeze the ethyl linoleate, and the supernatant produced was collected. The precipitate was dissolved again in CH_3CN (120 mL) and then cooled at the same temperature to collect the next supernatant. This procedure was repeated once more, and all supernatants were combined and evaporated in vacuo. The residue was subjected to silica gel column chromatography (silica gel, BW-350, Fuji Silysia Chemical, Kasugai, Japan) developed with hexanes−ethyl acetate (88:12 v/v) to removed residual lipid and AIBN. The fraction, which contained the coupling products, was evaporated and next purified by preparative HPLC to give peak compounds A and B. [separation conditions: column, Cosmosil 5SL-II (10 × 250 mm); solvent, EtOAc−hexane (7:93 v/v); flow rate, 7.9 mL/min, detection, 280 nm; injection, 46 mg/injection and total 20 times] The recycle HPLC purification of the peak A was carried out to give compounds **1**−**3** in 2.9 mg, 6.2 mg, and 5.3 mg yields, respectively [separation conditions: column, Cosmosil 5C18-AR-II (20 × 250 mm); solvent, CH_3CN-H_2O (9:1 v/v); flow rate, 7.9 mL/min; detection, 260 nm; injection, 17 mg/injection and total 4 times; recycle number of times, 8−9 times]. On the other hand, peak B was purified by preparative HPLC on an octadecyl silica gel column to give compound **4** in 1.9 mg yield [separation conditions: column, Cosmosil 5C18-AR-II (20

× 250 mm); solvent, CH_3CN-H_2O (95:5 v/v); flow rate, 7.9 mL/min; detection, 260 nm; injection, 8 mg/injection and total 4 times].

Analytical Data for Products 1−4: Compound 1. HR-FABMS (*m*/*z*) $[M + H]^+$ calcd for $C_{31}H_{47}O_8$, 547.3271; found, 547.3309. FABMS (*m*/*z*) 547, 517, 327, 307, 154; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.12 (d, J = 15.5 Hz, 1H, H-2), 7.40 (d, J = 15.5 Hz, 1H, H-3), 3.21 (dd, J = 3.0 and 1.7 Hz, 1H, H-2′), 3.23 (dd, J = 6.5 and 3.0 Hz, 1H, H-5′), 6.46 (dd, J = 6.5 and 1.7 Hz, 1H, H-6′), 2.29 (t, J = 7.5 Hz, 2H, H-2″), 1.61 (m, 2H, H-3″), 1.24−1.38 (complex, 14H, H-4″, H-5″, H-6″, H-7″, H-15″, H-16″, and H-17″), 2.04 (m, 2H, H-8″), 5.35 (dt, J = 10.5 and 8.0 Hz, 1H, H-9″), 5.07 (tt, J = 10.5 and 1.0 Hz, 1H, H-10″), 3.13 (dt, J = 10.5 and 3.0 Hz, 1H, H-11″), 1.88 (dt, J = 3.0 and 1.0 Hz, 1H, H-12″), 4.34 (ddd, J = 7.5, 5.5, and 1.0 Hz, 1H, H-13″), 1.38 (m, 1H, H-14″a), 1.53 (m, 1H, H-14″b), 0.87 (br t, J = 7.0 Hz, 3H, H-18″), 4.13 (q, J = 7.5 Hz, 2H, H-1‴), 1.26 (t, J = 7.5 Hz, 3H, H-2‴), 3.78 (s, 3H, 1-OCH_3), 3.46 (s, 3H, 3′-OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.3 (C-1), 118.0 (C-2), 141.3 (C-3), 140.1 (C-1′), 43.9 (C-2′), 95.2 (C-3′), 201.5 (C-4′), 55.7 (C-5′), 132.3 (C-6′), 173.9 (C-1″), 34.3 (C-2″), 24.9 (C-3″), 29.7, 29.5, 29.2, and 29.1 (C-4″, C-5″, C-6″, and C-7″), 27.5 (C-8″), 131.6 (C-9″), 129.9 (C-10″), 36.4 (C-11″), 42.1 (C-12″), 84.9 (C-13″), 30.2 (C-14″), 25.5 (C-15″), 31.6 (C-16″), 22.4 (C-17″), 13.9 (C-18″), 60.2 (C-1‴), 14.3 (C-1‴), 51.8 (1-OCH_3), 53.5 (3′-OCH_3).

Compound 2. HR-FABMS (*m*/*z*) $[M + H]^+$ calcd for $C_{31}H_{47}O_8$, 547.3271; found, 547.3256. FABMS (*m*/*z*) 547, 515, 455, 327, 281, 154; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.11 (d, J = 15.5 Hz, 1H, H-2), 7.40 (d, J = 15.5 Hz, 1H, H-3), 3.17 (dd, J = 3.0 and 1.7 Hz, 1H, H-2′), 3.31 (dd, J = 6.5 and 3.0 Hz, 1H, H-5′), 6.46 (dd, J = 6.5 and 1.7 Hz, 1H, H-6′), 2.28 (t, J = 7.8 Hz, 2H, H-2″), 1.60 (m, 2H, H-3″), 1.22−1.33 (complex, 14H, H-4″, H-5″, H-6″, H-7″, H-15″, H-16″, and H-17″), 1.93 (complex, 2H, H-8″), 5.46 (dt, J = 15.0 and 6.8 Hz, 1H, H-9″), 5.18 (br dd, J = 15.0 and 8.6 Hz, 1H, H-10″), 2.84 (dt, J = 8.6 and 3.0 Hz, 1H, H-11″), 1.93 (complex, 1H, H-12″), 4.33 (br t, J = 6.0 Hz, H-13″), 1.38 (m, 1H, H-14″a), 1.53 (m, 1H, H-14″b), 0.87 (br t, J = 7.0 Hz, 3H, H-18″), 4.12 (q, J = 7.0 Hz, 2H, H-1‴), 1.25 (t, J = 7.0 Hz, 3H, H-2‴), 3.78 (s, 3H, 1-OCH_3), 3.46 (s, 3H, 3′-OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.3 (C-1), 118.0 (C-2), 141.4 (C-3), 140.3 (C-1′), 43.9 (C-2′), 95.2 (C-3′), 201.7 (C-4′), 55.8 (C-5′), 132.4 (C-6′), 173.9 (C-1″), 34.3 (C-2″), 24.9 (C-3″), 29.1, 29.1, 29.0, and 28.9 (C-4″, C-5″, C-6″, and C-7″), 32.3 (C-8″), 132.5 (C-9″), 130.5 (C-10″), 41.8 (C-11″), 41.3 (C-12″), 85.0 (C-13″), 29.9 (C-14″), 25.4 (C-15″), 31.6 (C-16″), 22.4 (C-17″), 13.9 (C-18″), 60.2 (C-1‴), 14.3 (C-2‴), 51.8 (1-OCH_3), 53.5 (3′-OCH_3).

Compound 3. HR-FABMS (*m*/*z*) $[M + H]^+$ calcd for $C_{31}H_{47}O_8$, 547.3271; found, 547.3277. FABMS (*m*/*z*) 547, 517, 455, 391, 363, 287, 259, 227, 154; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.11 (d, J = 15.5 Hz, 1H, H-2), 7.40 (d, J = 15.5 Hz, 1H, H-3), 3.17 (dd, J = 3.2 and 1.5 Hz, 1H, H-2′), 3.31 (dd, J = 6.6 and 3.2 Hz, 1H, H-5′), 6.46 (br dd, J = 6.6 and 1.5 Hz, 1H, H-6′), 2.28 (t, J = 7.6 Hz, 2H, H-2″), 1.61 (m, 2H, H-3″), 1.22−1.39 (complex, 14H, H-4″, H-5″, H-6″, H-7″, H-15″, H-16″, and H-17″), 1.37 (m, 1H, H-8″a), 1.54 (m, 1H, H-8″b), 4.33 (br t, J = 6.0 Hz, 1H, H-9″), 1.93 (dt, J = 3.2 and 1.0 Hz, 1H, H-10″), 2.84 (dt, J = 9.0 and 3.2 Hz, 1H, H-11″), 5.18 (br dd, J = 15.2 and 9.0 Hz, 1H, H-12″), 5.46 (dt, J = 15.2 and 7.0 Hz, H-13″), 1.94 (br q, J = 7.0 Hz, 2H, H-14″), 0.87 (br t, J = 7.3 Hz, 3H, H-18″), 4.12 (q, J = 7.2 Hz, 2H, H-1‴), 1.25 (t, J = 7.2 Hz, 3H, H-2‴), 3.78 (s, 3H, 1-OCH_3), 3.46 (s, 3H, 3′-OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.3 (C-1), 118.0 (C-2), 141.4 (C-3), 140.2 (C-1′), 43.9 (C-2′), 95.2 (C-3′), 201.7 (C-4′), 55.8 (C-5′), 132.4 (C-6′), 173.8 (C-1″), 34.3 (C-2″), 24.9 (C-3″), 29.3, 29.0, 29.0, and 28.9 (C-4″, C-5″, C-6″, and C-15″), 25.7 (C-7″), 29.9 (C-8″), 85.0 (C-9″), 41.3 (C-10″), 41.8 (C-11″), 130.4 (C-12″), 132.7 (C-13″), 32.3 (C-14″), 31.3 (C-16″), 22.5 (C-17″), 14.1 (C-18″), 60.2 (C-1‴), 14.3 (C-2‴), 51.8 (1-OCH_3), 53.5 (3′-OCH_3).

Compound 4. HR-FABMS (*m*/*z*) $[M + H]^+$ calcd for $C_{31}H_{47}O_8$, 547.3271; found, 547.3309. FABMS (*m*/*z*) 547, 518, 455, 391, 363, 287, 259, 227,154; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.12 (d, J = 15.8 Hz, 1H, H-2), 7.40 (d, J = 15.8 Hz, 1H, H-3), 3.21 (dd, J = 3.3 and 2.0 Hz, 1H, H-2′), 3.24 (dd, J = 6.5 and 3.3 Hz, 1H, H-5′), 6.45 (br dd, J = 6.5 and 2.0 Hz, 1H, H-6′), 2.27 (t, J = 7.5 Hz, 2H, H-2″), 1.59 (m, 2H, H-3″), 1.24−1.40 (complex, 14H, H-4″, H-5″, H-6″, H-7″, H-15″, H-16″, and H-17″), 1.34 (m, 1H, H-8″a), 1.52 (m, 1H, H-8″b), 4.33 (br t, J = 6.0 Hz, 1H, H-9″), 1.87 (br dt, J = 3.3 and 1.0 Hz, 1H, H-10″), 3.13 (dt, J = 10.5 and 3.3 Hz, 1H, H-11″), 5.07 (br t, J = 10.5 Hz, 1H, H-12″), 5.36 (dt, J = 10.5 and 7.0 Hz, 1H, H-13″), 2.04 (m, 2H, H-14″), 0.89 (t, J = 7.0 Hz, 3H, H-18″), 4.12 (q, J = 7.0 Hz, 2H, H-1‴), 1.25 (t, J = 7.0 Hz, 2H, H-2‴), 3.78 (s, 3H, 1-OCH_3), 3.46 (s, 3H, 3′-OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.3 (C-1), 118.0 (C-2), 141.3 (C-3), 140.1 (C-1′), 43.9 (C-2′), 95.2 (C-3′), 201.5 (C-4′), 55.8 (C-5′), 132.3 (C-6′), 173.8 (C-1″), 34.3 (C-2″), 24.9 (C-3″), 29.3, 29.2, 29.0, and 29.0 (C-4″, C-5″, C-6″, and C-15″), 25.7 (C-7″), 30.3 (C-8″), 84.8 (C-9″), 42.2 (C-10″), 36.4 (C-11″), 129.8 (C-12″), 131.8 (C-13″), 27.5 (C-14″), 31.5 (C-16″), 22.6 (C-17″), 14.0 (C-18″), 60.2 (C-1‴), 14.3 (C-2‴), 51.8 (1-OCH_3), 53.5 (3′-OCH_3).

RESULTS AND DISCUSSION

HPLC Detection of Radical Coupling Products from Methyl Ferulate and Linoleate. To detect the radical coupling products of methyl ferulate and ethyl linoleate, the AIBN-induced radical oxidation reaction of a large amount of ethyl linoleate in the presence of methyl ferulate was carried out. The amount of methyl ferulate relative to that of ethyl linoleate should be as low as possible because a higher amount of ferulate would dimerize (*11*). Therefore, the weight ratio of methyl ferulate to ethyl linoleate in the oxidation reaction was set to about 0.1% by referring to the cases of α-tocopherol (*16*) and curcumin (*17*, *18*). The radical oxidation reaction was carried out with 4 mg of methyl ferulate and 4 g of ethyl linoleate in a reaction vial (i.d. 40 mm, height 75 mm, SV-50, Nichiden-Rika, Kobe, Japan) (final concentration in the vial ca. 2 mM for methyl ferulate and ca. 1.3 M for ethyl linoleate) at 40 °C, and the reaction products were analyzed by HPLC on a silica gel column. The analytical data for the reaction mixture at 4 h and the control experiment at 3 h, which was carried out without methyl ferulate, are shown in **Figure 2**. From the data, new peaks were clearly observed at the 15.4 and 15.9 min retention times in the methyl ferulate-containing experiment along with a methyl ferulate peak at 21.0 min. Any other peaks were probably due to the oxidation products only from ethyl linoleate because they were also observed in the control experiment.

Isolation and Structural Identification of Coupling Products. To clarify that the 15.4 and 15.9 min peak substances (peaks A and B, respectively, as shown in **Figure 2**) were the coupling products of methyl ferulate and the lipid, we isolated them and determined their chemical structures. Forty vials, each of which contained 10 mg of methyl ferulate and 4 g of ethyl linoleate, were incubated at 40 °C for 15 h. After removal of most of the AIBN and ethyl linoleate, the reaction mixture was purified by silica gel column chromatography and subsequent preparative HPLC on the same type of column to collect the substances responsible for the 15.4 and 15.9 min peaks. An NMR analysis of the material collected from the 15.4 min peak revealed that it was a mixture of substances. These substances were completely separated by a recycle HPLC technique on a reversed-phase column as shown in **Figure 3**. The material collected from the 15.9 min peak was also purified by preparative HPLC on a reversed-phase column. These purifying techniques afforded pure compounds **1**−**4** in 1.6% total yield from methyl ferulate. Although this isolation yield is very low, it should be noted that this yield does not reflect the real conversion yield because of an unavoidable loss in the multistep purification used.

Compound **1** was isolated as a colorless oil. Its molecular formula was determined as $C_{31}H_{46}O_8$ from the FABMS result [*m*/*z* 547.3309 $(M + H)^+$]. The molecular formula indicated that **1** was an oxidative coupling product consisting of methyl

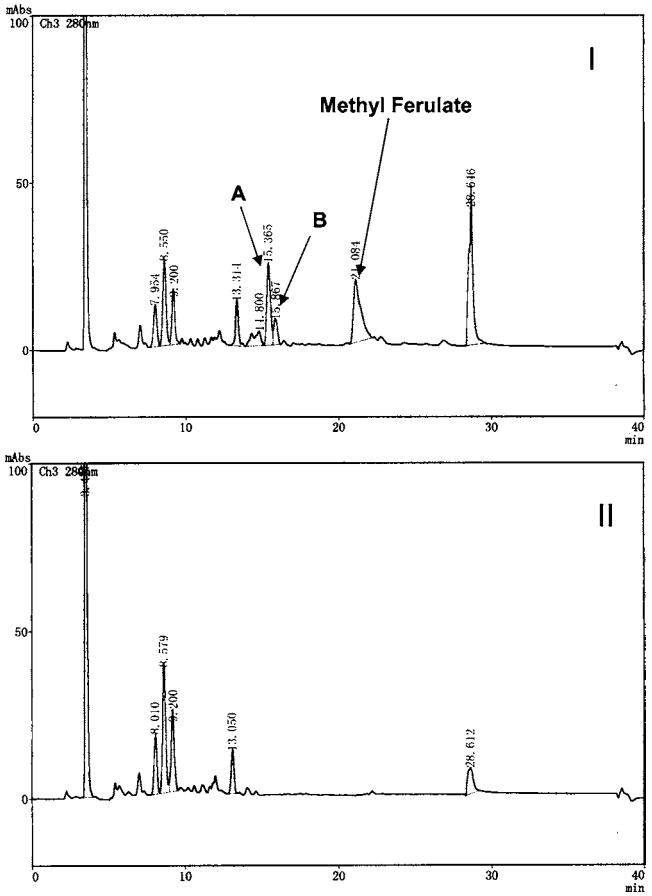

Figure 2. HPLC analytical data of AIBN- [2,2′-azobis(isobutyronitrile)-] induced oxidation products in ethyl linoleate with methyl ferulate (profile I, upper panel) or without methyl ferulate (profile II, lower panel).

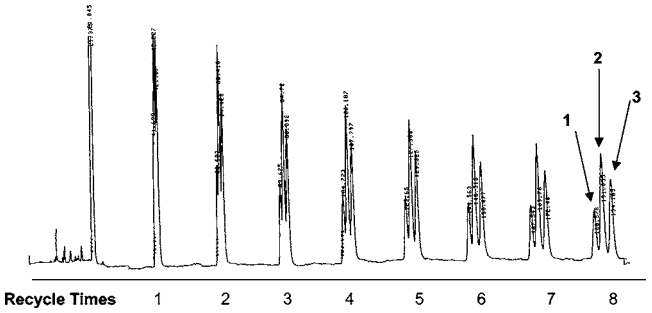

Figure 3. Separation pattern of products **1**–**3** in the 15.4 min peak by recycle HPLC.

ferulate, ethyl linoleate, and molecular oxygen. In the ^{1}H NMR of **1**, both signal sets due to the original methyl ferulate and ethyl linoleate were observed; however, several signals assignable to the aromatic part of methyl ferulate and the olefinic part of ethyl linoleate had disappeared, indicating that the double bond of ethyl linoleate reacted with the aromatic part of methyl ferulate. The fine structure of the coupled moiety of **1** was mainly elucidated by the 2D NMR technique. The analysis of the proton−proton couplings observed in the COSY spectrum and C−H long-range coupling connectivities around the coupled moiety in the HMBC spectrum revealed a bicyclo[2.2.2] structure including a carbonyl group at the 4′-position (δ 201.5) and an olefin at the 1′-position (**Figure 4A**). There should be an oxygen function between C-3′ and C-13″ on the basis of their carbon chemical shift values (C-3′ δ 95.2, C-13″ δ 84.9). Consideration of the oxygen count of the molecular formula indicated that a peroxyl group should exist between the two

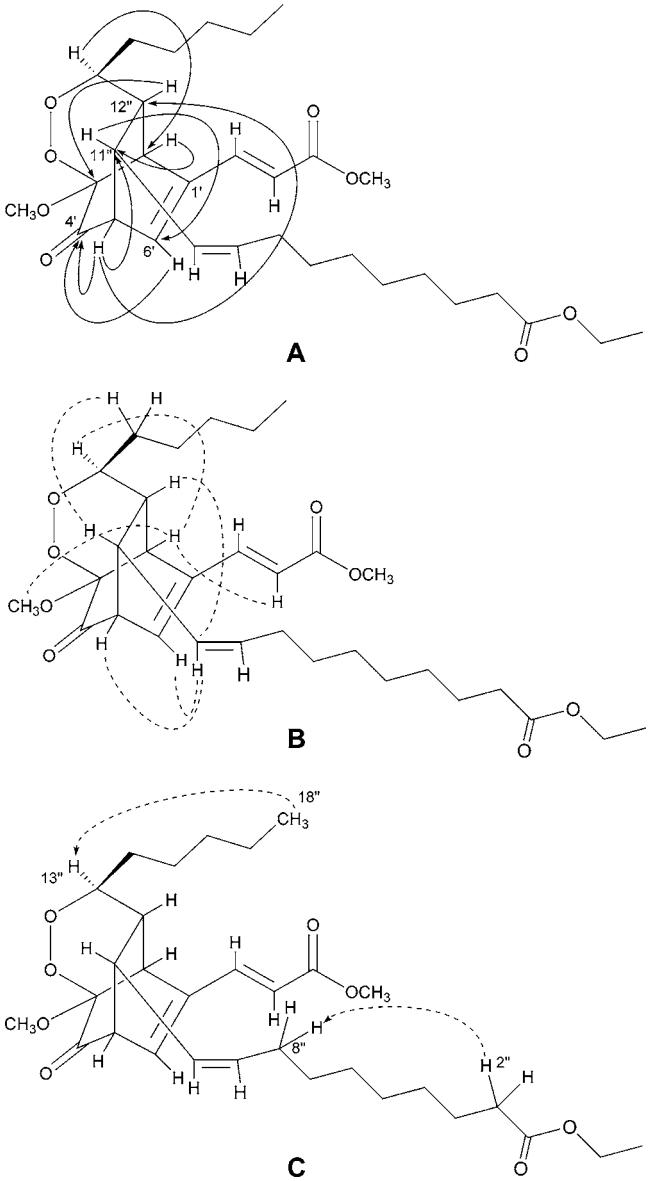

Figure 4. Selected proton–carbon long-range connectivities observed in HMBC (**A**), NOE correlations observed in NOESY (**B**), and proton–proton correlations observed in TOCSY (**C**) of product **1**.

carbons at the 3′- and 13″-positions and the tricyclic system as depicted. The stereochemistry around the tricyclic system was deduced from the proton coupling constants and NOE correlations (**Figure 4B**). The phase-sensitive NOESY spectrum of **1** showed a strong correlation between H-2′ and H-13‴, suggesting that H-13″, had an axial orientation in the conformationally restricted 1,2-dioxacyclohexane ring. The stereochemistry of H-11″ and H12″ was also deduced to be trans on the basis of the small coupling constant (J = 3.0 Hz) of the two protons. The groups attached to the tricyclic ring system at the 11″- and 13″-positions were elucidated. A cis olefin, which was deduced by the coupling constant between H-9″ and H-10″ (J = 10.5 Hz), was determined to be adjacent to the 11″-position by the chemical shift of H-11″ (δ 3.13). At the other end of the olefin (9″-position) and at the 13″-position, the remaining alkyl groups must be attached. These groups should be the pentyl and ethoxycarbonylheptyl groups on the basis of the starting ethyl linoleate structure. To determine the attached groups, a TOCSY spectrum was measured, which showed clear connectivities from the 18″-methyl proton to the H-13″ and from the α-protons of the ethyl ester to the allylic protons at the 8″-

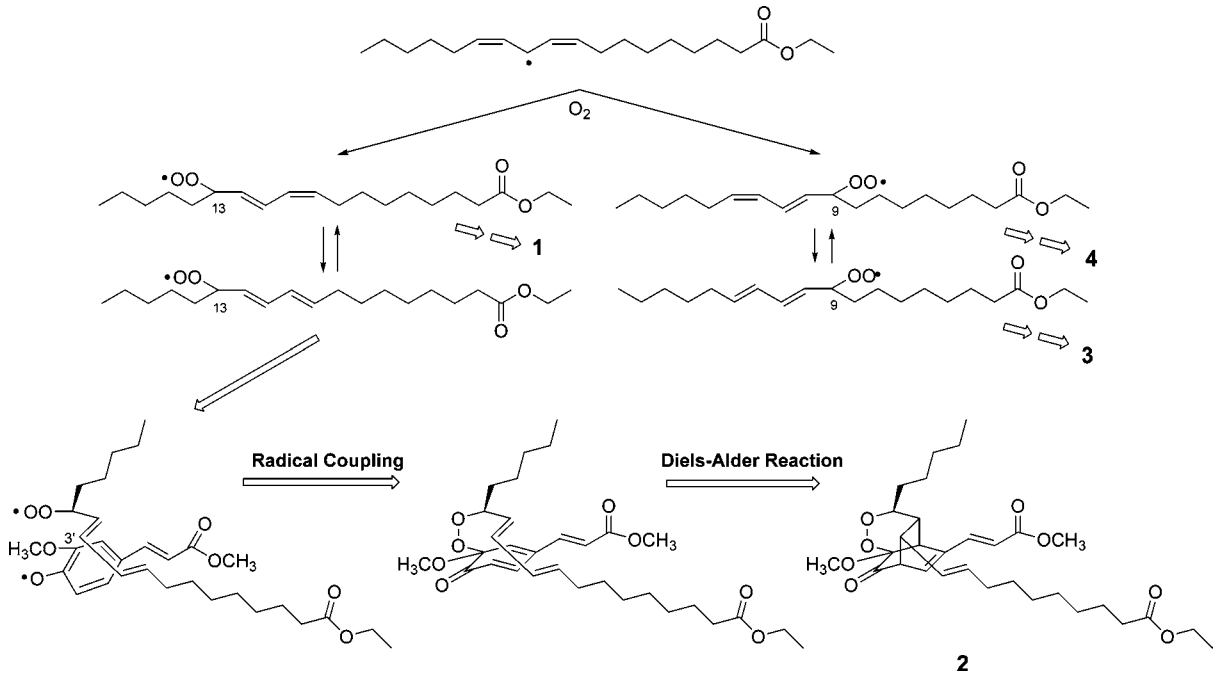

Figure 5. Scheme for proposed mechanism of antioxidation of methyl ferulate in ethyl linoleate.

position, clarifying that the pentyl group was attached to the 13″-position and the esteric alkyl moiety to the 9″-position (**Figure 4C**). Therefore, the compound has the structure **1** shown in **Figure 1**.

Compound **2** was isolated as a colorless oil, and its molecular formula was determined to be $C_{31}H_{46}O_8$ from FABMS [*m*/*z* 547.3256 $(M + H)^+$]. All spectroscopic data were very similar to those of **1**; however, only the NMR signal sets due to an olefin were different. The coupling constant (J = 15.0 Hz) between the protons on the olefin indicated that the olefin had trans geometry. The 2D NMR data of **2** including HMBC, NOESY, and TOCSY gave the same analytical results as those of **1**, indicating that the remainder of **2** was the same as **1**. Thus, **2** was the *trans*-olefinic isomer of **1** as illustrated in **Figure 1**. Compounds **3** and **4** were isolated as colorless oils and their molecular formulas were both determined to be $C_{31}H_{46}O_8$ from each FABMS result [*m*/*z* 547.3277 $(M + H)^+$ for **3** and 547.3309 $(M + H)^+$ for **4**]. The spectroscopic data (^1H and ^{13}C NMR) of **3** and **4**, except for the mass fragmentation pattern, gave almost the same results as those of **2** and **1**, respectively, which indicated that a similar structural relationship existed between **3** and **2** and between **4** and **1**. The TOCSY spectrum of **3** showed the opposite substitution pattern of the two alkyl groups on the tricyclic system to that of **2**, and the spectrum of **4** also showed a similar opposite pattern to that of **1**. Thus, compounds **3**and **4** were determined to be the corresponding isomeric compounds on the substitution of the alkyl chain groups of the tricyclic system to **2** and **1**, respectively. Thus, they have structures **3** and **4** as depicted in **Figure 1**.

Proposed Antioxidation Mechanism of Methyl Ferulate in Linoleate. From our elucidation of the chemical structures of the four isolated compounds, we propose the antioxidant mechanism of methyl ferulate in ethyl linoleate as illustrated in **Figure 5**. As shown in **Figure 5**, methyl ferulate traps a radical at the phenolic group and is converted to a ferulate radical. The radical reacts with a peroxyl radical of the ethyl linoleate at the 3′-position of methyl ferulate, affording four coupling products through a peroxyl linkage. This coupling is the important radical scavenging step of the antioxidation of the methyl ferulate. The coupling products are not very stable because the aromatic stability of the original benzene ring is disrupted by the coupling reaction. Thus, the subsequent Diels−Alder reaction smoothly occurs in the coupling products. It is well-known that the linoleic ester produces four isomeric peroxyl radicals during its autoxidation (*19*), whose chemical structures include the 9-*trans*-11-*trans*-diene-13-hydroperoxide, 9-*cis*-11-*trans*-diene-13-hydroperoxide, 10-*trans*-12-*cis*-diene-9-hydroperoxide, and 10-*trans*-12-*trans*-diene-9-hydroperoxide moieties. The coupling of these isomeric lipid peroxyl radicals with the methyl ferulate radical would produce the corresponding peroxides, and the geometric chemistry of the four peroxides was retained from that of the lipid peroxyl radicals. Although the peroxide bond is not very stable due to the ease of homolysis, it is well recognized that the peroxide in the six-membered ring is more stable than the straight-chain peroxides (*20*). The Diels−Alder reaction of the coupling products afforded the corresponding six-membered ring peroxides, which rendered some stability to the products. In our previous antioxidant mechanism study, curcumin also afforded this type of stabilized cyclic peroxide (*17*, *18*). α-Tocopherol is a very potent antioxidant; however, it does not afford cyclic peroxide. α-Tocopherol reacts with the peroxyl radical of linoleate at the 8a-position (*21*) as a suitable captodative position (*22*). The produced peroxide does not undergo Diels−Alder reaction because of the absence of diene in the tocopherol moiety of the coupling product. The effect of this difference in the antioxidant products between ferulate and tocopherol on the antioxidant efficacy is very interesting and should be further investigated soon.

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Supporting Information Available: Tables of correlation data of COSY, NOESY, HMBC, and TOCSY of products **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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