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ARTICLE

Identification of Cytotoxic Dimers in Oxidation Product from Sesamol, a Potent Antioxidant of Sesame Oil

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Phytophenols of edible plants have recently attracted much attention as potent antioxidants and related bioactive substances. These antioxidative phytophenols are very oxidizable due to their chemical properties, and their oxidation products must accumulate in the oxidizing foods when they are contained as active ingredients. In this investigation, several phytophenols, which are well known as potent antioxidants in food science, were oxidized by oxygen in the presence of a catalytic amount of Ferric ion. Caffeic acid, catechin, chlorogenic acid, rosmarinic acid, and sesamol were quickly oxidized, whereas eugenol, resveratrol, rutin, and quercetin were not under the stated conditions. The oxidation product mixtures of the quickly oxidized phytophenols were next subjected to a cytotoxic assay using normal cells. Among the products, the oxidation product from sesamol showed a remarkably high cytotoxic activity. To clarify the cytotoxic principle of the oxidation products, an assay-guided fractionation and subsequent isolation of the oxidation compound of sesamol was carried out. Structure analysis of the isolated compounds revealed that they are new dimeric compounds (**2**-**5**) of sesamol. The cytotoxic activity of the dimers was evaluated from the population of dead cells by a flow cytometric analysis of rat thymocytes in the presence of 100 μM of each compound. Compound **2** showed the most potent cytotoxic activity among them. Compound **2** has a typical conjugated carbonyl moiety and the moiety possibly contributes to its high toxicity from a structure−activity point of view.

KEYWORDS: Phytophenol; sesamol; oxidation; Fe ion; cytotoxic activity; antioxidant; dimer

INTRODUCTION

Phytophenols recently have attracted much attention as functional constituents in foods and food supplements for improving human health. Some phytophenols have a very potent antioxidant activity, which is thought to be closely linked to the beneficial actions of food ingested by humans. The antioxidative food phenols demonstrate the prevention of oxidative deterioration, inhibition of enzymatic browning, inhibition of off-flavor generation, etc., in foods (*1*), and also antiaging, prevention of cancer, cardiovascular disease, etc., in the human body (*2*). The antioxidation efficiency of the phenolic antioxidants depends on their potential for oxidation, which means that most of the potent antioxidants are oxidized much faster than other biomolecules, thus providing their potent antioxidant activity. This easily oxidizable property of the antioxidative phenols may lead to the accumulation of various oxidation products in foods (*3*−*10*). However, the functionality of the accumulated oxidation products has not yet been examined in detail (*11*, *12*). In this study, we have investigated the cytotoxic property of the oxidation products from widely noted antioxidative phytophenols from the viewpoint of food sanitation. Our screening study for the cytotoxicity of the oxidation products of the phytophenols revealed that the oxidation mixture from sesamol showed a potent cytotoxicity to normal mammalian cells.

Sesamol (3,4-methylenedioxyphenol) is one of the phenolic antioxidants of sesame oil (seed oil of *Sesamum indicum*) (*13*) (**Figure 1**). Many beneficial activities of sesamol, which include cancer chemoprevention (*14*), antimutagenicity (*15*), and antihepatoxic activity (*16*), in addition to its potent antioxidant activity (*17*), have also been reported. Although sesamol is one of the promising food-functional compounds, we recently determined that its oxidation product was easily produced in nature, including in foods and related systems, and had potent cytotoxic activity (*18*). In this study, the isolation and structural identification of new cytotoxic dimers from the oxidation mixture of sesamol were investigated in addition to the cytotoxic screening results of several phytophenol oxidation products.

MATERIALS AND METHODS

Chemicals and Instruments. Sesamol (purity ≥98%) and caffeic acid (purity ≥98%) were purchased from Tokyo Kasei (Tokyo, Japan). Catechin (purity ≥98%), chlorogenic acid (purity ≥95%), rosmarinic acid (purity ≥97%), and Chelex 100 resin were purchased from Sigma-Aldrich (St. Louis, MO). $FeCl_3 \cdot 6H_2O$ and all solvents were obtained from Nacalai Tesque (Kyoto, Japan). The NMR spectra were measured using a

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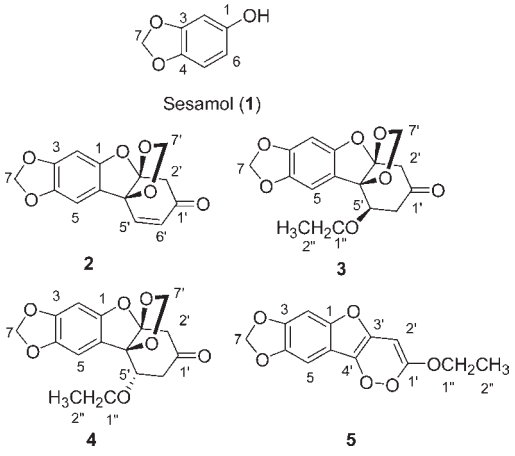

Figure 1. Chemical structures of sesamol (**1**) and its oxidative dimers (**2**−**5**). (Tentative position numbering is given on the basis of the numbering system of sesamol.)

Unity Plus 500 spectrometer (Varian, Palo Alto, CA), an ECX-400 spectrometer (JEOL, Tokyo, Japan) using the manufacturer-supplied pulse sequences [^{1}H, ^{13}C, correlated spectroscopy (HH−COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY)]. The mass spectra were measured with a XEVO Qtof spectrometer (Waters Japan, Tokyo, Japan) in ESI mode. IR spectra were measured with FTIR-8400 by film on NaCl (Shimadzu, Kyoto, Japan). A LC-10ATvp low pressure gradient system (Shimadzu, Kyoto, Japan) equipped with an SPD-M10AVP photodiode array detector and a DGU-12A degasser was employed for the analytical HPLC. A LC-6AD recycle system (Shimadzu) equipped with an UV-970 detector (JASCO, Tokyo, Japan) was used for preparative HPLC. Flow cytograms were obtained by a flow cytometer CytoACE-150 equipped with an argon laser (JASCO). Mass fragment analysis was performed by MassFragment (version 2.0) from Waters using the following parameters [DBE, 0−50; electron count, both; maximum H deficit, 6; fragment number of bonds, four; scoring (phenyl, 8; aromatic, 6; multiple, 4; ring, 2; single, 1; hetero modifier, 0.5; H-penalty, 0; max score, 16)].

Oxidation of Phytophenols. To an ethanol solution (10 mL) of each phytophenol (52 mM) in a screw-capped vial (i.d. 35 mm × h 78 mm) was added a 520 mM $FeCl_3$ aqueous solution (0.1 mL). The vial was incubated under oxygen atmosphere at 40 °C. Twenty microliters of the aliquot was removed at 1 day intervals and diluted with 60 μL of ethanol. Five microliters of the diluted solution was injected to analytical HPLC to measure the remaining nonoxidized phytophenol under the following conditions: column, a 250 mm × 4.6 mm i.d., 5 μm, Cosmosil 5C18-AR-II (Nacalai Tesque); flow rate, 1 mL/min; detection, 280 nm; solvent CH_3CN−1% AcOH aq, 15:85 for chlorogenic acid, 30:70 for rosmarinic acid, 20:80 for caffeic acid; 30:70 for sesamol, and 15:85 for catechin. After almost all phytophenol (≥80%) was consumed, each solution was passed through Chelex 100 (2 g) to remove the iron ion. The solution was evaporated in vacuo to give the oxidation mixture (yield, 212 mg from chlologenic acid; 317 mg from rosmarinic acid; 342 mg from caffeic acid; 206 mg from sesamol; and 335 mg from catechin).

Assay of Cytotoxic Acivity. Cell (rat thymocytes) preparation was carried out by the method similar to that described previously (*19*). Briefly, thymus glands were dissected from anesthetized Wistar rats, and they were sliced at a thickness of 400−500 μm. The slices were gently triturated in RPMI-1640 medium with glutamine (300 mg/L) and 10% fetal bovine serum to enable the dissociation of lymphocytes. The RPMI-1640 medium containing the cells was passed through a mesh (pore diameter, 53 μm) to remove the unwanted residue. The cell suspension was diluted to achieve approximately 5×10^5 cells/mL. The cells were cultured in 24-well Falcon tissue culture plates (2 mL in each well) at 37 °C in a CO_2 incubator (Sanyo, Tokyo). The compounds were respectively added to the cell suspensions in each well at 1 h after the commencement of incubation, and the incubation continued for the next 24 h. The cell suspension was examined by a flow cytometer CytoACE-150 (JASCO). A cytogram (forward scatter versus side scatter) was constructed from 2000 cells. To assess cell viability, propidium iodide was added to the cell suspension to achieve a final concentration of 10 μM because propidium stains dead but not living cells. Propidium fluorescence was measured from the cells 1−2 min after adding the dye. The excitation wavelength for propidium was 488 nm, produced by an argon laser, and emission (propidium fluorescence) was detected at 600 ± 20 nm. Statistical analysis was performed by using Tukey's multivariate analysis. A P value of < 0.05 was considered significant.

Isolation of Sesamol Dimers. To an ethanol solution (100 mL) of sesamol (1 g) in a 500 mL screw-capped glass bottle was added 720 mM $FeCl_3$ aqueous solution (1 mL). Ten bottles were incubated for 9 days at 40 °C under oxygen atmosphere. The solutions in the bottles were combined and evaporated. The residue was dissolved in ethyl acetate (1 L), then partitioned three times with saturated NaCl aqueous solution (1 L). The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to give the oxidation mixture (9.9 g). The oxidation mixture (9.8 g) was fractionated by silica gel chromatography (Cosmosil 75SL-II-Prep, 600 g), eluted with ethyl acetate in hexane (1.2 L each for 20%, 30%, 40%, 50%, and 60%) to afford 10 fractions (yield fr. 1, 0.23 g; fr. 2, 0.26 g; fr. 3, 1.25 g; fr. 4, 0.54 g; fr. 5, 0.55 g; fr. 6, 1.58 g; fr. 7, 1.80 g; fr. 8, 1.81 g; fr. 9, 1.04 g; fr. 10, 0.51 g). The cytotoxic fraction 4 (0.2 g) was further purified by HPLC under the following conditions [column, a 250 mm × 20 mm i.d., 5 μm, Cosmosil 5C18-AR-II (Nacalai Tesque); solvent, CH_3OH−1% AcOH aqueous solution, 70:30; flow rate, 4.0 mL/min; detection, 280 nm]. By the preparative HPLC, 4 major peaks (retention time, 22, 24, 30, and 35 min) were collected to give compounds **2**−**5** (yield: **2**, 13 mg; **3**, 7 mg; **4**, 30 mg; **5**, 24 mg).

Analytical Data for Dimers 2−5. *Compound 2*. HR-ESIMS (m/z) $[M + H]^+$, calcd for $C_{14}H_{11}O_6$, 275.0556; found, 275.0554; UV (λ_{max}, CH_3CN) nm 311.5, 236.2; IR (film on NaCl) ν cm^{-1} 2893 (−CH_2−), 1691 (C═O), 1462 ($COCH_2$−), 1294 (C−O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.10 (1H, d, J = 10.0 Hz, H5′), 6.80 (1H, s, H5), 6.43 (1H, s, H2), 6.15 (1H, d, J = 10.0 Hz, H6′), 5.97 (1H, brs, H7b), 5.96 (1H, brs, H7a), 5.25 (1H, brs, H7′b), 4.88 (1H, brs, H7′a), 3.24 (1H, d, J = 17.0 Hz, H2′b), 3.06 (1H, d, J = 17.0 Hz, H2′a); ^{13}C NMR (125 MHz, $CDCl_3$) δ 193.6 (C1′), 154.1 (C1), 150.8 (C3), 143.3 (C4), 140.6 (C5′), 128.5 (C6′), 114.1 (C6), 113.9 (C3′), 103.8 (C5), 101.9 (C7), 93.8 (C2), 93.6 (C7′), 84.2 (C4′), 45.0 (C2′).

Compound 3. HR-ESIMS (m/z) $[M + H]^+$, calcd for $C_{16}H_{17}O_7$, 321.0974; found, 321.0974; UV (λ_{max}, CH_3CN) nm 305.9 ; IR (film on NaCl) ν cm^{-1} 2978 (−CH_3), 2883 (−CH_2−), 1728 (C═O), 1475 ($COCH_2$−), 1290 (C−O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.97 (1H, s, H5), 6.39 (1H, s, H2), 5.97 (2H, brs, H7), 5.25 (1H, brs, H7′b), 4.88 (1H, brs, H-7′a), 4.10 (1H, dd, J = 8.0 and 4.0 Hz, H5′), 3.76 (1H, m, H1″b), 3.66 (1H, m, H1″a), 3.20 (1H, d, J = 16.0 Hz, H2′b), 2.84 (1H, d, J = 16.0 Hz, H2′a), 2.68 (1H, dd, J = 18.0 and 8.0 Hz, H6′b), 2.47 (1H, dd, J = 18.0 and 4.0 hz, H6′a), 1.29 (3H, t, J = 8.0 Hz, H2″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.2 (C1′), 154.6 (C1), 150.3 (C3), 143.1 (C4), 114.0 (C3′), 113.5 (C6), 104.8 (C5), 101.7 (C7), 93.7 (C7′), 92.9 (C2), 88.6 (C4′), 75.9 (C5′), 66.6 (C1″), 46.4 (C2′), 39.8 (C6′), 15.4 (C2″).

Compound 4. HR-ESIMS (m/z) $[M + H]^+$, calcd for $C_{16}H_{17}O_7$, 321.0974; found, 321.0996; UV (λ_{max}, CH_3CN) nm 306.4; IR (film on NaCl) ν cm^{-1} 2978 (−CH_3), 2883 (−CH_2−), 1724 (C═O), 1460 ($COCH_2$−), 1292 (C−O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.80 (1H, s, H5), 6.41 (1H, s, H2), 5.96 (1H, brs, H7a), 5.91 (1H, brs, H7b), 5.25 (1H, brs, H7′b), 4.88 (1H, brs, H7′a), 4.23 (1H, dd, J = 5.0 and 2.5 Hz, H5′), 3.60 (1H, m, H1″b), 3.52 (1H, m, H1″a), 3.08 (1H, d, J = 17.0 Hz, H2′b), 3.03 (1H, d, J = 17.0 Hz, H2′a), 2.68 (1H, dd, J = 18.0 and 2.5 Hz, H6′b), 2.47 (1H, dd, J = 18.0 and 5.0 Hz, H6′a), 1.07 (3H, t, J = 7.0 Hz, H2″); ^{13}C NMR (125 MHz, $CDCl_3$) δ 204.0 (C1′), 155.3 (C1), 150.1 (C3), 143.0 (C4), 113.7 (C3′), 112.4 (C6), 104.8 (C5), 101.7 (C7), 93.3 (C7′), 92.9 (C2), 89.8 (C4′), 74.4 (C5′), 65.9 (C1″), 45.5 (C2′), 39.5 (C6′), 15.2 (C2″).

Compound 5. HR-ESIMS (m/z) $[M + H]^+$, calcd for $C_{13}H_{11}O_4$, 263.0556; found, 263.0538; UV (λ_{max}, CH_3CN) nm 369.3, 265.4; IR (film on NaCl) ν cm^{-1} 3078 (=CH), 2921 (−CH_3), 1718 (not assignable), 1250 (C−O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 (1H,s, H5), 6.83 (1H, s, H2), 6.81 (1H, s, H2′), 6.09 (1H, s, H7), 4.44 (2H, q, J = 7.3 Hz, H1″), 1.43 (3H, t, J = 7.3 Hz, H2″); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.0 (C1′), 160.5 (C3′), 151.8 (C2′), 151.4 (C3), 145.2 (C4), 142.4 (C1), 109.8 (C6), 104.2 (C5), 102.5 (C7), 98.3 (C2), 62.5 (C1″), 14.0 (C2″).

Hydrogenation of Compound 5. To **5** (2 mg) and 10% Pd−C (5 mg) in a flask was added ethanol (2 mL). The mixture was stirred under H_2 atmosphere for 10 min at room temperature. The mixture was filtered, and

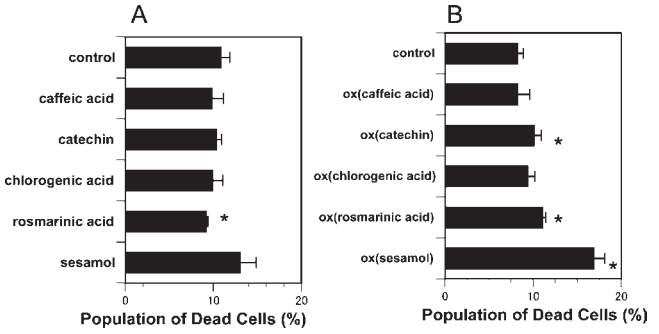

Figure 2. Cytotoxic activity of phytophenols (100 μM) (**A**) and their oxidation products (30 μg/mL) (**B**) to rat thymocytes. Data were expressed as the mean ± SD (n = 5−10). The asterisk (*) shows significant difference ($P < 0.05$) to the control experiment data.

then the filtrate was evaporated. The residue was purified by silica gel TLC (Merck, Darmstadt, Germany) developed with ethyl acetate−hexane (1:3) to give hydrogenated compound **6** (1 mg).

Compound **6**. HR-ESIMS (m/z) $[M + H]^+$, calcd for $C_{13}H_{13}O_6$, 265.0712; found, 265.0728; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.72 (1H, s, H2′), 6.60 (1H, s, H5′), 5.93 (1H, d, J = 2.0 Hz, H7′), 5.89 (1H, d, J = 2.0 Hz, H7′), 4.17 (2H, m, H1″), 3.79 (1H, dd, J = 6.8 and 3.6 Hz, H3), 3.08 (1H, dd, J = 16.6 and 3.6 Hz, H2), 2.79 (1H, dd, J = 16.6 and 6.8 Hz, H2), 1.24 (3H, t, J = 6.0 Hz, H2″).

RESULTS AND DISCUSSION

Cytotoxic Activity of Oxidation Products from Phytophenols. Several phytophenols, which have been reported to possess a potent antioxidant activity, were selected and oxidized. For the oxidation method, we employed the Fe-catalyzed oxidation as the possible oxidation reaction in foods and related bioresources. Each ethanol solution of the antioxidative phytophenol (caffeic acid, catechin, chlorogenic acid, eugenol, resveratrol, rosmarinic acid, rutin, sesamol, and quercetin) was oxidized by 10 mol % of $FeCl_3$ under an oxygen atmosphere for 10 days. The oxidative conversion of the phytophenols was monitored by HPLC analysis. Although eugenol, resveratrol, rutin, and quercetin were not oxidized or very slightly oxidized (< 20%), almost all of the caffeic acid, catechin, chlorogenic acid, rosmarinic acid, and sesamol were oxidized within 10 days under the above stated conditions. After the removal of the Fe ions, the oxidation mixtures from caffeic acid, catechin, chlorogenic acid, rosmarinic acid, and sesamol were subjected to a cytotoxic assay using rat thymocytes by a flowcytometric method (*19*). **Figure 2** shows the cytotoxic activity of five oxidation mixtures. Although all of the oxidized phenols showed a higher population of dead cells compared to that in the control experiment, the oxidation product from sesamol demonstrated a significantly greater activity than the other products (**Figure 2B**). However, all of the original phenols did not show any toxic activity, which was revealed by the statistical analysis (**Figure 2A**). These results demonstrate that sesamol should easily afford oxidation products in nature including foods and that the oxidation products have a potent cytotoxic activity in normal cells.

Isolation of Sesamol Dimers from a Cytotoxic Fraction of Oxidation Product. To identify the cytotoxic active compounds in the oxidation product of sesamol, we attempted the separation and isolation of the active compounds from the oxidation mixture. One gram of sesamol dissolved in ethanol was oxidized by oxygen in the presence of a catalytic amount of $FeCl_3$. After the oxidation was completed, the oxidation mixture was fractionated by silica gel column chromatography into 10 fractions. A review of the cytotoxic activity of the 10 fractions produced the following data [the population of dead cells (%) of each fraction (100 μg/mL): fr. 1, 10.8 ± 0.7; fr. 2, 13.7 ± 2.6; fr. 3, 12.4 ± 0.6; fr. 4, 75.3 ± 0.9; fr. 5, 28.5 ± 7.3; fr. 6, 32.0 ± 5.1; fr. 7, 38.9 ± 3.2; fr. 8, 18.3 ± 1.1; fr. 9, 49.8 ± 3.9; fr. 10, 15.6 ± 1.6; control, 7.5 ± 0.8]. The data show that fraction 4 was the most potent cytotoxic fraction among the 10 fractions, which made us perform a further purification of this fraction. The HPLC analysis of fraction 4 revealed that the fraction still had peaks corresponding to several compounds (**Figure 3**). We succeeded in isolating 4 compounds (**2**−**5**) as pure forms by a preparative HPLC technique.

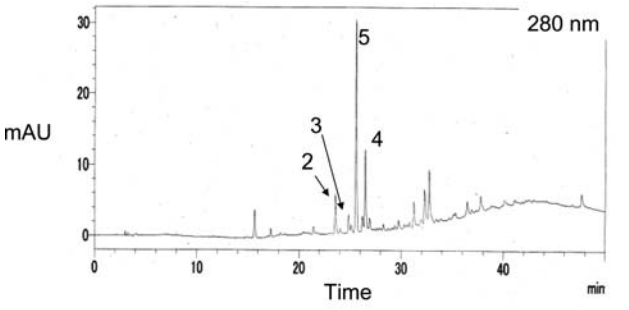

Figure 3. HPLC analytical profile of the most active fraction 4 from sesamol oxidation.

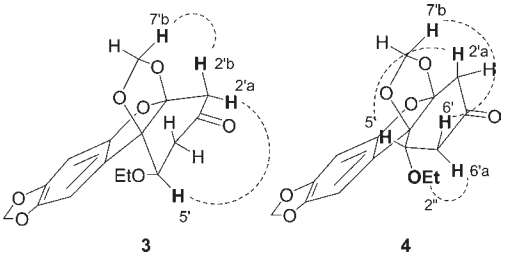

Figure 4. Selected NOE correlations (dashed lines) observed in NOESY of compounds **3** and **4**.

Structure Determination of the Sesamol Dimers. Compound **2** was isolated as a colorless viscous oil. The HR-ESI-MS of **2** revealed that the molecular formula of **2** was $C_{14}H_{10}O_6$. This molecular formula suggested that **2** was a dimeric compound of sesamol. The absorbance maxima at 1691 cm^{-1} in the IR spectrum of **2** indicated the presence of a conjugated carbonyl group in **2**. The ^{1}HNMR spectrum of **2** showed two olefin signals [7.10 (d, J = 10 Hz), 6.15 (d, J = 10 Hz)] in the downfield region, indicating the presence of a *cis*-double bond conjugated to the carbonyl group. The proton signal sets, which were assignable to two methylenedioxy groups, were observed at 5.97−5.96 ppm (2H), 5.25 (1H), and 4.88 (1H) ppm. The upfield shifted signals (δ 5.25 and δ 4.88) of one of the methylenedioxy groups strongly indicated that the aromaticity was disrupted in the one of the original sesamol rings. Further determination, especially for the dimeric position of two original sesamol rings, was carried out by analysis of the HMBC spectrum of **2**. The HMBC correlations clarified the dimerization linkage of two sesamol rings that existed between the 6 and 4′-positons. The HMBC data also indicated that a conjugated carbonyl system existed at 1′, 5′, and 6′-positions. The 3′-positon should be an acetal carbon between 1-O and 7′-CH_2O by considering its carbon chemical shift (δ 113.9), and another dimeric linkage was thus estimated to be between the 1 and 3′-positions. On the basis of these data, the structure of the sesamol dimer **2** was determined to be structure **2** as depicted in **Figure 1**.

Compounds **3** and **4** were isolated as colorless oils. The HR-ESI-MS of **3** and **4** (**3**, m/z 321.0974 $[M + H]^+$ and **4**, m/z 321.0996

$[M + H]^+$) revealed the same molecular formula, $C_{16}H_{16}O_7$. The ^1H and ^{13}C NMR of **3** and **4** were very similar to each other and also similar to those of **2**. Although their NMR data were similar to those of **2**, both NMR spectra of **3** and **4** indicated the presence of an ethoxy group instead of the conjugated carbonyl group of **2**. The attached position of the ethoxy group was determined by their HMBC data to be at the 5′-position of both **3** and **4**. These results indicated that **3** and **4** have the same planar structure with an ethoxy group and that they were stereoisomers based on the stereochemistry of the ethoxy group. The stereochemistry of the ethoxy group in **3** and **4** were determined from the correlations obtained by NOESY as shown in **Figure 4**. In the NOESY of **3**, there are two correlations between H2′b and H7′b and between H2′a and H5′. H7′b is one of the protons of the 3′,4′-methylendioxy group; therefore, H2′b had the same orientation, whereas H2′a and H5′ had the opposite orientation to that of the methylenedioxy group. However, a typical correlation was observed between H7′b and H6′b in the NOESY of **4**. The NOESY showed two other correlations between H2′a and H5′ and between H6′a and the ethoxy protons. These results indicated that the ethoxyl group of **3** had an up-orientation at the 7-position, while the ethoxy group of **4** had a down-orientation as shown in structures **3** and **4** in **Figure 1**.

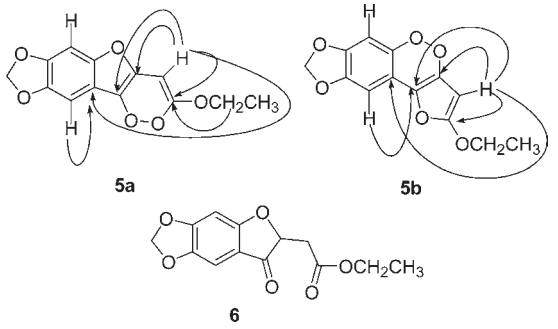

Figure 5. The C-H long-range correlations (arrows) observed in HMBC of compound **5**, on two possible structures (**5a** and **5b**) for **5** and the structure of hydrogenated product **6** from **5**.

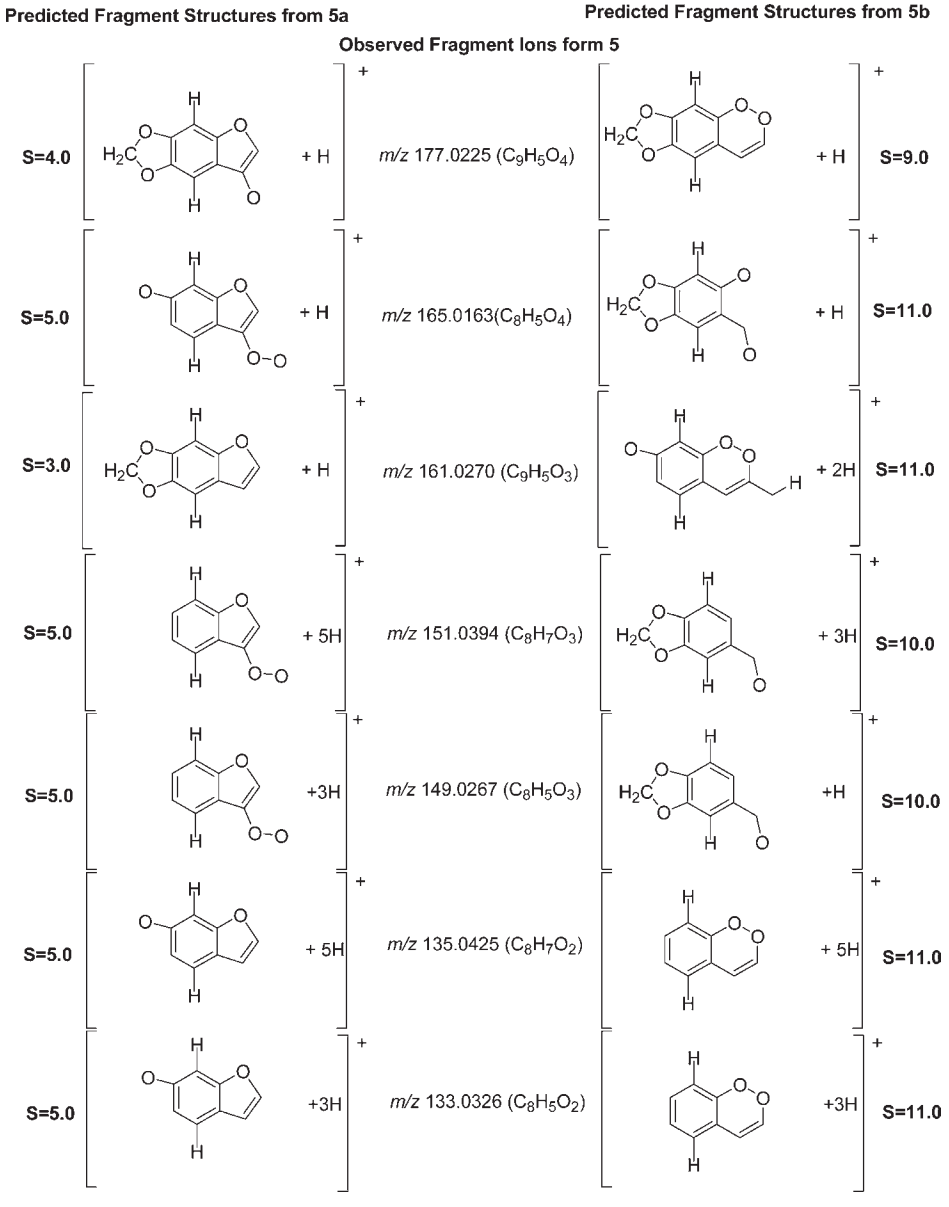

Figure 6. Observed fragment ions from compound **5** by ESI-MS-MS and predicted fragment ion structures and their formation difficulty scores (**S**) from precursor structures **5a** and **5b**.

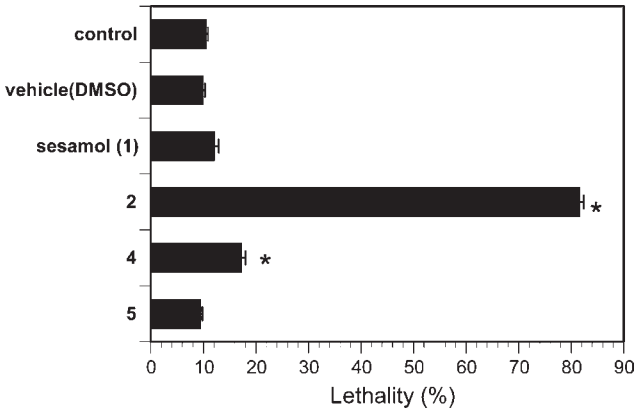

Figure 7. Cytotoxic activity of sesamol dimers (100 μM) to rat thymocytes. Data were expressed as the mean ± SD (n = 5−10). The asterisk (*) shows significant difference ($P < 0.05$) to the control experiment data.

Compound **5** was isolated as yellow needles (mp 123.0−123.5 °C). The HR-ESI-MS of **5** revealed that its molecular formula was $C_{13}H_{10}O_4$. The UV absorption maxima at 369 nm indicated that **5** had a higher conjugated structure compared to the other compounds. The ^{1}H NMR data of **5** showed three isolated aromatic proton signals and signal sets due to an ethoxy group and methylenedioxy group. An additional structural analysis was carried out using the HMBC spectrum of **5**. On the basis of the correlations obtained from the HMBC and molecular formula of **5**, two possible structures containing a peroxide bond (**5a** and **5b**) could be postulated for the exact structure of **5** (**Figure 5**). The HMBC correlations and other NMR data indicated that the peroxy bond should be between the 1- and 3′-positions (structure **5a**) or between 1′- and 4′-positions (structure **5b**); however, determination of the position of such a peroxy bond was one of the difficult problems of this structure determination. Hill and Mortishire-Smith (*20*) recently reported a new analytic method for mass fragmentation, which was based on the high-resolution mass data of fragment ions, systematic bond disconnection of the proposed precursor structure, and ranking of the energy demand for the formation of the resulting fragment structures. This mass fragmentation analysis method was used for the elucidation of the structure for **5**. The calculated formation scores of the fragments, which was the sum of the difficulty rating for each bond dissociation, from each precursor structure, **5a** and **5b**, are summarized in **Figure 6**. A comparison of the score value of each fragment clearly indicated that more low score fragments could be derived from **5a** than **5b**; thus, **5a** was expected to be the structure of compound **5**. To confirm structure **5a** as that of **5**, compound **5** was reduced by hydrogen on palladium charcoal. This reduction afforded the keto-ester compound **6** (**Figure 5**). This result clearly demonstrated that structure **5a** was the exact structure for compound **5** as shown in **Figure 1**.

Cytotoxic Activity of the Sesamol Dimers. We succeeded in isolating three major dimers (**2**, **4**, and **5**) and a structurally related minor dimer (**3**) from the most active fraction of the sesamol oxidation products. For the next investigation, the cytotoxic activity of the isolated compounds was examined using rat thymocytes as the normal cells, which were suitable for the flow cytometric analysis (*19*). **Figure 7** shows the cell lethality percent (dead cell population of the cells) in the presence of 100 μM of each sesamol dimer (**2**, **4**, and **5**). Although sesamol at this concentration did not affect the lethality percent compared with that of the control experiment, compounds **2** and **4** showed a cytotoxic effect, and the effect of **2** was very strong. Compound **2** has the typical conjugated carbonyl moiety in its structure. It is well-known that sesquiterpene lactones having a conjugated carbonyl moiety possess a high cytotoxic activity and that the conjugated carbonyl system is believed to be essential for their cytotoxicity (*21*). Compound **2** has a conjugated carbonyl system and lipophilicity similar to those of the cytotoxic sesquiterpene lactones. These similarities would be one of the reasons for the high cytotoxicity of **2** to the living cells. However, **4** is an ethanol-bearing derivative of **2** without the conjugated carbonyl system. The structural difference would weaken the cytotoxicity of **4**. In this investigation, we employed ethanol, which had satisfactory solubility for various phenols and inorganic salts and was usable for food, as a reaction media. When other solvents were employed, no **4** and **5** should be produced. Further studies for solvent effect on the sesamol oxidation are necessary to understand the oxidation in various food systems. In our previous preliminary paper, we reported the very rare structures of the cytotoxic trimer and tetramer of sesamol (*18*). These compounds were also found to be in fractions 5 and 6 in this investigation, which was revealed by the HPLC analysis of the silica gel chromatography fractions.

In conclusion, sesamol, the potent antioxidant of sesame oil, is very easily oxidized in the presence of Fe ions and produces cytotoxic oxidation products, which are structurally oligomeric compounds of sesamol. These compounds possibly accumulate in the oxidizing food or related biomaterials that contain sesamol as an antioxidative ingredient.

Supporting Information Available: Four tables of correlation data of 2D NMR spectra of compounds **2**, **3**, **4**, and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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