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Structure and Function of the Oxidation Products of Polyphenols and Identification of Potent Lipoxygenase Inhibitors from Fe-Catalyzed Oxidation of Resveratrol

Yoshimi Shingai, Aya Fujimoto, Mitsuhiro Nakamura, and Toshiya Masuda*

Graduate School of Integrated Arts and Sciences, University of Tokushima, Tokushima 770-8502, Japan

Supporting Information

ABSTRACT: Polyphenols have recently attracted much attention as potent antioxidants and related bioactive substances. These potent antioxidative polyphenols are very oxidizable due to their chemical properties, and their oxidation products must accumulate in the oxidizing foods when they are contained as the active ingredients. In this investigation, 30 polyphenols and related phenolics were oxidized with oxygen in the presence of a catalytic amount of Fe ions. Piceatannol, catechin, epicatechin, hydroxytyrosol, carnosol, and carnosic acid were oxidized very quickly. Sinapic acid, caffeic acid, chlorogenic acid, rosmarinic acid, gallic acid, propyl gallate, α-tocopherol, quercetin, and nordihydroguaiaretic acid were moderately oxidized. Protocatechuic acid, syringic acid, taxifolin, resveratrol, gentisic acid, secoisolariciresinol, and ellagic acid were oxidized for 19−20 days; however, their oxidation was very slow and did not complete. The other phenolics were not oxidized. The obtained oxidation products were next subjected to a lipoxygenase inhibition assay and the results compared to those of the corresponding phenols. Very interestingly, the oxidation product from resveratrol showed a high inhibitory activity, whereas resveratrol itself had no activity and its oxidation efficiency was low. To clarify the inhibition principle of the oxidation product, an LC-MS analysis was carried out on the oxidation product. The analytical results showed that they are the oligomeric and degraded compounds of resveratrol. Among them, the structures of three dimeric compounds were successfully identified, and their activity data clarified that the closed ring dimers were potent lipoxygenase inhibitors, whereas the opened ring dimer was not. It should be noted that resveratrol had almost no lipoxygenase inhibitory activity, contrary to some researchers' findings.

KEYWORDS: polyphenol, oxidation, antioxidant, resveratrol, lipoxygenase inhibitor, dimer

INTRODUCTION

Polyphenols, natural phenols bearing at least two phenolic functional groups, have currently attracted much attention as active constituents in functional foods and food supplements, which promise to improve human health. Polyphenols show very potent antioxidant activity, and this activity is closely linked to various beneficial actions, including antiaging, prevention of cancer, cardiovascular disease, etc. Most polyphenols are not very stable, especially under oxidative conditions. Their easily oxidizable property contributes to their efficient antioxidant activity. The antioxidant is oxidized much more quickly than other biomolecules, thus providing potent antioxidant activity. It should be noted that the oxidation of polyphenol afforded new products,[1] and the products might accumulate in foods and in the human body. The functionality of the oxidation products, regardless of being beneficial or nonbeneficial, has not yet been intensively examined. Recently, we started a project to investigate the chemical structures and functional property of the oxidation products. Subsequently, we found a potent cytotoxic activity of the oxidation products from sesamol, an antioxidant of sesame oil.[2] We also reported a new tyrosinase inhibitor from the oxidation of rosmarinic acid.[3] We have now screened the lipoxygenase inhibitory activity of the products from the Fe-catalyzed oxidation of various polyphenols. Lipoxygenase is widely distributed in plants and animals.[4] In foods, lipoxygenase accelerates lipid oxidation, which results in the formation of an unpleasant smell from the degradation products of unsaturated lipids.[5] In humans, lipoxygenase is responsible for inflammation,[6] which is one of the first responses to various diseases. Therefore, lipoxygenase inhibitors have attracted much interest not only in food science but also in medical science.[7]

The Fe-catalyzed air oxidation, which we employed, was one of the autoxidation reactions found in nature including foods. To an ethanol solution of each polyphenol was added a catalytic amount of $FeCl_3$, and the mixture was stored under an oxygen atmosphere. The lipoxygenase inhibitory activity of the resulting oxidation mixtures was measured and compared to that of the original polyphenols. Although the activity of almost all of the polyphenols was decreased by the oxidation, only resveratrol was found to remarkably enhance the activity. Chemical evidence for the enhanced activity of the resveratrol oxidation was investigated by an LC-MS analysis of the oxidation product and succeeded in identifying two active compounds from the oxidation product.

MATERIALS AND METHODS

Chemicals and Instruments. Caffeic acid (purity ≥ 98%), sinapic acid (purity ≥ 98%), ferulic acid (purity ≥ 98%), gallic acid (purity ≥ 98%), umbelliferone (purity ≥ 98%), esculetin (purity ≥ 98%),

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resveratrol (purity ≥ 98%), kaempferol (purity ≥ 98%), hydroxytyrosol (purity ≥ 98%), γ-oryzanol (purity not specified), morin (purity ≥ 90% as hydrate), and propyl gallate (purity ≥ 98%) were purchased from Tokyo Kasei (Tokyo, Japan). Chlorogenic acid (purity ≥ 95%), rosmarinic acid (purity ≥ 97%), syringic acid (purity ≥ 98%), taxifolin (purity ≥ 90% as hydrate), nordihydroguaiaretic acid (purity ≥ 90%), and Chelex 100 resin were purchased from Sigma-Aldrich (St. Louis, MO). (−)-Epicatechin (purity ≥ 97%), ellagic acid (purity ≥ 90%), vanillic acid (purity ≥ 95%), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Wako Pure Chemicals (Osaka, Japan). Luteolin (purity not specified) was purchased from Kanto Chemicals (Tokyo, Japan). Piceatannol (purity ≥ 95%) and (+)-secoisolariciresinol (purity ≥ 95%) were synthesized.[8] Carnosol (purity ≥ 95%) and carnosic acid (purity ≥ 95%) were isolated from sage (*Salvia officinalis*).[9] Protocatechuic acid (purity ≥ 97%), quercetin (purity not specified), (+)-catechin (purity = 98% as hydrate), α-tocopherol (purity ≥ 96%), gentisic acid (purity ≥ 98%), $FeCl_3 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, and all solvents were obtained from Nacalai Tesque (Kyoto, Japan). NMR spectra were measured with an ECX-400 spectrometer (JEOL, Tokyo, Japan) using the manufacturer-supplied pulse sequences [^1H, ^{13}C, correlated spectroscopy (HH−COSY), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC)]. Mass spectra were measured with a XEVO QtofMS spectrometer (Waters Japan, Tokyo, Japan) in the positive ESI mode. An 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. An LC-6AD system (Shimadzu) equipped with a UV-970 detector (JASCO, Tokyo, Japan) was used for preparative HPLC.

Oxidation of Polyphenols. To an ethanol solution (10 mL) of each phenol (0.5 mM) in a 78 mm × 35 mm i.d. screw-capped vial was added a 50 μM $FeCl_3$ aqueous solution (100 μL). The vial was incubated under an oxygen atmosphere at 40 °C. Ten microliter aliquots were removed at 1 day intervals and diluted with 30 μL of ethanol. Ten microliters of the diluted solution was injected into an analytical HPLC to measure the remaining nonoxidized phenol under the following conditions: column, 250 mm × 4.6 mm i.d., 5 μm, Cosmosil 5C18-AR-II (Nacalai Tesque); flow rate, 0.5 mL/min; detection, 280 nm; solvent, CH_3OH/1% CH_3CO_2H in H_2O (10:90, v/v) for gallic acid; chlorogenic acid, hydroxytyrosol, protocatechuic acid CH_3OH/1% CH_3CO_2H in H_2O (40:60, v/v) for syringic acid, (+)-catechin, (−)-epicatechin, caffeic acid, and esculetin; CH_3OH/1% CH_3CO_2H in H_2O (50:50, v/v) for sinapic acid, ferulic acid, umbelliferone rosmarinic acid, vanillic acid, resveratrol, piceatannol, and taxifolin; CH_3OH/1% CH_3CO_2H in H_2O (60:40, v/v) for luteolin, quercetin, morin, propyl gallate, gentisic acid, (+)-secoisolariciresinol, and ellagic acid; CH_3OH/1% CH_3CO_2H in H_2O (70:30, v/v) for kaempferol; CH_3OH/1% CH_3CO_2H in H_2O (80:20, v/v) for carnosol, carnosic acid, and nordihydroguaiaretic acid; CH_3OH for γ-oryzanol and α-tocopherol. After almost all of the phenol (>80%) was consumed or incubated for 19−20 days, each solution was then passed through Chelex 100 (2−4 g) to remove the iron ions. The solution was evaporated in vacuo to give the following yields of oxidized mixture (starting weight of polyphenol in parentheses): 125 mg from caffeic acid (91 mg), 66 mg from sinapic acid (112 mg), 110 mg from ferulic acid (98 mg), 213 mg from chlorogenic acid (177 mg), 243 mg from rosmarinic acid (181 mg), 122 mg from gallic acid (94 mg), 69 mg from protocatechuic acid (77 mg), 111 mg from syringic acid (99 mg), 98 mg from vanillic acid (84 mg), 103 mg from umbelliferone (82 mg), 85 mg from esculetin (98 mg), 82 mg from resveratrol (115 mg), 164 mg from piceatannol (122 mg), 129 mg from luteolin (143 mg), 59 mg from taxifolin (51 mg), 53 mg from kaempferol (48 mg), 207 mg from quercetin (170 mg), 172 mg from (+)-catechin (147 mg), 60 mg from (−)-epicatechin (48 mg), 109 mg from hydroxytyrosol (78 mg), 395 mg from γ-oryzanol (310 mg), 184 mg from morin (160 mg), 145 mg from propyl gallate (107 mg), 233 mg from α-tocopherol (216 mg), 90 mg from gentisic acid (77 mg), 190 mg from (+)-secoisolariciresinol (182 mg), 74 mg from ellagic acid (152 mg), 215 mg from carnosol (165 mg), 201 mg from carnosic acid (166 mg), and 218 mg from nordihydroguaiaretic acid (152 mg).

Lipoxygenase Inhibiton Assay.FOX method. The FOX assay was carried out following a previous procedure[10] with a slight modification. Briefly, to a 50 mM Tris-HCl buffer (pH 7.5, 3.9 mL) solution were added the sample in methanol (30 μL) and a 5 mM linoleic acid methanol solution (30 μL). After thorough stirring, 7 μL of lipoxygenase in the Tris-HCl buffer (6650 units/mL) was added to the mixture. The solution was incubated for 20 min at 25 °C with shaking, and then the FOX reagent (1 mL) was added. The FOX reagent consisted of 0.5 mM of xylenol orange (Dojindo, Kumamoto, Japan), 0.5 mM ferrous sulfate (Nacalai Tesque), and 550 mM perchloric acid (Wako Pure Chemicals) in distilled water. After incubation at 45 °C for 30 min, the absorbance of the solution was measured at 586 nm. The inhibition was evaluated as the percent inhibition, which was calculated using the following equation:

$$\%\ \text{inhibition} = (\textit{ABS of expt without sample} - \textit{ABS of sample expt}) \times 100/(\textit{ABS of expt without sample})$$

Spectrophotometric Method. The method of Ha et al.[11] was slightly modified. The mixture of 1 mM linoleic acid in methanol (50 μL) and the sample of the methanol solution (20 μL) in 0.1 M Tris-HCl buffer (pH 8.0, 2.9 mL) was preincubated at 25 °C for 10 min in a crystal cuvette (1 cm). Five microliters of the lipoxygenase buffer solution (6,650 units/mL) was added to the solution and quickly stirred. An increase in the 234 nm absorbance of the solution was measured for 5 min at 25 ± 0.1 °C with a UVmini-124 spectrophotometer (Shimadzu) equipped with a temperature-controlled cell holder TCC-240A (Shimadzu). The inhibitory activity was evaluated by the percent inhibition calculated by the following equation:

$$\%\ \text{inhibition} = (\textit{ABS increase of expt without sample} - \textit{ABS increase of sample expt}) \times 100/(\textit{ABS increase of expt without sample})$$

LC-MS Analysis of the Oxidation Product from Resveratrol. Ten microliters of the acetonitrile solution (0.2 mg/mL) of the resveratrol oxidation product was injected into an LC-MS instrument, Acquity UPLC, and Xevo QtofMS (Waters Japan, Tokyo, Japan), through a sample injector, Acquity sample manager (Waters). The LC-MS analysis was carried out under the following conditions. Separation conditions: column, 150 × 2.0 mm i.d., 5 μm, Cosmosil 5C18-AR-II (Nacalai Tesque); flow rate, 0.2 mL/min; solvent A, ultrapure water; solvent B, acetonitrile (LC-MS grade, Merck, Darmstadt, Germany). Gradient conditions: linear gradient from 25% solvent B (0 min) to 75% solvent B (40 min). UV absorbance detection: 280 nm (from 0 to 26 min). MS conditions: mode, ESI positive; capillary voltage, 3.0 kV; cone voltage, 40 V; source temperature, 120 °C; desolvation temperature., 450 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 800 L/h, MS^E low collision energy, 6 V; MS^E high collision energy, from 20 to 30 V. The elemental composition of each peak compound was calculated from the high-resolution MS data of the protonated or ion-adducted molecular ion by MassLynx software (V. 4.1, Waters).

Resveratrol Dimer (7) Prepared by $CuCl_2$. A previous method[12] was employed using $CuCl_2$ instead of the $FeCl_3$ because oxidation by Cu^{2+} was faster than that by Fe^{3+}. To a solution of resveratrol (100 mg) in ethanol (10 mL) was added 0.63 mL of a 4.2 M $CuCl_2$ aqueous solution. The solution was kept at 40 °C for 3 h. The mixture was poured into saturated NaCl aqueous solution, extracted with ethyl acetate, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography eluted with acetone/hexane (1:1, v/v) to give 13 mg of a solid. Further purification was carried out by preparative

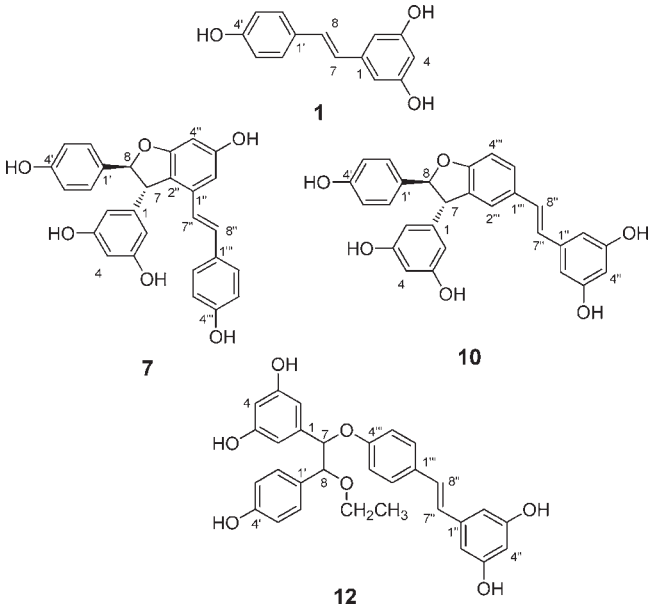

Figure 1. Chemical structures of resveratrol (**1**) and its dimers (**7**, **10**, and **12**) Position numbering is tentative and is based on the numbering system of resveratrol.

HPLC [column, 250 × 20 mm i.d., 5 μm, Cosmosil 5C18 AR-II (Nacalai Tesque); solvent, CH_3OH/1% CH_3CO_2H in H_2O (1:1, v/v); flow rate, 6 mL/min; detection, 250 nm] to give **7** (2 mg, retention time, 20 min). The spectroscopic data of **7** (see the Supporting Information) were identical to those of *trans*-ε-viniferin in the literature.[13] Identification of compound **7** to the HPLC peak 7 compound of the Fe-catalyzed resveratrol oxidation mixture was carried out by comparison of their retention times, UV spectra, HR-MS spectra, and MS/MS fragmentation spectra obtained by the MS^E method (Waters).

Reveratrol Dimers (10 and 12) Prepared by DPPH. The method of Wang et al.[14] was employed with modification. To a solution of resveratrol (100 mg) in ethanol (10 mL) was added a DPPH (345 mg) ethanol solution (180 mL) at room temperature. The mixture was evaporated and subjected to Cosmosil SL-II Prep silica gel column chromatography (30 g, Nacalai Tesque) eluted with acetone/hexane (1:1, v/v) to afford 89 mg of the reaction product. The product was further purified by preparative HPLC [column, 250 × 20 mm i.d., 5 μm, Cosmosil 5C18 AR-II (Nacalai Tesque); solvent, CH_3OH/1% CH_3CO_2H in H_2O (55:45, v/v); flow rate, 5 mL/min; detection, 280 nm] to give **10** (25 mg) and **12** (13 mg). **12**: HR-ESIMS, m/z $[M + H]^+$, calcd for $C_{30}H_{29}O_7$, 501.1891; found, 501.1912; ESI-MSMS, m/z $[M + H]^+$ 501, 455, 361, 273, 227; UV (λ_{max}, CH_3CN) 319.6 nm; ^1H NMR (400 MHz, acetone-d_6), δ 7.37 (2H, d, J = 8.6 Hz, H2‴ and H6‴), 7.04 (2H, d, J = 8.4 Hz, H2′ and H6′), 6.96 (1H, d, J = 16.0 Hz, H8″), 6.87 (2H, d, J = 8.6 Hz, H3‴ and H5‴), 6.85 (1H, d, J = 16.0 Hz, H7″), 6.68 (2H, d, J = 8.4 Hz, H3′ and H5′), 6.50 (2H, d, J = 2.0 Hz, H2″ and H6″), 6.24 (1H, br s, H4″), 6.20 (2H, d, J = 1.6 Hz, H2 and H6), 6.13 (1H, br s, H4), 5.14 (1H, d, J = 6.4 Hz, H7), 4.53 (1H, d, J = 6.8 Hz, H8), 3.41(2H, q, J = 3.2 Hz, H1⁗), 1.10 (3H, t, J = 3.2 Hz, H2⁗); ^{13}C NMR (100 MHz, acetone-d_6), δ 159.5 (C3″ and C5″), 159.3 (C4‴), 158.8 (C3 and C5), 157.6 (C4′), 141.7 (C1), 140.7 (C1″), 130.8 (C1‴), 130.6 (C1′), 129.9 (C2′ and C6′), 128.7 (C8″), 128.3 (C2‴ and C6‴), 127.4 (C7″), 117.0 (C3‴ and C5‴), 115.3 (C3′ and C5′), 107.0 (C2 and C6), 105.5 (C2″ and 6″), 102.5 (C4″), 102.3 (C4), 85.7 (C8), 84.5 (C7), 65.1 (C1⁗), 15.7 (C2⁗). The chemical structure of **10** was deduced as structure **10** in Figure 1 from its spectroscopic data (see the Supporting Information), which were identical to those reported in the literature.[15] Identification of compounds **10** and **12** to the HPLC peaks 10 and 12, respectively, of the Fe-catalyzed resveratrol oxidation mixture was carried out by comparison of their retention times, UV spectra, HR-MS spectra, and MS fragmentation spectra obtained by the MS^E method (Waters).

RESULTS AND DISCUSSION

Oxidation of Polyphenols. It is well-known that many polyphenols possess very strong antioxidant activities, which is one of the typical functions of phenolics. During the antioxidation, the phenolic antioxidant first traps the peroxyl radical of a biomolecule, and then it turns to the phenolic radical species. The produced phenolic radical next undergoes a termination reaction to afford a stable nonradical compound. These radical trapping and terminating stages should be recognized as the entire oxidation process of the phenol, suggesting that the more potent antioxidant is oxidized more rapidly. The potent antioxidant must be converted to another structurally different compound from the original antioxidant during the oxidation process. In this investigation, the oxidation of polyphenols and related phenolic compounds was carried out under air oxidation conditions in the presence of a catalytic amount of ferric ions, which was employed as one of the common oxidations in nature including foods. Thirty kinds of polyphenols were subjected to the oxidation and 22 oxidizable polyphenols were selected and are summarized in Table 1. The oxidation stage of each polyphenol was monitored by the decrease of its peak intensity in the HPLC chromatogram of the reaction mixture. The oxidation reaction was continued at most for 20 days or until 80% of the polyphenol was consumed. Oxidation of piceatannol, catechin, epicatechin, hydroxytyrosol, carnosol, and carnosic acid was very fast and completed in a day. Sinapic acid, caffeic acid, chlorogenic acid, rosmarinic acid, gallic acid, propyl gallate, α-tocopherol, quercetin, and nordihydroguaiaretic acid were completely oxidized in 10 days. Up to 50% of protocatechuic acid, syringic acid, resveratrol, gentisic acid, secoisolariciresinol, taxifolin, and ellagic acid were oxidized within 19−20 days. Almost none of the ferulic acid, vanillic acid, umbelliferone, esculetin, luteolin, kaempferol, γ-oryzanol, and morin was oxidized for 19−20 days under the stated conditions. The Fe-catalyzed air oxidation is a mild oxidation; therefore, only phenolics bearing the catechol structure without any electron-withdrawing group on the same aromatic ring showed highly oxidizable properties, which is one of the structural requirements for the efficient antioxidant. Antolovich and co-workers[16] intensively investigated the oxidation products from phenolic antioxidants and reported various oxidation products; however, they employed periodic acid oxidation or the Fenton oxidation, which were much stronger reagents than ferric ion. Catechin can be easily oxidized by the Fe-catalyzed oxidation, which agreed with the report by Oszmianski et al.[17] The second group, including sinapic acid, caffeic acid, etc., showed moderate oxidizable property, but these compounds belong to the potent antioxidants in food science. Cillier and Singleton[18] reported that the autoxidation of caffeic acid occurred readily; however, they employed alkaline conditions to accelerate the oxidation. Although the third group including protocatechuic acid is also recognized as antioxidative compounds in the literature, their antioxidant potency is probably much lower on the basis of these results.

Lipoxygenase Inhibitory Activity. Twenty-two phenolics could be oxidized under our Fe-catalyzed oxidation conditions. Next, we planned to carry out a screening for the beneficial

Table 1. Lipoxygenase Inhibitory Acitivty of 22 Polyphenols and Their Oxidation Products

phenolic sample	% inhibition of nonoxidized sample (100 μM)[a]	% inhibition of oxidized sample (corresponding concn for 100 μM of the nonoxidized sample)[a]
caffeic acid	−2.7 ± 4.8	−5.5 ± 3.1
(+)-catechin	9.0 ± 2.0	−4.4 ± 2.8
carnosic acid	87.3 ± 10.2	−14.7 ± 13.5
carnosol	4.3 ± 3.5	−1.4 ± 2.9
chlorogenic acid	32.7 ± 0.7	2.5 ± 4.7
ellagic acid	−16.5 ± 7.8	−16.3 ± 3.5
(−)-epicatechin	19.2 ± 2.8	−13.7 ± 2.1
gallic acid	11.7 ± 4.6	−0.9 ± 9.5
gentisic acid	33.4 ± 6.1	−0.7 ± 0.6
hydroxytyrosol	51.6 ± 4.0	−19.2 ± 1.6
nordihydroguaiaretic acid	92.8 ± 2.1	−8.1 ± 4.6
piceatannol	117.7 ± 0.3[b]	49.8 ± 3.9
propyl gallate	53.6 ± 1.7	1.0 ± 2.3
protocatechuic acid	−4.1 ± 1.8	2.2 ± 3.9
quercetin	101.9 ± 4.3[b]	45.4 ± 4.3
resveratrol	4.5 ± 3.4	79.5 ± 1.9
rosmarinic acid	90.6 ± 3.9	56.0 ± 6.1
secoisolariciresinol	56.9 ± 6.8	−35.1 ± 1.3
sinapic acid	83.0 ± 5.8	8.8 ± 2.8
syringic acid	2.9 ± 0.8	−4.7 ± 2.4
taxifolin	17.6 ± 5.3	−19.5 ± 4.1
α-tocopherol	−21.3 ± 2.5	−20.7 ± 1.9

[a] Mean ± SD (n = 3). [b] Reduction of Fe^{3+}, which was contained in FOX reagent as an impurity, might take place.

function of the oxidation products. Lipoxygenase is a nonheme dioxygenase widely distributed in both animals and plants.[4] It catalyzed the oxidation of essential fatty acids to form the corresponding conjugated hydroperoxides. In food, the hydroperoxides are responsible for the loss of vitamins and for the development of undesired flavors. In living cells, they are responsible for inflammation and are very active against biomolecules to induce cell death. Therefore, the inhibitory activity of lipoxygenase is important not only in food science but also in human health. Table 1 shows the lipoxygenase inhibitory activity of 100 μM of 22 kinds of nonoxidized polyphenols and an equal amount of their oxidation products, these data being obtained by the FOX method. The inhibitory activity at 100 μM showed a strong activity in sinapic acid, rosmarinic acid, piceatannol, quercetin, carnosic acid, and nordihydroguaiaretic acid; however, each oxidation product showed a weaker activity than the nonoxidized one. It should be noted that resveratrol did not have any inhibitory activity at the same concentration; however, the oxidized resveratrol exhibited a very strong inhibitory activity at the corresponding concentration. Reducing compounds interfere with the FOX method, and some polyphenols have a reducing ability to ferric ion. Therefore, strong inhibitory active products were evaluated again by the spectrophotometric method, and a similar inhibitory efficiency for the resveratrol oxidation product was obtained (98% inhibition at 50 μM). In addition to the observed strong activity, the oxidative conversion rate of resveratrol was relatively low (33%). These data indicated that the Fe-catalyzed oxidation of resveratrol should produce a small amount of a very potent lipoxygenase inhibitory compound in the oxidation mixture. Resveratrol (**1**) is an antioxidative polyphenol distributed in edible fruits including nuts, berries, grapes, and

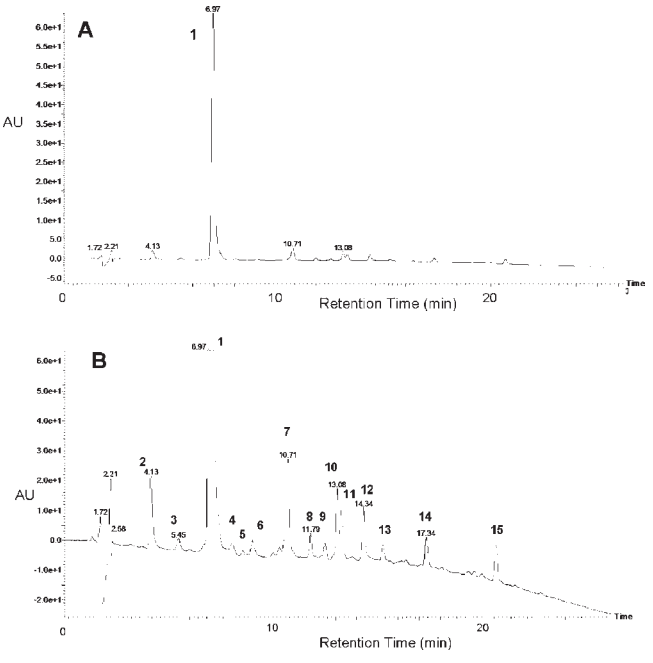

Figure 2. HPLC profile (A) of oxidation products from resveratrol and an expanded profile (B). Detection was carried out at 280 nm. Each peak was numbered, and MS was obtained for the peak directly by LC-MS.

their derived foods and beverages. Resveratrol was first found as a phytoalexin; it is now proposed as a life-extending, cancer-preventing, neuroprotective, cardioprotective, and antidiabetic

Table 2. LC-MS Analytical Results of Oxidation Products from Resveratrol

peak	retention time (min)	observed molecular related ion (m/z)	expected formula	calculated molecular related ion (m/z)
2	4.13	123.0438	$C_7H_7O_2$	123.0446
3	5.45	361.1070	$C_{22}H_{17}O_5$	361.1076
1 (resveratrol)	6.97	229.0859	$C_{14}H_{13}O_3$	229.0865
4	8.02	681.2175	$C_{35}H_{37}O_{14}$	681.2183
5	8.29	333.1099	$C_{21}H_{17}O_4$	333.1127
6	9.00	681.2172	$C_{35}H_{37}O_{14}$	681.2183
7	10.71	455.1498	$C_{28}H_{23}O_6$	455.1495
8	11.79	273.1660	$C_{12}H_{26}O_5Na$	273.1678
9	12.50	455.1473	$C_{28}H_{23}O_6$	455.1495
10	13.08	455.1515	$C_{28}H_{23}O_6$	455.1495
11	13.27	455.1488	$C_{28}H_{23}O_6$	455.1495
12	14.34	501.1910	$C_{30}H_{29}O_7$	501.1913
13	15.28	481.1649	$C_{30}H_{25}O_6$	481.1651
14	17.34	333.1109	$C_{21}H_{17}O_4$	333.1127
15	20.71	255.1020	$C_{16}H_{15}O_3$	255.1021

agent.[19] Resveratrol should now be one of the promising multifunctional polyphenols (Figure 1).

LC-MS Analysis of the Oxidation Product from Resveratrol. The Fe-catalyzed oxidation of resveratrol was not a very efficient reaction and afforded a very complex mixture. To identify the active compound from the oxidation product, direct isolation of the active compound from the product seemed to be impossible. Therefore, we adopted an LC-MS method to identify the active compound. The UV absorption (280 nm) chromatography of the oxidation mixture (Figure 2A) shows that the predominant peak in the chromatography is the remaining resveratrol peak 1, and the other peaks are numerous and very small compared to that of resveratrol. However, the peaks at retention times of 4.13 (peak 2), 10.71 (7), 13.08 (10), 13.27 (11), and 14.34 (12) min are predominant even among the small peaks (Figure 2B). An MS analysis for these peaks in the chromatography gave the corresponding molecular-related ion as shown in Table 2. These data indicated that the major peak compounds (**7**, **10**, **11**, and **12**) were dimeric derivatives of resveratrol except for that associated with the peak at 4.13 min (**2**).

Identification of the Oxidation Compounds. There are five predominant peaks (2, 7, 10, 11, and 12) for the oxidation products from resveratrol in the HPLC data. Among them, the 4.13 min peak compound (m/z 123.0438 $[M + H]^+$, $C_7H_7O_2$) was thought to be a degraded compound of resveratrol from the MS data and identical to *p*-hydroxybenzaldehyde (**2**) by comparison with an authentic sample. It should be noted that various resveratrol dimers and related oligomers have been found in plant families including Genetaceae.[20] Kim and co-workers[21] isolated the lipoxygenase inhibitory resveratrol dimer from *Paeonia* seeds. Ngoc and co-workers[13] and Ha et al.[22] also recently reported that some natural resveratrol dimers exerted a soybean lipoxygenase inhibitory activity. These reports and our LC-MS analysis results strongly indicated that some dimers of resveratrol might contribute to the potent inhibitory activity of the Fe-catalyzed oxidation product of resveratrol. Many research groups have already reported the synthesis of resveratrol dimer.[12,23−27] Therefore, we carried out the preparation of the same resveratrol dimers by the reported synthetic method. After applying many conditions to prepare the dimers, we found that the treatment of resveratrol with an excess amount of cupric chloride in ethanol

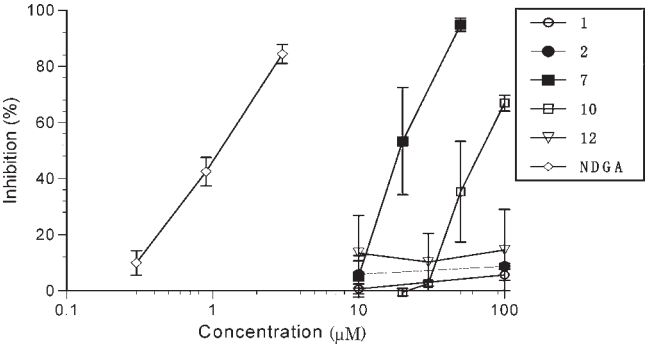

Figure 3. Lipoxygenase inhibitory activity of resveratrol (**1**), a degraded product (**2**), and dimers (**7**, **10**, and **12**). Data are expressed as the mean ± SD (n = 3). NDGA (nordihydroguaiaretic acid) was employed as a reference potent inhibitor.

gave the dimer (**7**), the conditions of which were similar to those reported by Yao et al.[12] The structure of the dimer (**7**) was identical to the structure **7** in Figure 1 by spectroscopic analysis and also identical to peak 7 by comparison of their HPLC retention time and MS/MS spectrometric data. We also found that the DPPH oxidation of resveratrol gave a compound (**10**), which was reported by Wang et al.[14] Under the same conditions, a minor dimer (**12**) corresponding to peak 12 was obtained. The structure of compound **10** was also identical to the structure **10** in Figure 1 based on a spectroscopic analysis. The structure of the dimer (**12**) was determined by an instrumental analysis. The HR-ESIMS data indicated that its molecular formula was $C_{30}H_{28}O_7$. This formula indicated that **12** was a dimeric derivative of resveratrol with the addition of one molecule of ethanol. The coupling position of two resveratrol moieties was deduced by the C−H long-range correlation between C4‴ and H7 in the HMBC spectrum of **12**. The carbons at the 7- and 8-positions (δ 84.5 and 85.7, respectively) were oxygenated carbons altered from the olefinic carbons of the original resveratrol structure. The HMBC spectrum of **12** also clarified that an ethoxyl group was attached at the 8-position from the correlation of H8 and C1⁗. The other correlation data in the HMBC of **12** suggested the structure **12** to

be the compound **12**; however, the stereochemical relationship between the 7- and 8-positions is still unclear from the data.

Activity of the Identified Dimers in the Oxidation Product of Resveratrol. The lipoxygenase inhibitory activities of our four identified oxidation compounds of resveratrol were measured (Figure 3), the data showing that two resveratrol dimers (**7** and **10**) exert a concentration-dependent inhibitory activity from 10 to 100 μM, and their IC_{50} values were estimated to be 17 and 62 μM, respectively, whereas the dimer (**12**), the aldehyde (**2**), and resveratrol (**1**) did not show any activity under the same conditions. Although strong lipoxygenase inhibitory activities of the various resveratrol dimers and related oligomers have been reported,[13,20,21] we found that the opened-ring dimer (**12**) did not show any activity. These data indicated that the typical dihydrofuran ring system that existed in the dimers and oligomers of resveratrol was essential for the potent inhibitory activity. Some researchers have reported the potent lipoxygenase inhibitory activity of resveratrol itself;[13,21,22,28] however, we did not observe the same potency. Pinto and co-workers[29] reported a very interesting phenomenon concerning the lipoxygenase inhibitory activity of resveratrol.[29] They indicated that the oxidized resveratrol, which was produced from an in situ enzymatic reaction, contributed to the strong activity. Recently, Yang et al.[30] pointed out that the oxidation of resveratrol occurred in a cell culture medium and the oxidized resveratrol might alter the efficiency of some biological activities. We also observed the lipoxygenase inhibitory activity of the 2 week stock solution of resveratrol (data not shown), whereas the fresh solution of resveratrol had no activity. These data indicated that the oxidation of resveratrol, even if its oxidation efficiency was very low, dramatically changed its biological activity. Resveratrol has a very simple structure; however, it possesses a great variety of biological activities in vitro and in vivo. Pezzuto[31] recently suggested that further studies on the activity of its metabolites were required for biological understanding of resveratrol. Even in this lipoxygenase inhibitory activity in vitro, some oxidation products of resveratrol contributed the potent activity. Therefore, researchers should note these phenomena when using resveratrol.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Spectroscopic data for known compounds (**7** and **10**) and 2D NMR data for new compound (**12**). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Fax: 81-88-656-7244. E-mail: masuda@ias.tokushima-u.ac.jp.

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■ REFERENCES

(1) Masuda, T.; Akiyama, J.; Fujimoto, A.; Yamauchi, S.; Maekawa, T.; Sone, Y. Antioxidation reaction mechanism studies of phenolic lignans, identification of antioxidation products of secoisolariciresinol from lipid oxidation. *Food Chem.* **2010**, *123*, 442–450.

(2) Masuda, T.; Shingai, Y.; Fujimoto, A.; Nakamura, M.; Oyama, Y.; Maekawa, T.; Sone, Y. Identification of cytotoxic dimers in oxidation product from sesamol, a potent antioxidant of sesame oil. *J. Agric. Food Chem.* **2010**, *58*, 10880–10885.

(3) Fujimoto, A.; Shingai, Y.; Nakamura, M.; Maekawa, T.; Sone, Y.; Masuda, T. A novel ring-expanded product with enhanced tyrosinase inhibitory activity from classical Fe-catalyzed oxidation of rosmarinic acid, a potent antioxidative Lamiaceae polyphenol. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 7393–7396.

(4) Brash, A. R. Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *J. Biol. Chem.* **1999**, *274*, 23679–23682.

(5) Ridolfi, M.; Terenziani, S.; Patumi, M.; Fontanazza, G. Characterization of the lipoxygenases in some olive cultivars and determination of their role in volatile compounds formation. *J. Agric. Food Chem.* **2002**, *50*, 835–839.

(6) O'Donnell, V. B.; Maskrey, B.; Taylor, G. W. Eicosanoids: generation and detection in mammalian cells. *Methods Mol. Biol.* **2009**, *462*, 5–23.

(7) Menna, C.; Olivieri, F; Catalano, A.; Procopio, A. Lipoxygenase inhibitors for cancer prevention: promises and risks. *Curr. Pharm. Des.* **2010**, *16*, 725–733.

(8) Yamauchi, S.; Masuda, T.; Sugahara, T.; Kawaguchi, Y.; Ohuchi, M.; Someya, T.; Akiyama, J.; Tominaga, S.; Yamawaki, M.; Kishida, T.; Akiyama, K.; Maruyama, M. Antioxidant activity of butane type lignans, secoisolariciresinol, dihydroguaiaretic acid, and 7,7′-oxodihydroguaiaretic acid. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 2981–2986.

(9) Masuda, T.; Kirikihira, T.; Takeda, Y.; Yonemori, S. Thermal recovery of antioxidant activity from carnosol quinone, the main antioxidation product of carnosol. *J. Sci. Food Agric.* **2004**, *84*, 1421–1427.

(10) Gay, C. A; Gebicki, J. M. Perchloric acid enhances sensitivity and reproducibility of the ferric-xylenol orange peroxide assay. *Anal. Biochem.* **2002**, *304*, 42–46.

(11) Ha, T. J.; Kubo, I. Lipoxygenase inhibitory activity of anacardic acids. *J. Agric. Food Chem.* **2005**, *53*, 4350–4354.

(12) Yao, C.-S.; Lin, M.; Wang, Y.-H. Synthesis of the active stilbenoids by photoxidation reaction of *trans*-ε-viniferin. *Chin. J. Chem.* **2004**, *22*, 1350–1355.

(13) Ngoc, T. M.; Minh, P. T. H.; Hung, T. M.; Thuong, P. T.; Lee, I.; Min, B.-S.; Bae, K. Lipoxygenase inhibitory constituents from rhubarb. *Arch. Pharm. Res.* **2008**, *31*, 598–605.

(14) Wang, M.; Jin, Y.; Ho, C.-T. Evaluation of resveratrol derivatives as potential antioxidants and identification of a reaction product of resveratrol and 2,2-diphenyl-1-picrylhydrazyl radical. *J. Agric. Food Chem.* **1999**, *47*, 3974–3977.

(15) Nicotra, S.; Cramarossa, M. R.; Mucci, A.; Pagnoni, U. M.; Riva, S.; Forti, L. Biotransformation of resveratrol: synthesis of *trans*-dehydrodimers catalyzed laccases from *Myceliophtora thermophyla* and from *Trametes oubescens*. *Tetrahedron* **2004**, *60*, 595–600.

(16) Antolovich, M.; Bedgood, D. R., Jr.; Bishop, A. G.; Jardine, D.; Prenzler, P. D.; Robards, K. LC-MS investigation of oxidation products of phenolic antioxidants. *J. Agric. Food Chem.* **2004**, *52*, 962–971.

(17) Oszmianski, J.; Cheynier, V.; Moutounet, M. Iron-catalyzed oxidation of (+)-catechin in model system. *J. Agric. Food Chem.* **1996**, *44*, 1712–1715.

(18) Cilliers, J. J. L.; Singleton, V. L. Characterization of the products of nonenzymatic autoxidative phenolic reactions in a caffeic acid model system. *J. Agric. Food Chem.* **1991**, *39*, 1298–1303.

(19) Pirola, L; Fröjdö, S. Resveratrol: one molecule, many targets. *IUBMB Life* **2008**, *60*, 323–332.

(20) Cheng, K.-W.; Wang, M.; Chen, F.; Ho, C.-T. Oligostilbenes from *Gnetum* species and anticarcinogenetic and anti-inflammatory activities of oligostilbenes. In *Dietary Supplements*; Ho, C.-T., Simon, J. E., Shahidi, F., Shao, Y., Eds.; ACS Symposium Series 987; American Chemical Society: Washington, DC, 2008; pp 36−58.

(21) Kim, H.-J.; Chung, S.-K.; Choi, S.-W. Lipoxygenase inhibitors from *Paeonia lactiflora* seeds. *J. Food Sci. Nutr.* **1999**, *4*, 163–166.

(22) Ha, D. T.; Kim, H.; Thuong, P. T.; Ngoc, T. M.; Lee, I.; Hung, N. D.; Bae, K. Antioxidant and lipoxygenase inhibitory activity of oligostilbenes from leaf and stem of *Vitis amurensis*. *J. Ethnopharmacol.* **2009**, *125*, 304–309.

(23) Sako, M.; Hosokawa, H.; Ito, T.; Iinuma, M. Regioselective oxidation coupling of 4-hydroxystilbenes: synthesis of resveratrol and ε-viniferin (*E*)-dehydrodimers. *J. Org. Chem.* **2004**, *69*, 2598–2600.

(24) Takaya, Y.; Terashima, K.; Ito, J.; He, Y.-H.; Tateoka, M.; Yamaguchi, N.; Niwa, M. Biomimic transformation of resveratrol. *Tetrahedron* **2005**, *61*, 10285–10290.

(25) Yu, B.-B.; Han, X.-Z.; Lou, H.-X. Oligomers of resveratrol and ferulic acid prepared by peroxidase-catalyzed oxidation and their protective effects on cardiac injury. *J. Agric. Food Chem.* **2007**, *55*, 7753–7757.

(26) Wilkens, A.; Paulsen, J.; Wray, V.; Winterhalter, P. Structure of two novel trimeric stilbenes obtained by horseradish peroxidase catalyzed biotransformation of *trans*-resveratrol and (−)-ε-viniferin. *J. Agric. Food Chem.* **2010**, *58*, 6754–6761.

(27) Li, W.; Li, H.; Luo, Y.; Yang, Y.; Wang, N. Biosynthesis of resveratrol dimers by regioselective oxidative coupling reaction. *Synlett* **2010**, 1247–1250.

(28) Fan, X.; Matteis, J. P. Inhibition of oxidative and antioxidative enzymes by *trans*-resveratrol. *Food Chem. Toxicol.* **2001**, *66*, 200–203.

(29) Pinto, M. C.; Garcia-Barrado, J. A.; Macias, P. Resveratrol is a potent inhibitor of the dioxygenase activity of lipoxygenase. *J. Agric. Food Chem.* **1999**, *47*, 4842–4846.

(30) Yang, N.-C.; Lee, C.-H.; Song, T.-Y. Evaluation of resveratrol oxidation in vitro and the crucial role of bicarbonate ions. *Biosci., Biotechnol., Biochem.* **2010**, *74*, 63–68.

(31) Pezzuto, J. M. The phenomenon of resveratrol: redefining the virtues of promiscuity. *Ann. N.Y. Acad. Sci.* **2011**, *1215*, 123–130.