Synthesis of Selenium-Containing Polyphenolic Acid Esters and Evaluation of Their Effects on Antioxidation and 5-Lipoxygenase Inhibition

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Six novel selenium-containing polyphenolic acid esters were synthesized and evaluated as antioxidants and 5-lipoxygenase inhibitors. Synthesis of the title compounds involved the Mitsunobu reaction of polyphenolic acids (4—8, 14) with 2-phenylselenoethanol (3). Compounds 22, 23, and 25 were found to be very effective antioxidants and 5-lipoxygenase inhibitors with activity comparable to or better than caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE).

Key words polyphenolic acid ester; organoselenium compound; antioxidant; free radical scavenging activity; 5-lipoxygenase inhibition

Free radicals are highly reactive molecules generated predominantly during cellular respiration and normal metabolism. Imbalance between the cellular production of free radicals and the ability of cells to defend against them is referred to as oxidative stress (OS). Biological systems use enzymatic (superoxide dismutase, glutathione peroxidase, *etc.*) and nonenzymatic (uric acid, creatinine, polyamine, retinal, *etc.*) antioxidant systems to prevent OS. However, once the systems are disturbed the uncontrolled oxidative stresses initiate a series of harmful biochemical events or generate them as a consequence of earlier tissue injury, thus aggravating the final damages. Such damages include brain dysfunction, cancer, and cardiovascular disease and inflammation.[1,2]

Selenium is an essential trace element and has been known to be intimately involved in the activity of enzymes such as glutathione peroxidase (GSH Px) and thioredoxin reductase, which catalyze chemistry essential to the protection of biomolecules against OS and free radical damage.[3] Although possessing potent antioxidative activity, like other protein drugs GSH Px is limited for clinical use due to the instability and immunogenicity of the endogenous forms. To circumvent the intrinsic difficulties of using natural enzymes as drugs, several attempts have been made to produce synthetic compounds that mimic the properties of GSH Px. Ebselen,[4] 2-phenylbenzisoselenazole-3(2*H*)-one, was the most successful compound and showed anti-inflammatory, anti-atherosclerotic, and cytoprotective activities. These biological activities are attributed partly to the strong tendency of selenium to undergo redox reactions. Therefore, in the design of novel antioxidants as therapeutic agents there are a number of approaches utilizing the redox properties of selenium and incorporating selenium into the molecule skeleton. Review articles have summarized the synthesis and properties of a considerable number of organoselenium compounds prepared as potential pharmaceuticals.[5—7]

Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE, **1**; Fig. 1), a kind of polyphenolic acid, is an active component of propolis from honeybee hives. It has antiviral, anti-inflammatory, and immunomodulatory properties[8] and has been shown to inhibit the growth of different types of transformed cells.[8—13] In transformed cells, CAPE alters the redox state and induces apoptosis.[14] It has been reported that CAPE suppresses lipid peroxidation,[15] displays antioxidant activity,[16] and inhibits ornithine decarboxylase, protein tyrosine kinase (PTK), and lipoxygenase activities.[17—20] CAPE can also inhibit phorbol ester-induced H_2O_2 production and tumor promotion.[21,22]

In recent years, studies in our laboratory have been directed at synthetic antioxidants with diverse anti-oxidative functionalities.[23—25] Based on the unique anti-oxidative properties of selenium, we speculated that ester analogues of polyphenolic acid and phenylselenoethanol, such as **2** (Fig. 1) might provide synergistic antioxidative activity. In this study, a series of selenium-containing polyphenolic acid esters were prepared and their efficacies as radical scavengers, antioxidants, and inhibitors of 5-lipoxygenase were investigated as well.

Chemistry

Selenium-containing aliphatic alcohol (2-phenylselenoethanol; **3**) was prepared from 2-chloroethanol with phenylslenol produced *in situ* by a reduction of diphenyl diselenide with sodium borohydride (Chart 1). Although Fischer esteri-

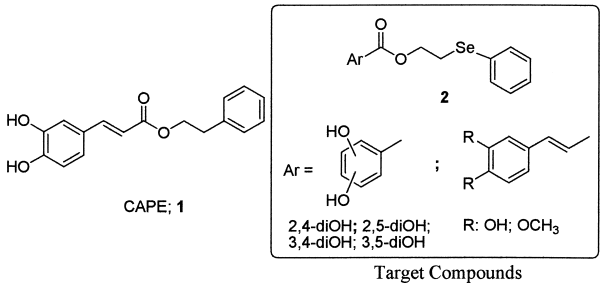

Fig. 1

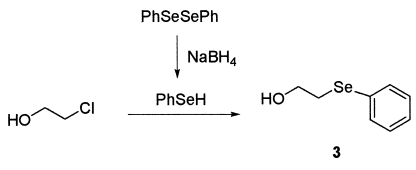

Chart 1

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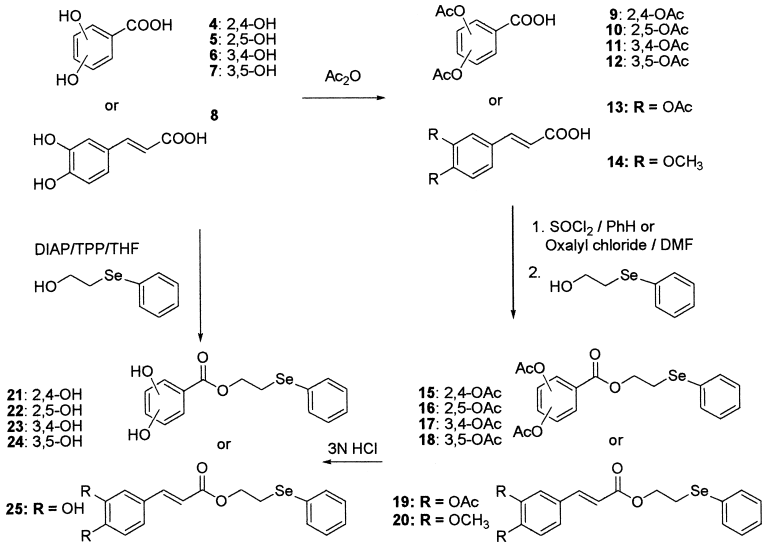

Chart 2

fication[9] of polyphenolic acid and aliphatic alcohol provides the ester products in one step, alternative synthetic methods are still needed because the harsh reaction conditions and the low yield make the esterification of limited applicability. A general synthetic route for the preparation of target compounds (**20**—**25**) is depicted in Chart 2. The phenolic hydroxyl groups of polyphenolic acids were protected from commercially available polyphenolic acids (**4**—**8**) by acetylation with acetic anhydride in pyridine in the dark at room temperature because acids activated *ex situ* (chlorides, anhydrides, and mixed anhydrides) in the next step showed poor chemoselectivity between phenolic and selenium-containing aliphatic alcohol. The acids were activated to the corresponding acid chlorides by reaction of **4**—**8** with freshly distilled thionyl chloride in dichlomethane at reflux temperature. The acid chloride intermediates were also obtained by using a reagent of oxalyl chloride instead of thionyl chloride. We though that oxalyl chloride should be superior to thionyl chloride because the boiling point of oxalyl chloride (63 °C) is lower than that of thionyl chloride (79 °C), so that after reaction the excess oxalyl chloride is easily removed. Without purification, the acid chlorides reacted with selenium-containing aliphatic alcohol to give the ester products. Removal of protective groups was then achieved in a process initiated by 3 N HCl hydrolysis of the esters to yield target compounds (**21**—**25**). Although the synthesis *via* acid chloride was straightforward, a one-step esterification in which acids react directly with alcohol is practical because of the troublesome protection and deprotection steps. The recent study by Appenedino *et al.*[26] indicated that the Mitsunobu reaction is especially suitable for the esterification of phenolic acids and alcohols. Because phenolic carbons are not substrates for the *SN*2-type reaction, the Mitsunobu reaction shows good chemoselectivity between hydroxyl groups bound to aliphatic and aromatic carbons. Thus, polyphenolic acids (**4**—**8**, **14**) were reacted directly with **3** in the presence of triphenylphosphine (TPP), diisopropyl azodicarboxylate (DIAD), and tetrahydrofuran to give the target compounds (**20**—**25**).

Table 1. Scavenging Activity of Antioxidants for DPPH· Radicals

Compound	SC_{50} (μM)[a]	Inhibition (%)[b]
20	610	5.6
21	490	6.7
22	70.5	40.9
23	100	27.8
24	290	8.9
25	76.3	36.9
CAPE	38	75.3

Data are shown as SC_{50} (μM) and % inhibition at 50 μM of antioxidants from two independent experiments. *a*) SC_{50}, The concentration of test compounds needed to reduce DPPH absorption by 50% at 517 nm. The values were calculated from the slope equation of the dose response curves. Values are means of three independent determinations. *b*) Inhibition (%), indicates the percent inhibition at 50 μM of antioxidant.

Results and Discussion

The reducing abilities of the examined compounds at different concentrations were determined by their interaction with 0.5 mM of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Antioxidants can react with DPPH and produce 1,1-diphenyl-2-picryl-hydrazine.[27] Due to its odd electron DPPH gives a strong absorption band at 517 nm. As this electron becomes paired in the presence of a free radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The change in absorbance produced in this reaction is assessed to evaluate the antioxidant potential of test samples, and this assay is useful as a primary screening system. The free radical scavenging profiles of the six novel selenium-containing polyphenolic acid esters and reference compound CAPE are shown in Table 1. The activities of these compounds, in the order of SC_{50} values from low to high was CAPE>**22**≈**25**>**23**>**24**≈**21**≈**20**. Chen *et al.*[28] reported that the structural requirement of polyphenolic acid for potent scavenging activity were compounds containing an *o*-dihydroxy group; and when the *o*-dihydroxy group was substituted with a methoxy group, the activity decreased dramatically. We also found this trend in activity in our study (**25**>**23**>**24**>**21**>**20**). It was of interest to note that without the *o*-dihydroxy group, **22** also showed an inhibition of free

Table 2. Antioxidant Activity of the Compounds against 2,2′-Azobis(2-amidinopropane) Dihydrochloride (AAPH)-Induced Lipid Peroxidation of a Tween-Emulsified Linoleic Acid System

Compound	Rate of peroxide formation (ΔA_{500}/min)	Inhibition (%)[a)]
CAPE	7.6×10^{-4}	76.9
22	6.7×10^{-4}	87.7
23	1.7×10^{-3}	43.3
25	9.3×10^{-4}	80.2

a) Inhibition of peroxidation (%)=(1−rate of test compound/rate of control)×100%. Peroxidation was initiated by the addition of AAPH (0.1 M) solution to the Tween-emulsified linoleic acid mixture. Degree of peroxidation was estimated by measuring absorption at 500 nm for the formation of complex $[Fe(SCN)]^{2+}$. All tests were performed in triplicate. Data shown here represent the slope of the time course-absorption curves of each compound analyzed. The control for the assay was carried out identically but in the absence of the test compound and the slope set as 100%.

radicals with activity similar to that of **25**.

To verify the antioxidative activity of these compounds, compounds **22**, **23**, and **25** were further analyzed for their capacity to inhibit the lipid peroxidation induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The *in vitro* model using AAPH-induced lipid peroxidation of Tween-emulsified linoleic acid is a common method[29)] used to measure the antioxidative activity of synthetic antioxidants. In this assay, the oxidation is carried out under conditions relatively similar to *in vivo* biological systems. The inhibitory effects on lipid peroxidation or the antioxidative activity of these compounds are listed in Table 2. The relative rate of lipid peroxidation initiated by AAPH radical is defined the antioxidative activity of test compounds. The smaller the rate of lipid peroxidation, the stronger the antioxidative activity. As shown in Table 2, the inhibitory activity of these compounds in decreasing order was **22**≈**25**≈CAPE>**23**, and compounds **22** and **25** exhibited similar anti-oxidative activity comparable to that of CAPE. Generally, these results are in accordance with the DPPH radical scavenging activity of these synthetic analogues.

The increasing evidence for a role of peroxynitrite in biological processes prompted us to investigate the reaction of compounds with peroxynitrite. Studies[30)] also demonstrated that polyphenolic and flavonoid compounds exhibited efficient peroxynitrite-scavenging activity and prevented oxidation of macromolecules elicited by peroxynitrite. In this study, the peroxynitrite-scavenging activities were determined according to the method reported by Balavoine and Geletii[31)] and Radi *et al.*[32)] Briefly, peroxynitrite induced the bleaching of Pyrogallol red (PR) dye, which was measured at 550 nm. Consumption of PR in the presence and absence of the test compounds was measured over a range of peroxynitrite concentrations (0—62.5 μM). Figure 2 shows the D_0/D_A data for different concentrations of antioxidant plotted against $[\text{antioxidant}]_0/[PR]_0$. D_0 and D_A are the stoichiometries for the reaction of peroxynitrite with PR in the absence and presence of the tested antioxidant compounds, respectively. k_A and k_{PR} are the rate constants for the reaction of peroxynitrite with the tested antioxidants and PR, respectively. The ratio k_A/k_{PR}, which represents the relative antioxidant activities, was calculated from the slope of the straight line plotting D_0/D_A=against $[A]_0/[PR]_0$. The greater the ratio of k_A/k_{PR}, the more potent the peroxynitrite-scavenging activity of the test compounds. In this study, compound **25** exhibited greater or comparable k_A/k_{PR} values than the standard compound CAPE (Fig. 2). The peroxynitrite-scavenging potency was in the order of **25**>CAPE>**22**>**23**, and is listed in Table 3. The data shown here for these compounds and CAPE are similar to the previously published data[31)] for caffeic acid, with $k_A/k_{PR}=0.29$.

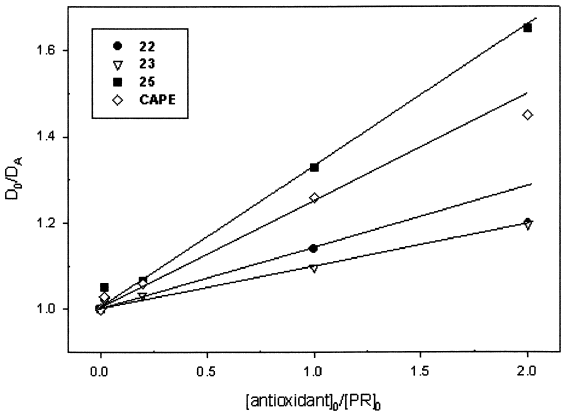

Fig. 2. Plot of D_0/D_A against $[\text{Antioxidant}]_0/[PR]_0$

Reactions were carried out at room temperature by adding peroxynitrite (12.5 μM) to tubes containing phosphate buffer (50 mM, pH 7.0) and 50 mM of $[PR]_0$. Results shown are the mean of two independent experiments.

Table 3. Inhibition of Test Compounds Peroxynitrite-Mediated Oxidation

Test compound	k_A/k_{PR}[a)]	Activity relative to CAPE
CAPE	0.25	1.00
22	0.15	0.60
23	0.1	0.40
25	0.34	1.36

a) The ratio k_A/k_{PR} represents the relative antioxidant activities and was calculated from the slope of the straight line plotting D_0/D_A against $[A]_0/[PR]_0$ from Fig. 1.

Caffeic acid and its synthetic derivatives, including CAPE, had been shown[19,33)] to inhibit efficiently the 5-, 12-, and 15-lipoxygenase activities that are suggested to be involved in cellular inflammation. To understand the anti-inflammatory effects of our Se-CAPE analogues, we analyzed the 5-lipoxygenase inhibitory activity of these compounds. We showed that our compounds, similar to CAPE, displayed typical uncompetitive inhibitory patterns for 5′-lipoxygenase. As shown in Fig. 3 with test compounds as inhibitor and linoleate as the varied-concentration substrate, a series of parallel lines were obtained, conforming to an uncompetitive inhibition pattern. A straight line can be obtained by replotting the concentration of the inhibitory test compounds and the vertical intercept ($1/V_{max}$), and the K_i value was determined from the horizontal intercept of the replotted figure and listed in Table 4. The representative inhibitory pattern and kinetic replot were shown in Fig. 3 for CAPE and compound **25**. Compounds **22** and **23** displayed almost identical results to that of compound **25** or CAPE. Our results demonstrate that compounds **22**, **23**, and **25** exhibit an inhibitory constant (K_i) against 5′-lipoxygenase that is similar to that of CAPE and the IC_{50} are almost identical to those of caffeic acid derivatives as described.[33)] The uncompetitive inhibitory patterns of these series of compounds demonstrate that they are nonspecific antioxidants. Caffeic acid derivatives were shown to be effective inhibitors of 5-, 12-, and 15-lipoxyge-

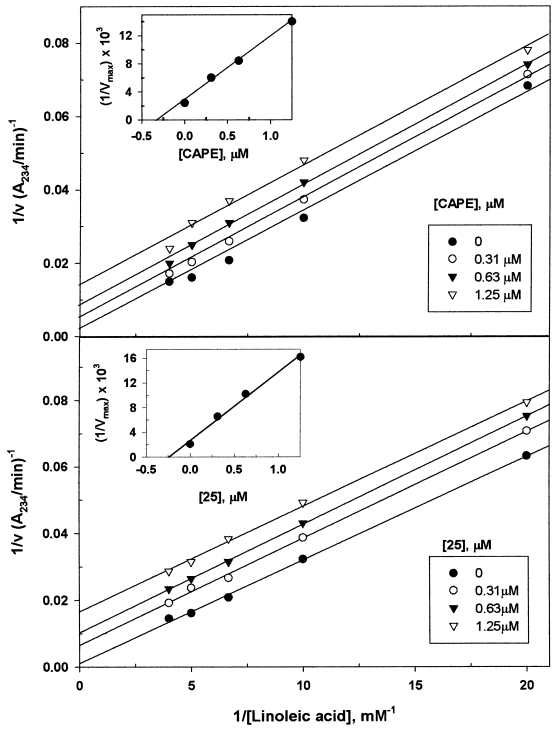

Fig. 3. Inhibition of 5-Lipoxygenase by CAPE and Compound **25**

Enzymatic activity was assayed as described in Experimental except that linoleate and test compounds were varied as indicated. From bottom to top, the test compound concentrations were as indicated. The inset shows the vertical intercept replotted against concentrations of test compounds. Compounds **22** and **23** exhibited identical inhibitory patterns and kinetic parameters against 5′-lipoxygenase as shown in this figure.

Table 4. IC_{50} and K_i Values of the Iinhibitory Effects against 5-Lipoxygenase

Compound	IC_{50} (μM)[a)]	K_i (μM)[b)]
22	0.18	0.27
23	0.21	0.29
25	0.24	0.22
CAPE	0.22	0.30

a) The test compound concentration that gave 50% inhibition of enzyme activity. *b*) The horizontal intercept was obtained from the plot of inhibitor concentrations against $1/V_{max}$, as shown in the inset of Fig. 2.

nases.[19] Therefore, these compounds are lipoxygenase inhibitors with antioxidant activity and with action mechanisms similar to that of CAPE. Based on this point, it is justified to observe that these compounds showed almost identical inhibitory constants and IC_{50} values.

Conclusion

Interest in the health-promoting effects of polyphenolic acid esters, including CAPE, and the unique chemopreventive properties of selenium prompted us to prepare ester analogues of polyphenolic acid and phenylselenoethanol and determine their antioxidant activities. We measured the antioxidant capacity of the six test compounds in terms of their effects on DPPH free radical scavenging, AAPH-induced lipid peroxidation as well as scavenging of peroxynitrite radical. The inhibitory effects of these compounds against 5-lipoxygenase were also analyzed in addition to the antioxidative activity. Among the compounds analyzed, **22**, **23**, and **25** are the most potent, with antioxidant activities comparable to or better than those of CAPE. These three compounds also had inhibitory activity and acted in an uncompetitive manner on 5-lipoxygenase. The role of selenium in the enhanced activities on inhibiting AAPH-induced lipid peroxidation and peroxynitrite-scavenging activities requires further investigation.

Experimental

Melting points (mp) were recorded on a BUCHI 530 apparatus and are uncorrected. Merck Art No.105554 plates precoated with Silica gel 60 containing a fluorescent indicator were used for thin-layer chromatography, and Silica gel 60 (Merck Art No 109385, 230—400 mesh) was employed for column chromatography. Evaporations were carried out at <50 °C using a rotary evaporator at reduced pressure (water aspirator). ^{1}H- and ^{13}C-NMR spectra were obtained with a Varian 300 NMR spectrometer at 300 and 75 MHz, respectively. Where necessary, deuterium exchange experiments were used to obtained proton shift assignments. Mass spectra were recorded on a JEOL J.M.S-300 spectrophotometer. Analytical samples were dried under reduced pressure at 78 °C in the presence of P_2O_5 for at least 12 h unless otherwise specified. Elemental analyses were obtained using a Perkin-Elmer 2400 Elemental Analyzer.

2-Phenylselenoethanol (3) Sodium borohydride (0.5 g, 15 mmol) was added in portions to a stirred solution of diphenyl diselenide (1.5 g, 5 mmol) in absolute ethanol at 0 °C. To the resulting colorless solution was added a solution of the appropriate 2-chloroethanol (0.7 ml, 10 mmol) dissolved in the minimum quantity of ethanol. The mixture was stirred under reflux for 3 h. Solvent was filtered and removed in a vacuum. The residue was purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (4/1) to give 2-phenylselenoethanol (0.96 g, 96%) as pale yellow oil: *Rf*: 0.2 (*n*-hexane/ethyl acetate=4/1). ^{1}H-NMR (DMSO-d_6) δ: 7.487.21 (m, 5H, ArH), 4.95 (s, 1H, OH), 3.60 (t, 2H, J=6.4 Hz, CH_2), 3.00 (t, 2H, J=14.2 Hz, $SeCH_2$). ^{13}C-NMR (DMSO-d_6) δ: 131.2, 130.2, 129.2, 126.4, 60.7, 29.4. FAB-MS *m/z* 202 ($M+H^+$). *Anal.* Calcd for $C_8H_{10}OSe$: C, 47.77; H, 5.01. Found: C, 47.67; H, 5.03.

Acetylation of Phenols of Polyphenolic Acid, General Procedure to Obtain Compounds 9—13 To a solution of polyphenolic acid (20 mmol) was added acetic anhydride (6 eq) and pyridine (2 ml). The mixture was stirred at room temperature in the dark for 4 h and then poured onto 1 M H_3PO_4 (10 ml) cold solution. The mixture was extracted with ethyl acetate. The layers were washed with brine and aqueous saturated sodium bicarbonate. The combined organic phase was dried under magnesium sulfate, filtered, and the solvent was removed under a vacuum. The residue was crystallized from proper solvent to afford the corresponding acetoxy polyphenolic acids: 2,4-diacetoxy benzoic acid (**9**), 2,5-diacetoxy benzoic acid (**10**), 3,4-diacetoxy benzoic acid (**11**), 3,5-diacetoxy benzoic acid (**12**), and 3,4-diacetoxy cinnamic acid (**13**). These acetoxy polyphenolic acids were identified by melting point and ^{1}H-NMR. Data were in agreement with literature values.[34,35]

Esterification of Acetoxy Polyphenolic Acids with 2-Phenylselenoethanol *via* Thionyl Chloride, General Procedure to Obtain Compounds 15—20 To a solution of acetoxy polyphenolic acid (4 mmol) in dry dichlomethane (20 ml) was added thionyl chloride (5 ml) and the mixture was refluxed for 3 h. The mixture was concentrated, and was added dry dichlomethane (20 ml), dimethylamino pyridine (0.1 eq), pyridine (1 ml), and 2-phenylselenoethanol (1.2 eq) were added at 0 °C. The reaction mixture was stirred for 3 h and poured onto ice water. The mixture was extracted three times with ethyl acetate. The organic layers were washed with brine and water. The combined organic phase was dried under magnesium sulfate, filtered, and the solvent was removed in a vacuum. Without purification, the residue was used for the next reaction.

Esterification of Acetoxy Polyphenolic Acids with 2-Phenylselenoethanol *via* Oxalyl Chloride, General Procedure to Obtain Compounds 15—20 To a solution of acetoxy polyphenolic acid (5 mmol) and in dry dichlomethane (10 ml), oxalyl chloride (0.7 ml) was added and the mixture was stirred for 8 h at room temperature. The mixture was concentrated, and dry dichlomethane (10 ml), triethylamine (5 ml), and 2-phenylselenoethanol (1.2 eq) were added at 0 °C. The reaction mixture was stirred over night and then poured onto ice water. The mixture was extracted three times with ethyl acetate. The combined organic phase was dried under magnesium sulfate, filtered, and the solvent was removed in a vacuum. Without purification, the residue (**15**—**19**) was used for the next reaction.

3,4-Dimethoxy-cinnamic Acid-(2-phenylseleno-ethyl ester) (20) The residue was purified by flash chromatography on silica gel with CH_2Cl_2/*n*-hexane (9/1) to give the product as a pale yellow powder in a yield of 79%:

Rf: 0.34 (CH_2Cl_2/*n*-hexane=9/1). mp 113—115 °C. ^{1}H-NMR (DMSO-d_6): δ: 7.54—7.19 (m, 8H, ArH), 6.97 (d, 1H, J=9.9 Hz, COCH), 6.48 (d, 1H, J=15.9 Hz, CH), 4.33 (t, 2H, J=12 Hz, $COOCH_2$), 3.78 (s, 6H, CH_3), 3.22 (t, 2H, J=13.8 Hz, $SeCH_2$). ^{13}C-NMR (DMSO-d_6): δ: 166.3, 151.2, 149.1, 145.0, 131.7, 129.5, 126.9, 123.0, 115.3, 111.8, 110.7, 63.3, 55.8, 25.0. IR (KBr) cm^{-1}: 2954, 1701, 1134. UV λ_{max} (MeOH) nm (log ε): 207 (4.26). FAB-MS *m/z*: 392 (M+H$^+$). *Anal.* Calcd for $C_{15}H_{14}O_4Se$: C, 58.32; H, 5.15. Found: C, 58.15; H, 5.14.

Hydrolysis of the Acetate of the Polyphenolic Acid Esters, General Procedure to Obtain Compounds 21—25 A solution of the polyphenolic acid esters (5 mmol) was dissolved in a mixture of THF (30 ml) and aqueous 3 N HCl (30 ml) at room temperature. The reaction mixture was stirred for 2 d at room temperature. The mixture was extracted three times with ethyl acetate. The organic layers were washed with brine and water. The combined organic phase was dried under magnesium sulfate, filtered, and the solvent was removed in a vacuum to give the residue.

Mitsunobu Esterification of Acetoxy Polyphenolic Acids with 2-Phenylselenoethanol To a solution of polyphenolic acids (6 mmol) and 2-phenylselenoethanol (6 mmol) in dry tetrahydrofuran (15 ml) were added TPP (6 mmol) and DIAP (6 mmol) at 0 °C. After stirring at room temperature for 48 h, the reaction was worked up by removal of the solvent, and the residue was partitioned between ethyl acetate and saturated $NaHCO_3$. The organic phase was washed with brine and then dried over Na_2SO_4, and the solvent was evaporated. The residue was purified by flash chromatography on a silica gel column.

2,4-Dihydroxy-benzoic Acid-(2-phenylseleno-ethyl ester) (21) The residue was purified by flash chromatography on silica gel with dichlomethane/*n*-hexane (2/1) to give the product as pale yellow oil in a yield of 68%: *Rf*: 0.27 (dichlomethane/*n*-hexane=2/1). ^{1}H-NMR (DMSO-d_6) δ: 10.51 (s, 1H, OH), 10.42 (s, 1H, OH), 7.54—6.26 (m, 8H, ArH), 4.45 (t, 2H, J=13.2 Hz, $COOCH_2$), 3.29 (t, 2H, J=10.2 Hz, $SeCH_2$). ^{13}C-NMR (DMSO-d_6) δ: 168.8, 164.4, 162.8, 131.8, 129.4, 126.9, 108.4, 103.9, 102.5, 64.1, 24.9. IR (KBr) cm^{-1}: 3416, 3090, 1666, 1095. UV λ_{max} (MeOH) nm (log ε): 208 (4.41). FAB-MS *m/z*: 338 (M+H$^+$). *Anal.* Calcd for $C_{15}H_{14}O_4Se$: C, 53.42; H, 4.18. Found: C, 53.75; H, 4.51.

2,5-Dihydroxy-benzoic Acid-(2-phenylseleno-ethyl ester) (22) The residue was purified by flash chromatography on silica gel with *n*-hexane/dichlomethane/ethyl acetate (4/1/1) to give the product as pale yellow oil in a yield of 56%: *Rf*: 0.55 (*n*-hexane/dichlomethane/ethyl acetate=4/1/1). ^{1}H-NMR ($CDCl_3$) δ: 7.63—6.88 (m, 8H, ArH), 4.61 (t, 2H, J=13.8 Hz, $COOCH_2$), 3.26 (2 t, H, J=17.1 Hz, $SeCH_2$). ^{13}C-NMR ($CDCl_3$) δ: 169.2, 155.8, 147.7, 133.1, 129.3, 129.2, 127.4, 127.3, 125.1, 118.4, 114.6, 64.7, 25.2. IR (KBr) cm^{-1}: 3425, 3120, 1676, 1076. UV λ_{max} (MeOH) nm (log ε): 213 (4.35). FAB-MS *m/z*: 338 (M+H$^+$). *Anal.* Calcd for $C_{15}H_{14}O_4Se$: C, 53.42; H, 4.18. Found: C, 53.08; H, 4.30.

3,4-Dihydroxy-benzoic Acid-(2-phenylseleno-ethyl ester) (23) The residue was purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate/acetic acid (70/29/1) to give the product as pale yellow powder in a yield of 50%: *Rf*: 0.33 (*n*-hexane/ethyl acetate/acetic acid=70/29/1). mp 90—92 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.61—6.89 (m, 8H, ArH), 4.52 (t, 2H, J=14.4 Hz, $COOCH_2$), 3.23 (t, 2H, J=13.2 Hz, $SeCH_2$). ^{13}C-NMR ($CDCl_3$) δ: 166.0, 156.2, 148.8, 132.9, 129.1, 127.1, 123.6, 116.5, 114.6, 64.0, 25.6. IR (KBr) cm^{-1}: 3253, 3023, 1738, 1119. UV λ_{max} (MeOH) nm (log ε): 206 (2.84). FAB-MS *m/z*: 338 (M+H$^+$). *Anal.* Calcd for $C_{15}H_{14}O_4Se$: C, 53.42; H, 4.18. Found: C, 53.13; H, 4.02.

3,5-Dihydroxy-benzoic Acid-(2-phenylseleno-ethyl ester) (24) The residue was purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (9/1→4/1→1/1) to give the product as pale yellow oil in a yield of 52%: *Rf*: 0.45 (*n*-hexane/ethyl acetate=1/1). ^{1}H-NMR ($CDCl_3$) δ: 7.62—6.99 (m, 8H, ArH), 4.55 (t, 2H, J=14 Hz, $COOCH_2$), 3.24 (t, 2H, J=14 Hz, $SeCH_2$). ^{13}C-NMR ($CDCl_3$) δ: 165.9, 157.0, 156.4, 153.0, 133.0, 132.0, 129.1, 127.1, 108.9, 107.5, 64.5, 25.4. IR (KBr) cm^{-1}: 3410, 3080, 1650, 1080. UV λ_{max} (MeOH) nm (log ε): 207 (4.10). FAB-MS *m/z*: 338 (M+H$^+$). *Anal.* Calcd for $C_{15}H_{14}O_4Se$: C, 53.42; H, 4.18. Found: C, 53.49; H, 4.11.

3,4-Dihydroxy-cinnamic Acid-(2-phenylseleno-ethyl ester) (25) The residue was purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (9/1→4/1→1/1) to give the product as pale yellow powder in a yield of 57%: *Rf*: 0.45 (*n*-hexane/ethyl acetate=1/1). mp 70—72 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.61—7.09 (8m, H, ArH), 7.01 (d, 1H, J=10.2 Hz, COCH), 6.90 (d, 1H, J=10.2 Hz, CH), 4.45 (t, 2H, J=14.7 Hz, $COOCH_2$), 3.19 (2H, t, J=14.7 Hz, $SeCH_2$). ^{13}C-NMR ($CDCl_3$) δ: 166.9, 156.3, 146.4, 145.0, 144.0, 132.9, 129.1, 127.5, 127.2, 122.3, 115.4, 115.2, 114.2, 63.7, 25.5. IR (KBr) cm^{-1}: 3251, 2979, 1737, 1109. UV λ_{max} (MeOH) nm (log ε): 209 (3.15). FAB-MS *m/z*: 364 (M+H$^+$). *Anal.* Calcd for $C_{15}H_{14}O_4Se$: C, 56.21; H, 4.44. Found: C, 56.01; H, 4.08.

Determination of the Scavenging Effect on 1,1-Diphenyl-2-pycryl-hydrazyl Radical (DPPH·) The ethanolic solution of DPPH·[27] was added to 2 ml of the test compounds at different concentrations in ethanol (12.5, 25, 37.5, 50 μM). Each mixture was then shaken vigorously and kept for 30 min at room temperature in the dark. The decrease in absorption of DPPH· at 517 nm was measured. Ethanol was used as a blank solution and DPPH· solution in ethanol served as the control. The percentage of remaining DPPH· was then calculated, and the radical-scavenging effects of the test compounds were compared in terms of IC_{50} (the concentration needed to reduce 50% of the initial amount of DPPH· and expressed as the molar ratio of each compound to the radical). All tests were performed in triplicate.

Determination of Antioxidative Activity The antioxidative activity was evaluated using AAPH-induced lipid peroxidation of a Tween-emulsified linoleic acid system and measured by the ferric thiocyanate assay as described.[28] Briefly, 0.2 ml of distilled water, 0.5 ml of 0.2 M phosphate buffer (pH 7.0), and 0.5 ml of 0.25% Tween-20 (in buffer solution) were mixed with 0.5 ml of 2.5% (w/v) linoleic acid in ethanol. The mixture was then stirred for 1 min. The peroxidation was initiated by the addition of 50 μl of AAPH solution (0.1 M). The stock solution of antioxidant or test compounds in DMSO (final concentrations for the test compounds and DMSO are 10^{-4} M and 0.1%, respectively) was then added, and the reaction was carried out at 37 °C for 375 min in the dark. The degree of inhibition of oxidation was measured by the ferric thiocyanate method for each interval of 75, 150, 225, 300, and 375 min. To 0.1 ml of peroxidation reaction mixture at each interval, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 2×10^{-2} M freshly prepared $FeCl_2$ (in 3.5% aqueous HCl) were added. Precisely 3 min after addition, the absorbance of the red complex $[Fe(SCN)]^{2+}$ was measured at 500 nm. The control for the assay was prepared in the same manner by mixing all of the chemicals and reagents except the test compound. All tests were performed in triplicate.

Determination of the Scavenging Effect on Peroxynitrite Peroxynitrite synthesis was carried out as described by Radi *et al.*[32] Briefly, acidified hydrogen peroxide (1 M in 0.7 M HCl, 20 ml) and sodium nitrite (0.2 M, 20 ml) solution were drawn into two separate syringes. The contents of both syringes were simultaneously injected into an ice-cooled beaker containing 1.5 M potassium hydroxide (40 ml). Manganese dioxide was added to the solution to remove excess hydrogen peroxide. The solution was filtered and the concentration of the resulting stock was determined spectrophotometrically at 302 nm (ε=1670 M^{-1} cm^{-1}). The typical yield of freshly prepared peroxynitrite was 30 mM. The peroxynitrite was diluted in 0.1 M NaOH. Experiments were conducted at 25 °C in 50 mM phosphate-buffered saline containing 1 mM diethylenetriaminepentaacetic acid, 90 mM NaCl, and 5 mM KCl, pH 7.4. Blanks using DMSO alone in the absence of test compounds and peroxynitrite allowed to degrade for 5 min in phosphate-buffered saline, pH 7.4, were also examined. There was no interference by DMSO and degraded peroxynitrite on the PR. Peroxynitrite induced the bleaching of PR dye, which was measured at 542 nm (ε=24000 M^{-1} cm^{-1}). Consumption of PR (50 μM) in the presence and absence of test compounds (1.25—125 μM) was measured over a range of peroxynitrite concentrations (0—62.5 μM). Antioxidative activities were determined according to the methods reported by Balavoine *et al.*[31] The ratios of rate constants k_A/k_{PR}, which represent the relative antioxidant activities, were determined by plotting D_0/D_A against $[\text{antioxidant}]_0/[PR]_0$. k_A and k_{PR} are the rate constants for reaction of peroxynitrite with the antioxidants and PR, respectively. D_0 and D_A are the stoichiometries for the reaction of peroxynitrite with PR in the absence and presence of the antioxidant compounds, respectively.

Assay of 5′-Lipoxygenase Activity The rate of the 5′-lipoxygenase-catalyzed formation of linoleic acid hydroperoxides was monitored spectrophotometrically at 234 nm.[19] The reaction was started by addition of the substrate to the otherwise complete assay mixture. The standard reaction mixture contained 0.27 μg/ml of 5-lipoxygenase, 100 μM of ammonium linolete, and 50 mM sodium phosphate, pH 6.8. The inhibition study was performed by adding CAPE and its structural analogues to the reaction mixture, and the resulting enzyme activity was compared with that of the control without inhibitor added. Inhibition with the test compounds was analyzed by varying the concentration of linoleate substrate from 0.05 to 0.25 mM, and test compound from 0 to 1.25 μM. Concentrations of other components were held constant.

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