Evaluation of Resveratrol Derivatives as Potential Antioxidants and Identification of a Reaction Product of Resveratrol and 2,2-Diphenyl-1-picryhydrazyl Radical

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Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), an antioxidant from grapes, and five other polyhydroxystilbenes were synthesized. Their antioxidative properties were evaluated in two model systems [pure lipid oxidation using the Rancimat method and 2,2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging model.] 3,3′,4,5′-Tetrahydroxystilbene, 3,3′,4,5,5′-pentahydroxystilbene, and 3,4,4′,5-tetrahydroxystilbene were found to be more active than resveratrol in both models. A dimer of resveratrol was identified as the major radical reaction product when resveratrol was reacted with DPPH radicals.

Keywords: *Resveratrol derivatives; polyhydroxystilbene; antioxidant; 2,2-diphenyl-1-picryhydrazyl; free radical scavenging*

INTRODUCTION

It was suggested recently that generation of free radicals plays a major role in the progression of a wide range of pathological disturbance such as brain dysfunction, cancer, and cardiovascular disease and inflammation (Huong et al., 1998; Haraguchi et al., 1997). In the food industry, free radicals are also found to be responsible for lipid oxidation, which is a major determinant in the deterioration of foods during processing and storage (Nunez-Delicado et al., 1997; Chen and Ho, 1997; Dziedzic et al., 1985). Due to these facts, considerable interest has been given to the addition of antioxidants in food and biological systems to scavenge free radicals. A lot of natural compounds have been found to be antioxidants, including vitamin E, flavonoids, phenolic acids, chlorophyll derivatives, and carotenoids (Larson 1988).

Recently resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a natural product derived from grapes, was found to be antioxidative, antimutagenic, and an inducer of phase II drug-metabolizing enzymes (Jang et al., 1997). Resveratrol belongs to a class of compounds called stilbenes, which are widely distributed in nature. Interest in the synthesis of stilbene compounds stems from the discovery of many natural stilbene compounds as antioxidative, antifungal, ichthyotoxic, antinitotic, and antileukemic agents (Pettit et al., 1989, 1995; Cushman et al., 1991; Hata et al., 1979). In the present study, we synthesized resveratrol and five other polyhydroxystilbenes and evaluated their antioxidative properties. We used the Rancimat method to test antioxidative activity in pure lard and 2,2-diphenyl-1-picryhydrazyl (DPPH) to test free radical scavenging activity. The six stilbene compounds we studied were 3,5-dihydroxy-*trans*-stilbene (**1**), 3,4′,5-trihydroxy-*trans*-stilbene (**2**), 3,3′,4,5′-tetrahydroxy-*trans*-stilbene (**3**), 3,4,4′,5-tetrahydroxy-*trans*-stilbene (**4**), 3,3′,5,5′-tetrahydroxy-*trans*-stilbene (**5**), and 3,3′,4,5,5′-pentahydroxy-*trans*-stilbene (**6**). The goal of this study was to find antioxidative stilbene derivatives more active than resveratrol and to use them to prevent free radical-related problems, either to benefit pathological disturbance or to prevent lipid oxidation. To examine the scavenging free radical process of stilbene compounds, the stable radical termination product was purified and identified.

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MATERIALS AND METHODS

General Procedures. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Gemini-200 instrument (Varian Inc., Melbourne, Australia) at 200 and 50 MHz, respectively. CH_3-OH-d_4 was used as a solvent, and chemical shifts were expressed in parts per million (δ). ^{13}C NMR multiplicity was determined by APT experiment. Chemical shifts were expressed in ppm. Electron impact (EI) mass spectra were recorded on a Finnigan MAT-90 instrument (Finnigan Inc., San Jose, CA), and atmospheric pressure chemical ionization (APCI)-MS was obtained from a Fisons/VG Plaform II mass spectrometer. Thin-layer chromatograghy was performed on Sigma−Aldrich silica gel TLC plates (250 μm thickness, 2−25 μm particle size; Aldrich, Milwaukee, WI) with compounds visualized by spraying with 5% (v/v) H_2SO_4 in an ethanol solution. Benzyl alcohol, 4-methoxybenzyl alcohol, 3,4-dimethoxybenzyl alcohol, 3,5-dimethoxybenzyl alcohol, 3,5-dimethoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, triethyl phosphite, sodium methoxide, pyridine hydrochloride, benzene, DMF, 2,2-diphenyl-1-picryhydrazyl (DPPH), silica gel (130−270 mesh), and TLC plates were purchased from Aldrich Chemical Co. (Milwaukee, WI), All organic solvents and pure lard (pork fat) were obtained from Fisher Scientific (Springfield, NJ).

General Synthetic Procedure for Resveratrol. All of the stilbene derivatives were prepared by the method according to literature (Bachelor et al., 1970; Drewes et al., 1973) as shown in Figure 1.

Synthesis of Resveratrol. 4-Methoxybenzyl alcohol (10 g) was dissolved in dry benzene, and excess dry HBr was bubbled into the solution with stirring over a period of 20 min. Then the solution was heated to 78 °C and allowed to stand for 1 h. Water (100 mL) was added to remove HBr, and the benzene

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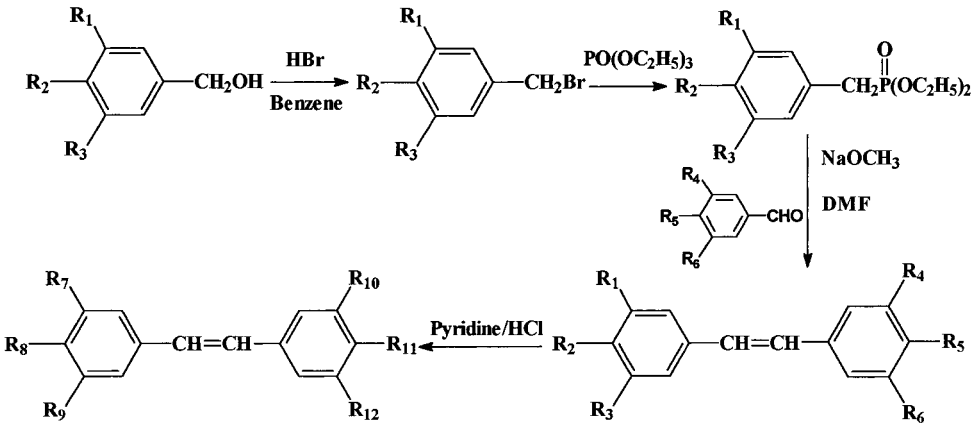

Figure 1. General synthetic scheme for stilbene derivatives.

layer was dried by $MgSO_4$. Evaporation of the benzene gave a quantitative yield of slightly colored oil of 4-methoxybenzyl bromide.

4-Methoxybenzyl bromide (3.8 g) was heated with excess triethyl phosphite (4.5 mL) to 130 °C until the evolution of ethyl bromide had ceased. Then the solution was cooled to 0 °C, and 25 mL of DMF and 1.1 g of sodium methoxide were added to it. To this solution 2.1 g of 3,5-dimethoxybenzaldehyde was added and allowed to stand at room temperature for 1 h. The reaction mixture was then heated to 100 °C, allowed to stand at this temperature for 1 h, and then kept at room temperature overnight. Water−methanol (2:1, 40 mL) was added, and the precipitated stilbene was collected by filtration and washed with water. The purity of this stilbene was detected by TLC (hexane:ethyl acetate 4:1); yield 3.2 g (95%).

Excess pyridine hydrochloride (11 g) and 3,4′,5-trimethoxystilbene (2 g) were mixed and heated to 190 °C for 4 h. The hot dark syrup was poured into 50 mL of 2 N HCl, and the reaction mixture was extracted with ethyl acetate (3 × 100 mL). The ethyl acetate layer was dried with $MgSO_4$, and then ethyl acetate was removed under reduced pressure. The residual was purified by column chromatography on silica gel eluted with chloroform−methanol (15:1) to get 3,4′,5-trihydroxystilbene 0.76 g (45%). MS *m/z*: 228 (M^+), 211, 199, 181, 157, 115.

Other stilbene compounds were synthesized in the same manner as 3,4′,5-trihydroxystilbene using the corresponding benzyl alcohol and benzaldehyde instead of 4-methoxybenzyl alcohol and 3,5-dimethoxybenzaldehyde.

*3,5-Dihydroxystilbene (**1**).* ^{1}H NMR (CD_3OD): 7.50 (2H, d, J = 7.3 Hz), 7.33 (2H, t, J = 7.3 Hz), 7.22 (1H, t, J = 7.3 Hz), 7.04 (1H, d, J = 16.6 Hz), 7.00 (1H, d, J = 16.6 Hz), 6.49 (2H, d, J = 2.1 Hz), 6.20 (1H, t, J = 2.1 Hz). MS *m/z*: 212 (M^+), 197, 165, 141, 115, 77.

*3,3′,5, 5′-Tetrahydroxystilbene (**3**).* ^{1}H NMR (CD_3OD): 6.86 (2H, s), 6,44 (4H, d, J = 2.1 Hz), 6.18 (2H, t, J = 2.1 Hz). MS *m/z*: 244 (M^+), 226, 197, 173, 160, 160, 141, 115.

*3,3′,4,5′-Tetrahydroxystilbene (**4**).* ^{1}H NMR (CD_3OD): 6.98 (1H, d, J = 2.1 Hz), 6.88 (1H, d, J = 16.3 Hz), 6.83 (1H, dd, J = 2.1, 8.0 Hz), 6.73 (1H, d, J = 16.3 Hz), 6.72 (1H, d, J = 8.0 Hz), 6.44 (2H, d, J = 2.1 Hz), 6.15 (1H, t, J = 2.1 Hz). MS *m/z*: 244 (M^+), 197, 173, 149, 123, 115.

*3,4,4′,5-Tetrahydroxystilbene (**5**).* ^{1}H NMR (CD_3OD): 7.30 (2H, d, J = 8.4 Hz), 6.80 (1H, d, J = 16.0 Hz), 6.74 (1H, d, J = 8.4 Hz), 6.72 (1H, d, J = 16.4 Hz), 6.50 (2H, s). MS *m/z*: 244 (M^+), 197, 169, 141, 115.

*3,3′,4,5,5′-Pentahydroxystilbene (**6**).* ^{1}H NMR (CD_3OD): 6.80 (1H, d, J = 16.2 Hz), 6.69 (1H, d, J = 16.2 Hz), 6.52 (2H, s), 6.41 (2H, d, J = 2.1 Hz), 6.14 (1H, t, J = 2.1 Hz). MS *m/z*: 260 (M^+), 244, 213, 197, 123, 108, 80.

Evaluation of the Inhibitory Effect on Lipid Oxidation by the Rancimat Method. Pure lard (pork fat) was used as the lipid substrate to evaluate the lipid oxidation inhibitory activity of the six stilbene derivatives and BHT. A Metrohm 679 Rancimat instrument (Herisan, Switzerland) was used in this experiment. Two micromoles (100 μL of 20 mM methanol solution) of the above compounds was mixed with 2.5 g of lipid in different glass cylinders. A total of 100 μL of methanol was also added to all cylinders, including the control. The air supply was maintained at 20 mL/min, and the temperature was kept at 110 °C. All tests were run in triplicate, which were averaged during data analysis.

Determination the Scavenging Effect on DPPH Radicals. In the 1.0×10^{-4} M ethanol solution of DPPH, test compounds and six stilbenes were added, and their final concentrations were 20 μM. Then the samples were shaken vigorously and kept in the dark for 0.5 h. The absorption of the samples was measured on a spectrophotometer (Milton Roy, model 301) at 517 nm against a blank of ethanol without DPPH. All tests were run in triplicate and averaged (Chen and Ho, 1995).

Analysis of Radical Reaction Product of DPPH and Resveratrol. One millimole of resveratrol and 1 mmol of DPPH were added to 50 mL of methanol. The mixture was shaken vigorously and kept in the dark for 0.5 h. Then methanol was removed under reduced pressure at 50 °C. The residue was subjected to silica gel (50 g) chromatography, which was performed with chloroform (200 mL), followed by chloroform−methanol (20:1, 10:1, 7:1, 5:1, and 1:1 each 200 mL). The fraction obtained by using chloroform−methanol (7:1) as the mobile phase and was further subfractionated by a column chromatography (20 × 50 mm) on Sephadex LH-20 (50 g, Pharmacia Biotech, Piscataway, NJ) using methanol as the mobile phase. The major compound (40 mg) was obtained after elution of 800 mL of methanol.

^{1}H NMR (200 MHz, in CD_3OD): 7.36 (1H, d, J = 8.4 Hz, H-6D), 7.19 (1H, s, H-2D), 7.17 (2H, d, J = 8.4 Hz, H-2A and H-6A), 6.98 (1H, d, J = 16.5 Hz, H-β), 6.82 (4H, m, H-3A, 5A, 5D, α), 6.46 (2H, d, J = 2.1 Hz, H-2E, 6E), 6.22 (2H, m, H-4B, 4E), 6.14 (2H, d, J = 2.1 Hz, H-2B and 6B), 5.39 (1H, d, J = 8.4 Hz, H-1C), 4.40 (1H, d, J = 8.4 Hz). ^{13}C NMR (50 MHz, in CD_3OD): 161.3 (s, C-4D), 160.2, 159.9 (4C, s, C-3B, 5B, 3E, 5E), 159.0 (s, C-4A), 146.7 (s, C-1B), 141.5 (s, C-1E), 133.1, 132.7, 132.6 (3C, s, C-1A, 1D, 3D), 129.7 (d, C-α), 129.0 (3C, d, C-2A, 6A, 2D), 127.7 (d, C-β), 124.5 (d, C-6D), 116.6 (2C, d, C-3A, 5A), 110.7 (d, C-5D), 108.1 (2C, d, C-2B, 6B), 106.1 (2C, d, C-2E, 6E), 103.0; 102.8 (2C, d, C-4B, 4E), 95.2 (d, C-1C), 59.0 (d, C-2C).

RESULTS AND DISCUSSION

Scavenging Effect on DPPH Radicals. The model of scavenging DPPH free radicals is a simple method to evaluate the antioxidative activity of antioxidants. Antioxidants serve hydrogen to free radicals and scavenge radicals. The scavenging effect of six stilbenes is shown in Table 1. All these compounds exhibited free radicals scavenging ability at the concentration of 20 μM as compared with control samples without additives. Specially 3,3′,4,5,5′-pentahydroxystilbene and 3,4,4′,5-

Table 1. Scavenging Effects of Antioxidants on the 2,2-Diphenyl-1-picrylhydrazyl Radical[a]

compound	absorption at 517 nm[b](SD)[c]	inhibition % (SD)
control	1.415 (0.001)	
3,5-dihydroxystilbene (**1**)	0.994 (0.008)	29.8 (0.57)
3,4′,5-trihydroxystilbene (**2**)	0.636 (0.036)	55.1 (2.54)
3,3′,5,5′-tetrahydroxystilbene (**3**)	0.757 (0.007)	46.5 (0.49)
3,3′,4,5′-tetrahydroxystilbene (**4**)	0.465 (0.008)	67.1 (0.57)
3,4,4′,5-tetrahydroxystilbene (**5**)	0.100 (0.008)	92.9 (0.21)
3,3′,4,5,5′-pentahydroxystilbene (**6**)	0.100 (0.008)	92.9 (0.21)

[a] The concentration of DPPH ethanolic solution was 1.0×10^{-4} M. [b] The concentration of compounds was 20 μM. [c] Each value is the mean of triplicate measurement, and SD means standard derivation of measurement.

tetrahydroxystilbene exhibited 92.9% inhibition. As shown in Table 1, 3,3′,4,5,5′-pentahydroxystilbene = 3,4,4′,5-tetrahydroxystilbene > 3,3′,4,5′-tetrahydroxystilbene > 3,4′,5-trihydroxystilbene > 3,3′,5,5′-tetrahydroxystilbene > 3,5-dihydroxystilbene. It is well-accepted that the DPPH radical scavenging of phenolic compounds is due to their hydrogen-donating ability; the more the number of hydroxyl group, the higher the possibility of free radicals scavenging ability (Chen et al., 1995). Part of the explanation for this is that pentahydroxystilbene is the most active antioxidant in this model. Phenol compounds are not active as antioxidants unless substitution at either the ortho or para position has increased the electron density of the hydroxyl group and lowered the oxygen−hydrogen bond energy. The effect of this is to increase the reactivity toward the free radicals (Madsen et al., 1997), so 3,4,4′,5-tetrahydroxystilbene and 3,3′,4,5′-tetrahydroxystilbene are more reactive than 3,3′,5,5′-tetrahyydroxystilbene. From the result of these tests, we also know that just like the highest activities shown by flavonols with group in the B ring with a 3′,4′,5′-substitution (Larson et al., 1988), 3,3′,4,5,5′-pentahydroxystilbene and 3,4,4′,5-tetrahydroxystilbene are the most active antioxidants.

Evaluation of the Inhibitory Effect on Lipid Oxidation by the Rancimat Method. The Rancimat method is a common method used to measure the potential antioxidative abilities of synthetic and natural antioxidants. The mechanism of this method is reported to be based on measuring the changes of electrical conductivity of water caused by the formation of short-chain compounds when fats and oils are oxidized under elevated temperature and accelerated aeration.

The effects of 2 μmol of stilbenes and BHT on the oxidative stability of lard are shown in Table 2. The effects in decreasing order were 3,3′,4,5′-tetrahydroxystilbene > 3,3′,4,5,5′-pentahydroxystilbene > 3,4,4′,5-tetrahydroxystilbene ≫ 3,4′,5-trihydroxystilbene ≫ BHT > 3,3′,5,5′-tetrahydroxystilbene > 3,5-dihydroxystilbene. 3,3′,4,5′-Tetrahydroxystilbene, 3,3′,4,5,5′-pentahydroxystilbene, and 3,4,4′,5-tetrahydroxystilbene showed higher lipid oxidation activity. In this model, 3,3′,5,5′-tetrahydroxystilbene and 3,5-dihydroxystilbene showed poor oxidation−inhibition activity, which suggests the importance of the presence of either an ortho or a para hydroxyl group in the tested compounds for their antioxidative activity.

The Rancimat method was used previously to measure the lipid oxidation−inhibition activity of carnosol, carnosic acid, EGCG, and ECG (the most powerful antioxidants of the flavonoids and flavonoid-related

Table 2. Induction Time of Lipid Oxidation Measured by Rancimat Method

compound[a]	induction time for lard (h) (SD)[b]
control	2.05 (0.10)
BHT	6.00 (0.20)
3,5-dihydroxystilbene (**1**)	2.25 (0.10)
3,4′,5-trihydroxystilbene (**2**)	12.3 (0.65)
3,3′,5,5′-tetrahydroxystilbene (**3**)	3.68 (0.20)
3,3′4,5′-tetrahydroxystilbene (**4**)	60.3 (1.5)
3,4,4′,5-tetrahydroxystilbene (**5**)	43.0 (1.0)
3,3′,4,5,5′-pentahydroxy-stilbene (**6**)	48.1 (0.70)

[a] The concentration of added compounds was 2 μmol in 2.5 g of lard. [b] Each value is the mean of triplicate measurement, and SD means standard derivation of measurement.

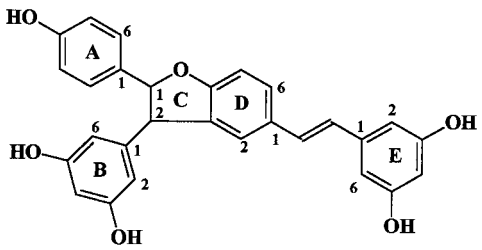

Figure 2. Structure of the oxidative dimerization product of resveratrol.

compounds) (Chen and Ho, 1995, 1997). They all showed excellent oxidation−inhibition activity. Specially, EGCG and ECG showed the induction time of 55.30 and 55.30 h, respectively (Chen et al., 1995). In our test, we found one compound (3,3′,4,5′-tetrahydroxystilbene) even better than EGCG. It is also interesting to notice that this compound is better than 3,3′,4,5,5′-pentahydroxystilbene. The present study showed that three compounds (3,3′,4,5′-tetrahydroxystilbene, 3,3′,4,5,5′-pentahydroxystilbene, and 3,4,4′,5-tetrahydroxystilbene) are more active than resveratrol in both models; especially 3,3′,4,5′-tetrahydroxystilbene deserves further investigation.

Analysis of a Radical Reaction Product. By using chromatographic methods, one major compound was isolated from the reaction between resveratrol and DPPH free radicals. This compound was isolated as white powders. The positive APCI-MS showed a molecular peak at 455 $[M + 1]^+$, suggesting that this compound is a possible dimer of resveratrol. By comparing the ^1H and ^{13}C NMR data with these known dimers of resveratrol (Langcake and Pryce, 1977; Lins et al., 1991; Sotheeswaran et al., 1985), the spectral data of this compound were found to be identical with those of a dimer formed during the oxidative dimerization of resveratrol with horseradish peroxidase−hydrogen peroxide (Langcake and Pryce, 1977). The structure of this compound is shown in Figure 2. The bioactivity and toxicity of this compound still remain unknown. This compound can be used to study the antioxidant mechanism.

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