Communication

Effect of Methyl Substitution on the Antioxidative Property and Genotoxicity of Resveratrol

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Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a natural phytoalexin with various biological activities including inhibition of lipid peroxidation and free radical scavenging properties. In addition to its beneficial effects, resveratrol also has significant genotoxicity that leads to a high frequency of chromosome aberration together with micronucleus and sister chromatid exchanges. To enhance the radical scavenging activities and to reduce the genotoxicity of resveratrol, we designed 4′-methyl resveratrol analogues where a methyl group was introduced at the ortho position relative to the 4′-hydroxy group, which is responsible for both antioxidative activities and genotoxicity of resveratrol. These synthesized methyl analogues of resveratrol showed increased antioxidative activities against galvinoxyl radical as an oxyl radical species. Furthermore, the methyl analogues also surprisingly showed reduced in vitro genotoxicities, suggesting that methyl substitution may improve resveratrol efficacy.

Introduction

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is a natural phytoalexin present in grapes and wine that has been shown to play an essential role in the prevention of several human pathological processes including inflammation (*1*), atherosclerosis (*2*), and carcinogenesis (*3*). The cancer preventive activity of resveratrol is linked to its ability to eliminate free radicals and to reduce oxidative and mutagenic stress. Lipid peroxidation is one of the basic mechanisms of cell and tissue damage leading to various diseases. So far, the protective effect toward lipid peroxidation has proved to be a typical antioxidative event of resveratrol (*4*). It has been demonstrated that resveratrol suppresses lipid peroxidation by both scavenging of free radicals and chelation of copper (*5*). Among three hydroxyl groups in resveratrol, the 4′-hydroxyl group is essential for radical scavenging activities (*6*). Besides, the 4′-hydroxyl group has been primarily responsible for the copper binding property (*7*). In addition to its beneficial effects, resveratrol is also reported to be genotoxic, inducing a high frequency of chromosomal aberrations (CA), micronucleus, and sister chromatid exchanges (SCE) in vitro (*8*). Structure–activity relationship studies of resveratrol analogues revealed that the 4′-hydroxyl group, besides being essential for antioxidative activity, is also responsible for the in vitro cytogenetic activity of resveratrol (*7, 9, 10*). In this regard, our challenge is to create novel resveratrol analogues that not only exert enhanced antioxidative abilities but also have reduced in vitro genotoxicity. Such analogues could lead to the development of new drugs against various diseases, particularly those related to oxidative stress. In our attempt to design new resveratrol analogues, we focused on the methyl groups of the tocopherol due to their proven antioxidative effects on the aromatic ring (*11*). In particular, methyl groups at the ortho position to the hydroxyl group contribute to delocalization of the unpaired electron of the corresponding phenoxyl radical, which is generated in the reaction with radical species, due to hyperconjugation. In this study, we describe resveratrol analogues where methyl groups are introduced into the ortho position of the 4′-hydroxyl group. Their designs are relatively simple, but in comparison

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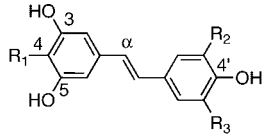
Scheme 1. Structure of Resveratrol and Methyl Derivatives



	R_1	R_2	R_3
resveratrol	H	H	H
1	H	CH_3	H
2	H	CH_3	CH_3
4-methylresveratrol	CH_3	H	H
3	CH_3	CH_3	H
4	CH_3	CH_3	CH_3

with resveratrol, their antioxidative abilities are significantly increased, and surprisingly, in vitro genotoxicities are also decreased.

Experimental Procedures

General. The reagents and solvents used were of commercial origin (Wako Chemicals, Tokyo Chemical Industry, Sigma, and Aldrich) and were employed without further purification. The ^{1}H NMR spectra and ^{13}C NMR spectra were recorded with a Varian AS 400 Mercury spectrometer. Chemical shifts are expressed in ppm downfield shift from TMS (δ scale). Low- and high-resolution mass spectra were obtained on a JEOL MS700 mass spectrometer. The progress of all reactions was monitored by thin-layer chromatography on silica gel 60 F_{254} (0.25 mm, Merck). Column chromatography was performed on silica gel 60 (0.063–0.200 mm, Merck). The purity of all synthetic compounds was approximately >98% (based on ^{1}H NMR spectra).

Diethyl 3,5-bis(benzyloxy)benzylphosphonate (5a). Triethyl phosphite (2.49 g, 15mmol) was added to a mixture of 3-benzyloxy-4-methylbenzylbromide (**11a**) (3.83 g, 10 mmol) and tetrabutylammonium iodide (73 mg, 0.2 mmol), and the resulting mixture was heated for 8 h. Excess triethyl phosphite was removed by heating for 3 h at 60 °C under vacuum to yield 4.32 g of **5a** (98% yield) as a light yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.21 (t, 6H), 3.90 (q, 4H), 5.09 (s, 4H), 6.55 (d, 2H), 6.57 (s, 1H), 7.4 (m, 12H). MS (EI *m*/*z*) 440 [M^+].

Methyl 3,5-dihydroxy-4-methylbenzoate (8). To a solution of 3,5-dihydroxy-4-methylbenzoic acid (10.08 g, 60 mmol) in methanol (150 mL), sulfuric acid (3.0 mL) was added. The resulting mixture was stirred for 18 h and poured into ice water (200 mL), and then, the product was extracted with ethyl acetate (3 × 300 mL). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (*n*-hexane:ethyl acetate = 1:1) to afford 9.40 g of **8** (86% yield) as a colorless oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.96 (3H, s), 3.75 (3H, s), 6.91 (2H, s), 9.28 (2H, br). MS (EI *m*/*z*) 182 [M^+].

Methyl 3,5-bis(benzyloxy)-4-methylbenzoate (9). To a well-stirred solution of **8** (6.64 g, 36.5 mmol) in DMF (100 mL), potassium carbonate (23.49 g, 170 mmol) was added under argon. After 1 h, benzyl bromide (15.6 g, 2.5 equiv, 91.3 mmol) was added dropwise over a period of 30 min and the reaction was allowed to proceed for 16 h. The reaction mixture was poured into ice water (200 mL) and extracted with CH_2Cl_2 (3 × 200 mL). The organic fractions were combined and washed with water and brine and then dried over anhydrous Na_2SO_4. The residue was purified by silica gel column chromatography (*n*-hexane:ethyl acetate = 2:1) to afford 10.44 g of **9** (79% yield) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.15 (3H, s), 3.82 (3H, s), 5.19 (4H, s), 7.27 (2H, s), 7.40 (10H, m). MS (EI *m*/*z*) 362 [M^+].

3,5-Bis(benzyloxy)-4-methylbenzyl alcohol (10). A solution of **9** (7.25 g, 20.0 mmol) in anhydrous diethyl ether (50 mL) was added to a cold suspension of lithium aluminum hydride (1.14 g, 30 mmol) in anhydrous diethyl ether (100 mL) over the period of 30 min. The reaction mixture was stirred for 6 h. Excess lithium aluminum hydride was decomposed by successive dropwise addition of 2-propanol and 10% potassium hydroxide. The organic layer was then passed through a Celite filter pad, and the filtrate was dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum to afford 6.09 g of **10** (91% yield) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.06 (3H, s), 4.43 (2H, d, J = 5.6 Hz), 5.08 (4H, s), 5.15 (1H, t, J = 5.6 Hz), 6.69 (2H, s), 7.40 (10H, m). MS (EI *m*/*z*) 334 [M^+].

3,5-Bis(benzyloxy)-4-methylbenzyl bromide (11b). Phosphorus tribromide (2.71 g, 10 mmol) was added to a stirred solution of **10** (3.34 g, 10 mmol) in dry CH_2Cl_2 (50 mL) at 0 °C under argon. The stirring was continued for 2 h at 0 °C and at room temperature for 2 h. The reaction mixture was poured onto ice water and extracted with diethyl ether (3 × 100 mL). The ether layers were combined, washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum, and the residue was purified by silica gel column chromatography (*n*-hexane:CH_2Cl_2 = 1:1) to afford 10.44 g of **11b** (3.62 g, 91% yield) as white needles. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.06 (3H, s), 4.64 (2H, s), 5.09 (4H, s), 6.85 (2H, s), 7.40 (10H, m). MS (EI *m*/*z*) 397 [M^+].

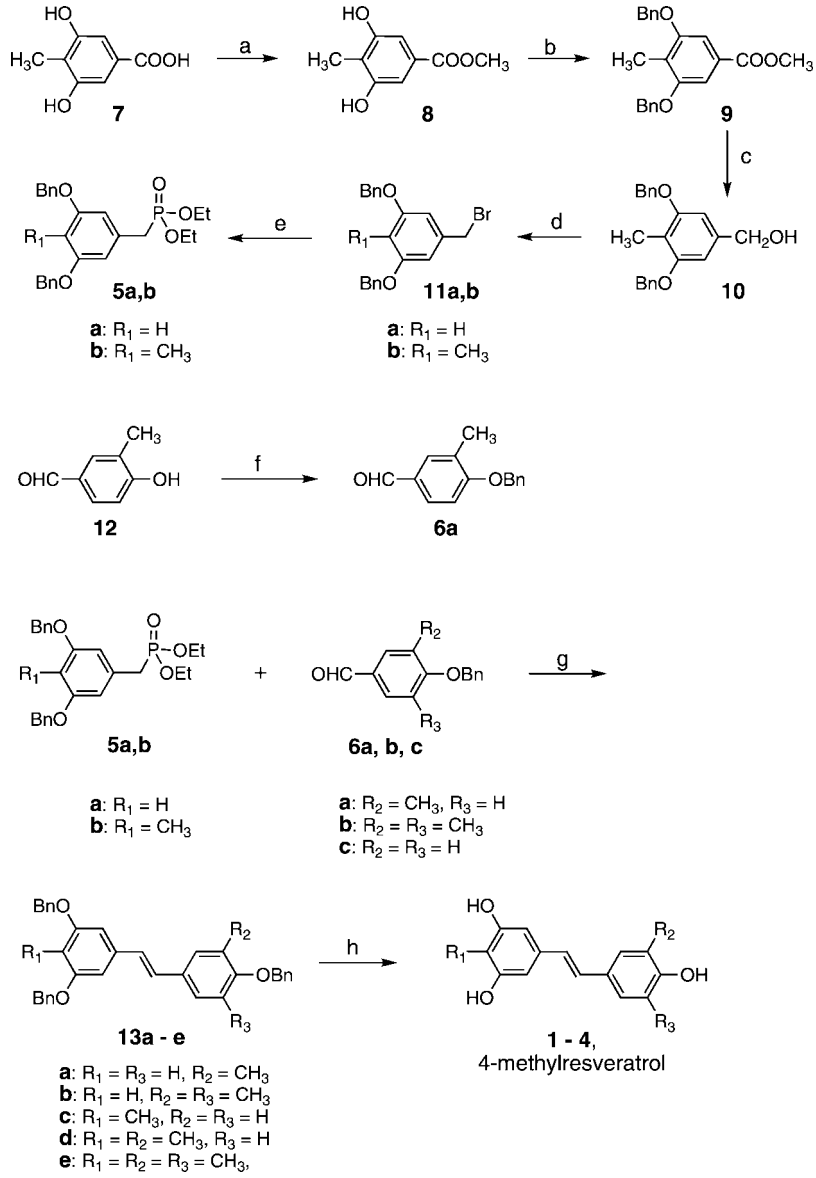
Diethyl 3,5-dibenzyloxybenzyl phosphonate (5b). Triethyl phosphite (3.0 g, 18 mmol) was added to **11b** (4.77 g, 12 mmol) containing a catalytic amount of tetrabutylammonium iodide, and the resulting mixture was heated at 120 °C for 12 h. Excess triethyl phosphite was removed by evaporater at 50 °C to afford **5b** (5.18 g, 95% yield) as a light yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.24 (6H, t, J = 7.2 Hz), 2.05 (3H, s), 3.86 (4H, quint, J = 7.2 Hz), 5.06 (4H, s), 6.66 (2H, s), 7.40 (12H, m). MS (EI *m*/*z*) 454 [M^+].

4-Benzyloxy-3-methylbenzaldehyde (6a). To a well-stirred solution of 4-hydroxy-3-methylbenzaldehyde (**12**) (3.40 g, 25 mmol) in DMF (100 mL), potassium carbonate (11.05 g, 80 mmol) was added under argon. After 1 h, benzyl bromide (10.68 g, 2.5 equiv, 62.5 mmol) was added dropwise over a period of 30 min and the reaction was allowed to proceed for 14 h. The reaction mixture was poured on ice water (200 mL) and extracted with CH_2Cl_2 (3 × 200 mL). The organic fractions were combined and washed with water and brine and dried over anhydrous Na_2SO_4. The residue was purified by silica gel column chromatography (*n*-hexane:ethyl acetate, 2:1) to afford 4.98 g of **6a** (88% yield) as a colorless oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.15 (3H, s), 3.82 (3H, s), 5.19 (4H, s), 7.27 (2H, s), 7.40 (10H, m). MS (EI *m*/*z*) 226 [M^+].

General Procedure for the Preparation of Stilbenes (13). Sodium hydride (0.2 g, 4 mmol) was added to a well-stirred suspension of the phosphate esters **5a**,**b** (2 mmol) in dry THF(10 mL) at −8 °C under argon. After 30 min, the aldehydes **6a**−**c** (2 mmol) in dry THF (15 mL) were added dropwise, and the reaction mixture was allowed to stir at room temperature for 16 h. The mixture was then cooled to 0 °C, and the excess sodium hydride was quenched with water (20 mL). The reaction mixture was then poured on ice, followed by addition of 2 M HCl (5 mL), and the products were extracted with ethyl acetate (2 × 100 mL). The organic layers were combined and were washed with brine. The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated. The purification of the crude product was performed by silica gel chromatography (*n*-hexane:CH_2Cl_2 = 2:1).

(*E*)-1-(Benzyloxy)-4-[3,5-bis(benzyloxy)styryl]-2-methylbenzene (13a). From **5a** and **6a**. Yield, 62.2%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.21 (3H, s), 5.10 (4H, s), 5.13 (2H, s), 6.54 (1H, dd, J = 2.0, 2.0 Hz), 6.82 (2H, d, J = 2.0 Hz), 6.98 (1H, d, J = 16.0 Hz), 7.01 (1H, d, J = 8.4 Hz), 7.16 (1H, d, J = 16.0 Hz), 7.40 (17H, m). MS (EI *m*/*z*) 512 [M^+].

Scheme 2. Synthesis of Methyl Derivatives of Resveratrol[a]



[a] (a) H_2SO_4, MeOH, 25 °C (18 h). (b) $C_6H_6CH_2Br$, K_2CO_3, DMF, 25 °C (16 h). (c) $LiAlH_4$, Et_2O, 25 °C (6 h). (d) PBr_3, CH_2Cl_2, 25 °C (2 h). (e) $P(OEt)_3$, $(n\text{-}Bu)_4N^+I^-$, 120 °C (12 h). (f) $C_6H_6CH_2Br$, K_2CO_3, DMF, 25 °C (14 h). (g) NaH, THF, 25 °C (16 h). (h) $AlCl_3$, $(CH_3)_2NC_6H_6$, CH_2Cl_2, 0 °C (16 h).

(*E*)-2-(Benzyloxy)-5-[3,5-bis(benzyloxy)styryl]-1,3-dimethylbenzene (13b). From **5a** and 4-benzyloxy-3,5-dimethylbenzaldehyde (**6b**). Yield, 78.9%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (6H, s), 4.80 (2H, s), 5.12 (4H, s), 6.57 (1H, dd, J = 2.0, 2.0 Hz), 6.85 (2H, d, J = 2.0 Hz), 7.04 (1H, d, J = 16.4 Hz), 7.17 (1H, d, J = 16.4 Hz), 7.28 (2H, s), 7.40 (15H, m). MS (EI m/z) 526 [M^+].

(*E*)-1,3-Bis(benzyloxy)-5-[4-(benzyloxy)styryl]-2-methylbenzene (13c). From **5b** and 4-(benzyloxy)benzaldehyde (**6c**). Yield, 75.7%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.08 (3H, s), 5.11 (2H, s), 5.15 (4H, s), 6.95 (2H, s), 7.01 (1H, d, J = 16.0 Hz), 7.19 (1H, d, J = 16.0 Hz), 7.40 (19H, m). MS (EI m/z) 512 [M^+].

(*E*)-1,3-Bis(benzyloxy)-5-[4-(benzyloxy)-3-methylstyryl]-2-methylbenzene (13d). From **5b** and **6a**. Yield, 62.4%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.07 (3H, s), 2.21 (3H, s), 5.13 (2H, s), 5.15 (4H, s), 6.94 (2H, s), 7.01 (1H, d, J = 16.0 Hz), 7.15 (1H, d, J = 16.0 Hz), 7.40 (18H, m). MS (EI m/z) 526 [M^+].

(*E*)-1,3-Bis(benzyloxy)-5-[4-(benzyloxy)-3,5-dimethylstyryl]-2-methylbenzene (13e). From **5b** and **6b**. Yield, 67.9%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.09 (3H, s), 2.26 (6H, s), 4.81 (2H, s), 5.17 (4H, s), 6.97 (2H, s), 7.06 (1H, d, J = 16.0 Hz), 7.17 (1H, d, J = 16.0 Hz), 7.28 (2H, s), 7.40 (15H, m). MS (EI m/z) 540 [M^+].

General Procedure for the Cleavage of the Benxyloxy Groups to Afford Methyl Resveratrols (1–4 and 4-Methylresveratrol). To a well-stirred solution of stilbene **13** (1 mmol) in dry CH_2Cl_2 (10 mL), *N*,*N*-dimethylaniline (3 mmol) was added under argon atmosphere at 0 °C. After 5 min, anhydrous $AlCl_3$ (4 mmol) was added to the reaction mixture. After 16 h, the reaction mixture was quenched with water at 0 °C. The reaction mixture was poured into a 1.0 M solution of HCl (20 mL). The resulting mixture was extracted with ethyl acetate (2 × 100 mL), and combined extracts were washed with brine (2 × 50 mL). The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated. The purification of the crude product was done by thin-layer chromatography under argon atmosphere (silica gel 60, 2 mm coated silica plate, CH_2Cl_2:MeOH = 10:1).

(*E*)-5-(4-Hydroxy-3-methylstyryl)benzene-1,3-diol (1). From **13a**. Yield, 50.4%; mp 217–219 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.12 (3H, s), 6.09 (1H, dd, J = 2.0, 2.0 Hz), 6.35 (2H, d, J = 2.0 Hz), 6.74 (1H, d, J = 8.0 Hz), 6.78 (1H, d, J = 16.4 Hz), 6.87 (1H, d, J = 16.4 Hz), 7.18 (1H, dd, J = 2.0, 8.0 Hz), 7.29 (1H, d, J = 2.0 Hz), 9.18 (2H, s), 9.44 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.2, 153.1, 140.3, 131.5, 128.6,

127.7, 126.9, 123.6, 115.1, 104.7, 102.5, 19.8. MS (EI *m/z*) 242 [M^+]. HRMS (EI *m/z*) calcd for $C_{15}H_{14}O_3$ [M]$^+$, 242.0943; found, 242.0944.

(*E*)-5-(4-Hydroxy-3,5-dimethylstyryl)benzene-1,3-diol (2). From **13b**. Yield, 68.6%; mp 205–208 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.16 (6H, s), 6.09 (1H, dd, J = 2.0, 2.0 Hz), 6.35 (2H, d, J = 2.0 Hz), 6.74 (1H, d, J = 8.0 Hz), 6.78 (1H, d, J = 16.0 Hz), 6.84 (1H, d, J = 16.0 Hz), 7.13 (2H, s), 8.37 (1H, s), 9.19 (2H, s). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.4, 152.0, 138.8, 135.5, 129.6, 125.3, 122.2, 114.3, 105.1, 102.9, 16.5. MS (EI *m/z*) 256 [M^+]. HRMS (EI *m/z*) calcd for $C_{16}H_{16}O_3$ [M]$^+$, 256.10995; found, 256.10997.

(*E*)-5-(4-Hydroxystyryl)-2-methylbenzene-1,3-diol (4-Methylresveratrol). From **13c**. Yield, 59.8%; mp 239–242 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.91 (3H, s), 6.43 (2H, s), 6.72 (2H, d, J = 8.8 Hz), 6.76 (2H, d, J = 16.4 Hz), 6.79 (1H, d, J = 16.4 Hz), 7.36 (1H, d, J = 8.8 Hz), 9.07 (2H, s), 9.51(1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.6, 158.7, 138.1, 131.5, 130.2, 128.5, 114.7, 110.5, 105.5, 9.8. MS (EI *m/z*) 242 [M^+]. HRMS (EI *m/z*) calcd for $C_{15}H_{14}O_3$ [M]$^+$, 242.0943; found, 242.0943.

(*E*)-5-(4-Hydroxy-3-methylstyryl)-2-methylbenzene-1,3-diol (3). From **13d**. Yield, 54.7%; mp 228–229 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.91 (3H, s), 2.11 (3H, s), 6.42 (2H, s), 6.72 (1H, d, J = 8.4 Hz), 6.74 (1H, d, J = 16.4 Hz), 6.75 (1H, d, J = 16.4 Hz), 7.16 (1H, dd, J = 2.0, 8.0 Hz), 7.28 (1H, d, J = 2.0 Hz), 9.08 (2H, s), 9.40 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.6, 154.5, 138.8, 132.3, 129.8, 128.8, 127.0, 123.2, 116.9, 108.3, 104.1, 19.6, 9.4. MS (EI *m/z*) 256 [M^+]. HRMS (EI *m/z*) calcd for $C_{16}H_{16}O_3$ [M]$^+$, 256.10995; found, 256.10996.

(*E*)-5-(4-Hydroxy-3,5-dimethylstyryl)-2-methylbenzene-1,3-diol (4). From **13e**. Yield, 73.0%; mp 205–208 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.91 (3H, s), 2.16 (6H, s), 6.43 (2H, s), 6.71 (1H, d, J = 16.4 Hz), 6.76 (1H, d, J = 16.4 Hz), 7.12 (2H, s), 8.33 (1H, s), 9.09 (2H, s). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.1, 158.2, 139.8, 132.6, 130.0, 128.4, 114.4, 109.5, 106.6, 9.8, −1.6. MS (EI *m/z*) 270 [M^+]. HRMS (EI *m/z*) calcd for $C_{16}H_{16}O_3$ [M]$^+$, 270.1256; found, 270.1259.

Spectral and Kinetic Measurements. Typically, an aliquot of methyl resveratrol (1.0×10^{-2} M) in deaerated MeCN was added to a quartz cuvette (10 mm i.d.) that contained galvinoxyl radical (G$^\bullet$) (2.5×10^{-6} M) in deaerated MeCN (3.0 mL). This led to a hydrogen transfer reaction from methyl resveratrol to G$^\bullet$. Changes in the UV–vis spectrum associated with this reaction were monitored using a Hewlett-Packard 8453 photodiode array spectrophotometer. The reaction rates were determined by following the change in absorbance at 428 nm due to G$^\bullet$ ($\epsilon = 1.43 \times 10^5$ M^{-1} cm^{-1}). Pseudofirst-order rate constants (k_{obs}) were determined by a least-squares curve fitting using an Apple Macintosh personal computer. The first-order plots of $\ln(A - A_\infty)$ vs time (A and $A\infty$ are denoted to the absorbance at the reaction time and the final absorbance, respectively) were linear until three or more half-lives, with the correlation coefficient $\rho > 0.999$.

ESR Measurement. An aliquot of deaerated MeCN solution of G$^\bullet$ (1.0×10^{-3} M) was added to a LABOTEC LLC-04B ESR sample tube containing deaerated MeCN solution of **4** (1.0×10^{-3} M), and ESR spectra of phenoxyl radical **4**$^\bullet$ produced in the reaction between **4** and G$^\bullet$ were taken on a JEOL X-band spectrometer (JES-FA100). The ESR spectrum was recorded under nonsaturating microwave power conditions. The magnitude of modulation was chosen to optimize the resolution and the signal-to-noise (S/N) ratio of the observed spectra. The g value and hyperfine coupling constants were calibrated with a Mn^{2+} marker. Computer simulation of the ESR spectra was carried out using Calleo ESR Version 1.2 program (Calleo Scientific Publisher) on an Apple Macintosh personal computer.

In Vitro CA Test. CHL cells were established from the lung of a female newborn Chinese hamster by Koyama et al. (*12*) and cloned by Ishidate and Odashima (*13*). They were maintained in Eagle's minimum essential medium (MEM; Gibco 11095-080) supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 U/mL)−streptomycin (100 μg/mL) (Gibco 15140-122) in 5% CO_2 in air at 37 °C. The doubling time was around 13 h, and the modal chromosome number was 25. Cells were seeded at 1.5×10^5/plate (60 mm in diameter) and incubated at 37 °C for 17 h. They were then treated with a test sample for 48 h, and colcemid (0.2 μg/mL) was added for the final 2 h. Chromosome preparations were made as follows: Cells were trypsinized and incubated in hypotonic KCl solution for 15 min and fixed three times with ice-cold fixative (glacial acetic acid:methanol, 1:3). Two drops of the fixed cell suspension were spread on a clean glass slide, air-dried, and stained with Giemsa solution. All slides were coded, and the number of cells with structural CAs was counted for 100 well-spread metaphases with a modal chromosome number of 25 ± 2.

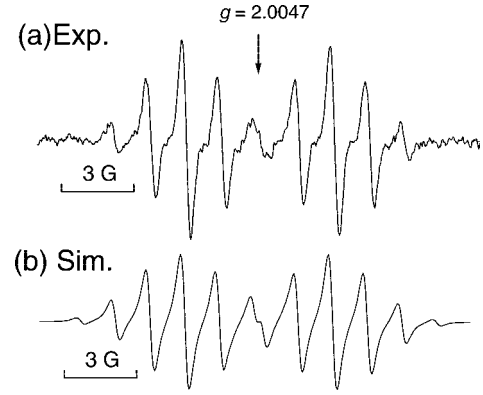


Figure 1. (a) ESR spectrum of phenoxyl radical generated in the reaction of **4** (5.0×10^{-4} M) with G$^\bullet$ (5.0×10^{-5} M) in deaerated MeCN at 298 K. (b) The computer simulation spectrum.

Table 1. Rate Constants (k_{HT}) of Hydrogen Transfer from Resveratrol and Methyl Analogues to G$^\bullet$

compd	k_{HT} (M^{-1} s^{-1})	compd	k_{HT} (M^{-1} s^{-1})
resveratrol	4.1	4-methylresveratrol	24.6
1	57.4	**3**	156
2	167	**4**	254

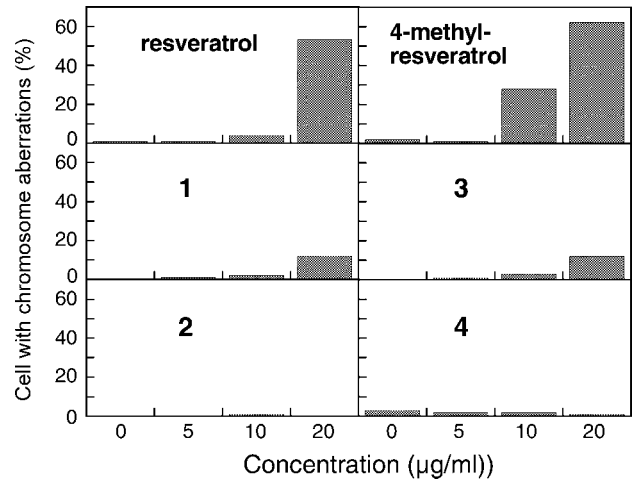


Figure 2. Chromosome aberrations induced in vitro by resveratrol and its methyl analogues. CHL cells were treated with the chemicals for 48 h.

Results and Discussion

Scheme 1 shows the structures of resveratrol and the designed methyl derivatives. Because the 4′-hydroxyl group is essential for both the antioxidative activity and the genotoxicity, resveratrol analogues were designed to introduce methyl groups ortho to the 4′-hydroxyl group. Methyl groups were also introduced at the 4-position para to the 4′-hydroxyl group. These

designed methyl derivatives (**1** and **2**) of resveratrol, 4-methylresveratrol, and its methyl derivatives (**3** and **4**) were synthesized by means of Wittig–Horner reactions between appropriate dibenzyloxybenzylphosphonic acid diethylesters (**5a**,**b**) and benzyloxybenzaldehydes (**6a**−**c**), followed by deprotection using $AlCl_3$ and *N*,*N*-dimethylaniline as outlined in Scheme 2. Trans geometries of these compounds were confirmed by their coupling constants (16.0–16.4 Hz) for the olefinic proton.

The radical scavenging activities of resveratrol and its analogues were evaluated by the hydrogen transfer reaction using galvinoxyl radical (G$^{\bullet}$) as an oxyl radical species. The hydrogen abstraction from resveratrols by G$^{\bullet}$ in deaerated acetonitrile was monitored by the decrease of absorbance at 428 nm due to G$^{\bullet}$ that obeyed pseudofirst-order kinetics, when the concentration of resveratrols was maintained at more than a 10-fold excess of the G$^{\bullet}$ concentration. The second-order rate constant (k_{HT}) for hydrogen abstraction was determined from the linear plot of pseudofirst-order rate constant vs the resveratrol concentration. As shown in Table 1, 3′-methylresveratrol (**1**), where one methyl group was introduced at the ortho position relative to the 4′-hydroxyl group, showed a significantly increased radical scavenging activity ascompared to resveratrol. A greater k_{HT} value was also obtained in compound **2**, which has methyl groups at both positions ortho to the 4′-hydroxyl group. In comparison to resveratrol, a 6-fold greater k_{HT} value was observed with 4-methylresveratrol, indicating that the 4-methyl group also affects the radical scavenging activities of the 4′-hydroxyl group. Similar to the methyl analogues (**1** and **2**) of resveratrol, the k_{HT} value of 4-methylresveratrol was increased by the introduction of methyl ortho to the 4′-hydroxyl group. Among resveratrol and its derivatives, compound **4** had the strongest antioxidative activity with a 60-fold greater k_{HT} value than that of resveratrol.

The strong antioxidative activity of tocopherol is attributed to the delocalization of phenoxyl radical, which is generated in the reaction with radical species, due to hyperconjugation with the *o*-methyl group (*11*). Therefore, to verify that the *o*-methyl group of **4** also contributes to delocalization of the unpaired electron in the corresponding phenoxyl radical, the ESR spectrum was measured for a solution containing **4** and G$^{\bullet}$ (Figure 1a). The observed ESR signals were characterized as the phenoxyl radical derived from **4** by computer simulation with the hyperfine splitting (hfs) values [$a_{CH_3}{}^{3'}$(3H), $a_{CH_3}{}^{5'}$(3H) = 0.141 mT, $a_H\alpha$(1H) = 0.601 mT] as shown in Figure 1b. We clearly demonstrated that the delocalization of the unpaired electron to the *o*-methyl groups by hyperconjugation results in the stronger antioxidative ability of *o*-methyl derivatives as compared to resveratrol.

We next considered the effect of the *o*-methyl group on the genotoxicity of resveratrol by means of the frequency of CA. Chinese hamster lung (CHL) cells were incubated with resveratrol and its analogues for 48 h, and the number of cells with structural CAs was counted after chromosome preparation. In agreement with a previous report (*8*), resveratrol induced a high frequency of CA consisting of obvious chromatid gaps and chromatid breaks. The frequency of CA is summarized in Figure 2. 4-Methylresveratrol showed a slightly higher frequency of CA as compared to resveratrol. Remarkably, *o*-methyl groups relative to 4′-hydroxyl group resulted in reduced genotoxicity. It is noteworthy that the frequency of CA induced by **1** and **3** was low, while CA induced by **2** and **4** was almost insignificant. These results suggest that two methyl substitutions reduce CA even more significantly than a single methyl substitution.

In conclusion, we have described the synthesis, antioxidative ability, and in vitro genotoxicity of resveratrol analogues with methyl groups ortho to the 4′-hydroxyl group. We demonstrated enhanced antioxidative activity coupled with reduced genotoxicity, rendering the methyl analogues **1**–**4** potentially valuable for the development of drugs effective for various types of diseases caused by oxidative stress. The genotoxicity of resveratrol has been attributed to the scavenging of tyrosyl free radicals in the R2 subunit of ribonucleotide reductase that catalyzes the rate-limiting step of de novo DNA synthesis (*14*). We previously reported that the 4′-hydroxyl group is responsible for scavenging tyrosyl radicals, which cause SCE and CA (*15*). Therefore, it is possible that the lower CA frequency for **1**–**4** as compared to resveratrol could be explained by the steric hindrance of the *o*-methyl group with respect to the radical scavenging reaction between the 4′-hydroxyl group and the tyrosyl radical. On the other hand, comparison of resveratrol and its *o*-methyl analogue (**1** and **2**) to the 4-methyl analogues (4-methyresveratrol, **3** and **4**), which have increased CA, shows a potential functional relationship between structure and enhanced radical scavenging activity. That is, slight increasing CA frequency in the corresponding 4-methyl analogues may be attributed to their enhanced radical scavenging activities that are responsible for theinhibition of ribonucleotide reductase. Further detailed insight and in vivo studies to fully exploit these potential benefits are currently underway.

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