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Structure–activity relationship studies of resveratrol and its analogues by the reaction kinetics of low density lipoprotein peroxidation

Jin-Chun Cheng, Jian-Guo Fang, Wei-Feng Chen, Bo Zhou *, Li Yang *, Zhong-Li Liu

National Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, Gansu 730000, China

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Abstract

Resveratrol (3,5,4′-*trans*-trihydroxystibene) is a natural phytoalexin present in grapes and red wine, which possesses a variety of biological activities including antioxidative activity. To find more active antioxidants, with resveratrol as the lead compound, we synthesized resveratrol analogues, i.e., 3,4,3′,4′-tetrahydroxy-*trans*-stilbene (3,4,3′,4′-THS), 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS), 2,4,4′-trihydroxy-*trans*-stilbene (2,4,4′-THS), 3,3′-dimethoxy-4,4′-dihydroxy-*trans*-stilbene (3,3′-DM-4,4′-DHS), 3,4-dihydroxy-*trans*-stilbene (3,4-DHS), 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), 3,5-dihydroxy-*trans*-stilbene (3,5-DHS) and 2,4-dihydroxy-*trans*-stilbene (2,4-DHS). Antioxidative effects of resveratrol and its analogues against free-radical-induced peroxidation of human low density lipoprotein (LDL) were studied. The peroxidation was initiated either by a water-soluble initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH), or by cupric ion (Cu^{2+}). The reaction kinetics were monitored either by the uptake of oxygen and the depletion of α-tocopherol (TOH) presented in the native LDL, or by the formation of thiobarbituric acid reactive substances (TBARS). Kinetic analysis of the antioxidation process demonstrates that these *trans*-stilbene derivatives are effective antioxidants against both AAPH- and Cu^{2+}-induced LDL peroxidation with the activity sequence of 3,4,3′,4′-THS ∼ 3,3′-DM-4,4′-DHS > 3,4-DHS ∼ 3,4,4′-THS > 2,4,4′-THS > resveratrol ∼ 3,5-DHS > 4,4′-DHS ∼ 2,4-HS, and 3,4,3′,4′-THS ∼ 3,4-DHS ∼ 3,4,4′-THS > 3,3′-DM-4,4′-DHS > 4,4′-DHS > resveratrol ∼ 2,4-HS > 2,4,4′-THS ∼ 3,5-DHS, respectively. Molecules bearing *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl groups possess significantly higher antioxidant activity than those bearing no such functionalities.

* Corresponding authors. Fax: +86 931 8625657 (B. Zhou).
E-mail address: bozhou@lzu.edu.cn (B. Zhou).

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1. Introduction

In the pathogenesis of atherosclerosis, growing evidence suggests that free-radical-induced oxidative modification of low density lipoprotein (LDL) may be involved [1–5]. Therefore, inhibition of LDL peroxidation by addition of antioxidants becomes an attractive therapeutic strategy to prevent and possibly to treat atherosclerosis and related diseases in human. This leads to a great deal of research devoted to the prevention of lipid peroxidation of LDL by antioxidants [6–9].

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a naturally occurring phytoalexin present in grapes, nuts and other plants. It is believed that the high level of this compound in red wine ($0.1 \sim 15$ mg/l) [10] is linked to the low incidence of heart diseases in some regions of France, the so-called ''French paradox,'' i.e., despite high fat intake, mortality from coronary heart disease is lower due to the regular drinking of wine [11,12]. This compound has attracted considerable attention due to its various biological and pharmacological activities, including antioxidative [13–16] and anticancer activities [17–20]. As a part of our ongoing research project on antioxidative effects of natural antioxidants and their derivatives [21–24] we found recently that some synthetic resveratrol analogues bearing *ortho*-dihydroxyl functionality exhibit significantly higher antioxidant and cytotoxic activity against HL-60 cancer cells than resveratrol and other analogues bearing no such functionality [25–27]. Therefore, it is desirable to see if the same structure–activity relationship is also valid in LDL peroxidation. We report herein an in vitro study on the antioxidative effect of resveratrol and related *trans*-stilbene analogues on free-radical-induced LDL peroxidation. The compounds studied are resveratrol, 3,4,3′,4′-tetrahydroxy-*trans*-stilbene (3,4,3′,4′-THS), 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS), 2,4,4′-trihydroxy-*trans*-stilbene (2,4,4′-THS), 3,3′-dimethoxy-4,4′-dihydroxy-*trans*-stilbene (3,3′-DM-4,4′-DHS), 3,4-dihydroxy-*trans*-stilbene (3,4-DHS), 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), 3,5-dihydroxy-*trans*-stilbene (3,5-DHS) and 2,4-dihydroxy-*trans*-stilbene (2,4-DHS) (Fig. 1). The peroxidation was initiated by a water-soluble azo initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and cupric ion (Cu^{2+}), and measured by oxygen uptake and formation of thiobarbituric acid reactive substances (TBARS). It was found that resveratrol and its analogues, especially 3,4,3′,4′-THS, 3,4,4′-THS, 3,4-DHS, and 3,3′-DM-4,4′-DHS, are good antioxidants for both AAPH- and cupric ion-initiated LDL peroxidation. The structure–activity relationship is discussed.

2. Experimental

2.1. Materials

Resveratrol, 3,4-DHS, 3,4,4′-THS, 2,4,4′-THS, 3,5-DHS, 4,4′-DHS and 2,4-DHS were synthesized with reference to the modified Wittig reaction [28,29] using diethylbenzylphosphonate, which was prepared from methoxyl substituted benzyl chloride

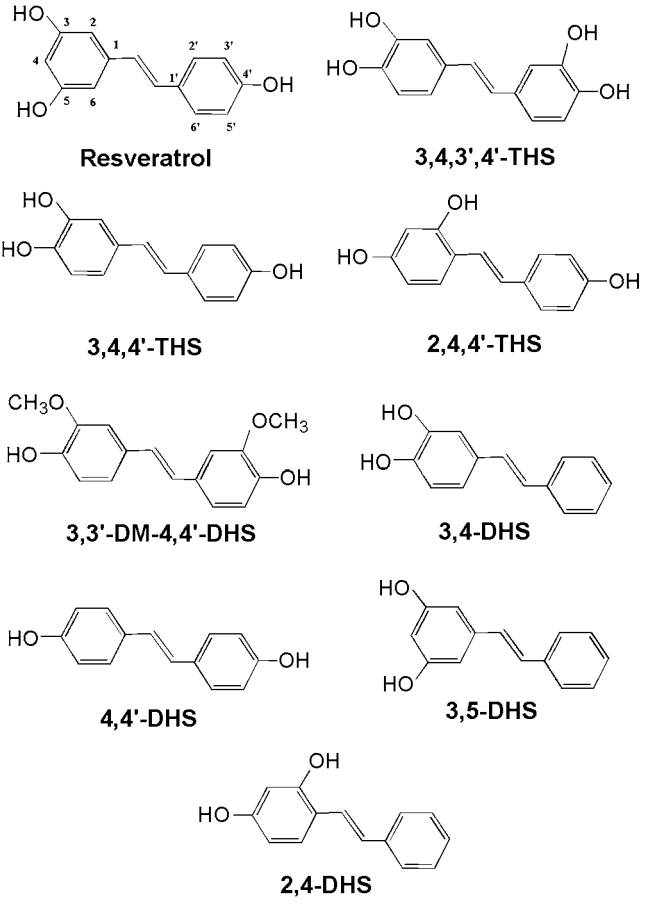


Fig. 1. Molecular structures of resveratrol and its analogues.

and triethyl phosphate, reacted with methoxyl substituted benzaldehyde and followed by removing the methyl protecting group with pyridine hydrochloride. This procedure gave exclusively the *trans*-isomers and good or moderate yields depending predominantly on the demethylation reaction. 3,4,3′,4′-THS and 3,3′-DM-4,4′-DHS were synthesized with reference to the modified McMurry reaction [30,31]. Briefly, zinc powder (2.0 g, 0.031 mol) was added to a stirred slurry of $TiCl_4$ (1.5 ml, 0.014 mol) in dry tetrahydrofuran (THF) at −78 °C under an inert atmosphere. After refluxing for 40 min, the black mixture was cooled and a solution of vanillin (4-hydroxy-3-methoxybenzaldehyde, 0.01 mol) in THF was added. After refluxing for 3–5 h, the mixture was poured into ice-water, and extracted with ether. The ether layer was washed with brine, dried over anhydrous sodium sulfate. and concentrated. Removal of the solvent under reduced pressure and purification of the residue by column chromatography gave the symmetrical stilbene (3,3′-DM-4,4′-DHS). Removing the methyl protecting group by pyridine hydrochloride gave 3,4,3′4′-THS. Their structures were fully identified with ^{1}H NMR and EI-MS or FAB-MS and

the data are consistent with those reported in the literature [28,29]. The purity of the compounds was all checked by HPLC being > 98%.

Resveratrol: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 6.22 (1H, t, $J = 2$ Hz, H-4), 6.49 (2H, d, $J = 2$ Hz, H-2 and H-6), 6.75 (2H, d, $J = 8.4$ Hz, H-3′ and H-5′), 6.77, 6.97 (each 1H, d, $J = 16.3$ Hz, —CH=CH—), 7.36 (2H, d, $J = 8.4$ Hz, H-2′ and H-6′); MS (*m*/*z*): 228 [M^+], 211, 199, 181, 157, 115.

3,4,3′,4′-THS: ^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ 6.80 (2H, d, $J = 8.1$ Hz, H-5 and H-5′), 6.84 (2H, s, —CH=CH—), 6.87 (2H, dd, $J = 8.1$, 1.8 Hz, H-6 and H-6′), 7.05 (2H, d, $J = 1.8$ Hz, H-2 and H-2′); MS (*m*/*z*): 244 [M^+], 221, 207, 165.

3,4,4′-THS: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 6.79 (1H, d, $J = 8.1$ Hz, H-5), 6.81 (2H, d, $J = 8.5$ Hz, H-3′ and H-5′), 6.87 (1H, dd, $J = 8.1$, 2.1 Hz, H-6), 6.79, 6.96 (each 1H, d, $J = 16$ Hz, —CH=CH—), 7.05 (1H, d, $J = 2.1$ Hz, H-2), 7.37 (2H, d, $J = 8.5$ Hz, H-2′ and H-6′); MS (*m*/*z*): 228 [M^+], 211, 199, 181, 157, 115.

2,4,4′-THS: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 6.73 (2H, d, $J = 9$ Hz, H-3′ and H-5′), 7.36 (2H, d, $J = 9$ Hz, H-2′ and H-6′), 6.30 (1H, d, $J = 2$ Hz, H-3), 6.32 (1H, dd, $J = 9$, 2 Hz, H-5), 6.77, 6.95 (each 1H, d, $J = 16$ Hz, —CH=CH—), 7.35 (1H, d, $J = 9$ Hz, H-6); MS (*m*/*z*): 228 [M^+], 211, 199, 181, 157, 115.

3,3′-DM-4,4′-DHS: ^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ 6.80 (2H, d, $J = 8.1$ Hz, H-5 and H-5′), 6.98 (2H, s, —CH=CH—), 6.97 (2H, dd, $J = 2.1$, 8.1 Hz, H-6 and H-6′), 7.16 (2H, d, $J = 1.8$ Hz, H-2 and H-2′); MS (*m*/*z*): 272 [M^+], 259, 218.

3,4-DHS: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 6.79 (1H, d, $J = 8$ Hz, H-5), 6.89 (1H, dd, $J = 8$, 2 Hz, H-6), 6.98, 7.01 (each 1H, d, J= 16.4 Hz, —CH=CH—), 7.09 (1H, d, $J = 2$ Hz, H-2), 7.19 (1H, t, $J = 7.4$ Hz, H-4′), 7.31 (2H, t, $J = 7.4$ Hz, H-3′ and H-5′), 7.51 (2H, d, $J = 7.4$ Hz, H-2′ and H-6′); MS (*m*/*z*): 212 [M^+], 197, 165, 141, 115, 77.

4,4′-DHS: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 6.83 (4H, d, $J = 8$ Hz, H-3, H-5, H-3′ and H-5′), 6.95 (2H, s, —CH=CH—), 7.40 (4H, d, $J = 8$ Hz, H-2, H-6, H-2′ and H-6′); MS (*m*/*z*): 212 [M^+], 197, 165, 141, 115, 77.

3,5-DHS: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 6.27 (1H, t, $J = 2.1$ Hz, H-4), 6.56 (2H, d, $J = 2.1$ Hz, H-2 and H-6), 7.05, 7.07 (each 1H, d, $J = 16.6$ Hz, —CH=CH—), 7.22 (1H, t, $J = 7.3$ Hz, H-4′), 7.33 (2H, t, $J = 7.3$ Hz, H-3′ and H-5′), 7.53 (2H, d, $J = 7.3$ Hz, H-2′ and H-6′); MS (*m*/*z*): 212 [M^+], 197, 165, 141, 115, 77.

2,4-DHS: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 6.30 (1H, d, $J = 2$ Hz, H-3), 6.32 (1H, dd, $J = 9$, 2 Hz, H-5), 6.97, 7.37 (each 1H, d, $J = 17$ Hz, —CH=CH—), 7.15 (1H, tt, $J = 8$, 1 Hz, H-4′), 7.28 (2H, d, $J = 8$ Hz, H-3′ and H-5′), 7.35 (1H, d, $J = 9$ Hz, H-6), 7.47 (2H, d, $J = 8$ Hz, H-2 and H-6); MS (*m*/*z*): 212[M^+], 197, 165, 141, 115, 77.

dl-α-Tocopherol (Merck, Biochemical reagent, > 99.9%) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, Aldrich) were kept under nitrogen in a refrigerator and used as received.

The LDL was isolated from human plasma of a healthy donor by the discontinuous density gradient centrifugation procedure as described in the literature [32] at 45,000 rpm (140,000*g*) for 6 h using a HITACHI 55P-72 ultracentrifuge at 4 °C. The isolated LDL fraction was then dialyzed with phosphate buffered saline (PBS, composed of 137 mM of NaCl, 2.7 mM of KCl, 8.1 mM of Na_2HPO_4 and 1.5 mM of KH_2PO_4 in distilled water, pH 7.4) containing 0.1 mM sodium ethylenediaminetetraacetate (EDTA) to prevent oxidation during the isolation. EDTA was removed by dial-

ysis with PBS before the oxidation experiments. The concentration of protein was determined by the method of Lowry [33].

2.2. Oxygen uptake measurements

The rate of oxygen uptake was measured in a closed glass vessel of ca. 2 ml in volume, at constant temperature and that was stirred, using a 5946-50 oxygen meter (Cole-Parmer Instruments, USA) which was able to record oxygen concentrations as low as 10^{-8} M. LDL was suspended in PBS (pH 7.4) to the final concentration of 0.2 mg protein/ml equivalent to approximately 0.4 μM of LDL (mass $= 2.5 \times 10^6$ g/M and protein content of ca. 20%) [34] under air. Resveratrol and its analogues were dissolved in DMSO to the concentration of 2 mM as stock solutions. The final concentration of DMSO in the suspension was less than 0.1% (v/v) of the LDL suspension to avoid disturbance of the system. AAPH was directly dissolved in PBS (pH 7.4) and injected into the LDL suspension to initiate the peroxidation. Every experiment was repeated three times and the results were reproducible to within 10% experimental deviation.

2.3. High performance liquid chromatographic (HPLC) measurements

A Gilson model 702 liquid chromatograph was used to separate α-tocopherol (TOH) with a Sychropack KPP-100 reversed-phase column (4×250 mm) and eluted with methanol–iso-propanol–formic acid (80:20:1 v/v/v) containing 50 mM of sodium perchlorate as a supporting electrolyte at a flow rate of 1 ml/min. Aliquots of 0.6 ml of reaction mixture were taken out from three identical reaction vessels of 2 ml in volume at appropriate time intervals, and TOH was extracted by hexane–ethanol partitioning (hexane : EtOH : LDL = 12:3:1 v/v/v) which yielded > 97% of TOH [34]. TOH was electrochemically detected by using a Gilson Model 142 electrochemical detector by setting the oxidation potential at +700 mV.

2.4. Malonodialdehyde (MDA) formation measurements

The formation of MDA was determined by thiobarbituric acid assay as a thiobarbituacid reactive substances (TBARS) to monitor LDL peroxidation [35]. The LDL was incubated at 37 °C in 0.1 M potassium phosphate buffer, pH 7.5, and made up to a final protein concentration of 0.2 mg/ml. The peroxidation was initiated by 10 μM of $CuSO_4$ and inhibited by 2 μM of resveratrol or its analogues which was added as a DMSO solution and the final concentration of DMSO in the suspension was less than 0.1% v/v that did not show appreciable interference to the reaction as evidenced by control experiments. The reaction mixture was gently shaken at 37 °C and aliquots of the reaction mixture were taken out at specific intervals and a TCA–TBA–HCl stock solution (15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N HCl) was added to the reaction mixture, together with 0.02% w/v butylated hydroxytoluene (BHT). This amount of BHT completely prevented the formation of any nonspecific TBARS [36]. The solution was then heated in a boiling water bath for 15 min. After cooling, the precipitate was removed by centrifugation. MDA in the supernatant was determined at 532 nm using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} [35].

3. Results and discussion

3.1. The antioxidant activity of resveratrol and its analogues in AAPH-induced LDL peroxidation

The important event of lipid peroxidation is the uptake of oxygen and formation of lipid peroxides [37]. In the present work oxygen uptake was used to measure the reaction kinetics of the LDL peroxidation. Fig. 2 shows oxygen uptake curves recorded during the water-soluble radical initiator AAPH-induced LDL peroxidation in the absence and in the presence of exogenous resveratrol. In the absence of resveratrol the oxygen uptake did not take place immediately as in the case of lipid peroxidation conducted in model membranes [21,25,38], but was still inhibited for ca. 20 min (Fig. 2, line a). This demonstrates the presence of endogenous antioxidants in LDL, such as α-tocopherol (TOH), ubiquinol-10 and carotenoids (Eq. (1)), which can trap the propagating radicals to inhibit the peroxidation. The oxygen uptake rate during the inhibition period is designated as R_{inh}. After the inhibition period the oxygen uptake became faster, indicating depletion of the endogenous antioxidants. The turning point from the inhibition period to the restoration of oxygen uptake is the inhibition time, t_{inh}. The slope of the oxygen uptake curve after the inhibition period represents the intrinsic peroxidation rate, R_p, of the LDL in the absence of antioxidants. After a short time of the inhibition period, different amounts of resveratrol were added. It was found that addition of resveratrol inhibits the LDL peroxidation, produce a new inhibition period and the inhibition time is proportional to the concentration of resveratrol (Fig. 2, lines b–d and the inset). This demonstrates that the peroxidation of LDL is inhibited dose-dependently by resveratrol in the absence of endogenous antioxidants. Other resveratrol analogues also show the dose-dependent inhibition for the LDL peroxidation (Figures not shown).

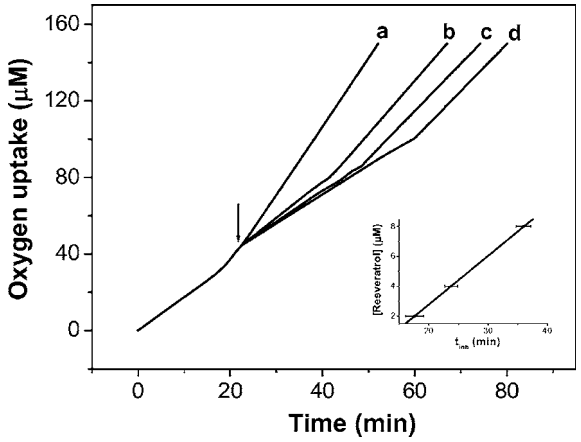


Fig. 2. Representative oxygen uptake curves recorded during the AAPH-initiated and resveratrol inhibited LDL peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [LDL] = 0.2 mg/ml; $[AAPH]_0 = 10.0$ mM. The arrow shows the time of addition of resveratrol. (a) Native LDL containing 2.47 μM of TOH; (b) $[resveratrol]_0 = 2$ μM; (c) $[resveratrol]_0 = 4$ μM; (d) $[resveratrol]_0 = 8$ μM. The inset shows the concentration dependence of the inhibition time (t_{inh}).

Fig. 3 shows representative oxygen uptake curves recorded during the AAPH-induced LDL peroxidation in the presence of the same concentration (4 μM) of resveratrol and its analogues which were added after the depletion of endogenous antioxidants. It is seen that after depletion of the endogenous antioxidants, addition of all of these tested compounds produces a new inhibition period, indicating that LDL peroxidation could be inhibited by these compounds in the absence of endogenous antioxidants. The inhibition time, t_{inh}, is significantly different for the different resveratrol analogues and follows the efficacy sequence of 3,4,3′,4′-THS ~ 3,3′-DM-4,4′-DHS > 3,4-DHS ~ 3,4,4′-THS > 2,4,4′-THS > resveratrol ~ 3,5-DHS > 4,4′-DHS ~ 2,4-DHS (the curve for 4,4′-DHS is not shown for clarity).

When resveratrol or its analogues was added before the AAPH-initiation the intrinsic inhibition period of the native LDL peroxidation was remarkably prolonged (Fig. 4) and the overall inhibition time was approximately equal to the sum of the intrinsic inhibition time of the native LDL and the inhibition time induced by resveratrol or its analogues when it was used after depletion of the endogenous antioxidants (compare Figs. 3 and 4). The efficacy sequence is 3,4,3′,4′-THS ~ 3,3′-DM-4,4′-DHS > 3,4-DHS > 3,4,4′-THS > 2,4,4′-THS ~ resveratrol > 4,4′-DHS ~ 2,4-DHS > 3,5-DHS, similar to that obtained when these compounds were used after depletion of the antioxidants in native LDL. This suggests that resveratrol and its analogues serve as chain-breaking antioxidants independently and do not have synergistic interaction with the intrinsic antioxidants, e.g., α-tocopherol (TOH), in the native LDL. It has been reported previously that if an exogenous antioxidant, such as vitamin C or green tea polyphenols, could react with TOH in a synergistic fashion in the native LDL, the overall inhibition time would be significantly longer than the sum of the intrinsic inhibition time of the native LDL and the inhibition time due to the exogenous antioxidant [8,39].

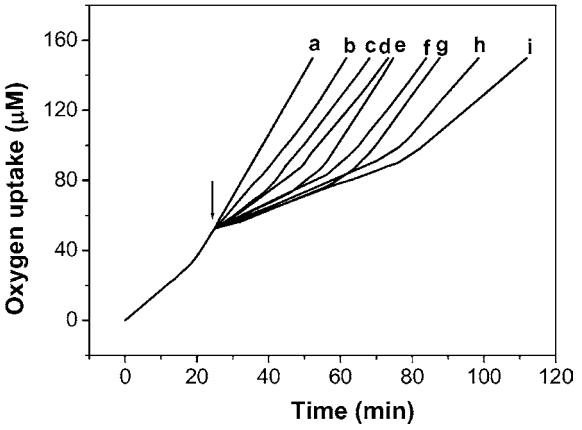


Fig. 3. Representative oxygen uptake curves recorded during the AAPH-initiated and resveratrol and its analogues (ROH) inhibited LDL peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [LDL] = 0.2 mg/ml; [AAPH] = 10 mM; $[ROH]_0$ = 4 μM. The ROHs were added after the intrinsic inhibition period. The arrow shows the time of addition of ROH. (a) Native LDL containing 2.47 μM of TOH; (b) 3,5-DHS; (c) 2,4-DHS; (d) resveratrol; (e) 2,4,4′-THS; (f) 3,4,4′-THS; (g) 3,4-DHS; (h) 3,4,3′,4′-THS; (i) 3,3′-DM-4,4′-DHS. Curve for 4,4′-DHS is not shown for clarity.

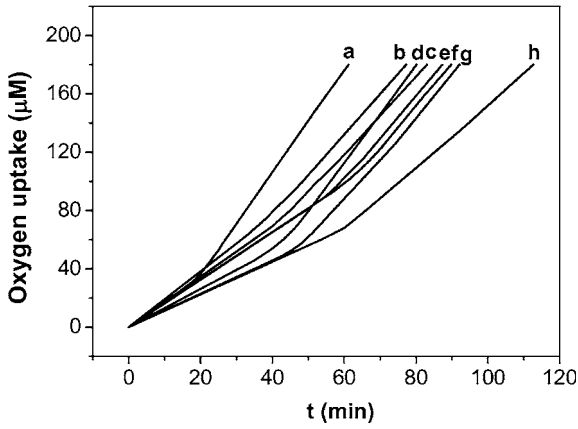


Fig. 4. Representative oxygen uptake curves recorded during the AAPH-initiated and resveratrol and its analogues (ROH) inhibited LDL peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [LDL] = 0.2 mg/ml; $[AAPH]_0 = 10$ mM; $[ROH]_0 = 4$ μM. The ROHs were added before the initiation. (a) Native LDL containing 2.47 μM of TOH; (b) 2,4-DHS; (c) resveratrol; (d) 2,4,4′-THS; (e) 3,4-DHS; (f) 3,4,4′-THS; (g) 3,4,3′,4′-THS; (h) 3,3′-DM-4,4′-DHS. Curves for 3,5-DHS and 4,4′-DHS are not shown for clarity.

3.2. Decay kinetics of α-tocopherol during the peroxidation

The decay kinetics of TOH during the antioxidation reaction has been used to determine the rate of initiation, R_i, of LDL peroxidation [34] and to study the synergism of TOH with other antioxidants [8,21–25]. In the present work the decay kinetics of TOH was studied by HPLC separation of the reaction mixture components followed by electrochemical determination of TOH. It was found that in the native LDL TOH decayed approximately linearly with time in the initial two half-lives (Fig. 5, line a), in accordance with the kinetic demand for antioxidant reactions (Eq. (7), vide infra). The decay rate was 2.3 nM/s. Addition of 3,4,3′,4′-THS decreased significantly the decay rate of TOH, but no

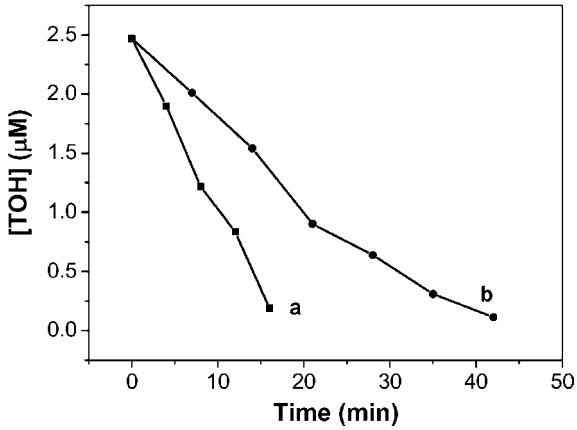


Fig. 5. Decay of α-tocopherol (TOH) during the AAPH-initiated and 3,4,3′,4′-THS inhibited peroxidation of LDL in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [LDL] = 0.2 mg/ml; $[AAPH]_0 = 10.0$ mM. (a) Native LDL containing 2.47 μM of TOH; (b) in the presence of 4 μM of 3,3′4,4′-THS.

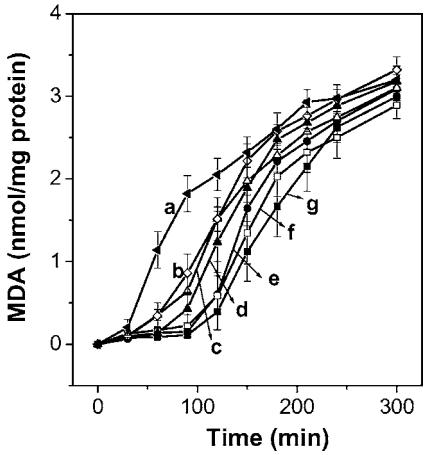


Fig. 6. Inhibition of MDA formtion during the Cu^{2+}-induced peroxidation of LDL by resveratrol and its analogues (ROH) at 37 °C. The LDL was suspended in PBS (pH 7.4) at a protein concentration of 0.2 mg/ml. $[CuSO_4] = 10$ μM; $[ROH]_0 = 2$ μM. (a) Native LDL; (b) resveratrol; (c) 3,3′-DM-4,4′-DHS; (d) 4,4′-DHS; (e) 3,4-DHS; (f) 3,4,4′-THS; (g) 3,4,3′,4′-THS. Curves for other resveratrol analogues are not shown for clarity. Every experiment was repeated three times.

apparent turning point was observed (Fig. 5, line b). This is in contrast to the case of vitamin C and green tea polyphenols which exhibited clear turning point in the decay curves of TOH, demonstrating the antioxidant synergism with TOH [8,22,39]. Therefore, it is believed that TOH and 3,4,3′,4′-THS work independently as chain-breaking antioxidants in LDL and they do not interact with each other, in accordance with the experimental results mentioned in the previous section.

3.3. Inhibition of Cu^{2+}-induced LDL peroxidation

Cu^{2+}-induced LDL peroxidation is considered to be more relevant to in vivo situation than the AAPH-induced peroxidation [1]. Therefore, Cu^{2+} was also used to induce LDL peroxidation and the formation of malondialdehyde (MDA), which is the final oxidation product of lipid peroxidation, was used to monitor LDL peroxidation [35]. Fig. 6 shows the MDA formation during the Cu^{2+}-induced peroxidation of LDL. Similar to the oxygen uptake experiments mentioned earlier, formation of MDA is inhibited for a short period of time due to the presence of endogenous antioxidants in the LDL. It is seen that addition of resveratrol and its analogues significantly suppresses the rate of MDA formation and increases the inhibition period of the native LDL. The antioxidative activity of resveratrol and its analogues is assessed by their inhibition period, t_{inh}, and it follows the sequence similar to that observed in the oxygen uptake assay mentioned above, i.e., 3,4,3′,4′-THS ~ 3,4-DHS ~ 3,4,4′-THS > 3,3′-DM-4,4′-DHS > 4,4′-DHS > resveratrol ~ 2,4-DHS > 2,4,4′-THS ~ 3,5-DHS (Table 1).

3.4. Antioxidation kinetics

It has been proven that lipid peroxidation induced by AAPH in model biomembranes follows the same classical rate law for auto-oxidation as that in homogeneous solutions

Table 1
Inhibition times (t_{inh}) of resveratrol and its analogues (ROH) in Cu^{2+}-induced peroxidation of human LDL[a]

ROH	TOH[b]	Resveratrol	3,3′,4,4′-THS	3,4,4′-THS	2,4,4′-THS	3,3′-DM-4,4′-DHS	3,4-DHS	4,4′-DHS	3,5-DHS	2,4-DHS
t_{inh} (min)	30 ± 1.5	58 ± 0.6	107 ± 4.0	105 ± 3.6	43 ± 0.6	88 ± 2.6	106 ± 3.2	77 ± 1.6	43 ± 1.2	53 ± 2.0

[a] Determined in PBS (pH 7.4) at 37 °C; [LDL] = 0.2 mg/ml; [$CuSO_4$] = 10 μM; $[ROH]_0$ = 2 μM. Every experiment was repeated three times and the SD are shown in the table.

[b] Intrinsic TOH (2.47 μM) and other antioxidants in native LDL.

[38], and that TOH is the principal lipid-soluble chain-breaking antioxidant in biomembranes [37] and in LDL [40]. Although TOH behaves as an antioxidant under normal experimental conditions in LDL, it is also reported that TOH might become a prooxidant to accelerate lipid peroxidation in LDL by the so-called tocopherol mediated peroxidation (TMP) when the radical flux is low and the concentration of TOH was high [5,34,41]. In the present study, since the radical flux was high ([AAPH] = 10 mM) and the concentration of TOH was low ($[TOH]_0 = 2.47$ μM), TOH behaved as a typical antioxidant as evidenced by the oxygen uptake kinetics (Figs. 2–4) and its decay kinetics (Fig. 5). Similar antioxidant behaviour of TOH in LDL has also been reported previously [8,42–44]. Therefore, the rate of oxygen uptake during the peroxidation of LDL initiated by 10 mM of AAPH can be expressed as follows:

$$-\mathrm{d}[O_2]/\mathrm{d}t = R_p = (k_p/(2k_t)^{1/2})R_i^{1/2}[LH], \tag{1}$$

where k_p and k_t are rate constants for the chain propagation and termination respectively, LH represents a lipid molecule with an abstractable hydrogen, i.e., polyunsaturated fatty acids in LDL, and R_i is the apparent rate of initiation

$$R_i = 2k_g \times e[R{-}N{=}N{-}R]. \tag{2}$$

Although the radical generation rate of AAPH is known as 1.3×10^{-6} [AAPH]/s at 37 °C for protein-containing solutions and liposomal dispersions [1,34], the cage effect parameter e varies appreciably on the medium and the concentration of the antioxidant and the initiator [34]. Therefore, the R_i value is generally determined by the inhibition period and/or by the decay rate of TOH (Eqs. (6) and (7), vide infra).

In the presence of an antioxidant molecule, AH, the peroxyl radical can be trapped and a new antioxidant radical, A$^{\cdot}$, produced (Eq. (3)). If the A$^{\cdot}$ is a stabilized radical (e.g., α-tocopheroxyl radical or Vitamin C radical anion) which can promote the rate-limiting hydrogen abstraction reaction (3) and undergo fast termination reaction (Eq. (4)), the peroxidation would be inhibited.

$$LOO^{\cdot} + AH \xrightarrow{k_{inh}} LOOH + A^{\cdot}, \tag{3}$$

$$LOO^{\cdot} + A^{\cdot} \xrightarrow{\text{fast}} LOOA, \tag{4}$$

where k_{inh} is the rate of inhibition, representing the activity of the antioxidant. During the inhibition period the rate of peroxyl formation by the initiation equals the rate of peroxyl trapping, therefore

$$R_i = R_{inh} = k_{inh} \times n[AH] \times [LOO^{\cdot}], \tag{5}$$

where n is the stoichiometric factor that designates the number of peroxyl radicals trapped by each antioxidant molecule and is given by

$$n = R_i t_{inh}/[AH]_0. \tag{6}$$

From Eqs. (3)–(5) we have Eq. (7)

$$-\mathrm{d}[AH]/\mathrm{d}t = R_i/n. \tag{7}$$

The n value of TOH is generally taken as 2 [34,37] according to Eqs. (3) and (4), hence R_i can be determined from the inhibition period (Eq. (6)) or from the decay rate of TOH (Eq. (7)). In the present experiment the R_i value calculated from the inhibition period is

4.2 nM/s. This value is in good agreement with the value of 4.6 nM/s obtained from the decay of TOH. Similar an R_i value of 4.5 nM/s has been reported [34] by measuring the decay of TOH with the same concentration of AAPH (10 mM).

The rate of oxygen uptake during the inhibition period is related to k_p and k_{inh} according to the steady-state treatment of the above equations as:

$$-d[O_2]/dt = R_{inh} = k_p R_i [LH]/(nk_{inh}[AH]). \quad (8)$$

The kinetic chain length defines the number of chain propagations by each initiating radical and is given by Eqs. (9) and (10) for inhibited and uninhibited peroxidations respectively. The kinetic data obtained from Figs. 2–4 are listed in Table 2.

$$kcl_{inh} = R_{inh}/R_i, \quad (9)$$

$$kcl_p = R_p/R_i. \quad (10)$$

It can be seen from Figs. 2–4 and Table 2 that in the present experimental conditions the endogenous TOH which contributes > 95% of endogenous antioxidants in native LDL [1,43] acts as a chain-breaking antioxidant, in accordance with the previous reports

Table 2
Kinetic parameters for the AAPH-initiated peroxidation of human LDL and its inhibition by resveratrol and its analogues (ROH)[a]

ROH	R_{inh} (10^{-8} M s^{-1})	R_p (10^{-8} M s^{-1})	t_{inh} (min)	kcl_{inh}	kcl_p	k_{inh} (10^5 M^{-1} s^{-1})	n[e]
Native[b]	2.9 ± 0.3	5.9 ± 0.4	19.6 ± 0.8	6.9	14.0	5.7	2[f]
Resveratrol[c]	2.5 ± 0.3	4.1 ± 0.5	23.8 ± 1.3	5.9	9.8	4.4	1.5
3,4,3′,4′-THS[c]	1.5 ± 0.4	4.0 ± 0.4	51 ± 2.1	3.5	9.5	3.7	3.2
3,4,4′-THS[c]	1.8 ± 0.5	4.5 ± 0.5	37.2 ± 1.2	4.3	10.7	4.2	2.3
2,4,4′-THS[c]	1.8 ± 0.1	4.2 ± 0.2	27.5 ± 1.6	4.3	10.0	6.1	1.7
3,3′-DM-4,4′-DHS[c]	1.3 ± 0.2	3.0 ± 0.4	51 ± 2.3	3.2	7.1	4.1	3.2
3,4-DHS[c]	1.4 ± 0.1	4.6 ± 0.6	39 ± 2.1	3.3	11.0	4.9	2.5
4,4′-DHS[c]	1.8 ± 0.3	4.4 ± 0.2	18.5 ± 0.5	4.3	10.5	8.2	1.2
3,5-DHS[c]	4.0 ± 0.2	5.8 ± 0.6	21.7 ± 1.2	9.5	13.8	3.3	1.4
2,4-DHS[c]	2.7 ± 0.4	4.5 ± 0.7	17.5 ± 1.9	6.4	10.7	6.2	1.1
Resveratrol[d]	2.9 ± 0.5	4.5 ± 0.4	45.0 ± 0.6	6.9	10.7	2.1	1.8
3,4,3′,4′-THS[d]	2.7 ± 0.4	5.0 ± 0.5	64.2 ± 2.6	6.5	11.9	1.76	2.5
3,4,4′-THS[d]	2.0 ± 0.3	5.0 ± 0.4	52.5 ± 2.1	4.7	11.9	3.11	2.0
2,4,4′-THS[d]	2.2 ± 0.3	5.5 ± 0.5	43.0 ± 1.1	5.2	13.1	3.05	1.7
3,3′-DM-4,4′-DHS[d]	1.9 ± 0.2	3.5 ± 0.3	62.4 ± 2.5	4.5	8.3	2.57	2.4
3,4-DHS[d]	2.7 ± 0.2	4.9 ± 0.5	58.0 ± 1.6	6.4	11.7	1.82	2.3
4,4′-DHS[d]	3.2 ± 0.2	5.0 ± 0.4	41.0 ± 1.1	7.6	11.9	2.32	1.6
3,5-DHS[d]	4.3 ± 0.6	5.3 ± 0.8	35.3 ± 1.1	10.2	12.6	1.93	1.4
2,4-DHS[d]	3.2 ± 0.4	4.6 ± 0.3	40.0 ± 1.9	7.6	11.0	2.46	1.6

[a] Determined in PBS (pH 7.4) at 37 °C; [LDL] = 0.2 mg/ml; [AAPH] = 10 mM; $[ROH]_0 = 4$ μM. Every experiment was repeated three times and the SD are shown in the table. R_i is taken as 4.2 nM/s by using Eq. (6). k_{inh} is calculated by using Eq. (8).

[b] Intrinsic TOH (2.47 μM) and other antioxidants in native LDL.

[c] ROH added after the depletion of the intrinsic antioxidants (see Fig. 3).

[d] ROH added before the initiation (see Fig. 4).

[e] $n = R_i t_{inh}/([ROH]_0 + [TOH]_0)$.

[f] Assuming that TOH is the major antioxidants in native LDL and each TOH molecule traps two peroxyl radicals (see text).

[8,42–44]. The inhibition rate constant, k_{inh}, of α-tocopherol is calculated to be $5.7 \times 10^5\ M^{-1}\ s^{-1}$ according to Eq. (8), taking the k_p as $16.6\ M^{-1}\ s^{-1}$ in phospholipid membranes [45] and the concentration of polyunsaturated fatty acids, [LH], in LDL as 0.55 M [1]. This value is close to the value of $5.9 \times 10^5\ M^{-1}\ s^{-1}$ reported recently by measuring the *cis*, *trans*/*trans*, *trans* product ratios of the cholesteryl linoleate hydroperoxides in LDL [44], but it is significantly higher than the value of $8 \times 10^4\ M^{-1}\ s^{-1}$ reported previously for liposomes [38] and $4.7 \times 10^4\ M^{-1}\ s^{-1}$ for 1,2-dimyristoylphosphatidyl-choline (DMPC) bilayers [45], due to the very high local concentration of LH in LDL particles.

All these compounds produce clear inhibition periods and have significantly decreased kinetic chain lengths in the absence of endogenous antioxidants, demonstrating that they are good antioxidants in LDL. The inhibition rate constant, k_{inh}, of these compounds is comparable to that of TOH and the stoichiometric factor, *n*, of 3,4,3′,4′-THS is 3.2, implying that each molecule of 3,4,3′4′-THS might be able to trap about three peroxyl radicals, hence more than one hydroxyl group must be involved in the antioxidation process. These facts make 3,4,3′,4′-THS the most active antioxidant among these compounds. Based on the inhibition time and/or stoichiometric factor the antioxidant efficacy of resveratrol and its analogues follows the sequence of 3,4,3′,4′-THS ∼ 3,3′-DM-4,4′-DHS > 3,4-DHS ∼ 3,4,4′-THS > 2,4,4′-THS > resveratrol ∼ 3,5-DHS > 4,4′-DHS ∼ 2,4-DHS. The similar activity of 3,4,3′,4′-THS and 3,3′-DM-4,4′-DHS is most likely due to the higher lipophilicity of 3,3′-DM-4,4′-DHS that makes it easier partition into LDL particles. We have found previously that making vitamin C lipophilic could enhance its antioxidant activity in LDL [7].

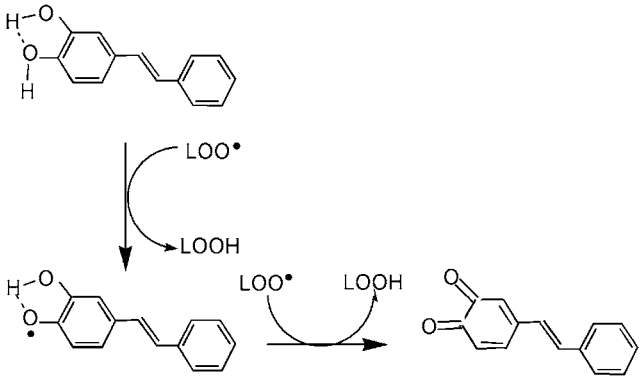
It is well-known that TOH, the most abundant and active form of vitamin E, is a principal lipid-soluble chain-breaking antioxidant in biomembranes [37] and in LDL [40]. Its synergistic antioxidative effect with other antioxidants, such as L-ascorbic acid (vitamin C) [39] and green tea polyphenols [8,22], has been well documented and proven to be due to the reduction of α-tocopheroxyl radical (TO•) by the co-existent antioxidant (ArOH) to regenerate TOH [Eq. (11)]. We have proven recently by using stopped-flow electron paramagnetic resonance (EPR) spectroscopy that resveratrol and 3,4-DHS could reduce α-tocopheroxyl radical (TO•) to regenerate TOH (Eq. (11)) with bimolecular rate constants of 0.23×10^2 and $3.0 \times 10^2\ M^{-1}\ s^{-1}$, respectively, in cetyl trimethylammonium bromide (CTAB) micelles [25].

$$TO^{\cdot} + ArOH \rightarrow TOH + ArO^{\cdot} \tag{11}$$

It can be seen from Table 2 that the k_{inh} decreases remarkably when resveratrol or its analogue is added before the initiation, and the overall inhibition time is approximately equal to the sum of the inhibition time of the endogenous antioxidants (principally TOH) in native LDL and that of resveratrol or its analogue when it is added after depletion of the endogenous antioxidants. It is also seen from Fig. 5 that no appreciable turning point is observed in the decay curve of TOH in the presence of 3,4,3′,4′-THS. These facts suggest that TOH and the exogenous resveratrol analogue might act independently, i.e., no synergistic antioxidant interaction takes place between the resveratrol analogues and TOH in LDL. This is probably due to the fact that the reaction rate of resveratrol analogues with peroxyl radicals in LDL, k_{inh}, is remarkably faster ($10^5\ M^{-1}\ s^{-1}$) than the rate of the TOH regeneration reaction (10^1–$10^2\ M^{-1}\ s^{-1}$). Hence the reaction (11) can not compete with reaction (3).

3.5. The structure–activity relationship and antioxidant mechanism

The important event of lipid peroxidation is oxygen absorption and the final product of the peroxidation is MDA [35]. In the present work the two indexes were used to evaluate the antioxidative activity of resveratrol and its analogues to find the structural determinant responsible for the antioxidant activity in vitro. Comparison of the data of Tables 1 and 2 demonstrates that the antioxidant activity of resveratrol and its analogues follows a similar pattern, whether the peroxidation is initiated by AAPH or by Cu^{2+}, and in spite of the difference in activity being monitored by oxygen uptake or by MDA formation. It is clearly seen that the antioxidative activity of 3,4,3′,4′-THS, 3,4-DHS, 3,4,4′-THS and 3,3′-DM-4,4′-DHS is significantly higher than that of the other compounds. That is, molecules bearing *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl functionality, are appreciably more active than those bearing no such functionalities. It has been proven that the *ortho*-methoxyl group can form an intramolecular hydrogen bond with the phenolic hydrogen, making the H-atom abstraction from the *ortho*-methoxyphenols surprisingly easy [46]. It was also known that the *ortho*-hydroxyl substitution on phenol would make the oxidation intermediate, *ortho*-hydroxyphenoxyl radical, more stable due to the intramolecular hydrogen bonding interaction as reported recently from both experiment by spectrophotometric measurements [47] and theoretical calculations [48] (Scheme 1). The theoretical calculation showed that the intramolecular H-bond in *ortho*-OH phenoxyl radical is ca. 4 kcal/mol stronger than that in the parent molecule catechol and the bond dissociation energy (BDE) of catechol is 9.1 kcal/mol lower than that of phenol [48]. In addition, *ortho*-OH phenoxyl radical and/or *ortho*-semiquinone radical anion will be more easily further oxidized to form the final product *ortho*-quinone as exemplified in Scheme 1 [47]. The fact that the stoichiometric factor, *n*, of 3,4,3′,4′-THS, 3,4-DHS, 3,4,4′-THS is larger than two (Table 2) suggests that the second peroxyl radical must be involved in the antioxidation reaction leading to the formation of the corresponding *ortho*-quinone as shown in Scheme 1. We have recently found the significantly higher antioxidant activity of molecules bearing *ortho*-diphenoxyl functionality in flavonols [9,21] and curcumin analogues [49,50].



Scheme 1. Mechanism of 3,4-DHS inhibited peroxidation.

4. Conclusion

The present investigation demonstrates that resveratrol and its analogues are effective antioxidants against AAPH- and Cu^{2+}-induced LDL peroxidation. Of particular interest is the finding that the compounds bearing *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl functionality exhibit remarkably higher antioxidative activity than the ones bearing no such functionalities. It gives us useful information for antioxidant drug design. In addition, synergistic antioxidant interaction between resveratrol analogues and α-tocopherol is not observed in LDL.

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