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Bioorganic & Medicinal Chemistry 12 (2004) 4017–4023
Bioorganic & Medicinal Chemistry

Inhibitory effects of multi-substituted benzylidenethiazolidine-2,4-diones on LDL oxidation

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Received 13 April 2004; revised 31 May 2004; accepted 1 June 2004

Abstract—Multi-substituted benzylidenethiazolidine-2,4-diones **3a**–**h** were synthesized by Knoevenagel condensation of di- or tri-substituted 4-hydroxybenzaldehydes [or 1-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethanone] **1** with thiazolidine-2,4-dione (**2**) and evaluated for antioxidant activities of Cu^{2+}-induced oxidation of human low-density lipoproteins (LDL). Among compounds **3a**–**h**, **3a** was superior to probucol in LDL-antioxidant activities and found to be ninefold more active than probucol. Due to its potency, compound **3a** was tested for complementary in vitro investigations, such as TBARS assay ($IC_{50} = 0.1\,\mu M$), lag time (240 min at 1.5 μM), relative electrophoretic mobility (REM) of ox-LDL (inhibition of 83% at 10 μM), fragmentation of apoB-100 (inhibition of 61% at 5 μM), and radical DPPH scavenging activity on copper-mediated LDL oxidation. In macrophage-mediated LDL oxidation, the TBARS formation was also inhibited by compound **3a**.

1. Introduction

The syntheses and biological activities of thiazolidinones have been extensively reviewed.[1] Especially, thiazolidinediones (TZDs) were found to exhibit antidiabetic activities as PPARγ ligands[2] and anti-inflammatory activities as 5-lipoxygenase and cyclooxygenase inhibitors.[3] In addition, it has been known that PPARγ is highly expressed in macrophage foam cells of human and mouse atherosclerotic lesions and TZDs inhibit the development of atherosclerosis in $Ldlr^{-/-}$ mice.[4] In general, oxidized low-density lipoproteins (ox-LDLs) play a key role in the early stages of atherosclerosis.[5] In connection with our ELISA system (based on the interaction between PPARγ and SRC-1) for searching a PPARγ ligand, we found that 3,5-di-*tert*-butyl-4-hydroxybenzylidene-thiazolidin-2,4-dione (**3a**) acted as a PPARγ ligand.[6] Therefore, compound **3a** and its analogues acting as both an antioxidant and a PPARγ ligand may be of value for the prevention and/or the treatment of atherosclerosis by attenuating foam cell formation.

For the last two decades, antioxidants have exhibited anti-atherogenic activities by inhibiting the foam cell formation in animal model.[7] Among various antioxidants, probucol and vitamin E are well known to lower lipid levels and the risk of coronary heart disease incidence.[8–13] However, both probucol and vitamin E have an untoward effect of lowering serum high-density lipoprotein (HDL)-cholesterol levels.

This paper deals with the study of antioxidant activities of **3a**–**h** on LDL using thiobarbituric acid reactive substances (TBARS) formation. Also, oxidation of LDL for **3a** was monitored by various systems, such as conjugated diene formation, relative electrophoretic mobility (REM) of ox-LDL, fragmentation of apoB-100, radical DPPH scavenging activity, and macrophage-mediated LDL oxidation.

Keyword: Antioxidant.
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doi:10.1016/j.bmc.2004.06.001

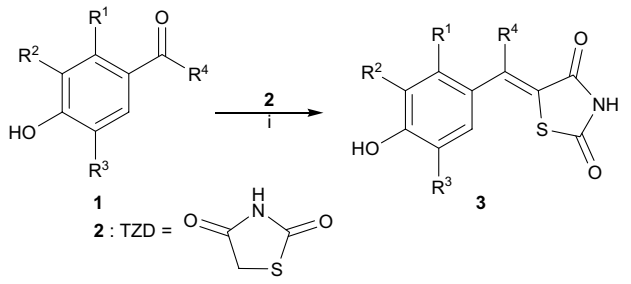
2. Results and discussion

2.1. Chemistry

5-(3,5-Di-*tert*-butyl-4-hydroxybenzylidene)thiazolidine-2,4-dione (**3a**) was prepared by Knoevenagel condensation of 3,5-di-*tert*-butylbenzaldehyde (**1**) with thiazolidine-2,4-dione (**2**) in 73% yield. Similar reactions of 3,5-di-*i*-propyl-, methyl-, or 2,3,5-trimethylbenzaldehydes **1b–d** with **2** gave benzylidenethiazoles **3b–d** in moderate yields. Benzaldehydes **1e–g** substituted with electron rich functional groups, such as hydroxy, fluoro, and methoxy groups, were subjected to Knoevenagel condensation conditions to give benzylidenethiazoles **3e–g** in good yields (method A) (Scheme 1). In order to prepare an analog containing a methyl substituent on the benzylidene double bond, Knoevenagel condensation (method B) of the di-*tert*-butyl ketone **1h** with **2** gave 5-[1-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethylidene]thiazolidine-2,4-dione (**3h**) in 78% yield. The geometry of all benzylidenethiazolidine-2,4-diones **3a–h** was determined to be (*Z*)-olefinic products by comparing their spectroscopic analysis with reported data previously.[3]

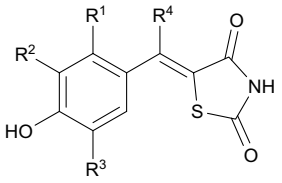
2.2. Biology

2.2.1. Lipid peroxidation. Various di-, tri-, or tetra-substituted benzylidenethiazoles **3a–h** were synthesized and examined in vitro for their abilities to protect human LDL against Cu^{2+}-induced peroxidation. The prescreening test as preliminary evaluation to select the best candidate among **3a–h** is based on a malondialdehyde (MDA) dosage. The results are expressed as IC_{50} (concentration inhibiting 50% of the Cu^{2+}-induced lipid peroxidation), determined by the TBARS assay, and expressed as a MDA equivalent. Then, initial concentration of α-tocopherol in a native LDL was 1.8 μM, which was determined by HPLC.[14] The LDL-antioxidant activities of the compounds **3a–h** were confirmed by the positive control with probucol. Compounds **3a–e** containing functional groups (*i*-Pr, Me, and OH) showed a potent antioxidant activities, whereas **3f** and **3g** involving electron donor groups (fluoro and methoxy) exhibited less antioxidant activity. Compound **3h** including an additional methyl group at benzylidene double bond exhibited a high inhibitory activity (Table 1). According to Lichtenberg's results,[15] antioxidants



Scheme 1. Reagents and reaction conditions: (i) method A: piperidine (cat.), EtOH, reflux; method B: NH_4OAc, toluene, reflux.

Table 1. Inhibition of copper-induced lipid peroxidation in the TBARS assay by benzylidenethiazlidine-2,4-diones **3a–h**



Compds	R^1	R^2	R^3	R^4	IC_{50} (μM)
3a	H	*t*-Bu	*t*-Bu	H	0.1
3b	H	*i*-Pr	*i*-Pr	H	0.9
3c	H	Me	Me	H	0.3
3d	Me	Me	Me	H	4.5
3e	H	OH	H	H	2.3
3f	H	F	F	H	16.0
3g	H	OMe	OMe	H	25.5
3h	H	*t*-Bu	*t*-Bu	Me	0.3
P[a]					0.9

[a] Probucol was used as a reference antioxidant.

are reducing agents that reduce transition metal or interact with free radicals to result in the formation of antioxidant-derived free radicals. Also, Ziouzenkova et al.[16] reported that copper ions are bound to high as well as to low affinity sites to generate lipid peroxyl radicals ($LOO^{\cdot}$), which can be effectively inhibited by α-tocopherol. Therefore, these results may be rationalized that bulky substituents on phenol ring may stabilize the phenoxy radical formed to show more antioxidant activity against LDL, which agreed with earlier work.[17] It also demonstrated that 2,6-di-*tert*-butylphenol is more reactive toward radical source to afford 2,6-di-*tert*-butylphenoxy radical in inhibition of free radical reaction.[18] Even though **3e** has more reactive to DPPH (Fig. 4), **3e** showed less antioxidant activity than **3a**. It may conclude that **3e** might protect Cu^{2+}-mediated LDL oxidation as a Cu^{2+}-chelator,[19] not free radical scavenger. In order to confirm, which part of phenol or amide moiety of **3a** concerns in free radical reaction, **1a** and thizolidine-2,4-dione **2** were tested for their antioxidant activities to show the values with an IC_{50} of 0.4 μM for **1a** and an IC_{50} of >100 μM for **2**. However, hybrid molecule **3a** appeared to be a remarkably potent antioxidant with an IC_{50} of 0.1 μM, which observed in the MDA test (at least nine times more potent than probucol itself). Furthermore, the steric and electronic factors of substituents to stabilize phenoxy radical formed from phenolic hydroxy group may influence antioxidant activities for human LDL.

2.2.2. Detection of conjugated diene formation. The extent of lag time was interpreted as oxidation resistant capacity of LDL. The formation of conjugated dienes during LDL oxidation represents the early peroxidation of LDL. The oxidation of LDL was determined by measuring conjugated diene formation at 234 nm every 10 min for 4 h. The effect of compound **3a** on the prolonging of lag time was shown in Figure 1. First, the LDL was incubated with $CuSO_4$ alone to have a lag time (80 min). While 1.0 μM of **3a** appeared to have moderate

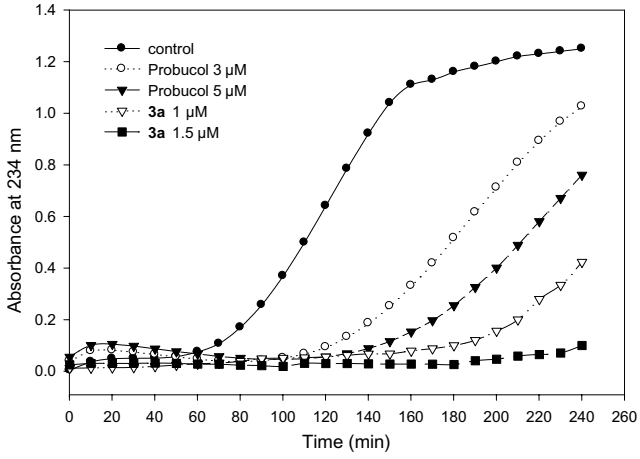


Figure 1. Effect of **3a** on Cu^{2+}-mediated LDL oxidation. LDL (100 μg protein/mL) was incubated with 5 μM of $CuSO_4$ in the presence or absence of antioxidant, **3a**. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 4 h. Probucol was used as a reference antioxidant.

effect on the lag time extension (190 min), 1.5 μM of **3a** strongly delayed the lag time to 240 min. However, 3.0 and 5.0 μM of probucol mildly extended the lag time to 132 and 162 min, respectively. Thus, **3a** inhibited much more the formation of conjugated diene formation during Cu^{2+}-induced LDL oxidation than did probucol.

2.2.3. Relative electrophoretic mobility (REM) of ox-LDL. The effects of various concentration of **3a** on Cu^{2+}-mediated oxidation of LDL were determined by REM assay. As shown in Figure 2, the 10 μM of $CuSO_4$ alone was employed to oxidize LDL for 12 h (lane 2). The REM of ox-LDL was lowered to 83%, 83%, and 10% (lanes 3–5), respectively, in the presence of 20, 10, and 5 μM of **3a**, whereas treatment of 1 μM of **3a** did not protect LDL oxidation (lane 6), compared to that of oxidized LDL. The mobility of LDL in probucol as a positive control was reduced dose-dependently to 86% (20 μM), 70% (10 μM), and 7% (5 μM) (lanes 7–9), whereas treatment with 1 μM of probucol did not protect LDL oxidation (lane 10).

2.2.4. ApoB-100 fragmentation. Since it has been reported that radical reaction of LDL causes fragmentation of apoB-100 that is a major component of LDL,[20]

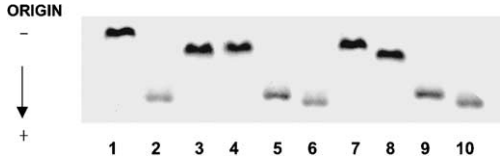


Figure 2. Effect of **3a** on electrophoretic mobility of LDL. LDL (240 μg protein/mL) was incubated with 10 μM $CuSO_4$. Lane 1: native LDL (absence of $CuSO_4$), lane 2: ox-LDL, lane 3: **3a** (20 μM), lane 4: **3a** (10 μM), lane 5: **3a** (5 μM), lane 6: **3a** (1 μM), lane 7: probucol (20 μM), lane 8: probucol (10 μM), lane 9: probucol (5 μM), lane 10: probucol (1 μM). Probucol is a positive control.

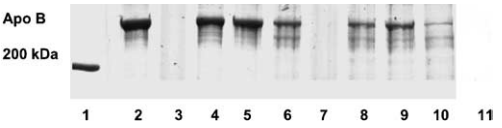


Figure 3. Inhibition of Cu^{2+}-mediated apoB-100 fragmentation in LDL by **3a**. LDL (240 μg protein/mL) was incubated for with 10 μM $CuSO_4$. Lane 1: standard marker, lane 2: native LDL (absence of $CuSO_4$), lane 3: ox-LDL, lane 4: **3a** (20 μM), lane 5: **3a** (10 μM), lane 6: **3a** (5 μM), lane 7: **3a** (1 μM), lane 8: probucol (20 μM), lane 9: probucol (10 μM), lane 10: probucol (5 μM), lane 11: probucol (1 μM).

the LDL oxidation has been evaluated by the fragmentation of apoB-100 through the electrophoretic analysis on polyacrylamide gel in the presence of 4% sodium dodecylsulfate (SDS-PAGE). The band of apoB-100 was observed on the native LDL (lane 2), but the band completely disappeared when the LDL was incubated with $CuSO_4$ (lane 3). The fragmentation of apoB-100 was inhibited in 86%, 80%, and 61%, respectively, in the presence of 20, 10, and 5 μM of **3a** (lanes 4–6), whereas addition of 1 μM of **3a** did not protect apoB-100 fragmentation (lane 7). At the same concentration of probucol, as a positive control, the fragmentation of apoB-100 was inhibited in 61%, 58%, and 36%, respectively, (lanes 8–10), whereas 1 μM of probucol also did not protect apoB-100 fragmentation (lane 11), as shown in Figure 3. As a result, **3a** was more potent than probucol in protection of LDL against Cu^{2+}-induced peroxidation.

2.2.5. Radical DPPH scavenging activity. 1,1-Diphenyl-2-picrylhydrasyl (DPPH) is a very stable free radical and widely used for evaluation of antioxidant activities.[21] In order to confirm whether compounds **3a**–**h** act as a free radical scavenger, we tested radical DPPH scavenging activity of representative **3a**, **3e**, **3f**, and **3g** at 100 μM. Then, activities of **3a**, **3e**, **3f**, and **3g** were measured as decolorizing activity following the trapping of the unpaired electron of DPPH.[22] These results are shown in Figure 4. At the earliest time, the DPPH radical was decreased rapidly by 100 μM of **3a** and **3e**, but decreased slowly by 100 μM of **3f** and **3g**. After 20 min, 34% and 9% DPPH radicals, respectively, were remained at the presence of compounds **3a** and **3e**, whereas **3f** and **3g** having electron donor groups such as fluoro and methoxy exhibited 93% and 87% DPPH radicals remaining at 100 μM, respectively. Therefore, these compounds **3a**–**h** have proven to have radical DPPH scavenging capacity.

2.2.6. Macrophage-mediated LDL oxidation. Next, we were interested to perform an antioxidant activity of **3a** in macrophage-mediated oxidation of LDL. The cellular oxidative modification of LDL to a form recognized by the scavenger receptor requires the presence of transition metal ions in the medium.[23] In macrophage-mediated LDL oxidation, the TBARS formation in the harvested medium was also inhibited by **3a** at a similar order of activity to that obtained in Cu^{2+}-induced LDL oxidation. These results are summarized in Table 2. The

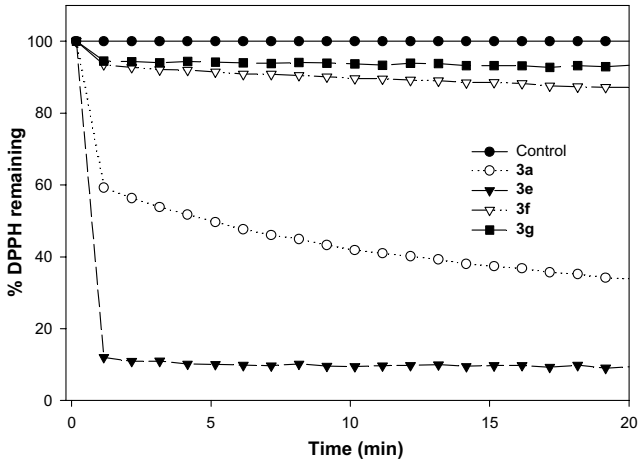


Figure 4. Effects of **3a**, **3e**, **3f**, and **3g** on radical DPPH scavenging. Compounds **3a**, **3e**, **3f**, and **3g** (100 μM) were incubated with 100 μM of DPPH in methanol at room temperature for 20 min. The absorbance at 517 nm of each compound solution was measured. The antiradical activity was expressed by the remaining DPPH percentage.

Table 2. Effects of **3a** and probucol on LDL oxidation by macrophages

Incubation conditions[a]	MDA nmol/mg LDL protein[b]
LDL + Cu^{2+}	10.4 ± 0.7
LDL + cell + Cu^{2+}	80.0 ± 2.6
LDL + cell + Cu^{2+} + 40 μM **3a**	2.0 ± 0.2
LDL + cell + Cu^{2+} + 20 μM **3a**	3.5 ± 0.1
LDL + cell + Cu^{2+} + 10 μM **3a**	4.8 ± 0.1
LDL + cell + Cu^{2+} + 5 μM **3a**	10.9 ± 1.3
LDL + cell + Cu^{2+} + 40 μM probucol	15.2 ± 0.7
LDL + cell + Cu^{2+} + 20 μM probucol	20.1 ± 2.1
LDL + cell + Cu^{2+} + 10 μM probucol	22.3 ± 1.6
LDL + cell + Cu^{2+} + 5 μM probucol	31.0 ± 2.0

[a] LDL (120 μg protein/mL) was incubated for 24 h at 37 °C in RPMI 1640 medium with 2 μM of Cu^{2+} in 12-well plate containing macrophages, in the absence (control) or presence of increasing concentrations of compound **3a** tested (5–40 μM).

[b] The extent of LDL oxidation was determined by directly in the harvested medium using the TBARS assay. Data are shown as means ± SD from two independent experiments performed in duplicate.

LDL was only incubated with 2 μM of $CuSO_4$ to give the value of ox-LDL (10.4 ± 0.7 MDA nmol/mg LDL protein), whereas the content of ox-LDL by incubation of LDL and 2 μM $CuSO_4$ in the presence of macrophage was 80.0 ± 2.6 MDA nmol/mg LDL protein. The value of ox-LDL by macrophage-mediated LDL oxidation has proven to be much higher than that by Cu^{2+}-induced LDL oxidation. This result coincides with the previous reports.[24] Therefore, antioxidant activities of **3a** and probucol were tested by macrophage-mediated LDL oxidation at dose-dependent concentration ranging from 5 to 40 μM. In the presence of 5, 10, 20, and 40 μM of **3a**, the content of ox-LDL was 10.9 ± 1.3, 4.8 ± 0.1, 3.5 ± 0.1, and 2.0 ± 0.2 MDA nmol/mg LDL protein, respectively. At the same concentration of probucol, the content of ox-LDL was 31.0 ± 2.0, 22.3 ± 1.6, 20.1 ± 2.1, and 15.2 ± 0.7 MDA nmol/mg LDL protein, respectively. As a result, antioxidant activity of **3a** was much higher than that of probucol on macrophage-mediated LDL oxidation (Table 2).

3. Conclusions

In this study, we synthesized multi-substituted benzylidenethiazolidine-2,4-diones **3a**–**h** by Knoevenagel condensation of di- or tri-substituted 4-hydroxybenzaldehydes [or 1-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethanone] **1** with thiazolidine-2,4-dione (**2**) and demonstrated that they exhibited antioxidant activities. Previously, Unangst et al.[3] reported that **3a** and **3h** were novel dual 5-lipoxygenase and cyclooxygenase inhibitors with anti-inflammatory activities. Furthermore, we demonstrated the most potent antioxidant activity of **3a** in various LDL oxidation tools (e.g., TBARS assay, conjugated diene formation, REM of ox-LDL, fragmentation of apoB-100 by SDS-PAGE, radical DPPH scavenging activity, and macrophage-mediated LDL oxidation). Further studies on in vivo efficacy test of cholesterol-lowering and anti-atherogenic activities and the mechanism of antioxidant action of compound **3a** are underway.

4. Experimental

4.1. Chemistry

Melting points were measured on a Thomas–Hoover capillary apparatus and are uncorrected. Proton and carbon NMR spectra were measured downfield relative to tetramethylsilane in $CDCl_3$ and *d*-DMSO unless otherwise noted (value in ppm); coupling constants *J* are reported in Hertz; ^1H NMR and ^{13}C NMR were conducted on Bruker avance 300 spectrometer. High resonance mass spectra (HRMS) were recorded on JMS-700 (Jeol, Japan) and measured at Gyeongsang National University (Chinju, Korea). HPLC analyses were performed on Shimadzu CLASS-VP using a porasil (silica, Waters) column eluted with hexane/*i*-PrOH (9:1) at a flow rate of 1 mL/min.

4.1.1. Typical procedure for the preparation of (*Z*)-5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-thiazolidine-2,4-dione (3a) [method A]. To a solution of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (**1a**) (7 g, 29.9 mmol) in EtOH (100 mL) was added thiazolidine-2,4-dione (**2**) (4.2 g, 35.8 mmol) in the presence of catalytic amounts of piperidine (0.9 mL, 9.0 mmol) at room temperature. After heating at 70 °C for 24 h, the reaction mixture was cooled to 0 °C, and 1 N HCl (0.5 mL) and H_2O (100 mL) were added to precipitate a yellow powder, which was filtered to give the crude compound **3a**. The collected **3a** was washed with EtOAc and dried on air to obtain the pure compound **3a** (7.3 g, 73%) as a yellow powder. Mp 240–242 °C (lit.[3] mp 238–240 °C); ^1H NMR (300 MHz, $CDCl_3$) δ 1.47 (18H, s), 5.71 (1H, s, –OH), 7.37 (2H, s), 7.85 (1H, s), 9.22 (1H, br, –NH); ^{13}C NMR (75 MHz,

$CDCl_3$) δ 167.8, 167.2, 156.7, 136.9, 135.9, 128.3, 124.4, 118.0, 34.5, 30.1; HPLC analysis [hexane/*i*-PrOH (9:1)] $t_R = 1.0$ min (99.96%); HRMS calcd for $C_{18}H_{23}NO_3S$: 333.1399; found: 333.1393.

4.1.2. (*Z*)-5-(4-Hydroxy-3,5-diisopropylbenzylidene)-thiazolidine-2,4-dione (3b). 89%, a yellow powder, mp 202–203 °C (lit.[3] mp 201–203 °C); ^{1}H NMR (300 MHz, *d*-DMSO) δ 1.18 (12H, d, $J = 6.6$ Hz), 3.32 (2H, m), 7.26 (2H, s), 7.73 (1H, s), 9.01 (1H, s, –OH), 12.44 (1H, br, –NH); ^{13}C NMR (75 MHz, *d*-DMSO) δ 22.7, 26.1, 118.8, 124.6, 126.1, 133.1, 135.9, 153.7, 167.4, 168.1. MS *m*/*z* 305 (M^+); HRMS calcd for $C_{16}H_{19}NO_3S$: 305.1086; found: 305.1085.

4.1.3. (*Z*)-5-(4-Hydroxy-3,5-dimethylbenzylidene)thiazolidine-2,4-dione (3c). 80%, a yellow powder, mp 266–268 °C; ^{1}H NMR (300 MHz, *d*-DMSO) δ 2.20 (6H, s), 7.17 (2H, s), 7.60 (s, 1H), 9.14 (1H, s, –OH), 12.42 (1H, br, –NH); ^{13}C NMR (75 MHz, *d*-DMSO) δ 16.6, 118.8, 123.9, 125.1, 130.9, 132.5, 156.1, 167.5, 168.1; MS *m*/*z* 249 (M^+); HRMS calcd for $C_{12}H_{11}NO_3S$: 249.0460; found: 249.0440.

4.1.4. (*Z*)-5-(4-Hydroxy-2,3,5-trimethylbenzylidene)-thiazolidine-2,4-dione (3d). 98%, a yellow powder, mp 257–259 °C; ^{1}H NMR (300 MHz, *d*-DMSO) δ 2.11 (3H, s), 2.18 (3H, s), 2.22 (3H, s), 7.01 (1H, s), 7.89 (1H, s), 8.88 (1H, s, –OH), 12.45 (1H, br, –NH); ^{13}C NMR (75 MHz, *d*-DMSO) δ 12.6, 15.6, 16.8, 121.3, 122.1, 123.3, 124.3, 127.1, 131.1, 136.6, 155.3, 167.3, 168.5; MS *m*/*z* 263 (M^+); HRMS calcd for $C_{13}H_{13}NO_3S$: 263.0616; found: 263.0619.

4.1.5. (*Z*)-5-(3,4-Dihydroxybenzylidene)thiazolidine-2,4-dione (3e). 78%, a yellow powder, mp >300 °C; ^{1}H NMR (300 MHz, *d*-DMSO) δ 6.85–7.00 (3H, m), 7.60 (1H, s), 9.46 (1H, s, –OH), 9.83 (1H, s, –OH), 12.42 (1H, br, –NH); ^{13}C NMR (75 MHz, *d*-DMSO) δ 98.0, 116.3, 118.7, 124.0, 124.3, 132.7, 145.9, 148.6, 167.5, 168.2; MS *m*/*z* 237 (M^+); HRMS calcd for $C_{10}H_7NO_4S$: 237.0096; found: 237.0090.

4.1.6. (*Z*)-5-(3,5-Difluoro-4-hydroxybenzylidene)thiazolidine-2,4-dione (3g). 75%, a yellow powder, mp 269–270 °C. ^{1}H NMR (300 MHz, *d*-DMSO) δ 7.28 (2H, dd, $J = 7.8$, 8.7 Hz), 7.68 (1H, s), 12.43 (1H, br, –NH); ^{13}C NMR (75 MHz, *d*-DMSO) δ 113.7, 122.8, 123.4, 130.1, 150.5, 153.7, 167.3, 167.6; MS *m*/*z* 257 (M^+); HRMS calcd for $C_{10}H_5F_2NO_3S$: 256.9958; found: 256.9955.

4.1.7. (*Z*)-5-(4-Hydroxy-3,5-dimethoxybenzylidene)thiazolidine-2,4-dione (3f). 78%, a yellow powder, mp 250–252 °C; ^{1}H NMR (300 MHz, *d*-DMSO) δ 3.81 (6H, s), 6.88 (2H, s), 7.71 (1H, s), 9.34 (1H, s), 12.47 (1H, br, –NH); ^{13}C NMR (75 MHz, *d*-DMSO) δ 56.0, 108.0, 119.6, 123.2, 132.9, 138.6, 148.2, 167.4, 168.0; MS *m*/*z* 281 (M^+); HRMS calcd for $C_{12}H_{11}NO_5S$: 281.0358; found: 281.0356.

4.1.8. (*Z*)-5-[1-[3,5-Bis(1,1-dimethylethyl)-4-hydroxyphenyl]ethylene]-2,4-thiazolidinedione (3h) [method B]. A mixture of **1h** (1.0 g, 4.03 mmol), **2** (0.94 g, 8.05 mmol), and NH_4OAc (0.6 g, 8.05 mmol) in toluene (4 mL) was refluxed for 48 h. The reaction mixture was cooled and evaporated. The residue was digested briefly in warm MeOH (10 mL), cooled, and filtered to give 1.1 g (78%) of **3h** as a yellow powder. Mp 254–255 °C (lit.[3] mp 253–254 °C); ^{1}H NMR (300 MHz, *d*-DMSO) δ 1.39 (18H, s), 2.65 (3H, s), 7.20 (2H, s), 7.45 (1H, br, –OH), 12.18 (1H, br, –NH); ^{13}C NMR (75 MHz, *d*-DMSO) δ 21.3, 30.1, 34.7, 120.0, 123.7, 132.9, 139.0, 150.5, 155.1, 166.6, 168.0.

4.2. Biology

4.2.1. Isolation of LDL. Blood was collected from normalipidemic volunteers. EDTA was used as anti-coagulant (1.5 mg/mL of blood). After low-speed centrifugation of the whole blood to obtain plasma, EDTA (0.1%), NaN_3 (0.05%), and PMSF (0.015%) were added to prevent lipoprotein modification. LDL was isolated from the plasma by discontinuous density gradient ultra centrifugation.[25] Briefly, the plasma was centrifuged at 100,000*g* at 4 °C for 20 h. After the top layers containing chylomicron and very low-density lipoprotein (VLDL) were removed, the density of remaining plasma fractions was increased to 1.064 with NaBr solution and they were recentrifuged at 100,000*g* for an additional 24 h. The LDL fraction in the top of the tube was collected and dialyzed overnight against three changes of phosphate buffer (pH 7.4) containing NaCl (150 mM), in the dark at 4 °C to remove NaBr and EDTA. The LDL in PBS was stored at 4 °C and used within 4 weeks. The purity of the fraction was confirmed by agarose gel electrophoresis and SDS-PAGE as described elsewhere.[26] Concentration of LDL protein was determined by using bovine serum albumin (BSA) as a standard.[26]

4.2.2. Measurement of α-tocopherol in LDL. LDL was added to the PBS buffer (10 mM, pH 7.4) at a final concentration of 150 μg/mL. The LDL solution (1 mL) was chilled in ice and 1 mL of ethanol containing 2 μg of α-tocopherol acetate as an internal standard was added to the LDL solution. The mixture was immediately extracted using 2 mL of hexane. After vortexing and centrifugation at 1000*g* for 5 min at 4 °C, 1.8 mL of organic phase (hexane) was removed under nitrogen stream to give the residue, which was dissolved in 80 μL of ethanol. Ethanol extracts (20 μL) was analyzed by HPLC (Shimadzu SCL-10A, Tokyo, Japan) on C-18 column (YMC-Pack Pro C_{18}, 150×4.6 mm I.D., S-5 μM, YMC Co., Ltd, Japan) with MeOH/H_2O (98:2) as the mobile phase at a flow rate 1 mL/min. α-Tocopherol was monitored using a diode array detector at 200 or 280 nm with retention time of 11.07 ± 0.27 min and quantified

the amount of α-tocopherol using a standard plot of peak area ratio (α-tocopherol/α-tocopherol acetate) against α-tocopherol concentration. The concentration of α-tocopherol in the native LDL solution (150 μg/mL) was found to be 1.8 μM.

4.2.3. Cu^{2+}-induced oxidation of LDL. Human plasma LDL (120 μg protein/mL) was oxidized in 10 mM phosphate-buffered saline with 10 μM $CuSO_4$ and then stopped by addition of 1 mM EDTA. In our experiments, oxidation was carried out in the presence of **3a**–**h**. After the incubation, TBARS formation, conjugated diene formation, relative electrophoretic mobility (REM), fragmentation of the apoB-100 of LDL, and radical DPPH scavenging activity were measured as described below.

4.2.4. TBARS assay. The LDL oxidation was determined spectrophotometrically by measuring the amount of thiobarbituric acid reactive substances (TBARS).[27] One milliliter of 20% (w/v) trichloroacetic acid (TCA) and 1 mL of 0.67% (w/v) thiobarbituric acid (TBA) were added to the post incubation mixture. The mixture was heated at 100 °C for 15 min and then cooled. After centrifugation at 1500*g* for 15 min to remove precipitated protein, the absorbance of the supernatant was measured at 540 nm. The concentration of TBARS was expressed as equivalents of 1,1,3,3-tetraethoxypropane that was used as standard.

The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio } (\%) = (A - A1)/A \times 100$$

where A was the absorbance of the control and $A1$ was the absorbance of the tested sample.

4.2.5. Detection of conjugated dienes. The formation of conjugated dienes was measured by monitoring of the absorbance at 234 nm using an UV–vis spectrophotometer (Hewlett Packard model 8453, Agilent Technologies, Germany).[28] Briefly, 3 mL of an LDL solution (120 μg/mL) in phosphate-buffered saline, at pH 7.4, was incubated 10 μM of $CuSO_4$ at 37 °C in the presence or absence of **3a**, thereafter the absorbance at 234 nm was measured every 10 min. Probucol was used as a reference antioxidant. The plot of absorbance against time produces three phases: (a) a lag phase, (b) a propagation phase, and (c) a decomposition phase. The lag time (the extent to which the compounds protected LDL from oxidation was reflected by the prolongation of the lag phase compared to that of control) was measured as the intercept between the baseline and the tangent of the absorbance curve during the propagation phase.[29]

4.2.6. Relative electrophoretic mobility (REM). The electrophoretic mobility of native or oxidized LDL was detected by agarose gel electrophoresis (Ciba Corning Diagnostics, Palo Alto, CA).[30] Briefly, the LDL (120 μg/mL) in phosphate-buffered saline, pH 7.4, was oxidized with 10 μM of $CuSO_4$ for 12 h at 37 °C with or without **3a** or probucol. Thereafter, the agarose gel (0.7% agarose) was electrophoresed (85 V) in buffer containing 40 mM/L Tris, 40 mM/L glacial acetic acid, and 1 mM/L EDTA for 1 h. After electrophoresis, lipoprotein bands were stained with Coomassie Brilliant Blue, and REM was defined as ratio of the distances migrated from the origin by ox-LDL versus native LDL.

4.2.7. Electrophoresis of the apoB-100 fragmentation on SDS-PAGE. After the oxidation with or without **3a**, sample were denatured with 3% SDS, 10% glycerol, and 2-mercaptoethanol at 95 °C for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 4%) was performed to detect the apoB-100 fragmentation. The electrophoresis was processed at 100 V for 80 min. After the electrophoresis, the gel was dried and stained with Coomassie Brilliant Blue R250.[31] Density of each spot was measured at 550 nm by a Shimadzu dual-wavelength flying-spot scanner CS-9000.

4.2.8. Radical DPPH scavenging activity. Radical DPPH scavenging activity was studied by methanol solution of compounds **3a**, **3e**, **3f**, and **3g** at 100 μM. Freshly made DPPH radical solution (2 mL) was added into each 1 mL of **3a**, **3e**, **3f**, and **3g** to result in 34%, 9%, 93%, and 87% DPPH radicals remaining. Then, the final concentration was 100 μM for DPPH radicals. The absorbance was measured at 517 nm against a blank of pure methanol including only DPPH radical for 20 min using the UV–visible spectrophotometer at room temperature. Radical DPPH scavenging capacity was calculated from the difference in absorbance with each concentration of **3a**, **3e**, **3f**, and **3g** and expressed as percent DPPH radical remaining, according to the following equation:

% DPPH remaining = 100 × (absorbance of sample/absorbance of control).

4.3. Cell culture

Human monocytic THP-1 cells (ATCC) were cultured in RPMI 1640 medium (Gibco/BRL) with phenol red containing 10% fetal bovine serum (Gibco/BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO_2 in air. Cells in RPMI 1640 medium with serum and antibiotics were plated in 12-well plate (1×10^6 cell/well in 1 mL). Differentiation of THP-1 cells to macrophage was induced by treatment of phobal 12-myristrate 13-acetate (PMA, 150 ng/mL, Sigma) for 3 days.[32]

4.3.1. Cell-mediated oxidation. THP-1 macrophage cells were washed three times with serum-free RPMI 1640 media. The LDL (120 μg/mL) was added to the culture medium and the medium was supplemented with 2 μM $CuSO_4$ to catalyze cell-mediated LDL oxidation in the

presence or absence of **3a** or probucol. Compound **3a** was dissolved in ethanol, in which ethanol concentration was not in excess at least 0.5% of the medium. Equivalent amounts of ethanol were added to control cell cultures. Incubation was then carried out at 37 °C for 24 h, in a humidified atmosphere containing 5% CO_2. After supernatants were collected and centrifuged, the extent of LDL oxidation was determined directly in the harvested medium using TBARS assay.

Acknowledgements

This work is supported by a grant (National Research Laboratory Program) from the Ministry of Science and Technology (M1-0302-00-0033), Korea.

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