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Antioxidant and anti-inflammatory activities of *N*-acetyldopamine dimers from Periostracum Cicadae

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Abstract—A known *N*-acetyldopamine dimer, (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethyl)-1,4-benzodioxane (**1**) and a new *N*-acetyldopamine dimer, (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethylene)-1,4-benzodioxane (**2**) were isolated from the methanolic extracts of Periostracum Cicadae. Compounds **1** and **2** inhibited the Cu^{2+}-mediated, 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH)-mediated, and 3-morpholinosydnonimine (SIN)-1-mediated LDL oxidation in the thiobarbituric acid-reactive substances (TBARS) assay. The antioxidant activities of **1** and **2** were tested with respect to other parameters, such as lag time of conjugated diene formation, relative electrophoretic mobility (REM), and apoB-100 fragmentation on copper-mediated LDL-oxidation. Compounds **1** and **2** also showed 1,1-diphenyl-2-picrylhydrasyl (DPPH) radical scavenging activity. Compound **2** was more efficient than compound **1** at inhibiting the reactive oxygen species (ROS) generation, nitric oxide (NO) production, and nuclear factor-κB (NF-κB) activity as well as the expression of pro-inflammatory molecules such as inducible nitric oxide synthase (iNOS), interleukin (IL)-6, tumor necrosis factor (TNF)-α, and cyclooxygenase (COX)-2 in LPS-induced RAW264.7 cells.

1. Introduction

Atherosclerosis is regarded as an inflammatory disease, characterized by the accumulation of macrophage-derived foam cells in the vessel wall and accompanied by the production of a wide range of chemokines, inflammatory cytokines, and growth factors.[1] The generation of reactive oxygen species (ROS) in phagocytic leukocytes (neutrophils, monocytes, macrophages, and eosinophils) is one of the most important hallmarks of the inflammatory process.[2] In addition to promoting general cytotoxicity, ROS may also act to up-regulate pro-inflammatory gene expression by activating nuclear factor-κB (NF-κB), a process that is itself sensitive to the cellular redox state.[3] NF-κB has been regarded as a proatherogenic factor, mainly because of its regulation of many of the pro-inflammatory genes linked to atherosclerosis. Oxidized low-density lipoprotein (LDL) also plays a key role in the generation of inflammatory processes in atherosclerotic lesions of all stages.[4,5] Much attention has been concentrated on the development of LDL-antioxidants from fruits, vegetables, and beverage plants,[6] or of newly designed synthetic antioxidants.[7] However, there is a few report of LDL-antioxidants from insects, which are the largest and most diverse groups of organisms. So, the inhibitory activities of the extracts of Asian medicinal insects were examined on LDL oxidation for screening of anti-atherogenic substances.[8] The methanolic extracts of Periostracum Cicadae showed significant inhibitory activity on copper-mediated LDL oxidation in thiobarbituric acid-reactive substances (TBARS) assay. Periostracum Cicadae, the cast-off shell of the *Cryptotympana pustulata* named 'Seontoi' in Korea, has been used for traditional Asian medicine as an antifebrile, a spasmolytic, and an antiphlogistic.[9] In this study, we describe the isolation, characterization of structure, and antioxidant activities of the two *N*-acetyldopamine dimers **1** and **2** (Fig. 1) isolated from Periostracum Cicadae. In addition, we studied

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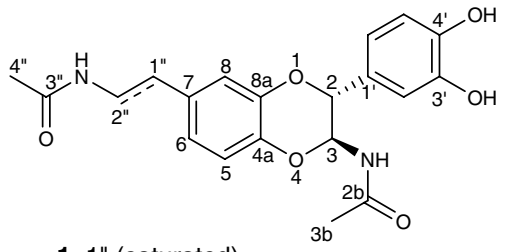

1 1" (saturated)
2 1" (unsaturated, *E*)

Figure 1. Chemical structures of **1** and **2** isolated from Periostracum Cicadae.

the effects of **1** and **2** on ROS generation, NO production, expression of pro-inflammatory molecules, such as iNOS, IL-6, TNF-α, and COX-2, and NF-κB activity in LPS-induced RAW264.7 cells.

2. Results and discussion

2.1. Isolation and structure elucidation

Using copper-mediated LDL oxidation in TBARS assay, bioactivity-guided fractionation of the methanolic extracts of Periostracum Cicadae led to the isolation of a known *N*-acetyldopamine dimer, (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethyl)-1,4-benzodioxane (**1**), and a new *N*-acetyldopamine dimer, (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethylene)-1,4-benzodioxane (**2**).

Compound **1** was isolated as white powder. The EIMS afforded the positive ion $[M]^+$ at *m/z* = 386, implying a molecular formula of $C_{20}H_{22}N_2O_6$, which was confirmed by the HREIMS ($[M+H]^+$ at *m/z* = 387.1553, calcd 387.1556). The $[\alpha]_D$ value of **1** was +20.0° (*c* 0.8, MeOH) {lit. $[\alpha]_D$ +28.2° (*c* 1.0, MeOH)}. Structure of **1** was elucidated by the study of MS and NMR (^1H, ^{13}C, COSY, HMQC, and HMBC) experiments and identified by comparison with the reported spectral data. The absolute stereochemistry was determined by the CD spectral data, showing a negative Cotton effect in 235 nm and a positive sign in 280 nm as showed in Figure 2. Thus, the structure of **1** was confirmed as (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethyl)-1,4-benzodioxane.[10]

Compound **2** was isolated as yellow powder. The molecular formula of **2** was determined to be $C_{20}H_{20}N_2O_6$ on the basis of its positive HRFABMS data (*m/z* $[M+Na]^+$, 407.1224, calcd 407.1219 for $C_{20}H_{20}N_2NaO_6$). The $[\alpha]_D$ value of **2** was +33.8° (*c* 0.1, MeOH). The UV spectrum of **2** exhibited λ_{max} at 282 nm ($\log\varepsilon = 4.32$, in MeOH) and the IR spectrum of **2** showed strong hydroxyl absorption band at 3428 cm^{-1}. The ^1H NMR spectrum of **2** showed two singlet peaks at δ_H 1.86 (s, 3H, H-3b) and 2.02 (s, 3H, H-4″), two methane protons at δ_H 4.69 (d, *J* = 7.1, 1H, H-2) and 5.68 (d, *J* = 7.1 Hz, 1H, H-3), two olefinic protons at δ_H 6.09 (d, *J* = 14.7 Hz, 1H, H-1″) and 7.29 (d, *J* = 14.7 Hz, 1H, H-2″), and multiplets at δ_H 6.73–6.89 corresponding to 6H were attributed to two phenyl rings. In the HMBC correlations, long-range couplings were observed from the methine proton at δ_H 5.68 to the methine carbon (C-3, δ 78.3) and *N*-acetylamino carbon (C-2b, δ 173.2). According to these results, the *N*-acetylamino and *N*-acetylamino-2-ethylene groups are located at the 3 and 7 positions in 1,4-benzodioxane moiety. Then, absolute stereochemistry of **2** was determined by comparing its coupling constant value with reported data of **1**[10] and CD spectrums of compounds **1** and **2** (Fig. 2). Therefore, the structure of **2** was determined as (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethylene)-1,4-benzodioxane.

2.2. Inhibitory effects of lipid peroxidation

N-Acetyldopamine dimer is known to be components of sclerotized insect cuticles and isolated from the femurs of locusts and beetles,[11] and from the cockroach belonging to Epilampridae.[12] Thereafter, Noda et al. isolated *N*-acetyldopamine dimers from *Cryptotympana* sp.[12] Oxenkrug et al. reported that *N*-acetyldopamine, a sepiapterin reductase inhibitor and the immediate precursor and metabolite of melatonin, inhibits rat brain lipid peroxidation induced by LPS.[13] However, there is no report about the potential of *N*-acetyldopamine dimer as antioxidant. Ability of **1** and **2** to attenuate LDL oxidation was measured by measuring the amount of TBARS.[14] The compounds **1** and **2** showed potent antioxidant activities on copper-mediated LDL-oxidation with IC_{50} values of 2.1 ± 0.1 and 1.5 ± 0.1 μM, respectively (probucol, IC_{50} = 2.8 ± 0.2 μM) (Fig. 3A). Under 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH; a free radical generator, that thermally decomposes to aqueous peroxy free radicals)-mediated oxidation,[15] **1** and **2** showed LDL-antioxidant activities with IC_{50} values of 3.1 ± 0.1 and 2.7 ± 0.1 μM, respectively (probucol, IC_{50} = 4.3 ± 0.1 μM) (Fig. 3B). Subsequently, under 3-morpholinosydnonimine (SIN-1; a peroxy nitrite generator)-mediated oxidation,[16] **1** and **2** exhibited LDL-antioxidant activities with IC_{50} values of 2.8 ± 0.1 and 1.6 ± 0.1 μM, respectively (probucol, IC_{50} = 3.3 ± 0.1 μM) (Fig. 3C). Probucol, a known antioxidant having anti-atherogenic activity,[17,18] is used as a positive control in a series of experiments.

2.3. Inhibitory effects of conjugated diene formation

Oxidation of the LDL was determined by measuring the conjugated diene formation at 234 nm[19] and typical effects of **1** and **2** are shown in Figure 4. The LDL (120 μg protein/mL) in PBS buffer (pH 7.4, 10 mM) was incubated with 5 μM $CuSO_4$ alone to have a lag time of 82 min. In the presence of each of 1.0 μM **1** and **2**, the lag time, indicating the resistance of LDL to oxidation, was extended to 101 and 120 min, respectively, whereas, the addition of 1.0 μM probucol extended the lag time to 103 min. Thus, **2** delayed oxidation of LDL much greater than that of **1** or probucol.

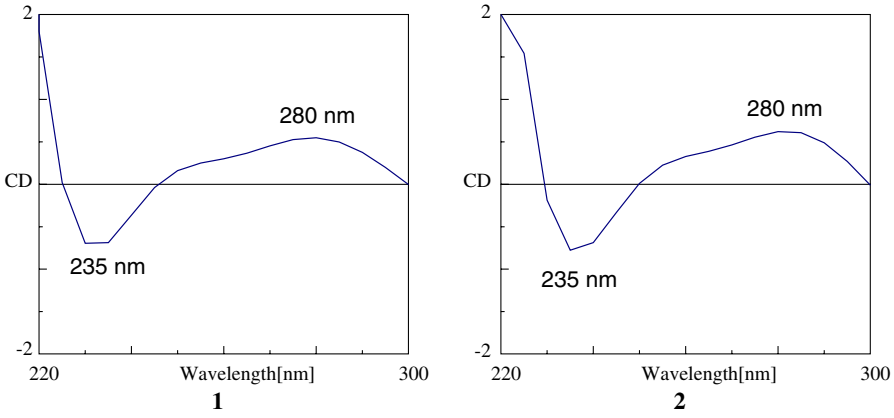

Figure 2. CD spectrums of **1** and **2**.

2.4. Effects on relative electrophoretic mobility (REM) of oxidized LDL

To evaluate another parameter that is affected by LDL oxidation, **1** and **2** were applied to REM assay (Fig. 5).[20] The LDL was incubated with 5 μM $CuSO_4$ alone to induce the oxidation of LDL for 12 h (lane 2). The LDL oxidation was almost protected in the presence of 20, 10, 5 μM of **1** and **2**, (Figs. 5A and B). Then, probucol inhibited the oxidation of LDL in dose-dependent manner. From this result, **1** and **2** showed much more protection of the LDL oxidation than probucol.

2.5. Inhibitory effects of ApoB-100 fragmentation

Radical reaction of LDL causes facial degradation of apoB-100 which is a major component of LDL.[21] The inhibition of the oxidative process by **1** and **2** was evaluated by the densitometric area of band of apoB-100 through SDS–PAGE.[22] The band of apoB-100 was observed on native LDL, which had been incubated without 5 μM $CuSO_4$ for 4 h at 37 °C, but the band completely disappeared when the LDL was incubated with 5 μM $CuSO_4$. The compounds **1** and **2** dose-dependently protected the fragmentation of apoB-100 (Table 1). At the same concentration, **2** was more active than **1** or probucol in the protection of apoB-100 fragmentation against copper-induced oxidation.

2.6. Radical 1,1-diphenyl-2-picrylhydrasyl (DPPH) scavenging activity

In order to explain the mechanism of antioxidant components, two kinds of mechanisms, free radical scavenging and metal chelation, are commonly proposed. The radical scavenging activity was measured as decolorizing activity following the trapping of the unpaired electron of DPPH (Fig. 6).[23] After 3 min of incubation at room temperature, only 5% and 16% of the DPPH radicals, respectively, were remained in the presence of 100 μM of **1** and **2**, whereas 72% DPPH radicals were remained in the presence of 100 μM probucol. Therefore, **1** and **2** showed strong radical scavenging capacity compared to probucol. The effect on metal chelating was measured by monitoring the change of maximum absorbance of test compound by the formation of metal-compound chelate.[24] Five micromolar of **1** and **2** was incubated with 100 μM $CuSO_4$ in PBS buffer (pH 7.4) for 10 min at room temperature and the spectra of UV absorbance were recorded either in the presence or absence of **1** and **2**. With the addition of copper ions, the characteristic band of compounds **1** and **2** with a maximum absorption at 282 nm was not changed (data not shown).

2.7. Anti-inflammatory effects in RAW264.7 macrophages

Many anti-inflammatory drugs, such as glucocorticoids, nonsteroidal anti-inflammatory drugs, and immunosuppressants, act as inhibitors of the NF-κB pathway and suppress expression of various inflammation-associated genes, such as iNOS, COX-2, IL-1β, and TNF-α. Therefore, inhibitors of the NF-κB pathway have been used for treatment of inflammatory diseases, including atherosclerosis.[25] The expression of iNOS has been found in atherosclerotic lesions and produces high amounts of NO, which contributes to the process of atherosclerosis and produces the powerful oxidant peroxynitrite.[26] Peroxynitrite has been strongly implicated as a cytotoxic effect or molecule contributing to cellular damage and promoting the formation of atherosclerotic lesions.[26] Basically, *N*-acetyldopamine attenuates LPS-stimulated TNF-α production and superoxide production in THP-1 cells.[27] Therefore, we hypothesized that the anti-inflammatory action of *N*-acetyldopamine dimers is mediated by their antioxidant action and attempted to investigate the antioxidative and anti-inflammatory action of *N*-acetyldopamine dimers using RAW264.7 macrophages. ROS have a causal role in atherosclerosis and other cardiovascular diseases. We first examined whether **1** and **2** inhibit ROS generation in LPS (1 μg/mL)-stimulated RAW264.7 cells using 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA), which is widely used to measure ROS level in cells. Treatment with 1 μg/ml of LPS enhanced ROS level to about 4-fold compared to that in the untreated cells. Pretreatment with various concentrations of **1** and **2** dose-dependently reduced the ROS level in LPS-stimulated RAW264.7 cells (Fig. 7). The concentrations of **1** and **2** used in these experiments had no effect on the viability as determined by MTT and morphology of macrophages (data not

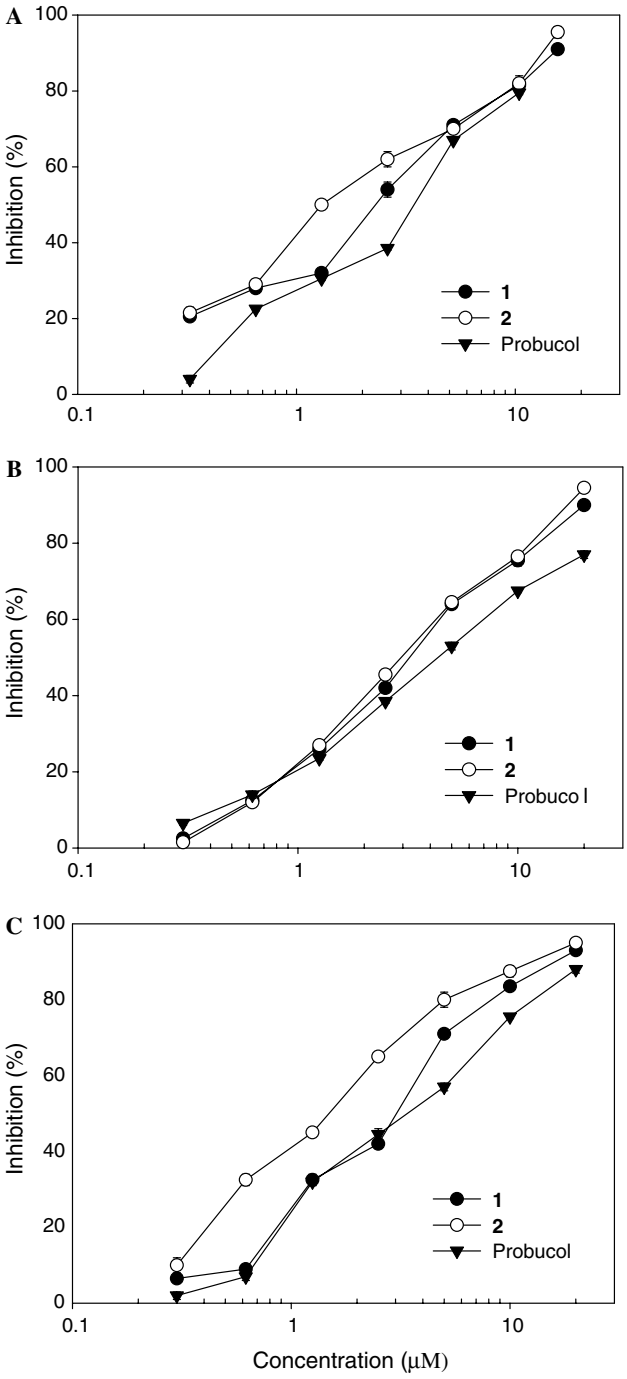

Figure 3. Effects of **1** and **2** on LDL oxidation mediated by Cu^{2+} (A), AAPH (B), and SIN-1 (C). LDL (120 μg protein/ml) in PBS (pH 7.4) was oxidized with 5 μM $CuSO_4$ (or 4 mM AAPH, 1 mM SIN-1) and test sample for 4 h at 37 °C. The extent of LDL oxidation was measured by TBARS assay. Data are shown as means ± SD of duplicate experiments.

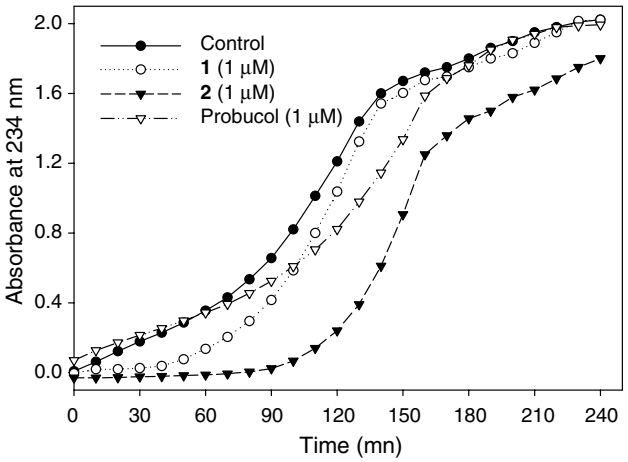

Figure 4. Effects of **1** and **2** on the generation of conjugated dienes during the copper-mediated oxidation of LDL. LDL (120 μg protein/mL) in PBS (pH 7.4) was incubated with 5 μM $CuSO_4$ at 37 °C in the presence or absence of **1** and **2**. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 4 h. Values shown are from a representative experiment.

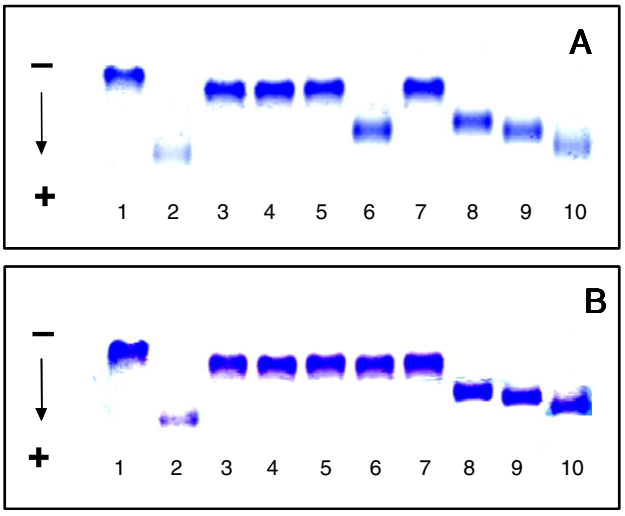

Figure 5. Effects of **1** (A) and **2** (B) on the REM of LDL during Cu^{2+}-mediated oxidation. LDL (120 μg protein/mL in PBS) was incubated for 12 h at 37 °C with 5 μM $CuSO_4$. After incubation, approximately 2 μg of LDL protein was loaded onto 0.7% agarose gel for electrophoresis. The gel was stained with Coomassie brilliant blue R-250. (A) Lane 1, native LDL (absence of $CuSO_4$); lane 2, ox-LDL; lane 3, **1** (20 μM); lane 4, **1** (10 μM), lane 5, **1** (5 μM); lane 6, **1** (2 μM); lane 7, probucol (20 μM); lane 8, probucol (10 μM); lane 9, probucol (5 μM); and lane 10, probucol (2 μM). (B) Lane 1, native LDL (absence of $CuSO_4$); lane 2, ox-LDL; lane 3, **2** (20 μM); lane 4, **2** (10 μM); lane 5, **2** (5 μM); lane 6, **2** (2 μM); lane 7, probucol (20 μM); lane 8, probucol (10 μM), lane 9, probucol (5 μM); and lane 10, probucol (2 μM).

shown). We next examined the effects of **1** and **2** on NO production and iNOS expression in RAW264.7 cells stimulated with LPS (1 μg/mL) in the presence of various concentrations of **1** and **2**. The NO production and iNOS protein expression were slightly decreased in concentration-dependent manner by **1** and **2** (Fig. 8). NF-κB regulates the transcription of various inflammatory cytokines, such as IL-1, -2, -6, and -8, and TNF-α, as well as genes encoding COX-2, iNOS, immunoreceptors, cell adhesion molecules, hematopoietic growth factors, and growth factor receptors.[28] We examined LPS-induced mRNA expression of representative pro-inflammatory molecules such as IL-6, TNF-α, and COX-2 in RAW264.7 cells by RT-PCR. The transcription of pro-inflammatory molecules was markedly induced by LPS treatment, and the treatment of **2** reduced more

Table 1. Antioxidant effects of **1** and **2** on the Cu^{2+}-mediated oxidation and apoB-100 fragmentation in LDL[a]

Compounds (μM)	% of remaining apoB-100[b]
Native LDL	100
Ox-LDL	0
1 (20)	58 ± 2
1 (10)	48 ± 5
1 (5)	30 ± 2
2 (20)	76 ± 4
2 (10)	58 ± 5
2 (5)	53 ± 3
Probucol (20)	42 ± 2
Probucol (10)	38 ± 3
Probucol (5)	24 ± 5

[a] LDL (120 μg protein/mL in PBS) was incubated for 4 h at 37 °C with 5 μM $CuSO_4$ in the absence or presence of 20, 10, and 5 μM **1**, **2**, and probucol. After incubation, approximately 2.0 μg of LDL protein was applied to SDS–PAGE (4%). After the electrophoresis, the gel was stained with Coomassie brilliant blue R250 and subjected to densitometric scanning by Bio-Rad Model GS-800 with Bio-Rad Quantity One-4.4.0 software to compare residual apoB-100 band intensity.

[b] Data are shown as means ± SD ($n = 3$).

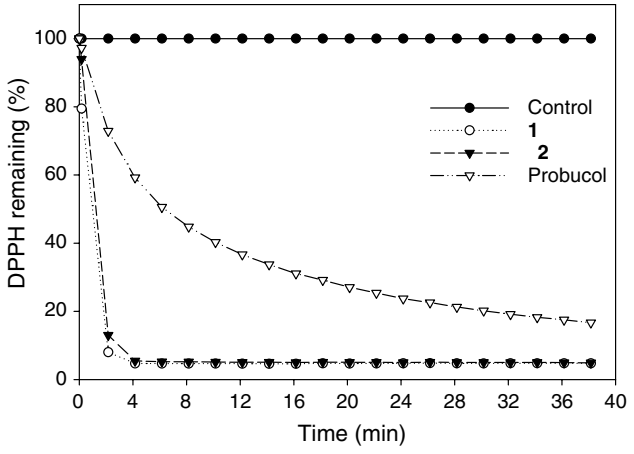

Figure 6. Effects of **1** and **2** on radical DPPH scavenging. Compounds **1** and **2** (100 μM) were incubated with 100 μM DPPH in methanol for 40 min at room temperature. The absorbance of each compound solution was measured at 517 nm. The antiradical activity was expressed by the percentage of remaining DPPH.

the mRNA levels than **1** in a concentration-dependent manner for all of IL-6, TNF-α, and COX-2 (Fig. 9). IL-6 is expressed within atherosclerotic lesions in macrophage-rich areas and may stimulate inflammatory responses in macrophages.[29] RAW264.7 macrophages did not constitutively express IL-6 in the absence of LPS treatment; however, LPS-stimulated RAW264.7 cells showed increased secretion of IL-6, and this increase was inhibited in a dose-dependent manner by treatment of **2** (Fig. 10), though not to a statistically significant degree. To investigate a molecular mechanism of the anti-inflammatory action by **1** and **2**, we examined the effects of **1** and **2** on the LPS-induced NF-κB activation using an NF-κB reporter gene assay system.[30,31] As shown in Figure 11, **1** and **2** inhibited the LPS-induced NF-κB activation in RAW264.7 cells.

3. Conclusion

In conclusion, a known *N*-acetyldopamine dimer, (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethyl)-1,4-benzodioxane (**1**), and a new *N*-acetyldopamine dimer, (2*R*,3*S*)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethylene)-1,4-benzodioxane (**2**), were isolated by bioassay-guided fractionation from the methanolic extracts of Periostracum Cicadae. Compounds **1** and **2** showed potent antioxidant activities in the Cu^{2+}-, AAPH-, and SIN-1-mediated lipid peroxidation of LDL in TBARS assay. In addition, **1** and **2** inhibited the conjugated diene formation (the early peroxidation of LDL), REM of ox-LDL, facial degradation of apoB-100 on Cu^{2+}-induced LDL oxidation. We hypothesize that **1** and **2** exert the antioxidant function by direct interaction with generated radicals or inhibition of radical generation. Compounds **1** and **2** also showed strong radical DPPH scavenging activity not metal-chelating activity. Pretreatment of **1** and **2** dose-dependently reduced the ROS level in LPS-stimulated RAW264.7 cells. Compound **2** more effectively inhibited than **1** the NO production and the iNOS protein expression. In addition, compound **2** was more efficient than compound **1** at inhibiting the LPS-induced NF-κB activation and the expressions of NF-κB target molecules such as IL-6, TNF-α, and COX-2. These results could be of value for development of **1** and **2** as anti-atherosclerotic agents which are worthy of further in vivo investigations. However, the mechanism of **1** and **2** on the inhibition of NF-κB activation was still unclear. Further studies remain to be elucidated how *N*-acetyldopamine dimers regulate the NF-κB activation.

4. Experimental

4.1. General

Optical rotations were measured with a DIP-370 Polarimeter (JASCO, Japan). The IR spectra were recorded on a FT-IR spectrometer MB-100 instrument (Bomen, USA) with KBr pellets. Circular dichroism (CD) spectra were recorded on a J-715 (JASCO) in MeOH (200 μM). One- and two-dimensional NMR spectra were recorded on a Bruker AMX-500 FT-NMR spectrometer (Bruker, Germany) with $CDCl_3$. HREIMS was recorded on a JMS-700 MStation (Jeol, Japan). HPLC apparatus was equipped with a Shimadzu model LC-10ADVP pump and SPD-M10AVP photodiode array detector using YMC Hydrosphere-C_{18} (4.6 × 250 mm i.d., 5 μm) column. Silica gel (230–400 mesh, Merck) for column chromatography and silica gel 60 F_{254} for TLC were supplied by the Merck Korea. Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Gibco-BRL Co. (Gaithersburg, MD, USA). Antibodies against iNOS and actin were obtained from BD Transduction Laboratories (Lexington, KY, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. 2′,7′-Dichloro-fluorescein diacetate ($DCFH_2$-DA) was purchased from Molecular Probe (Eugene, OR, USA). All the reagent grade chemicals were purchased from the Sigma–Aldrich Korea.

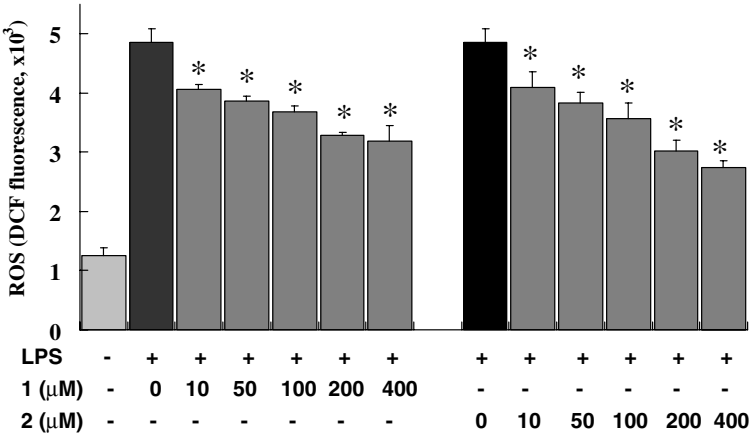

Figure 7. Compound **1** and **2** suppress the intracellular ROS level in RAW264.7 cells. RAW264.7 cells were pretreated with **1** and **2** for 2 h, followed by incubation with 1 μg/mL LPS for 16 h. Significantly different from the LPS alone-treated group, $*P < 0.01$. The data represent means ± SD ($n = 3$).

4.2. Extraction and isolation

The dried Periostracum Cicadae, which was imported from Yunnam, China, was purchased from a market of traditional Asian medicine in Daejeon, Korea. The powder of dried Periostracum Cicadae (200 g) was extracted two times with MeOH (2 L) for 2 days at room temperature. The combined MeOH extracts (20.8 g) were concentrated in vacuo, and the resulting aqueous suspension was partitioned with *n*-hexane, $CHCl_3$, and EtOAc, successively. Among these three fractions, the EtOAc fraction (12.0 g) inhibited strongly copper-mediated LDL oxidation (97% inhibition at 40 μg/mL) in TBARS assay. It was subjected to silica gel column chromatography (∅ 7 × 30 cm, 230—400 mesh, Merck) eluting a step gradient of $CHCl_3$/MeOH (95:1, 90:1, 60:1, 30:1, 10:1, 5:1, 1:1, (v/v) and 100% MeOH, each of 1.0 L). The active fraction ($CHCl_3$/MeOH, 10:1 fraction, 2.8 g) was rechromatographed on a silica gel column (∅ 5.5 × 24 cm) using *n*-hexane/EtOAc (95:1, 90:1, 60:1, 30:1, 10:1, 5:1, 1:1, (v/v) and 100% EtOAc, each of 500 mL). The one active fraction (*n*-hexane/EtOAc, 5:1 fraction, 860 mg) was performed by preparative TLC with development of *n*-hexane/EtOAc (1:1, v/v) to give five fractions. The active fraction (84 mg) was finally purified by a HPLC system (Hydrosphere-C_{18} (4.6 × 250 mm, YMC Co.); MeOH/H_2O = 9:1; 4.0 mL/min; 220 nm) to obtain compounds **1** (5.4 mg, t_R = 12 min) and **2** (6.3 mg, t_R = 9.0 min).

4.2.1. (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethyl)-1,4-benzodioxane (1). Yellow powder; EIMS: $[M]^+$ at m/z = 386; HREIMS: $[M+H]^+$ at m/z = 387.1553, (calcd for $C_{20}H_{23}N_2O_6$, 387.1556); $[\alpha]_D^{25}$ +20.2° (c = 0.8, MeOH) {lit. $[\alpha]_D^{25}$ +28.2° (c = 1.0, MeOH)}.[10]

4.2.2. (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2″-aminoethylene)-1,4-benzodioxane (2). Yellow powder; EIMS: $[M]^+$ at m/z = 384, HRFABMS: $[M+Na]^+$ atm/z = 407.1224 (calcd for $C_{20}H_{20}N_2NaO_6$, 407.1219); $[\alpha]_D^{25}$ +33.8 (c = 0.1, MeOH); UV (MeOH): λ_{max} at 282 nm ($\log\varepsilon$ = 4.32), IR (KBr): ν_{max} 3428 (OH) cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ_H 1.86 (3H, s, H-3b), 2.02 (3H, s, H-4″), 4.69 (1H, d, J = 7.1 Hz, H-2), 5.68 (1H, d, J = 7.1 Hz, H-3), 6.09 (1H, d, J = 14.7 Hz, H-1″), 6.73–6.81 (3H, m, H-5, H-6, H-8), 6.84–6.89 (3H, m, H-2′, H-5′, H-6′), 7.29 (1H, d, J = 14.7 Hz, H-2″); ^{13}C NMR (CD_3OD, 125 MHz): δc 22.5 (C-3b), 22.6 (C-4″), 78.3 (C-3), 78.4 (C-2), 113.9 (C-1″), 114.7 (C-2′), 115.6 (C-5′), 116.3 (C-5), 118.3 (C-8), 120.3 (C-6), 120.6 (C-6′), 122.9 (C-2″), 128.7 (C-1′), 131.9 (C-7), 142.6 (C-4a), 144.6 (C-8a), 146.5 (C-3′), 147.2 (C-4′), 170.6 (C-3″), 173.2 (C-2b).

4.3. In vitro antioxidant activities

Blood was collected from normalipidaemic volunteers with permission according to the 'Guidelines of Blood Donation Program for a Research' of the Korea Red Cross Blood Center and LDL was isolated from the plasma by preparative ultracentrifugation as described.[32] The copper-mediated, AAPH-mediated, and SIN-1-mediated LDL oxidation were performed as described.[33] The extent of LDL oxidation was measured by TBARS assay.[32,33] The formation of conjugated diene, REM, and fragmentation of ApoB-100 on LDL oxidation were measured as described.[32,33] The radical DPPH scavenging activity was measured as described.[32,33]

4.4. Culture of RAW264.7 cells

RAW264.7 cells (murine macrophage cell line) obtained from American Type Culture Collection (Manassas, VA) were cultured in DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/mL streptomycin, and 10% FBS in a humidified incubator with 5% CO_2 in air at 37 °C.

4.5. Measurement of intracellular ROS generation

To investigate the effects of compounds **1** and **2** on LPS-induced ROS production in RAW264.7 cells, a fluorometric assay using $DCFH_2$-DA (Molecular Probes) as a probe was used for the presence of hydroxyl radical. The intracellular production of ROS was measured as described[34] with modification. ROS have been

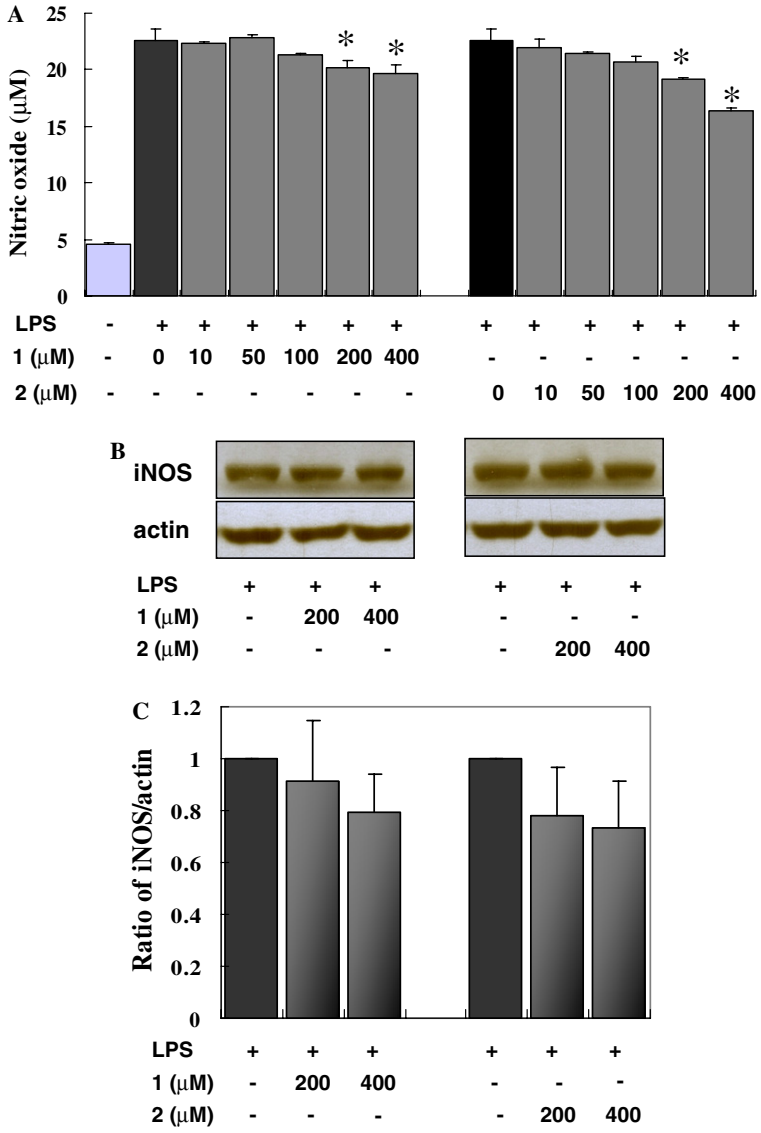

Figure 8. Effects of **1** and **2** on the NO production and expression of iNOS in RAW264.7 cells by LPS. RAW264.7 cells were plated at 1×10^6 cells/mL and pretreated with **1** and **2** for 2 h, followed by incubation with 1 μg/mL LPS for 18 h. (A) The inhibitory effect of **1** and **2** on NO production. The levels of nitrite were measured in the culture medium by Griess reagents. Significantly different from the LPS alone-treated group, $^*P < 0.05$. (B) Inducible NOS protein was determined by Western blot analysis. Cells were harvested, and cell lysates were separated on a 10% SDS–polyacrylamide gel. (C) Histogram showing densitometric analysis of iNOS expression. Each histogram shows the ratio of integrated density of iNOS protein with actin in the sample. This experiment has been repeated two times separately with similar observation.

implicated in the pathophysiology of many vascular disorders. Confluent RAW264.7 cells (10^4 cells/well) in 96-well plates were pretreated with **1** and **2** (0–400 μM) for 2 h, followed by incubation with 1 μg/mL LPS for 16 h. After the removal of media from wells, the cells were incubated with 10 μM of $DCFH_2$-DA for 1 h. The fluorescence was measured on a spectrofluorometer (Wallac 1420, Perkin-Elmer, Turku, Finland) at 485 nm excitation and 530 nm emission wavelength.

4.6. Measurement of NO production and iNOS expression

Nitrite production, as an assay of NO release, was measured as described.[35] RAW264.7 cells were plated at 1×10^6 cells/mL and pretreated with **1** and **2** (0–400 μM) for 2 h, followed by incubation with 1 μg/mL LPS for 18 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine in 2% phosphoric acid) and incubated for 10 min at room temperature. Sodium nitrite was used to generate a standard curve, and nitrite concentration was determined by measuring the optical density at 540 nm with the model 680 Microplate reader (Bio-Rad, Hercules, CA, USA). The cell lysates were prepared by suspending 1×10^6 cells in 100 μL lysis buffer (140 mM NaCl, 15 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, pH 7.5), and extracted for 30 min at 4 °C. Samples of equal amounts of proteins were separated through 10% SDS–PAGE. After electrophoresis, proteins were transferred to Immobilon-P

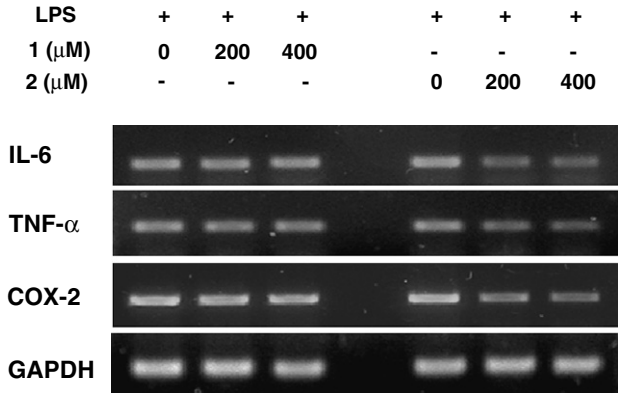

Figure 9. Effects of **1** and **2** on the LPS-induced expressions of various inflammation-associated genes, such as IL-6, TNF-α, and COX-2, in RAW264.7 cells by RT-PCR analysis. The cells were pretreated with **1** and **2** for 2 h, followed by incubation with 1 μg/mL LPS for 12 h. The total RNAs were isolated, and RT-PCR analyses were carried out.

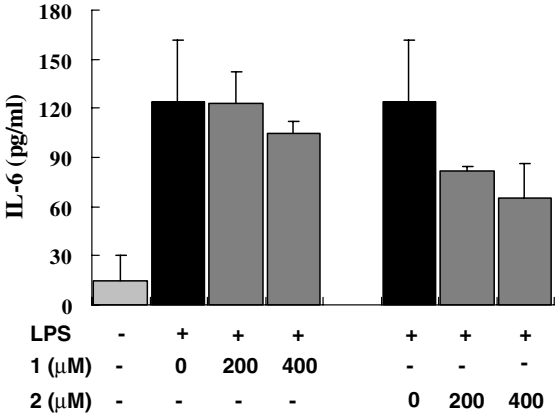

Figure 10. Effects of **1** and **2** on the production of IL-6 by LPS. The cells were pretreated with **1** and **2** for 2 h followed by incubation with 1 μg/mL LPS for 12 h. After incubation, the culture supernatants were recovered, and determination of IL-6 was carried out as protocols. Significantly different from the LPS alone-treated group, $^{*}P < 0.05$.

membrane (Millipore Co., Bedford, MA). The membrane was incubated for 2 h at room temperature with iNOS antibody. Immunoreactive proteins were detected with the ECL Western blotting kit (Amersham).

4.7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RAW264.7 cells were pretreated with **1** and **2** for 2 h, followed by incubation with 1 μg/mL LPS for 12 h. The cells were harvested and total RNA was isolated by RNeasy mini columns (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's instructions. A sample (1 μg) of total RNA was used for the synthesis of the first strand cDNA using the Omniscript (Qiagen, Santa Clarita, CA, USA) according to the manufacture's instructions. For amplification of IL-6, TNF-α, and COX-2, the following primers were used: for IL-6, 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′ (sense), 5′-CATCTGGCTAGGTAACAGAATATTT ATATC-3′ (anti-sense); for TNF-α, 5′-CTCAGATCATCTTCT

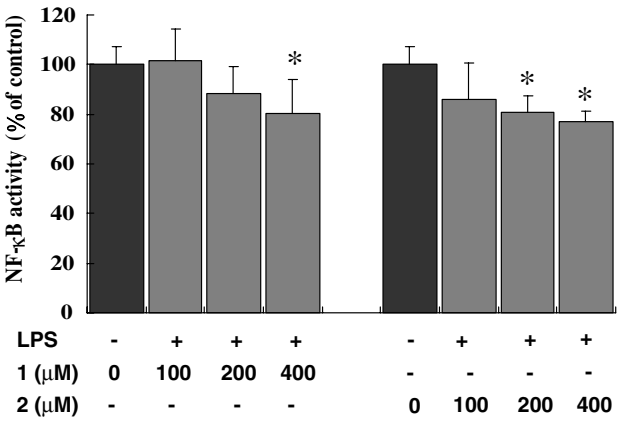

Figure 11. Inhibition of NF-κB-mediated transcription of the reporter gene by **1** and **2**. RAW264.7 cells transfected with a NF-κB reporter plasmid were pretreated with **1** and **2** for 2 h, and then stimulated with LPS (1 μg/mL). NF-κB activity in the culture medium was measured using SEAP assay. Data represent means ± SD of two independent experiments performed in triplicate; Significantly different from the LPS alone-treated group, $^{*}P < 0.05$.

CAAA ATTCGAGTGACA-3′ (sense), 5′-CTTCACAG AGCA ATGACTCCAAAGT-3′ (anti-sense); for COX-2, 5′-AACCGTGGGGAATGTATGAGCA-3′ (sense), 5′-AACTCTCTCCGTAGAAGAACCTTTTCCA-3′ (anti-sense). For PCR amplification, the following conditions were used; 95 °C for 2 min for one cycle; 95 °C for 1 min; 55 °C for 30 s; and 72 °C for 1 min for 20–33 cycles. The amplified PCR products were separated with 1.2% agarose gel, and then stained with ethidium bromide.

4.8. Measurement of IL-6 production

RAW264.7 cells were pretreated with different concentrations of **1** and **2** for 2 h and then stimulated by LPS (1 μg/mL) for 12 h. The IL-6 level of each culture supernatant was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D system, MN) according to the manufacturer's protocol.

4.9. NF-κB activity assay

NF-κB activity was determined by using RAW264.7 cells stably transfected with a plasmid containing 8 copies of κB elements linked to secreted alkaline phosphatase (SEAP) gene.[30,31] The cells were kindly provided by Dr. Jung Joon Lee (KRIBB, Daejeon, Korea). Cells were grown to 70% confluence in 96-well plate and medium was replaced with 200 μL of fresh DMEM containing 0.5% FBS, and then the cultures were incubated for 24 h. Cells were pretreated with **1** and **2** (0–400 μM), followed by incubation with 1 μg/ml of LPS for 24 h. One hundred microliters of each culture supernatant was transferred to a new 96-well plate, heated for 5 min at 65 °C, and then mixed with an equal volume of 2× SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM L-homoarginine). The reaction was initiated by the addition of 20 μL of 120 mM *p*-nitrophenyl phosphate dissolved in 1× SEAP assay buffer and incubated for 2 h at 37 °C. The absorbance of the reaction

mixture was measured at 405 nm with the model 680 Microplate reader.

4.10. Data analysis

All values are expressed as means ± SD. Statistical analysis was done using Student's *t*-test. A value of $P < 0.05$ was accepted as statistically significant.

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References and notes

1. de Winther, M. P.; Kanters, E.; Kraal, G.; Hofker, M. H. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 904.
2. D'Acquisto, F.; May, J. J.; Ghosh, S. *Mol. Interv.* **2002**, *2*, 22.
3. Schoonbroodt, S.; Piette, J. *Biochem. Pharmacol.* **2000**, *60*, 1075.
4. Witztum, J. L.; Steinberg, D. *J. Clin. Invest.* **1991**, *88*, 1785.
5. Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. *N. Engl. J. Med.* **1989**, *320*, 915.
6. (a) Gey, K. F. *J. Nutr. Biochem.* **1995**, *6*, 206; (b) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. *Free Radic. Biol. Med.* **1996**, *20*, 933; (c) Ahn, B. T.; Lee, S.; Lee, S. B.; Lee, E. S.; Kim, J. G.; Bok, S. H.; Jeong, T. S. *J. Nat. Prod.* **2001**, *64*, 1562; (d) Lee, W. S.; Back, Y. I.; Kim, J. R.; Cho, K. H.; Sok, D. E.; Jeong, T. S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5623; (e) Thuong, P. T.; Na, M.; Su, N. D.; Seong, R. S.; Lee, Y. M.; Sok, D. E.; Bae, K. *Biol. Pharm. Bull.* **2005**, *28*, 1095; (f) Lee, W. S.; Kim, J. R.; Im, K. R.; Cho, K. H.; Sok, D. E.; Jeong, T. S. *Planta Med.* **2005**, *71*, 295.
7. Kontogiorgis, A. C.; Pontiki, A. E.; Hadjipavlou-Litina, D. *Mini Rev. Med. Chem.* **2005**, *5*, 563.
8. Park, D. S.; Yoon, M. A.; Xu, M. Z.; Yu, H.; Kim, J. R.; Jeong, T. S.; Park, H. Y. *Kor. J. Pharmacogn.* **2004**, *35*, 233.
9. Okuda, T., Ed.; *Encyclopedia of Natural Medicine*; Hirokawa Publishing: Tokyo, 1986; p 244.
10. Noda, N.; Kubota, S.; Miyata, Y.; Miyahara, K. *Chem. Pharm. Bull.* **2000**, *48*, 1749.
11. Andersen, S. O.; Jacobsen, J. P.; Roepstorff, P. *Tetrahedron* **1980**, *36*, 3249.
12. Minami, Y.; Oumori, K.; Muranaka, Y.; Yamada, S.; Yamawaki, I.; Kaneda, M.; Kimura, H.; Hironaka, Y.; Fujita, T. Jpn. Kokai Tokyo Koho JP 07242543 (95242543) and *Chem. Abstr*. **1996**, *124*, 574.
13. Oxenkrug, G. F.; Requintina, P. J. *Ann. N. Y. Acad. Sci.* **2005**, *1053*, 394.
14. Liu, F.; Ng, T. B. *Life Sci.* **2000**, *66*, 725.
15. Devaraj, S.; Li, D. J.; Vazquez, M.; Jialal, I. *Free Radic. Biol. Med.* **1999**, *26*, 1064.
16. Gugliucci, A.; Menini, T. *Life Sci.* **2002**, *71*, 693.
17. Carew, T. E.; Schwenke, D. C.; Steinberg, D. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7725.
18. Kuzuya, M.; Kuzuya, F. *Free Radic. Biol. Med.* **1993**, *14*, 67.
19. Khursheed, P. N.; Enrique, B.; Charles, S. L. *Atherosclerosis* **2000**, *152*, 89.
20. Reid, V. C.; Mitchinson, M. J. *Atherosclerosis* **1993**, *98*, 17.
21. Tanaka, K.; Iguchi, H.; Taketani, S.; Nakata, R.; Tokumaru, S.; Sugimoto, T.; Kojo, S. *J. Biochem.* **1999**, *125*, 173.
22. Noguchi, N.; Niki, E. *Methods Enzymol.* **1994**, *233*, 490.
23. Bursa, S. Y.; Oleszek, W. *J. Agric. Food Chem.* **2001**, *49*, 2774.
24. Neudörffer, A.; Desvergne, J. P.; Bonnefont-Rousselot, D.; Legrand, A.; Fleury, M. B.; Largeron, M. . *J. Agric. Food Chem.* **2006**, *54*, 1898.
25. Hermanowski-Vosatka, A.; Balkovec, J. M.; Cheng, K.; Chen, H. Y.; Hernandez, M.; Koo, G. C., et al. *J. Exp. Med.* **2005**, *205*, 517.
26. Buttery, L. D.; Sprigall, D. R.; Chester, A. H.; Evans, T. J.; Standfield, E. N.; Parums, D. V.; Yacoub, M. H.; Polak, J. M. *Lab. Invest.* **1996**, *75*, 77.
27. Perianayagam, M. C.; Oxenkrug, G. F.; Jaber, B. L. *Ann. N. Y. Acad. Sci.* **2005**, *1053*, 386.
28. Baeuerle, P. A.; Henkel, T. *Annu. Rev. Immunol.* **1994**, *12*, 141.
29. Schieffer, B.; Schieffer, E.; Hilfiker-Kleiner, D.; Hilfiker, A.; Kovanen, P. T.; Kaartinen, M.; Nussberger, J.; Harringer, W.; Drexler, H. *Circulation* **2000**, *101*, 1372.
30. Koo, T. T.; Lee, J. H.; Park, Y. J.; Hong, Y. S.; Kim, H. S.; Kim, K. W., et al. *Planta Med.* **2001**, *67*, 103.
31. Lee, J. H.; Koo, T. H.; Hwang, B. Y.; Lee, J. J. *J. Biol. Chem.* **2002**, *277*, 18411.
32. Jeong, T. S.; Kim, J. R.; Kim, K. S.; Cho, K. H.; Bae, K. H.; Lee, W. S. *Bioorg. Med. Chem.* **2004**, *12*, 4017.
33. Yoon, M. A.; Jeong, T. S.; Park, D. S.; Xu, M. Z.; Oh, H. W.; Song, K. B.; Lee, W. S.; Park, H. Y. *Biol. Pharm. Bull.* **2006**, *29*, 735.
34. Hayakawa, M.; Miyashita, H.; Sakamoto, I.; Kitagawa, M.; Tanaka, H.; Yasuda, H.; Karin, M.; Kikugawa, K. *EMBO J.* **2003**, *22*, 3356.
35. Kim, Y. M.; de Vera, M. E.; Watkins, S. C.; Billiar, T. R. *J. Biol. Chem.* **1997**, *272*, 1402.