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Antioxidant Activities of Abietane Diterpenoids Isolated from *Torreya nucifera* Leaves

WOO SONG LEE,[†,‡] JU-RYOUNG KIM,[†,‡] JONG-MIN HAN,[†] KI CHANG JANG,[§] DAI-EUN SOK,[||] AND TAE-SOOK JEONG*,[†]

National Research Laboratory of Lipid Metabolism & Atherosclerosis, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea, National Institute of Subtropical Agriculture, R. D. A., Jeju 690-150, Republic of Korea, and College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea

Investigation on antioxidant compounds from the ethanolic extracts of *Torreya nucifera* leaves resulted in the isolation of abietane diterpenoids, a known 18-methylesterferruginol (**1**) and a new 18-dimethoxyferruginol (**2**). The structures of compounds **1** and **2** were elucidated on the basis of their spectroscopic analyses. Compounds **1** and **2** inhibited the Cu^{2+}-mediated, 2,2′-azobis(2-amidinopropane)hydrochloride-mediated and 3-morpholinosydnonimine-1-mediated low-density lipoprotein (LDL) oxidation in the thiobarbituric acid-reactive substances assay as well as the macrophage-mediated LDL oxidation. Compounds **1** and **2** exhibited the potent antioxidant activities in the conjugated diene production, relative electrophoretic mobility, and apoB-100 fragmentation on copper-mediated LDL oxidation. Compound **1** also suppressed nitric oxide production and inducible nitric oxide synthase expression in lipopolysaccharide-stimulated RAW264.7 cells.

KEYWORDS: ***Torreya nucifera*; abietane diterpenoids; LDL; antioxidant activity; NO; iNOS; atherosclerosis**

INTRODUCTION

Oxidized low-density lipoproteins (ox-LDL) and reactive oxygen species play important roles in early stages of atherosclerosis (*1*, *2*). The ox-LDL stimulates endothelial cells and macrophages to induce production of several kinds of cytokines such as monocyte chemotactic protein-1 and macrophage colony-stimulated factor (*3*) under pathological conditions. Furthermore, ox-LDL stimulates monocytes to cause expression of adhesion molecules on the cell surface (*4*). Monocyte-derived macrophages recognize ox-LDL through the scavenger receptors, resulting in massive accumulation of lipids and further foam cell formation (*5*).

Atherosclerosis is a complex and chronic inflammatory disease (*6*). Nitric oxide (NO), a radical produced from L-arginine via nitric oxide synthase (NOS) and an important cellular second messenger, plays a dual role as both a beneficial molecule and a detrimental molecule in the process of inflammation. The small amount of NO produced by constitutive NOS, including endothelial NOS and neuronal NOS (nNOS), is an important regulator of physiological homeostasis, whereas the large amount of NO produced by inducible NOS (iNOS) has been closely correlated with the pathophysiology in atherosclerosis (*7*, *8*).

To find out the crucial component of functional foods for prevention or treatment of atherosclerosis, many polyphenols having the LDL-antioxidant activity were isolated from various fruits, vegetables, and beverage plants (*9*). Recently, we also reported that diarylheptanoids, diarylbutane lignan, and neolignan isolated from *Alnus japonica* and *Saururus chinensis* showed the antioxidant effect on LDL oxidation (*10*−*12*). In the continued screening of cholesterol-lowering and antiatherosclerotic agents, we found that the ethanolic extracts of leaves of *Torreya nucifera* exhibited significant LDL-antioxidant activity.

T. nucifera is a Taxaceae tree found in snowy areas near the Sea of Jeju Island in Korea and has been used in traditional Asian medicine as a remedy for stomachache, hemorrhoids, and rheumatoid arthritis (*13*). Asakawa and Orihara groups reported that various abietane diterpenoids were isolated from the leaves of *T. nucifera* (*14*) and from the suspension-cultured cells of *T. nucifera* var. *radicans* (*15*), but the bioactivity of various abietane diterpenoids was not evaluated. The abietane diterpenoids isolated from rosemary (*Rosmarinus dfficinalis* L.), carnosol, carnosic, and rosmanol showed the antioxidant activity by the ferric thiocyanate and the thiobarbituric acid (TBA) methods (*16*−*18*). In general, abietane diterpenoids have been known to possess a variety of biological activities, such as antibacterial (*19*), cardiovascular (*20*), and aldose reductase and α-glucosidase inhibitory activities (*21*).

In this study, we isolated two abietane diterpenoids, a known 18-methylesterferruginol (**1**) and a new 18-dimethoxyferruginol (**2**), from *T. nucifera* leaves and characterized their structures

* To whom corresponding should be addressed. Tel: +82-42-860-4558. Fax: +82-42-861-2675. E-mail: tsjeong@kribb.re.kr.
[†] Korea Research Institute of Bioscience and Biotechnology.
[‡] Both authors contributed equally to the work.
[§] National Institute of Subtropical Agriculture.
[||] Chungnam National University.

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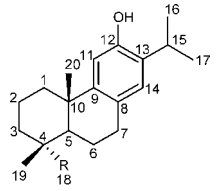

1 R = CO_2Me; **2** R = $CH(OMe)_2$

Figure 1. Chemical structures of abietane diterpenoids **1** and **2** isolated from the leaves of *T. nucifera*.

through spectroscopic methods (**Figure 1**). The antioxidant activities of two compounds **1** and **2** were also evaluated using several methods: Cu^{2+}-mediated, 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH)-mediated, 3-morpholinosydnonimine (SIN)-1-mediated, and macrophage-mediated LDL oxidation in the thiobarbituric acid-reactive substances (TBARS) assay; measurement of the formation of conjugated diene, relative electrophoretic mobility (REM), and fragmentation of ApoB-100 on LDL oxidation; and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity. In addition, we determined the inhibitory effect of compound **1** on NO production and iNOS expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.

MATERIALS AND METHODS

General Experimental Procedures. Melting points were determined on a Fisher−Johns melting point apparatus and are uncorrected. Optical rotations were measured a DIP-370 Polarimeter (JASCO, Japan). The IR spectra were taken on a Fourier transform infrared spectrometer MB-100 instrument (Bomen, United States) with KBr pellets and $CHCl_3$ solution. One- and two-dimensional NMR [^{1}H NMR, ^{13}C NMR, and two-dimensional NMR (^{1}H−^{1}H correlation spectroscopy, heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum coherence)] spectra were recorded on a Bruker AMX-500 FT-NMR spectrometer (Bruker, Germany) with $CDCl_3$. High-resolution electron impact (HREI)-MS was recorded on a JMS-700 MStation (JEOL, Japan). A high-performance liquid chromatography (HPLC) apparatus was equipped with a Shimadzu model LC-10ADVP pump and SPD-M10AVP photodiode array detector using YMC Hydrosphere-C_{18} (4.6 mm × 250 mm i.d., 5 μm) column. Silica gel (230−400 mesh, Merck) for column chromatography and silica gel 60 F_{254} for thin-layer chromatography (TLC) were supplied by Merck (Korea). All of the reagent grade chemicals were purchased from Sigma-Aldrich (Korea).

Plant Material. The leaves of *T. nucifera* were collected at Jeju Island, Korea, in October 2003. A voucher specimen (002-049) was deposited in the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Extraction and Isolation. The dried leaves of *T. nucifera* (2.3 kg) were ground and extracted two times with 95% EtOH (18 L × 2) for 1 week at room temperature. The combined ethanolic extracts were concentrated in vacuo, and the resulting aqueous suspension was partitioned with *n*-hexane, $CHCl_3$, and EtOAc, successively. The EtOAc fraction (18 g) exhibited a potent LDL-antioxidant effect (85 ± 0.4% inhibition at 40 μg/mL) in the TBARS assay and was chromatographed on silica gel (230−400 mesh, 1.0 kg) eluting a step gradient of *n*-hexanes−EtOAc (98:2, 97:3, 95:5, 10:1, 5:1, 3:1, 1:1, and EtOAc, v/v, each of 1.5 L) to give 17 fractions at the first column. The active fraction (*n*-hexanes−EtOAc, 5:1−3:1, fr. 8, 593 mg) was rechromatographed on silica gel column (230−400 mesh, 40 g) by eluting with $CHCl_3$−MeOH solvent pairs (98:2, 95:5, 10:1, 5:1, 3:1, 1:1, 0:1, v/v, each of 100 mL) to yield 11 fractions at the second column. The one active fraction ($CHCl_3$−MeOH, 5:1−1:1, fr. 8−10, 149 mg) was applied to preparative TLC with $CHCl_3$−MeOH (49:1, v/v) to yield three fractions. The active constituents (fr. 1, 36 mg) were finally purified by sephadex LH-20 column chromatography eluting with $CHCl_3$−MeOH (3:2, v/v) to obtain compound **1** (17.5 mg) as a white powder. Among the second column, the other active fraction ($CHCl_3$−MeOH, 1:1−0:1, fr. 11, 230 mg) was applied to sephadex LH-20 column chromatography eluting with $CHCl_3$−MeOH (1:1, v/v) to afford six fractions. The active fraction (fr. 5, 122 mg) was purified by preparative TLC developing with $CHCl_3$−MeOH (80:1, v/v) to obtain compound **2** (18.0 mg) as a yellow oil.

Table 1. NMR Data of **2** (125 MHz for ^{13}C and 500 MHz for ^{1}H in $CDCl_3$)

no.	^{13}C	^{1}H
1	38.2	1.42 d like (J = 12.5 Hz) 2.05 d like (J = 12.6 Hz)
2	18.3	1.53–1.62 m
3	30.4	1.27–1.40 m
4	42. 6	
5	42.8	1.81 dd (J = 1.7, 12.1 Hz)
6	19.4	1.64–1.75 m
7	29.3	2.68–2.78 m
8	126.9	
9	148.7	
10	37.3	
11	110.9	6.55 s
12	150.7	4.59 br
13	131.4	
14	126.5	6.75 s
15	26.7	3.04 m
16	22.5	1.15 d (J = 7.2 Hz)
17	22.7	1.16 d (J = 7.2 Hz)
18	113.3	3.96 s
18a	58.7	3.43 s
18b	59.0	3.45 s
19	16.7	0.9 s
20	25.2	1.12 s

Compound 1. White powder; mp 156−157 °C (CH_2Cl_2) {lit. 156−157 °C}; $[\alpha]_D^{25}$ +75.7° (*c* 0.28, EtOH) {lit. $[\alpha]_D^{25}$ +71.0° (*c* 1.2%, EtOH)} (*22*). IR (KBr): ν_{max} 1696 (C═O), 3442 (OH) cm^{-1}. HREI-MS *m*/*z* = 330.2195 (calcd for $C_{21}H_{30}O_3$ [M]$^+$, 330.2195). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.12 (3H, s, H-20), 1.16 (3H, t like, J = 7.3 Hz, H-16, 17), 1.19 (3H, s, H-19), 1.29 (1H, m, H-6a), 1.41 (1H, m, H-2a), 1.55−1.77 (5H, m, H-1a, H-3, H-2b, H-6b), 2.12 (1H, d like, J = 12.7 Hz, H-1b), 2.14 (1H, dd, J = 1.7, 12.6 Hz, H-5), 2.74 (2H, m, H-7), 3.04 (1H, m, H-15), 3.59 (3H, s, CO_2Me), 4.58 (1H, s, −OH), 6.55 (1H, s, H-11), 6.74 (1H, s, H-14). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 16.5 (C-19), 18.5 (C-2), 21.8 (C-6), 22.5 (C-16), 22.7 (C-17), 25.0 (C-20), 26.8 (C-15), 29.2 (C-7), 36.6 (C-1), 36.9 (C-10), 38.0 (C-3), 44.8 (C-4), 47.7 (C-5), 51.9 (−OMe), 110.8 (C-11), 126.7 (C-14), 127.0 (C-8), 131.7 (C-13), 147.9 (C-9), 150.8 (C-12), 179.2 (C-18); purity 95% (HPLC analysis conditions 90% aqueous MeOH, 1 mL/min, λ = 190 nm, R_t = 5.3 min).

Compound 2. Yellow oil; $[\alpha]_D^{25}$ +75.7° (*c* 0.28, EtOH). IR ($CHCl_3$): ν_{max} 3377 (OH) cm^{-1}. HREI-MS *m*/*z* = 346.2511 (calcd for $C_{22}H_{34}O_3$ [M]$^+$, 346.2508); purity 96% (HPLC analysis conditions 90% aqueous MeOH, 1 mL/min, λ = 190 nm, R_t = 5.8 min). ^{1}H NMR (500 MHz, $CDCl_3$),^{13}C NMR (125 MHz, $CDCl_3$) data, see **Table 1**.

Isolation of LDL. Blood was collected from normalipidemic volunteers with permission according to the Guidelines of Blood Donation Program for a Research of the Korea Red Cross Blood Center. Ethylenediaminetetraacetic acid (EDTA) was used as anticoagulant (1.5 mg/mL of blood). After low-speed centrifugation of the whole blood to obtain plasma and to prevent lipoprotein modification, EDTA (0.1%), NaN_3 (0.05%), and phenylmethylsulfonyl fluoride (PMSF, 0.015%) were added. LDL was isolated from the plasma by preparative ultracentrifugation as described (*12*).

Cu^{2+}-Induced LDL Oxidation and TBARS Assay. A LDL solution (250 μL, 50−100 μg of protein) in phosphate-buffered saline (PBS) was supplemented with 10 μM $CuSO_4$ as an oxidation initiator. The oxidation was performed in a screw-capped 5 mL glass vial at 37 °C in the presence or absence of test compounds. After 4 h of incubation, the reaction was terminated by addition of 1 mL of 20% trichloroacetic acid. Following precipitation, 1 mL of 0.67% TBA in 0.05 N NaOH was added and vortexed, and the final mixture was heated for 5 min at 95 °C, cooled on ice, and centrifuged for 2 min at 1000*g*. The optical

density of the produced malondialdehyde was measured at 532 nm. Calibration was done with a malonaldehyde bis(dimethyl acetal) (MDA) standard prepared from tetramethoxypropane (*23*).

AAPH-Mediated or SIN-1-Mediated LDL Oxidation. Human plasma LDL (120 μg protein/mL) was oxidized in 10 mM PBS buffer (pH 7.4) with 4 mM AAPH (a free radical generator that thermally decomposes to aqueous peroxy free radicals) or 1 mM SIN-1 (a peroxy nitrite generator) at 37 °C for 4 h, and then, the reaction was stopped by addition of 1 mM EDTA (*24*, *25*). In this experiment, oxidation was carried out in the presence of compounds. After the incubation, the extent of LDL oxidation was measured by TBARS assay.

Another LDL Oxidation Assays. The formation of conjugated diene, REM, and fragmentation of ApoB-100 on LDL oxidation was measured as described previously (*26*).

Cell-Mediated Oxidation of LDL. 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 mg/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 plates (1×10^6 cell/well in 1 mL). Differentiation of THP-1 cells to macrophages was induced with phorbol 12-myristate 13-acetate (PMA, 150 ng/mL, Sigma) for 3 days. THP-1 macrophages were washed three times with serum-free RPMI 1640 media. To examine the effect of the compounds on macrophage-mediated LDL oxidation, cells were incubated with LDL (120 μg/mL) in the culture medium with or without compounds, supplemented with 2 μM $CuSO_4$ for 24 h at 37 °C. The extent of LDL oxidation was determined directly in the harvested medium using the TBARS assay (*27*).

Radical DPPH Scavenging Activity. The radical DPPH scavenging activity was measured according to the procedure described by Hatano et al. (*28*). In brief, freshly made radical DPPH solution (2 mL; final concentration, 100 μM) was added to 1 mL of test compound (final concentration, 100 μM) in methanol. The absorbance of DPPH radical remaining was measured at 517 nm against a blank of pure methanol including only DPPH radical for 35 min using the UV−visible spectrophotometer at room temperature. The radical DPPH scavenging capacity was calculated from the difference in the absorbance with tested compounds and expressed as percent DPPH radical remaining.

Culture of RAW264.7 Cells. RAW264.7 cells (murine macrophage cell line) obtained from American Type Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomiycin, and 10% fetal bovine serum in a humidified incubator with 5% CO_2 in air at 37 °C.

Measurement of NO. Nitrite production, as an assay of NO release, was measured in culture medium. RAW264.7 cells were plated at 1×10^6 cells/mL and pretreated with 5−50 μM compound **1** 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 the nitrite concentration was determined by measuring the optical density at 540 nm with the model 680 Microplate reader (Bio-Rad, Hercules, CA).

Western Blot Analysis. RAW 264.7 cells were incubated with various concentrations of compound **1** and 1 μg/mL LPS for 18 h. The cells were washed twice in ice-cold PBS. The cell lysates were prepared by suspending 1×10^6 cells in 100 μL of lysis buffer [140 mM NaCl, 15 mM ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 100 μM PMSF, and 20 μM leupeptin, pH 7.5], disrupted by sonication, and extracted for 30 min at 4 °C. The extracts were centrifuged at 15000*g* for 10 min. Samples of equal amounts of proteins were separated through 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). After electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA). The membrane was incubated for 2 h at room temperature with iNOS antibody (BD Transduction Laboratories, Lexington, KY). Immunoreactive proteins were detected with the ECL Western blotting kit (Amersham).

Data Analysis. All values are expressed as means ± standard deviations (SD) of two independent experiments performed in duplicate. Statistical analysis was done using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

As described in the extraction and isolation section, the EtOAc fraction of ethanolic extracts of *T. nucifera* leaves showed the high LDL-antioxidant activity. Bioassay-guided fractionation using an LDL-antioxidant assay (TBARS assay) led to the isolation of the abietane diterpenoids **1** and **2** (**Figure 1**).

The structures of isolated compounds **1** and **2** were determined by spectroscopic analysis of IR, MS, and NMR experiments and comparison with values previously reported (*22*). To confirm more exact structures, compound **1** was synthesized from starting material, dehydroabietic acid, by six steps (scheme and data not shown), according to the synthetic method of Fieser and Campbell (*29*). The spectroscopic data of synthetic compound were identical with those of compound **1** isolated from *T. nucifera*. Therefore, the structure of compound **1** was elucidated to be 12-hydroxy abietic-8,11,13-trien-18-oic acid methyl ester (**1**). The ^{1}H NMR spectrum of compound **2** revealed the presence of two aromatic protons (1H, s, H-11, δ 6.55 and 1H, s, H-14, δ 6.75), a dimethoxymethyl group (C18a-OMe, δ 3.43, C18b-OMe, δ 3.45, and 1H, s, H-18, δ 3.96), an isopropyl methine group (1H, m, H-15, δ 3.04), a benzylic methylene group (2H, m, H-7, δ 2.68−2.78), two tertiary methyl groups (3H, s, H-19, δ 0.90 and 3H, s, H-20, δ 1.12), and an isopropyl group (3H, d, $J_{16,15} = 7.2$ Hz, H-16, δ 1.15 and 3H, d, $J_{17,15} = 7.2$ Hz, H-17, δ 1.16), respectively. In the HMBC spectrum, long-range couplings were observed from the methine proton (δ 3.96) of dimethoxymethyl to the methylene carbon (C-3, δ 30.4), the two methoxy carbons (C-18a, δ 58.7 and C-18b, δ 59.0), and tertiary methyl carbon (C-19, δ 16.7). The HMBC correlations of H-20 (δ 1.12) with C-1 (δ 38.2), C-5 (δ 42.8), C-9 (δ 148.7), and C-10 (δ 37.3) and of H-5 (δ 1.81) with C-4 (δ 42.6), C-6 (δ 19.4), C-9 (δ 148.7), C-10 (δ 37.3), C-19 (δ 16.7), and C-18 (δ 113.3) were shown. These results supported the position and presence of dimethoxymethyl group at C-18 (δ 113.3) and two tertiary methyl groups at C-19 (δ 16.7) and C-20 (δ 25.2) (**Table 1**). The relative stereochemistry between C-19 and C-20 of compound **2** was established by nuclear Overhauser effect (NOE) experiments, namely, a NOE of 1.95% for the H-20 was observed upon irradiation of H-19. Therefore, NOE correlation signals between H-19 and H-20 indicated the 1,3-diaxial configurations. Similarly, the 12-OH/H-11 and 12-OH/H-15 correlations indicated that 12-OH was in an ortho position with each H-11 and H-15. The structure of compound **2** was elucidated to be a new compound, 12-hydroxy abietic-8,11,13-trien-18-dimethyl acetal (**2**).

Although compound **1** was synthesized from dehydroabietic acid (*29*) and isolated from the woody verdure of the Scotch pine (*30*), its potential as an antioxidant against LDL oxidation was not evaluated. In this study, compounds **1** and **2** were first evaluated for their potential to inhibit the Cu^{2+}-induced, AAPH-mediated, and SIN-1-mediated human LDL-oxidation on the TBARS assay. Compounds **1** and **2** showed potent Cu^{2+}-induced LDL-antioxidant activities with IC_{50} values of 1.1 ± 0.2 and 1.8 ± 0.1 μM, respectively (probucol, $IC_{50} = 3.1 \pm 0.1$ μM). Under AAPH-mediated oxidation, compounds **1** and **2** showed IC_{50} values of 2.4 ± 0.1 and 4.8 ± 0.3 μM, respectively (probucol, $IC_{50} = 2.8 \pm 0.3$ μM). Subsequently, under SIN-1-mediated oxidation, 5.0 μM compounds **1** and **2** inhibited 68.2 ± 1.2 and 61.5 ± 3.1% of LDL oxidation, respectively (5.0

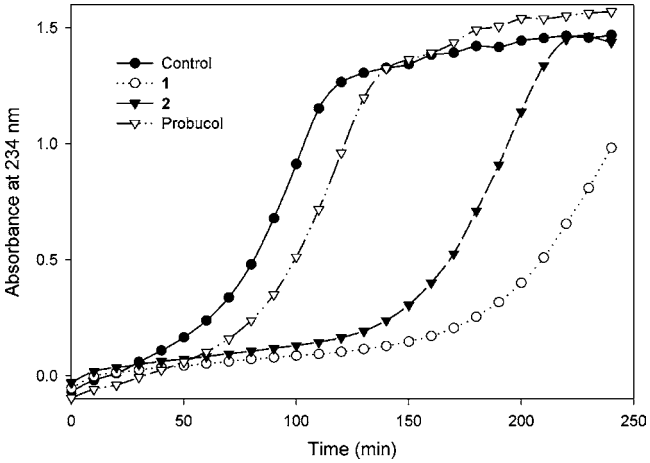

Figure 2. Effects of compounds **1** and **2** on the generation of conjugated diene. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 4 h. Probucol was used as a reference antioxidant. Values shown are a representative experiment.

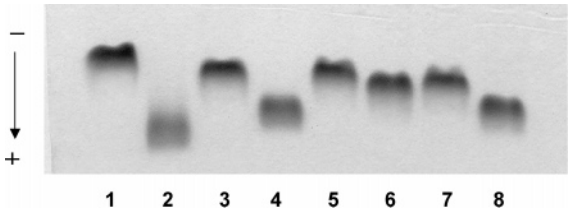

Figure 3. Effects of compounds **1** and **2** on changes in electrophoretic mobility of LDL. Lane 1, native LDL (absence of $CuSO_4$); lane 2, ox-LDL; lane 3, probucol (5.0 μM); lane 4, probucol (2.0 μM); lane 5, compound **1** (5.0 μM); lane 6, compound **1** (2.0 μM); lane 7, compound **2** (5.0 μM); and lane 8, compound **2** (2.0 μM).

μM probucol, 58.1 ± 5.3% inhibition). Probucol, a known antioxidant having antiatherogenic activity (*31*, *32*), is used as a positive control in a series of experiments.

Oxidation of LDL was determined by measuring the conjugated diene formation at 234 nm (*33*). The lag time of conjugated diene production, indicating of the resistance of LDL to oxidation, was prolonged when LDL was incubated with the compounds **1** and **2**. As shown in **Figure 2**, the LDL (100 μg/mL) was incubated with 5.0 μM $CuSO_4$ alone to have a lag time of 70 min. In the presence of each of 1.0 μM compounds **1** and **2**, the lag time was extended to 186 and 157 min, respectively, whereas the addition of 1.0 μM probucol extended the lag time to 84 min. Thus, compounds **1** and **2** delayed LDL oxidation much greater than that of probucol.

To evaluate another parameter that is affected by LDL oxidation, compounds **1** and **2** were applied to REM assay. As shown in **Figure 3**, native LDL (lane 1) and the ox-LDL with 5.0 μM $CuSO_4$ alone (lane 2) were subjected to electrophoresis. The mobility of ox-LDL in the addition of compounds **1** and **2** was reduced dose dependently. In the presence of each 5.0 and 2.0 μM compounds **1** and **2**, the LDL oxidation was protected in 77.1 ± 1.2 and 58.3 ± 0.8% for compound **1** and 54.0 ± 1.3 and 19.1 ± 0.7% for compound **2**, respectively, as compared to that of ox-LDL. Then, probucol inhibited the oxidation of LDL in 77.4 ± 2.1% at 5.0 μM and 22.1 ± 0.5% at 2.0 μM.

Radical reaction of LDL causes facial degradation of apoB-100, which is a major component of LDL (*34*). The inhibition of the oxidative process by compounds **1** and **2** was evaluated by the densitometric area of band of apoB-100 through SDS−PAGE (**Figure 4**). The band of apoB-100 was observed on native LDL, which had been incubated without 5 μM $CuSO_4$ for 12 h at 37 °C, but the band completely disappeared when the LDL was incubated with 5 μM $CuSO_4$. The compounds **1** and **2** at 10 μM protected the fragmentation of apoB-100 in 75 ± 1.4 and 72 ± 1.7%, respectively. Thus, compounds **1** and **2** were more active in the protection of apoB-100 fragmentation against copper-induced oxidation of LDL than probucol (22 ± 0.6%).

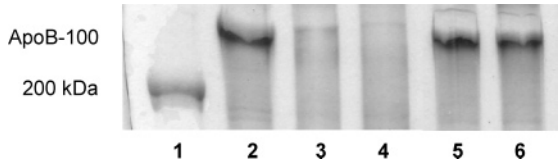

Figure 4. Antioxidant effects of compounds **1** and **2** on the apoB-100 fragmentation. Lane 1, molecular weight marker; lane 2, native LDL (absence of $CuSO_4$); lane 3, ox-LDL; lane 4, probucol (5.0 μM); lane 5, compound **1** (10 μM); and lane 6, compound **2** (10 μM).

Table 2. Effects of **1** and **2** on Macrophage-Mediated Oxidation of LDL[a]

incubation conditions	MDA nmol/mg LDL protein
LDL + cell	5.7 ± 0.8
LDL + Cu^{2+}	66.8 ± 2.1
LDL + cell + Cu^{2+} (control)	481.4 ± 4.8
LDL + cell + Cu^{2+} + 1.0 μM **1**	141.0 ± 3.6*
LDL + cell + Cu^{2+} + 1.0 μM **2**	137.6 ± 9.8*
LDL + cell + Cu^{2+} + 1.0 μM probucol	270.0 ± 11.2*

[a] *P < 0.05 vs control. Data are shown as means ± SD from two independent experiments performed in duplicate.

Next, the antioxidant activities of compounds **1** and **2** in macrophage-mediated oxidation of LDL were examined (**Table 2**). The cellular oxidative modification of LDL to a form recognized by the scavenger receptor requires the presence of transition metal ions in the medium (*35*, *36*). In the case of the LDL incubated in the presence of THP-1 macrophages without $CuSO_4$, the MDA-like product formation (5.7 ± 0.8 MDA nmol/mg LDL protein) was much too low. The 2 μM copper-induced LDL oxidation in the absence of the cells slightly increased the MDA-like product formation (66.8 ± 2.1 MDA nmol/mg LDL protein). Surprisingly, the copper-induced plus macrophage-mediated LDL oxidation (481.4 ± 4.8 MDA nmol/mg LDL protein) was 7-fold higher as compared with only the copper-induced LDL oxidation. Therefore, the antioxidant activity of the compounds **1** and **2** was tested by macrophage-mediated LDL oxidation with 2 μM $CuSO_4$. The content of the MDA-like product in the presence of 10 μM compounds **1** and **2** was reduced 70.7 and 71.4%, respectively. At the same concentration of probucol, it was reduced 43.9%. As a result, the antioxidant activities of compounds **1** and **2** were about 2-fold higher than that of probucol on macrophage-mediated LDL oxidation.

The radical DPPH scavenging activity is an important parameter to determine antioxidant activity. The radical scavenging capacities of compounds **1** and **2** were measured as decolorizing activity following the trapping of the unpaired electron of DPPH (**Figure 5**). After 35 min, 67 ± 3.8 and 78 ± 2.1% DPPH radicals, respectively, remained at the presence of each 100 μM compounds **1** and **2**, whereas only 18 ± 1.9% DPPH radicals remained at 100 μM probucol. Therefore, compounds **1** and **2** showed mild radical scavenging capacity as compared to probucol.

We determined whether compound **1** inhibited NO production and iNOS expression in activated macrophages. Prior to our

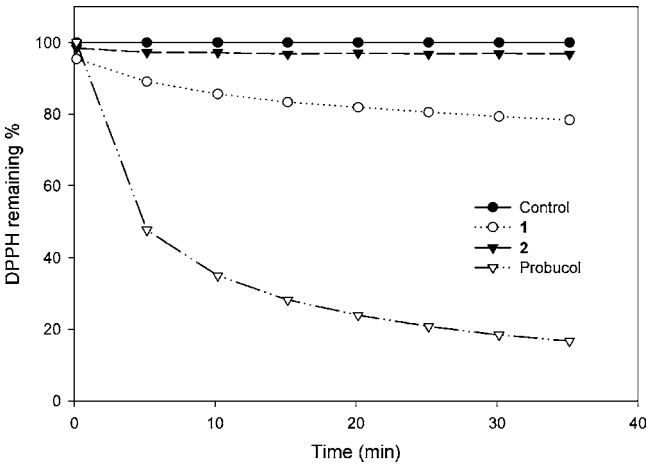

Figure 5. Effects of compounds **1** and **2** on radical DPPH scavenging. Compounds **1** and **2** (100 μM) were incubated with 100 μM DPPH in methanol for 35 min at room temperature. The absorbance of each compound solution was measured at 517 nm. The antiradical activity was expressed by the remaining DPPH percentage.

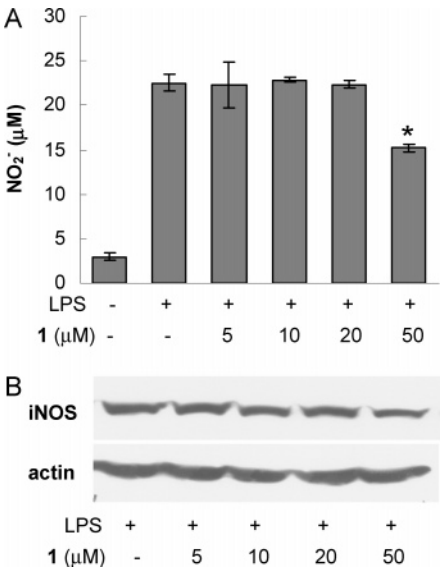

Figure 6. Effect of compound **1** on the induction of iNOS by LPS. (**A**) Inhibition of NO production by compound **1** in RAW264.7 cells. RAW264.7 cells were treated with 5–50 μM compound **1** dissolved in dimethyl sulfoxide for 2 h prior to the addition of LPS (1 μg/mL), and the cells were further incubated for 18 h. (**B**) Inhibition of LPS-inducible iNOS protein expression by compound **1**. The level of iNOS protein was monitored for 18 h after treatment of cells with LPS (1 μg/mL). The data represent the mean ± SD ($n = 4$). *$P < 0.05$ indicates statistically significant differences from LPS alone-stimulated group.

study on the effect of compound **1**, the methylthiazolyldiphenyltetrazolium bromide (MTT) assay was performed to determine if compound **1** has cytotoxicity in RAW264.7 macrophages. Results showed that compound **1** at a concentration up to 50 μM had no effect on cell viability (data not shown). NO production was monitored in RAW264.7 cells stimulated by LPS in the presence or absence of compound **1** for 18 h. LPS (1 μg/mL) increased the level of nitrite in culture medium by 10-fold as compared to control (LPS absence), and 50 μM compound **1** inhibited 30% NO production (**Figure 6A**). Western blot analysis confirmed that 50 μM compound **1** markedly suppressed the expression of iNOS protein stimulated by LPS (**Figure 6B**).

In conclusion, **1** and a new compound, **2** were isolated by bioassay-guided fractionation from the ethanolic extracts of *T. nucifera* leaves. Compounds **1** and **2** showed potent antioxidant activities in the Cu^{2+}-, AAPH-, and SIN-1-mediated LDL oxidation in TBARS assay as well as the macrophage-mediated LDL oxidation. In addition, compounds **1** and **2** inhibited the conjugated diene formation, REM of ox-LDL, and fragmentation of apoB-100 on Cu^{2+}-induced LDL oxidation and showed mild radical DPPH scavenging activity. Compound **1** is able to reduce iNOS expression in LPS-stimulated RAW264.7 cells.

LITERATURE CITED

(1) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol: Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915−923.

(2) Steinberg, D. Atherogenesis in perspective: Hypercholesterolemia and inflammation as partners in crime. *Nat. Med*. **2002**, *8*, 1211−1217.

(3) Glass, C. K.; Witzum, J. L. Atherosclerosis: The road ahead. *Cell* **2001**, *104*, 503−516.

(4) Liao, L.; Starzyk, R. M.; Granger, D. N. Molecular determinants of oxidized low-density lipoprotein-induced leukocyte adhesion and microvascular dysfunction. *Arterioscler., Thromb., Vasc. Biol*. **1997**, *17*, 437−444.

(5) Dejager, S.; Mietus-Synder, M.; Pitas, R. E. Oxidized low-density lipoproteins bind to the scavenger receptor expressed by rabbit smooth muscle cells and macrophages. *Arterioscler. Thromb*. **1993**, *13*, 371−378.

(6) Ross, R. Atherosclerosis—An inflammatory disease. *N. Engl. J. Med*. **1999**, *340*, 115−126.

(7) Moncada, S.; Palmer, R. M.; Higgs, E. A. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev*. **1991**, *43*, 109−142.

(8) MacMicking, J.; Xie, Q. W.; Nathan, C. Nitric oxide and macrophage function. *Annu. Rev. Immunol*. **1997**, *15*, 323−350.

(9) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med*. **1996**, *20*, 933−956.

(10) Ahn, B. T.; Lee, S.; Lee, S. B.; Lee, E. S.; Kim, J. G.; Bok, S. H.; Jeong, T. S. Low-density lipoprotein-antioxidant constituents of *Saururus chinensis*. *J. Nat. Prod.* **2001**, *64*, 1562−1564.

(11) Lee, W. S.; Back, Y. I.; Kim, J. R.; Cho, K. H.; Sok, D. E.; Jeong, T. S. Antioxidant activities of a new lignan and a neolignan from *Saururus chinensis*. *Bioorg. Med. Chem. Lett*. **2004**, *14*, 5623−5628.

(12) Lee, W. S.; Kim, J. R.; Im, K. R.; Cho, K. H.; Sok, D. E.; Jeong, T. S. Antioxidant effects of diarylheptanoid derivatives from *Alnus japonica* on human LDL oxidation. *Planta Med*. **2005**, *71*, 295−299.

(13) Bae, K. H. *The Medicinal Plants of Korea*; Kyo-Hak Publishing Co. Ltd.: Seoul, 2000; p 45.

(14) Harrison, L. J.; Asakawa, Y. 18-Oxoferruginol from the leaf of *Torreya nucifera*. *Phytochemistry* **1987**, *26*, 1211−1212.

(15) Orihara, Y.; Yang, J.-W.; Komiya, N.; Koge, K.; Yoshikawa, T. Abietane diterpenoids from suspension cultured cells of *Torreya nucifera* var. *radicans*. *Phytochemistry* **2002**, *59*, 385−389.

(16) Nakatani, N.; Inatani, R. Structure of rosmanol, a new antioxidant from rosemary (*Rosmarinus officinalis* L.). *Agric. Biol. Chem*. **1981**, *45*, 2385−2386.

(17) Inatani, R.; Nakatani, N.; Fuwa, H. Antioxidative effect of the constituents of rosemary (*Rosmarinus* officinalis L.) and their derivatives. *Agric. Biol. Chem*. **1983**, *47*, 521−528.

(18) Nakatani, N.; Inatani, R. Two antioxidative diterpenes from rosemary (*Rosmarinus officinalis* L.) and a revised structure for rosmanol. *Agric. Biol. Chem*. **1984**, *48*, 2081−2085.

(19) Kobayashi, K.; Masako, N.; Fukushima, M.; Shiobara, Y.; Kodama, M. Antibacterial activity oif pisiferic acid and its derivatives against Gram-negative and -positive bacteria. *Agric. Biol. Chem*. **1988**, *52*, 77−83.

(20) Ulubelen, A.; Öksü, S.; Kolak, U.; Birman, H.; Voelter, W. Cardioactive terpenoids and a new rearranged diterpene from *Salvia syriaca*. *Planta Med*. **2000**, *66*, 627−629.

(21) Matsuda, H.; Murakami, T.; Yashiro, K.; Yamahara, J.; Yoshikawa, M. Antidiabetic principles of natural medicines. IV. Aldose reductase and α-glucosidase inhibitors from the roots of *Salacia oblonga* WALL. (Celastraceae): Structure of a new friedelane-type triterpene, kotalagenin 16-acetate. *Chem. Pharm. Bull*. **1999**, *47*, 1725−1729.

(22) Tahara, A.; Shimagaki, M.; Itoh, M.; Harigaya, Y.; Onda, M. Diterpenoids. XXXVIII. Conversion of l-abietic acid into steroidal skeletons: Formation of the D-ring (1). *Chem. Pharm. Bull.* **1975**, *23*, 3189−3202.

(23) Yagi, K. *Lipid Peroxides in Biology and Medicine*; Yagi, K., Ed.; Academic Press: Orlando, FL, 1982; p 223.

(24) Devaraj, S.; Li, D. J.; Vazquez, M.; Jialal, I. Cyclosporin A does not increase the oxidative susceptibility of low-density lipoprotein in vitro. *Free Radical Biol. Med.* **1999**, *26*, 1064−1068.

(25) Gugliucci, A.; Menini, T. Three different pathways for human LDL oxidation are inhibited in vitro by water extracts of the medicinal herb *Achyrocline satureoides*. *Life Sci.* **2002**, *71*, 693−705.

(26) Lee, J. H.; Lee, B. W.; Kim, J. H.; Jeong, T. S.; Kim, M. J.; Lee, W. S.; Park, K. H. LDL-antioxidant pterocarpans from roots of *Glycine max* (L.) Merr. *J. Agric. Food Chem.* **2006**, *54*, 2057−2063.

(27) Lesnik, P.; Dachet, C.; Petit, L.; Moreau, M.; Griglio, S.; Brudi, P.; Chapman, M. J. Impact of a combination of a calcium antagonist and a beta-blocker on cell- and copper-mediated oxidation of LDL and on the accumulation and efflux of cholesterol in human macrophages and murine J774 cells. *Arterioscler., Thromb., Vasc. Biol*. **1997**, *17*, 979−988.

(28) Bursa, S. Y.; Oleszek, W. Antioxidant and antiradical activities of flavonoids. *J. Agric. Food Chem*. **2001**, *49*, 2774−2779.

(29) Fieser, L. F.; Campbell, W. P. Hydroxyl and amino derivatives of dehydroabietic acid and dehydroabietinol. *J. Am. Chem. Soc*. **1939**, *61*, 2528−2534.

(30) Roshchin, V. I.; Skachkova, N. M.; Lyandres, G. V.; Maksimchuk, P. L. Carbon dioxide extract from woody verdure of the Scotch pine. Group composition and acids. *Chem. Nat. Compd. (Engl. Transl.)* **1988**, *24*, 447−452.

(31) Carew, T. E.; Schwenke, D. C.; Steinberg, D. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low-density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidaemic rabbit. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7725−7729.

(32) Kuzuya, M.; Kuzuya, F. Probucol as an antioxidant and antiatherogenic drug. *Free Radical Biol. Med.* **1993**, *14*, 67−77.

(33) Esterbauer, H.; Stiegl, G.; Puhl, H.; Rotheneder, M. Continuous monitoring of in vitro oxidation of human low-density lipoprotein. *Free Radical Res. Commun.* **1989**, *6*, 67−75.

(34) Tanaka, K.; Iguchi, H.; Taketani, S.; Nakata, R.; Tokumaru, S.; Sugimoto, T.; Kojo, S. Facial degradation of apolipoprotein B by radical reactions and the presence of cleaved proteins in serum. *J. Biochem.* **1999**, *125*, 173−176.

(35) Heinecke, J. W.; Baker, L.; Rosen, H.; Chait, A. Iron and copper promote modification of low-density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest*. **1984**, *74*, 1890−1894.

(36) Steinbrecher, U. P.; Parthasarathy, S.; Leake, D. S.; Witztum, J. L.; Steinberg, D. Modification of low-density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low-density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3883−3887.

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