Planta Medica

Journal of Medicinal Plant and Natural Product Research

Publishers

Georg Thieme Verlag KG
Stuttgart · New York
Rüdigerstraße 14
D-70469 Stuttgart
Postfach 30 11 20
D-70451 Stuttgart

Thieme Publishers
333 Seventh Avenue
New York, NY 10001, USA
www.thieme.com

New Benzamide Derivatives and NO Production Inhibitory Compounds from *Limonia acidissima*

Authors Ki Hyun Kim[1], Il Kyun Lee[1], Kyung Ran Kim[1], Sang Keun Ha[2], Sun Yeou Kim[2], Kang Ro Lee[1]

Affiliations
[1] Natural Products Laboratory, College of Pharmacy, Sungkyunkwan University, Suwon, Korea
[2] Graduate School of East-West Medical Science, Kyung Hee University, Yongin-City, Kyungki-Do, Korea

Key words
- *Limonia acidissima*
- Rutaceae
- benzamide derivative
- NO production inhibitory activity

Abstract

Three new benzamide derivatives, *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-octadecanoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**1**), *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**2**), and *N*-{[*p*-(3,7-dimethyl-6*R*,7-epoxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**3**), together with 10 known compounds (**4**–**13**), were isolated from the bark of *Limonia acidissima*. The structures of these new compounds were determined through spectral analyses, including extensive 2D NMR data. Among the isolates, 13*α*,14*β*,17*α*-lanosta-7,9,24-triene-3*β*,16*α*-diol (**8**), 4-methoxy-1-methyl-2(1*H*)-quinolinone (**10**), and 13*α*,14*β*,17*α*-lanosta-7,24-diene-3*β*,11*β*,16*α*-triol (**13**) potently inhibited nitric oxide (NO) production in microglia cells.

Introduction

The wood apple (*Limonia acidissima* L., Rutaceae) is a tropical fruit that is distributed in dry, warm regions of Burma, India, Malaya, and Sri Lanka [1]. The fruit and stem bark are well known for medicinal properties such as fungicide, bactericide and insecticide activities, and the fruit pulp is also applied externally as a remedy for certain insect bites [2]. Thanaka, a root paste made from the pulp of *L. acidissima*, is a facial cosmetic that is also used to remove small spots and lesions on the skin [3]. Previous phytochemical studies on different parts of this plant revealed the presence of coumarins, steroids, triterpenoids, benzoquinones, and tyramine derivatives [4–9]. Some coumarins isolated exhibit antifungal activities [6, 10]. In the course of our continuing search for biologically active compounds from natural medicinal sources, we investigated the bark of *L. acidissima*. Phytochemical investigation of the EtOAc-soluble fraction of the ethanol extract of this herb led to the isolation of three new benzamide derivatives, *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-octadecanoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**1**), *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**2**), and *N*-{[*p*-(3,7-dimethyl-6*R*,7-epoxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**3**), together with 10 known compounds (**4**–**13**). The structures of these new compounds were determined through spectral analysis, including extensive 2D NMR data. This paper describes the structural elucidation of the new compounds (**1**–**3**) and the NO production inhibitory activity of compounds **1**–**13** in BV-2 cells (**Fig. 1**).

Materials and Methods

General experimental procedures

All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Optical rotations were measured on a Jasco P-1020 polarimeter in MeOH. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. UV spectra were recorded with a Shimadzu UV-1601 UV-Visible spectrophotometer. FAB and HR-FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer. NMR spectra, including ^{1}H-^{1}H COSY, HMQC, and HMBC experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) using TMS as an internal standard and with chemical shifts given in ppm (δ). For preparative HPLC we used a Gilson 306 pump with a Shodex refractive index detector and an Econosil RP-18 10-μ column (250 × 22 mm). Silica gel 60 (Merck; 70–230 mesh and 230–400 mesh)

received July 9, 2008
revised January 12, 2009
accepted February 17, 2009

Bibliography
DOI 10.1055/s-0029-1185512
Published online April 2, 2009
Planta Med 2009; 75: 1146–1151 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

Correspondence
Prof. Dr. Kang Ro Lee
Natural Products Laboratory
College of Pharmacy
Sungkyunkwan University
300 Chonchon-dong
Jangan-ku, Suwon 440–746
Korea
Phone: + 82 31 290 77 10
Fax: + 82 31 292 88 00
krlee@skku.ac.kr

This is a copy of the author's personal reprint

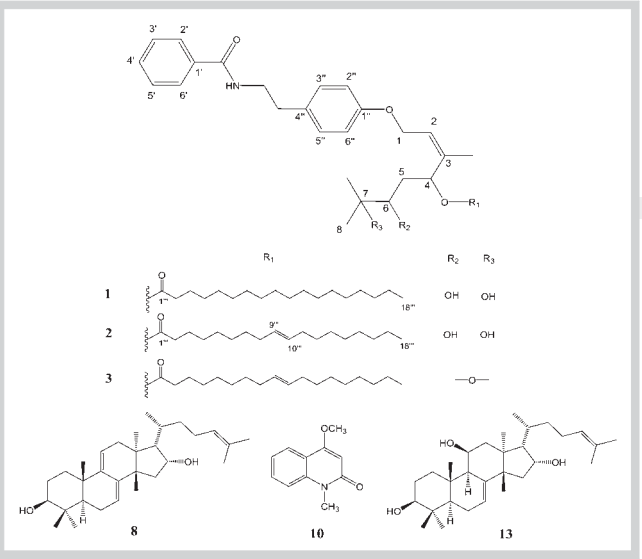

Fig. 1 Chemical structures of compounds **1–3, 8, 10**, and **13**.

and RP-C_{18} silica gel (Merck; 230–400 mesh) were used for column chromatography. Merck pre-coated silica gel F_{254} plates and RP-18 F_{254s} plates were used for TLC. Spots were detected under UV light or by heating after spraying with 10% H_2SO_4 in EtOH (v/v). The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.). Low-pressure liquid chromatography was performed over a Merck Lichroprep Lobar-A Si 60 (240 × 10 mm) or a Lichroprep Lobar-A RP-18 (240 × 10 mm) column with a FMI QSY-0 pump (ISCO).

Plant material

The dried bark of *L. acidissima* was imported from Yangon, Union of Myanmar, in October 2006 and identified by Dr. W. Bae. A voucher specimen (SKKU 2006–10) was deposited at the R & D Institute, Miwon Commercial Co., Ltd., Ansan, Korea.

Extraction and isolation

The dried bark of *L. acidissima* (3 kg) was extracted with 85% EtOH three times at 85 °C. The resulting ethanol extract (250 g) was suspended in distilled water (7.2 L) and then partitioned with EtOAc, yielding an EtOAc-soluble extract (50 g). The EtOAc-soluble fraction (50 g) was separated over silica gel column (230–400 mesh, 500 g) eluted gradiently with *n*-hexane-EtOAc [10 : 1 (1.5 L), 5 : 1 (1.0 L), 1 : 1 (1.0 L), and 0 : 1 (2.0 L)] to afford 12 fractions [Fr. A, 10 : 1, 0.5 L; Fr. B, 10 : 1, 0.5 L; Fr. C, 10 : 1, 0.5 L; Fr. D, 5 : 1, 0.3 L; Fr. E, 5 : 1, 0.3 L; Fr. F, 5 : 1, 0.4 L; Fr. G, 1 : 1, 0.2 L; Fr. H, 1 : 1, 0.2 L; Fr. I, 1 : 1, 0.3 L; Fr. J, 1 : 1, 0.3; Fr. K, 0 : 1, 1.0 L; and Fr. L, 0 : 1, 1.0 L]. Fr. I (760 mg) was separated further over silica gel column (230–400 mesh, 210 g, $CHCl_3$/MeOH 15 : 1) to give three subfractions [Fr. I1–Fr. I3 (each 2.0 L)]. Fr. I2 (320 mg) was separated over RP-C_{18} silica gel column (230–400 mesh, 10 g, MeCN/H_2O 3 : 2) to give seven subfractions [Fr. I21, 0.5 L; Fr. I22, 0.5 L; Fr. I23, 0.5 L; Fr. I24, 0.5 L; Fr. I25, 0.5 L; Fr. I26, 0.5 L; and Fr. I27, 1.0 L]. Fr. I21 (50 mg) was separated by preparative reversed-phase HPLC, over 30 min at a flow rate of 2.0 mL/min (Econosil RP-18 10-μ column; 250 × 22 mm; 10-μ particle size; Shodex refractive index detector) to yield **5** (5 mg, 43% MeCN, t_R = 20.5 min). Fr. I23 (130 mg) was separated further by preparative reversed-phase HPLC, as described above, to obtain **6** (6 mg, 43% MeCN, t_R = 21.5 min) and **7** (5 mg, 43% MeCN, t_R = 24.0 min). Fr. I25 (35 mg) was separated by preparative reversed-phase HPLC, as described above, to obtain **1** (6 mg, 100% MeOH, t_R = 17.5 min) and **4** (6 mg, 100% MeOH, t_R = 20.0 min). Fr. I26 (35 mg) was separated by preparative reversed-phase HPLC, as described above, to obtain **2** (6 mg, 100% MeOH, t_R = 18.0 min) and **3** (16 mg, 100% MeOH, t_R = 21.5 min). Fr. I27 (35 mg) was separated by preparative reversed-phase HPLC, as described above, to give **8** (5 mg, 95% MeOH, t_R = 13.5 min) and **13** (4 mg, 95% MeOH, t_R = 17.0 min). Fr. J (2.7 g) was separated over silica gel column (230–400 mesh, 300 g, $CHCl_3$/MeOH 15 : 1) to give two subfractions [Fr. J1–Fr. J2, (each 3.0 L)]. Fr. J1 (1.7 g) was separated over Sephadex LH-20 column (150 g; Pharmacia Co.; CH_2Cl_2/MeOH 1 : 1) to obtain **9** (40 mg) and subfraction J11 (900 mg). Fr. J11 (900 mg) was separated over RP-C_{18} silica gel column (230–400 mesh, 30 g, MeOH/H_2O 3 : 2) to give three main fractions (Fr. J111, 1.5 L; Fr. J112, 1.5 L; and Fr. J113, 2.0 L). Fr. J111 (150 mg) was separated by using a Lobar-A RP-18 (240 × 10 mm) column (45% MeCN) and further separated by preparative reversed-phase HPLC, as described above, to get **12** (5 mg, 45% MeCN, t_R = 17.0 min). Fr. J112 (60 mg) was separated by using a Lobar-A RP-18 (240 × 10 mm) column (40% MeCN) and further separated by preparative reversed-phase HPLC, as described above, to obtain **11** (8 mg, 40% MeCN, t_R = 20.5 min). Fr. J113 (100 mg) was further separated by preparative reversed-phase HPLC, as described above, to yield **10** (30 mg, 40% MeCN, t_R = 15.0 min).

Isolated compounds

N-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-octadecanoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**1**): Amorphous gum; $[\alpha]_D^{25}$: + 72.5 (*c* 0.20, MeOH); IR (KBr): ν_{max} = 3379, 2948, 1661, 1554, 1115, 1026 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 225.3 (4.27), 283.2 (1.42), 298.5 (1.38) nm; FAB-MS: *m*/*z* = 694 $[M + H]^+$; HR-FAB-MS: *m*/*z* = 694.5049 $[M + H]^+$ (calcd. for $C_{43}H_{68}NO_6$: 694.5047). ^{1}H-NMR (CD_3OD, 500 MHz) data: see **Table 1**. ^{13}C-NMR (CD_3OD, 125 MHz) data: see **Table 2**.

N-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**2**): Amorphous gum; $[\alpha]_D^{25}$: + 97.0 (*c* 0.22, MeOH); IR (KBr): ν_{max} = 3384, 2950, 1658, 1556, 1114, 1025 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 225.2 (4.23), 282.5 (1.44), 298.3 (1.38) nm; FAB-MS: *m*/*z* = 691 $[M]^+$; HR-FAB-MS: *m*/*z* = 691.4823 $[M]^+$ (calcd. for $C_{43}H_{65}NO_6$: 691.4812). ^{1}H-NMR (CD_3OD, 500 MHz) data: see **Table 1**. ^{13}C-NMR (CD_3OD, 125 MHz) data: see **Table 2**.

N-{[*p*-(3,7-dimethyl-6*R*,7-epoxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**3**): Amorphous gum; $[\alpha]_D^{25}$: + 39.3 (*c* 0.15, MeOH); IR (KBr): ν_{max} = 3365, 2946, 1655, 1558, 1029 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 225.3 (4.25), 283.5 (1.42), 298.3 (1.40) nm; FAB-MS: *m*/*z* = 674 $[M + H]^+$; HR-FAB-MS: *m*/*z* = 674.4764 $[M + H]^+$ (calcd. for $C_{43}H_{64}NO_5$: 674.4784). ^{1}H-NMR (CD_3OD, 500 MHz) data: see **Table 1**. ^{13}C-NMR (CD_3OD, 125 MHz) data: see **Table 2**.

Hydrolysis of compounds 1 and 2

Compounds (**1**: 3.0 mg; **2**: 2.5 mg) were hydrolyzed with 10% EtOH/KOH (2 mL) at 40 °C for 1 h. The mixture was acidified with 0.1 N HCl, and then H_2O (5 mL) was added and the product was extracted twice with $CHCl_3$. The combined $CHCl_3$ layers were evaporated under reduced pressure to give the $CHCl_3$ extract (1.7 mg from **1**; 2.0 mg from **2**). This was purified over a silica gel Waters Sep-Pak Vac 6cc ($CHCl_3$/MeOH 30 : 1) to give **1a** (0.7 mg) from **1** and **2a** (1.4 mg) from **2**. The aqueous layer of the mixture

This is a copy of the author's personal reprint

Table 1 ^{1}H-NMR spectral data of compounds **1, 1a, 2, 3, 3a**, and **3b**[a].

H	1	1a	2	3	3a	3b
2′,6′	7.77 d (7.2)	7.77 d (7.0)	7.76 d (7.2)	7.77 d (7.2)	7.77 d (7.0)	7.77 d (7.0)
3′,5′	7.45 t (7.0)	7.45 t (7.0)	7.44 t (7.0)	7.44 t (7.0)	7.45 t (7.0)	7.45 t (7.0)
4′	7.51 t (7.0)	7.51 t (7.0)	7.50 t (7.0)	7.51 t (7.0)	7.52 t (7.0)	7.52 t (7.0)
N-CH_2	3.56 t (7.5)	3.68 t (7.0)	3.55 t (7.5)	3.55 t (7.5)	3.68 t (7.0)	3.68 t (7.0)
Ar-CH_2	2.84 t (7.5)	2.86 t (7.0)	2.84 t (7.5)	2.84 t (7.5)	2.86 t (7.0)	2.86 t (7.0)
2′′,6′′	6.84 d (8.5)	6.86 d (8.5)	6.85 d (8.3)	6.85 d (8.0)	6.86 d (8.5)	6.86 d (8.5)
3′′,5′′	7.16 d (8.5)	7.17 d (8.5)	7.16 d (8.3)	7.16 d (8.0)	7.17 d (8.5)	7.17 d (8.5)
1	4.60 d (6.5)	4.60 d (6.0)	4.59 d (6.2)	4.58 d (6.5)	4.61 d (6.0)	4.61 d (6.0)
2	5.80 t (6.0)	5.74 t (6.0)	5.80 t (6.0)	5.62 t (6.0)	5.76 t (6.0)	5.77 t (6.0)
3-Me	1.74 s	1.74 s	1.73 s	1.71 s	1.74 s	1.74 s
4	5.43 dd (10.0, 5.0)	4.31 t (6.5)	5.43 dd (10.0, 5.0)	5.08 dd (10.0, 2.0)	4.30 t (6.5)	4.28 t (6.5)
5	2.00 m	2.05 m	2.01 m	2.33 m	2.07 m	2.23 m
	1.61 m	1.84 m	1.63 m	1.63 m	1.61 m	1.77 m
6	3.19 dd (10.5, 1.0)	3.60 m	3.19 dd (10.5, 1.0)	3.93 t (6.5)	3.55 m	3.82 m
7-Me	1.10 s	1.13 s	1.05 s	1.12 s	1.16 s	1.16 s
8	1.14 s	1.16 s	1.14 s	1.16 s	1.17 s	1.17 s
2′′′	2.30 t (7.4)		2.31 t (7.4)	2.34 t (7.4)		
3′′′-17′′′	1.27 br s		[b]	[c]		
18′′′	0.88 t (6.9)		0.89 t (6.9)	0.88 t (6.9)		

[a] ^{1}H-NMR run at 500 MHz in CD_3OD. Chemical shifts are given in δ values. Proton coupling constants (J) in Hz are given in parentheses. [b] δH of 3′′′-17′′′ at 5.32 (2H, m, H-9′′′,10′′′), 2.03 (4H, m, H-8′′′,11′′′), and 1.27 (22H, br s, H-3′′′-H-7′′′, H-12′′′-H-17′′′), respectively. [c] δH of 3′′′-17′′′ at 5.33 (2H, m, H-9′′′,10′′′), 2.03 (4H, m, H-8′′′,11′′′), and 1.28 (22H, br s, H-3′′′-H-7′′′, H-12′′′-H-17′′′), respectively.

was also evaporated under reduced pressure to give the H_2O extract (1.2 mg from **1**; 0.5 mg from **2**), which was purified over a silica gel Waters Sep-Pak Vac 6cc ($CHCl_3$/MeOH 30 : 1) to afford octadecanoic acid (1.0 mg) from **1** and (*E*)-9-octadecenoic acid (0.4 mg) from **2** [11].

Hydrolysis of compound 3

Compound (**3**: 3.0 mg) was hydrolyzed with 5% EtOH/KOH (2 mL) at room temperature overnight. The mixture was acidified with 0.1 N HCl, and then H_2O (5 mL) was added and the product was extracted twice with $CHCl_3$. The combined $CHCl_3$ layer was evaporated under reduced pressure to give the $CHCl_3$ extract (2.0 mg). This was purified over a silica gel Waters Sep-Pak® Vac 6cc ($CHCl_3$/MeOH 30 : 1) to give **3a** (0.6 mg) and **3b** (0.8 mg). The aqueous layer of the mixture was also evaporated under reduced pressure to give the H_2O extract (0.9 mg). Purification of the water extract by a silica gel Waters Sep-Pak Vac 6cc ($CHCl_3$/MeOH 30 : 1) afforded (*E*)-9-octadecenoic acid (0.7 mg).

1a: Colorless gum; $[\alpha]_D^{25}$: + 62.1 (*c* 0.07, MeOH); IR (KBr): ν_{max} = 3378, 2948, 1659, 1554, 1115, 1026 cm^{-1}; FAB-MS: *m*/*z* = 428 $[M + H]^+$; ^{1}H-NMR (CD_3OD, 500 MHz) data: see **Table 1**.

3a: Colorless gum; ^{1}H-NMR (CD_3OD, 500 MHz) data: see **Table 1**.

3b: Colorless gum; $[\alpha]_D^{25}$: + 82.5 (*c* 0.04, MeOH); IR (KBr): ν_{max} = 3369, 2944, 1657, 1554, 1115, 1029 cm^{-1}; FAB-MS: *m*/*z* = 410 $[M + H]^+$; ^{1}H-NMR (CD_3OD, 500 MHz) data: see **Table 1**.

Octadecanoic acid: FAB-MS: *m*/*z* = 284 $[M]^+$; ^{1}H-NMR (CD_3OD, 500 MHz): δ = 0.88 (3H, t, *J* = 7.0 Hz, H-18′′′), 1.27 (30H, br s, H-3′′′-H-17′′′), 2.30 (2H, t, *J* = 7.2 Hz, H-2′′′).

(*E*)-9-octadecenoic acid (**4**): FAB-MS: *m*/*z* = 282 $[M]^+$; ^{1}H-NMR (CD_3OD, 500 MHz): δ = 0.88 (3H, t, *J* = 7.0 Hz, H-18′′′), 1.31 (22H, br s, H-3′′′-H-7′′′, H-12′′′-H-17′′′), 2.05 (4H, m, H-8′′′, H-11′′′), 2.20 (2H, t, *J* = 7.2 Hz, H-2′′′), 5.35 (2H, m, H-9′′′, H-10′′′).

Table 2 ^{13}C-NMR spectral data of compounds **1**–**3**[a].

C	1	2	3
1′	136.0	136.0	135.9
2′,6′	128.3	128.3	128.2
3′,5′	129.6	129.6	129.5
4′	132.7	132.7	132.5
CO-NH	170.3	170.4	170.2
N-CH_2	43.0	43.0	43.0
Ar-CH_2	35.9	35.9	36.6
1′′	158.8	158.8	158.8
2′′,6′′	116.0	116.0	116.0
3′′,5′′	130.9	130.9	130.9
4′′	133.0	133.0	132.7
1	65.6	65.6	65.7
2	126.9	126.9	124.3
3	138.0	138.0	141.3
3-Me	12.3	12.3	11.6
4	79.0	79.0	75.7
5	35.1	35.1	35.1
6	76.1	76.1	74.5
7	73.6	73.6	78.0
7-Me	24.7	24.7	26.0
8	26.3	26.3	26.1
4-O-CO-	174.7	174.7	175.3
2′′′	35.6	35.6	35.8
3′′′-17′′′	[b]	[c]	[d]
9′′′,10′′′ (in **2, 3**)		130.9/131.0	130.8/130.9
18′′′	14.5	14.5	14.4

[a] ^{13}C-NMR run at 125 MHz in CD_3OD. Chemical shifts are given in δ values. [b] δC of 3′′′-17′′′ at 26.1, 30.2, 30.4, 30.5, 30.6, 30.7, 30.8, 30.9, 33.2, and 23.8, respectively. [c] δC of 3′′′-8′′′, 11′′′-17′′′ at 26.1, 30.2, 30.4, 30.5, 30.6, 30.7, 30.8, 30.9, 33.2, and 23.8, respectively. [d] δC of 3′′′-8′′′, 11′′′-17′′′ at 26.1, 30.2, 30.4, 30.5, 30.6, 30.7, 30.8, 30.9, 33.0, and 23.7, respectively.

This is a copy of the author's personal reprint

Preparation of the (*R*)- and (*S*)-MTPA ester derivatives of 1a and 3a

Compound **1a** (0.7 mg) in deuterated pyridine (1.0 mL) was transferred into a clean NMR tube. (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 µL) was immediately added to the NMR tube under a N_2 gas stream, and then the NMR tube was shaken carefully to mix the sample and MTPA chloride evenly. The reaction NMR tube was permitted to stand at room temperature overnight. The reaction was completed to afford the (*R*)-MTPA ester derivative (**1r**) of **1a.** In the same manner described for **1r**, the (*S*)-MTPA ester derivative (**1s**) of **1a** was obtained. In the same manner described above, the MTPA ester derivatives (**3r** and **3s**) of **3a** (each 0.3 mg) were obtained. The ^{1}H-NMR spectra of **1s, 1r, 3s**, and **3r** were directly measured in the reaction NMR tubes.

1s: (pyridine-d_5, 500 MHz): δ = 1.393 (3H, s, 7-Me), 1.433 (3H, s, H-8), 1.953 (3H, s, 3-Me), 2.185 (1H, m, H-5a), 2.293 (1H, m, H-5b), 2.993 (2H, t, J = 7.0 Hz, Ar-CH_2), 3.836 (2H, t, J = 7.0 Hz, N-CH_2), 4.580 (2H, d, J = 6.0 Hz, H-1), 5.326 (1H, dd, J = 10.5, 2.0 Hz, H-6), 6.048 (1H, t, J = 7.5 Hz, H-4), 6.184 (1H, t, J = 6.0 Hz, H-2), 6.892 (2H, d, J = 8.5 Hz, H-2″, 6″), 7.188 (2H, d, J = 8.5 Hz, H-3″, 5″), 7.663 (2H, t, J = 7.0 Hz, H-3′, 5′), 7.707 (1H, d, J = 7.0 Hz, H-4′), 8.224 (2H, d, J = 7.0 Hz, H-2′, 6′).

1r: (pyridine-d_5, 500 MHz): δ = 1.421 (3H, s, 7-Me), 1.458 (3H, s, H-8), 1.957 (3H, s, 3-Me), 2.070 (1H, m, H-5a), 2.233 (1H, m, H-5b), 2.992 (2H, t, J = 7.0 Hz, Ar-CH_2), 3.836 (2H, t, J = 7.0 Hz, N-CH_2), 4.585 (2H, d, J = 6.0 Hz, H-1), 5.343 (1H, dd, J = 10.5, 2.0 Hz, H-6), 6.038 (1H, t, J = 7.5 Hz, H-4), 6.187 (1H, t, J = 6.0 Hz, H-2), 6.895 (2H, d, J = 8.5 Hz, H-2″, 6″), 7.185 (2H, d, J = 8.5 Hz, H-3″, 5″), 7.661 (2H, t, J = 7.0 Hz, H-3′, 5′), 7.709 (1H, d, J = 7.0 Hz, H-4′), 8.221 (2H, d, J = 7.0 Hz, H-2′, 6′).

3s: (pyridine-d_5, 500 MHz): δ = 1.389 (3H, s, 7-Me), 1.419 (3H, s, H-8), 1.934 (3H, s, 3-Me), 2.010 (1H, m, H-5a), 2.382 (1H, m, H-5b), 3.031 (2H, t, J = 7.0 Hz, Ar-CH_2), 3.843 (2H, t, J = 7.0 Hz, N-CH_2), 4.588 (2H, d, J = 6.0 Hz, H-1), 5.502 (1H, dd, J = 10.5, 2.0 Hz, H-6), 6.101 (1H, t, J = 7.5 Hz, H-4), 6.220 (1H, t, J = 6.0 Hz, H-2), 6.902 (2H, d, J = 8.5 Hz, H-2″, 6″), 7.196 (2H, d, J = 8.5 Hz, H-3″, 5″), 7.675 (2H, t, J = 7.0 Hz, H-3′, 5′), 7.746 (1H, d, J = 7.0 Hz, H-4′), 8.279 (2H, d, J = 7.0 Hz, H-2′, 6′).

3r: (pyridine-d_5, 500 MHz): δ = 1.376 (3H, s, 7-Me), 1.418 (3H, s, H-8), 1.938 (3H, s, 3-Me), 2.156 (1H, m, H-5a), 2.400 (1H, m, H-5b), 3.045 (2H, t, J = 7.0 Hz, Ar-CH_2), 3.846 (2H, t, J = 7.0 Hz, N-CH_2), 4.590 (2H, d, J = 6.0 Hz, H-1), 5.662 (1H, dd, J = 10.5, 2.0 Hz, H-6), 6.049 (1H, t, J = 7.5 Hz, H-4), 6.225 (1H, t, J = 6.0 Hz, H-2), 6.904 (2H, d, J = 8.5 Hz, H-2″, 6″), 7.196 (2H, d, J = 8.5 Hz, H-3″, 5″), 7.673 (2H, t, J = 7.0 Hz, H-3′, 5′), 7.759 (1H, d, J = 7.0 Hz, H-4′), 8.270 (2H, d, J = 7.0 Hz, H-2′, 6′).

Nitrite production inhibitory and cytotoxicity assay

To measure NO production, BV-2 cells were plated in 96-well plates (3×10^4 cells/well) and treated with 100 ng/mL LPS (Sigma), in the presence or absence of samples, for 24 h. Levels of nitrite, a soluble oxidation product of NO, in the culture media were determined using Griess reagent. The supernatant (50 µL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-napthyl)ethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate NO_2^- concentration. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay [12]. N^G-monomethyl-L-arginine (L-NMMA; Sigma), a well-known NOS inhibitor, was tested as a positive control.

Results and Discussion

Chromatographic purification of the EtOAc-soluble fraction of the ethanol extract of the dried bark of *L. acidissima* afforded three new benzamide derivatives (**1**–**3**) and 10 known compounds (**4**–**13**). The three new benzamide derivatives, *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-octadecanoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**1**), *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**2**), and *N*-{[*p*-(3,7-dimethyl-6*R*,7-epoxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide (**3**), have very similar patterns in their UV, IR, and NMR spectra. Based on the ^{1}H-NMR, ^{13}C-NMR (**Tables 1** and **2**), and ^{1}H-^{1}H-COSY spectra, the differences between compounds **1**–**3** lie in the substituted monoterpenes and fatty acid moieties: a 6,7-dihydroxy geranyloxy moiety and octadecanoic acid in **1**; a 6,7-dihydroxy geranyloxy moiety and (*E*)-9-octadecenoic acid in **2**; and a 6,7-epoxy geranyloxy moiety and (*E*)-9-octadecenoic acid in **3**.

Compound **1** was obtained as an amorphous gum. The molecular formula was determined to be $C_{43}H_{67}NO_6$ from the $[M + H]^+$ peak at m/z = 694.5049 (calcd. for $C_{43}H_{68}NO_6$: 694.5047) in the HR-FAB-MS. The IR spectrum indicated that **1** possessed hydroxy (3379 cm^{-1}), amide carbonyl (1661 cm^{-1}), and aromatic (1554 cm^{-1}) groups. The ^{1}H-NMR spectral data (**Table 1**) of **1** were similar to those of acidissimin [13], except for the replacement of a double bond at C-6 in acidissimin [13] with a 6,7-dihydroxy group in **1**, resulting in the upfield shifts of H-5 [δ = 2.00 (1H, m), 1.61 (1H, m)], H-6 [δ = 3.19 (1H, dd, J = 10.5, 1.0 Hz)], and Me-7 [δ = 1.10 (3H, s)]. The ^{13}C-NMR spectrum (**Table 2**) of **1** displayed 21 carbon signals, except for carbon signals assignable to the octadecanoyloxy moiety. An *N*-benzoyl-*O*-substituted tyramine was recognized from the presence of benzoyl, 1,4-disubstituted phenyl, and CO-NH-CH_2-CH_2- moieties [9]. The ^{1}H-NMR spectrum clearly indicated the appearance of three methyls at 7-Me (δ = 1.10), C-8 (δ = 1.14), and 3-Me (δ = 1.74). Two hydroxylated carbons at δ = 73.6 and δ = 76.1 were located at C-6 and C-7, respectively, by means of the ^{1}H-^{1}H COSY correlations between H-4/H-5 and H-5/H-6 and HMBCs between H-6/C-4, H-8/C-6, C-7, and 7-Me/C-6, C-7. The proton signal at δ = 4.60 was located at C-1 by ^{1}H-^{1}H COSY correlation between H-1/H-2 and an HMBC between H-1/C-3. The proton signal at δ = 5.43 was located at C-4 by HMBCs between H-4/3-Me and H-6/C-4. Based on these data, the 4-substituted 6,7-dihydroxy geranyloxy moiety is proposed as the partial structure in **1**. Finally, an upfield triplet at δ = 0.88 (H-17‴), a broad signal at δ = 1.27 (H-2‴-H-16‴) for 30 methylene protons, and a triplet at δ = 2.30 (H-1‴) suggested an octadecanoyloxy group. The partial structures were built into a complete planar structure from the HMBCs (**Fig. 2**). The octadecanoyloxy group was attached at C-4 of the 6,7-dihydroxy geranyl residue by the HMBC between H-4/4-O-CO. The HMBC between H-1/C-1″ confirmed the connection of the monoterpenoid stearate residue and the tyramine moiety. According to the above data, the planar structure of **1** was elucidated as *N*-{[*p*-(3,7-dimethyl-6,7-dihydroxy-4-octadecanoyloxy-2-octenyloxy)phenyl]ethyl} benzamide. Base saponification of **1** with 10% EtOH/KOH at 40 °C for 1 h furnished octadecanoic acid and compound **1a**, *N*-{[*p*-(3,7-dimethyl-4,6,7-trihydroxy-2-octenyloxy)phenyl]ethyl} benzamide. The structure of octadecanoic acid was confirmed through

This is a copy of the author's personal reprint

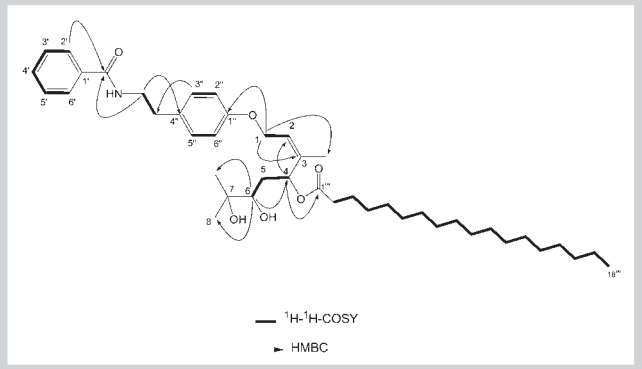

Fig. 2 Key HMBC and ^{1}H-^{1}H-COSY correlations of **1**.

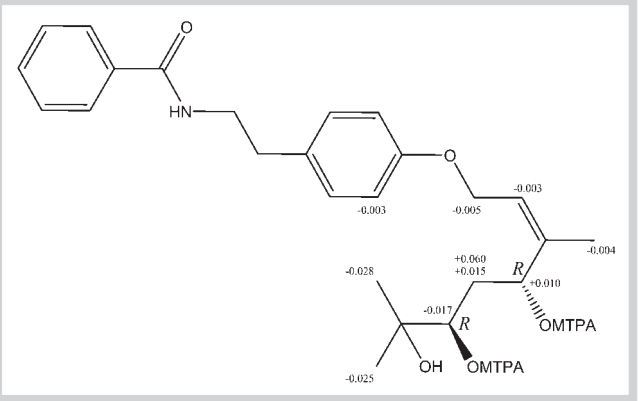

Fig. 3 Value differences ($\delta_S - \delta_R$) of the MTPA esters of **1a**.

direct comparison with its spectral data [13], and the structure of **1a** was determined on the basis of its ^{1}H-NMR data [8]. The absolute configuration of **1** was determined by a convenient Mosher ester procedure performed in an NMR tube [14]. The NMR data of MTPA ester derivatives of **1a** (**1r** and **1s**, ▶ **Fig. 3**) revealed the *R*-configurated C-4 and C-6. Finally, compound **1** was characterized as *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-octadecanoyloxy-2-octenyloxy)phenyl]ethyl} benzamide.

Compound **2** was obtained as an amorphous gum with the molecular formula of $C_{43}H_{65}NO_6$ from the $[M]^+$ peak at $m/z = 691.4823$ (calcd. for $C_{43}H_{65}NO_6$: 691.4812) in the HR-FAB-MS. The IR, ^{1}H-NMR, and ^{13}C-NMR spectra (▶ **Tables 1** and **2**) of **2** were similar to those of **1**, except that the octadecanoic acid moiety of **1** was replaced with the 9-octadecenoic acid moiety of **2**, resulting in the downfield shifts of H-8‴ and H-11‴ [$\delta = 2.03$ (4H, m)], H-9‴, and H-10‴ [$\delta = 5.32$ (2H, m)] in **2**. Base saponification of **2** with 10% EtOH/KOH at 40 °C for 1 h furnished (*E*)-9-octadecenoic acid and compound **2a**. The structure of **2a** was determined to be same as **1a** based on ^{1}H-NMR spectral measurement. The structure of (*E*)-9-octadecenoic acid was confirmed through co-separation of compound **4**, (*E*)-9-octadecenoic acid, and by direct comparison with the spectral data of **4** [11]. The (*E*)-9-octadecenoyloxy group was attached at the 4 position of the 6,7-dihydroxy geranyloxy moiety by the HMBC between H-4/4-O-CO. By detailed analysis of the ^{1}H-^{1}H COSY spectrum and the HMBCs, the remaining connectivities of **2** were confirmed. Thus, the structure of **2** was determined to be *N*-{[*p*-(3,7-dimethyl-6,7-dihydroxy-4–9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide. The absolute configuration of **2** was identical with **1** based on analysis of a convenient Mosher ester procedure performed in an NMR tube. Thus, compound **2** was characterized as *N*-{[*p*-(3,7-dimethyl-6*R*,7-dihydroxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide.

Compound **3** was obtained as an amorphous gum, whose molecular formula was determined to be $C_{43}H_{63}NO_5$ from the $[M + H]^+$ peak at $m/z = 674.4764$ (calcd. for $C_{43}H_{64}NO_5$: 674.4784) in the HR-FAB-MS. The IR, ^{1}H-NMR, and ^{13}C-NMR spectra (▶ **Tables 1** and **2**) of **3** were similar to those of **2**, except that the 6,7-dihydroxy group of the geranyl moiety in **2** was replaced by an 6,7-epoxy group in **3**. Alkaline hydrolysis of **3** afforded (*E*)-9-octadecenoic acid, **3a**, and **3b**. The structure of **3b**, *N*-{[*p*-(3,7-dimethyl-4-hydroxy-6,7-epoxy-2-octenyloxy)phenyl]ethyl} benzamide, was determined on the basis of its ^{1}H-NMR data [8]. The structure of (*E*)-9-octadecenoic acid was also confirmed by direct comparison with the spectral data of **4** [11]. The (*E*)-9-octadecenoyloxy group was attached at the 4 position of the epoxy geranyloxy moiety, based on the HMBC between H-4/4-O-CO. By detailed analysis of the ^{1}H-^{1}H COSY spectrum and the HMBCs, the remaining connectivities of **3** were confirmed. Thus, the planar structure of **3** was determined to be *N*-{[*p*-(3,7-dimethyl-6,7-epoxy-4–9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide. The absolute configuration at C-4 of **3** was the same as **1** based on the sign of optical rotations of **1** and **1a** ($[\alpha]_D^{25}$: + 72.5 and + 62.1, respectively) and **3** and **3b** ($[\alpha]_D^{25}$: + 39.3 and + 82.5, respectively). The compound **3a**, which was obtained from alkaline hydrolysis of **3**, was confirmed to be a stereoisomer of **1a** on the basis of the ^{1}H-NMR spectrum and co-TLC analysis with **1a**. During the alkaline hydrolysis reaction of **3**, the 6-epoxy group of **3b** was supposed to be split into the 6,7-dihydroxy group of **3a**. By Mosher ester procedure [14], the NMR data of MTPA ester derivatives of **3a** (**3 r** and **3 s**) revealed the *R*-configurated C-4 and *S*-configurated C-6. Because the absolute configuration at C-6 of **3a**, which was the decomposed product of the epoxy ring of **3b**, was *S*-configurated, the absolute configuration at C-6 of **3** was assigned an *R*-configuration. Finally, the structure of **3** was identified as *N*-{[*p*-(3,7-dimethyl-6*R*,7-epoxy-4*R*-9‴(*E*)-octadecenoyloxy-2-octenyloxy)phenyl]ethyl} benzamide.

The known compounds – (*E*)-9-octadecenoic acid (**4**) [11], syringaldehyde (**5**) [15], (*E*)-suberenol (**6**) [16], (*Z*)-suberenol (**7**) [16], 13*α*,14*β*,17*α*-lanosta-7,9,24-triene-3*β*,16*α*-diol (**8**) [17], limonin (**9**) [18], 4-methoxy-1-methyl-2(1*H*)-quinolinone (**10**) [19], marmesin (**11**) [20], rutaevin (**12**) [21], and 13*α*,14*β*,17*α*-lanosta-7,24-diene-3*β*,11*β*,16*α*-triol (**13**) [17] – were identified by comparison of physical and spectroscopic data (UV, IR, ^{1}H- and ^{13}C-NMR, and MS data) with those of literature values.

The ability of the isolated compounds (**1** – **13**) to inhibit NO production was evaluated in LPS-activated BV-2 cells, a microglial cell line. Among the isolates, 13*α*,14*β*,17*α*-lanosta-7,9,24-triene-3*β*,16*α*-diol (**8**), 4-methoxy-1-methyl-2(1*H*)-quinolinone (**10**), and 13*α*,14*β*,17*α*-lanosta-7,24-diene-3*β*,11*β*,16*α*-triol (**13**) exhibited the strongest inhibition of NO production. Compounds **8, 10**, and **13** inhibited NO production in a dose-dependent manner, with IC_{50} values of 10.7 µM, 9.03 µM, and 12.8 µM, respectively (▶ **Fig. 4**). N^G-monomethyl-L-arginine (L-NMMA), a well-known NOS inhibitor, was tested as positive control. The inhibitory effect of compounds **8, 10**, and **13** was higher than that of L-NMMA, compound **10** being the most potent. On the other hand, compounds **1**–**3** showed cytotoxicity at 10 µM. Compounds **4**–**7,**

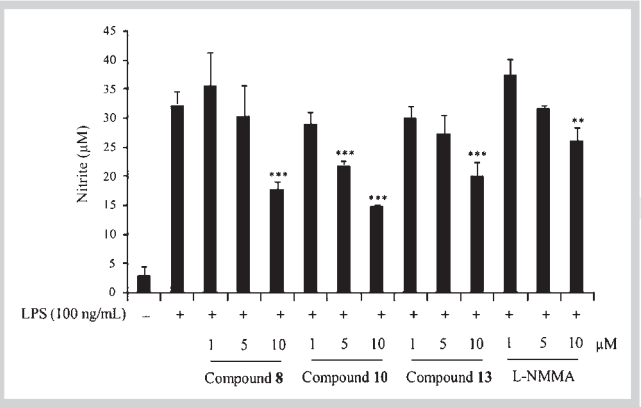

Fig. 4 Compounds **8, 10**, and **13** inhibited NO production in LPS-activated BV-2 cells. Nitrite was measured using Griess reagent at 24 h after treatment with LPS in the presence or absence of samples. Data are presented as the mean ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}p < 0.001$ versus LPS alone.

9, 11, and **12** did not show significant activity on NO production in the range of 1 to 10 μM.

Activated microglial cells produce excessive inflammatory substances, such as NO, cytokines, and prostaglandins. NO derived from inducible NOS in LPS-activated microglia is an important mediator of inflammation and neuronal cell death [22]. Compounds **8, 10**, and **13** may have therapeutic potential in diseases associated with increased NO production.

Acknowledgements

This work was supported by the R & D Institute, Miwon Commercial Co., Ltd. and the Cancer Preventive Material Development Research Center at Kyunghee University. The authors would like to thank Mr. Do Kyun Kim, Dr. Eun Jung Bang, and Dr. Jung Ju Seo at the Korea Basic Science Institute for the measurements of NMR and MS spectra.

References

1 *Allen BM.* Malayan fruits. Singapore: Donald Moore Press; 1967: 245
2 *Kirtikar KR, Basu BD.* Indian medicinal plants, 2nd edition, Vol. 1. India: Lalith Mohan Basu; 1933: 496–498
3 *Chopra RN, Nayar SL, Chopra IC.* Glossary of Indian medicinal plants. New Delhi: C.S.I.R.; 1956: 154
4 *Ghosh P, Sil P, Majumadar SG, Thakur S.* A coumarin from *Limonia acidissima.* Phytochemistry 1982; 21: 240–241
5 *Patra A, Misra SK, Chaudhuri SK.* Constituents of *Limonia acidissima*. Applications of two-dimentional NMR spectroscopy in structure elucidation. J Indian Chem Soc 1988; 65: 205–208
6 *Bandara BMR, Gunatilaka AAL, Wijeratne EMK, Adikaram NKB.* Antifungal constituents of *Limonia acidissima*. Planta Med 1988; 54: 374–375
7 *MacLeod JK, Moeller PDR, Bandara BMR, Gunatilaka AAL, Wijeratne EMK.* Acidissimin, a new limonoid from *Limonia acidissima*. J Nat Prod 1989; 52: 882–885
8 *Ghosh P, Ghosh MK, Thakur S, Datta JD, Akihisa T, Tamura T, Kimura Y.* Dihydroxy acidisiminol and acidissiminol epoxide, two tyramine derivatives from *Limonia acidissima*. Phytochemistry 1994; 37: 757–760
9 *Ghosh P, Sil P, Das S, Kokke WCMC, Kokke WCM, Akihisa T, Shimizu N, Tamura T, Matsumoto T.* Tyramine derivatives from the fruit of *Limonia acidissima.* J Nat Prod 1991; 54: 1389–1393
10 *Adikaram NKB, Abhayawardhane Y, Bandara BMR, Gunatilaka AAL, Wijeratne EMK.* Antifungal activity, acid and sugar content in the wood apple (*Limonia acidissima*) and their relation to fungal development. Plant Pathol 1989; 38: 258–265
11 *Miyake Y, Yokomizo K.* Determination of *cis*- and *trans*- 18: 1 fatty acid isomers in hydrogenated vegetable oils by high-resolution carbon nuclear magnetic resonance. JAOCS 1998; 75: 801–805
12 *Sargent JM, Taylor CG.* Appraisal of the MTT assay as a rapid test of chemosensitivity in acute myeloid leukaemia. Br J Cancer 1989; 60: 206–210
13 *Ghosh P, Bandyopadhyay AK, Thakur S.* Acidissimin, a new tyramine derivative from the fruit of *Limonia acidissima*. J Nat Prod 1989; 52: 1323–1326
14 *Su BN, Park EJ, Mbwambo ZH, Santarsiero BD, Mesecar AD, Fong HHS, Pezzuto JM, Kinghorn AD.* New chemical constituents of *Euphorbia quinquecostata* and absolute configuration assignment by a convenient Mosher ester procedure carried out in NMR tubes. J Nat Prod 2002; 65: 1278–1282
15 *Bohlmann F, Abraham WR.* Ein neues Syringaalkohol-derivat aus *Erechtites hieracifolia*. Phytochemistry 1980; 19: 469–470
16 *Motoharu JI, Mami I, Mika I.* New coumarins from *Citrus funadoko*. Heterocycles 1988; 27: 1451–1454
17 *Venkatraman G, Thombare PS, Sabata BK.* A tetracyclic triterpenoid from *Garuga pinnata*. Phytochemistry 1994; 36: 417–419
18 *Niu XM, Li SH, Peng LY, Lin ZW, Rao GX, Sun HD.* Constituents from *Limonia crenulata*. JANPR 2001; 3: 299–311
19 *Ito A, Shamon LA, Yu B, Mata-Greenwood E, Lee SK, Richard BB, Mehta RG, Farnsworth NR, Fong HHS, Pezzuto JM, Kinghorn AD.* Antimutagenic constituents of *Casimiroa edulis* with potential cancer chemopreventive activity. J Agric Food Chem 1998; 46: 3509–3516
20 *Joo SH, Lee SC, Kim SK.* UV absorbent, marmesin, from the bark of Thanakha, *Hesperethusa crenulata* L. J Plant Biol 2004; 47: 163–165
21 *Munehiro N, Hideki T, TakashiI, Hideo N.* The structure of graucin A, a new bitter limonoid from *Evodia grauca* Miq. (Rutaceae). Bull Chem Soc Jpn 1987; 60: 2503–2507
22 *Iadecola C.* Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci 1997; 20: 132–139

This is a copy of the author's personal reprint