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

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Abstract
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Three new benzamide derivatives, *N*-{[*p*-(3,7-di-methyl-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, 13α,14β,17α-lanosta-7,9,24-triene-3β,16α-diol (**8**), 4-methoxy-1-methyl-2(1*H*)-quinolinone (**10**), and 13α,14β,17α-lanosta-7,24-diene-3β,11β,16α-triol (**13**) potently inhibited nitric oxide (NO) production in microglia cells.

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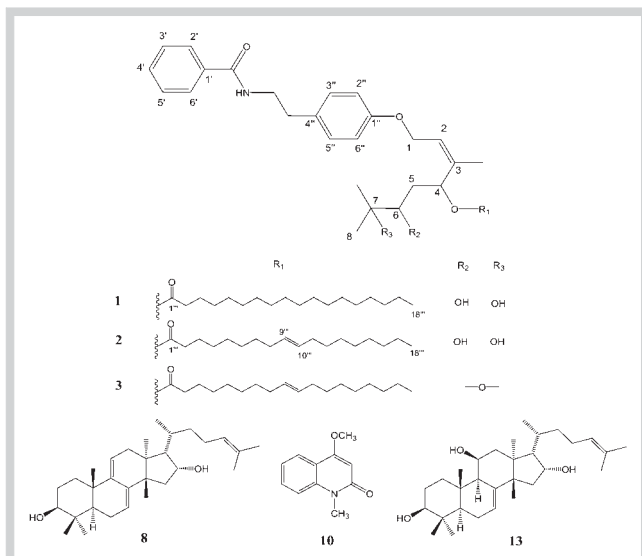
Fig. 1   Chemical structures of compounds **1–3**, **8**, **10**, and **13**.

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$_{254 s}$ plates were used for TLC. Spots were detected under UV light or by heating after spraying with 10 % H$_2$SO$_4$ 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$_2$O 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$_2$Cl$_2$/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$_2$O 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; [α]$_D^{25}$: + 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$_3$OD, 500 MHz) data: see ● **Table 1**. ^{13}C-NMR (CD$_3$OD, 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; [α]$_D^{25}$: + 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$_3$OD, 500 MHz) data: see ● **Table 1**. ^{13}C-NMR (CD$_3$OD, 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; [α]$_D^{25}$: + 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$_3$OD, 500 MHz) data: see ● **Table 1**. ^{13}C-NMR (CD$_3$OD, 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$_2$O (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

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$_3$OD. 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$_2$O 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$_2$O (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$_2$O 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$_3$OD, 500 MHz) data: see ● Table 1.
3a: Colorless gum; ^{1}H-NMR (CD$_3$OD, 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$_3$OD, 500 MHz) data: see ● Table 1.
Octadecanoic acid: FAB-MS: *m/z* = 284 [M]$^+$; ^{1}H-NMR (CD$_3$OD, 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$_3$OD, 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$_3$OD. 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.

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 ¹H-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-CH₂), 3.836 (2H, t, *J* = 7.0 Hz, N-CH₂), 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-CH₂), 3.836 (2H, t, *J* = 7.0 Hz, N-CH₂), 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-CH₂), 3.843 (2H, t, *J* = 7.0 Hz, N-CH₂), 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-CH₂), 3.846 (2H, t, *J* = 7.0 Hz, N-CH₂), 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 ¹H-NMR, ¹³C-NMR (○ **Tables 1** and **2**), and ¹H-¹H-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 ¹H-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 ¹³C-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-CH₂-CH₂- moieties [9]. The ¹H-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 ¹H-¹H 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 ¹H-¹H 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
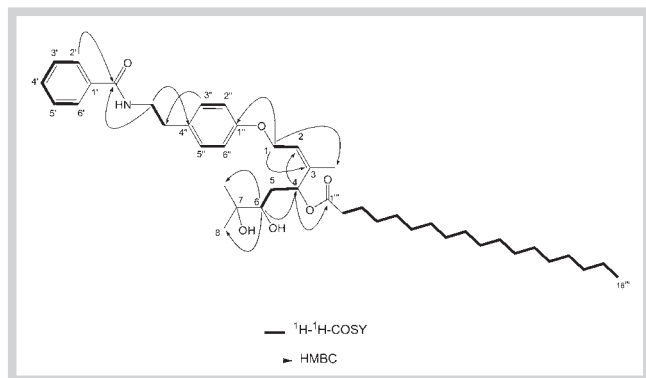
Fig. 2 Key HMBC and ^{1}H-^{1}H-COSY correlations of **1**.

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-octadece-

noyloxy 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** ([α]$_D^{25}$: + 72.5 and + 62.1, respectively) and **3** and **3b** ([α]$_D^{25}$: + 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.
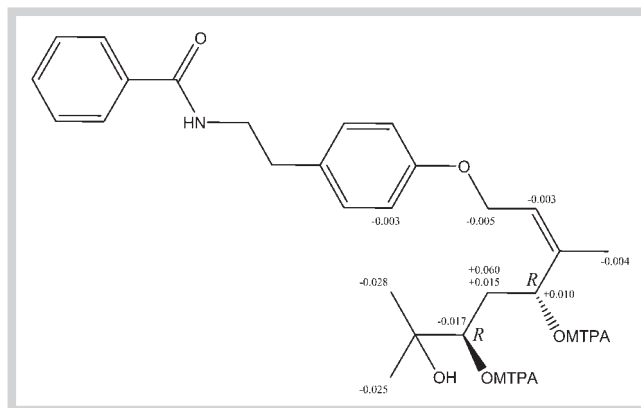
The known compounds – (*E*)-9-octadecenoic acid (**4**) [11], syringaldehyde (**5**) [15], (*E*)-suberenol (**6**) [16], (*Z*)-suberenol (**7**) [16], 13α,14β,17α-lanosta-7,9,24-triene-3β,16α-diol (**8**) [17], limonin (**9**) [18], 4-methoxy-1-methyl-2(1*H*)-quinolinone (**10**) [19], marmesin (**11**) [20], rutaevin (**12**) [21], and 13α,14β,17α-lanosta-7,24-diene-3β,11β,16α-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, 13α,14β,17α-lanosta-7,9,24-triene-3β,16α-diol (**8**), 4-methoxy-1-methyl-2(1*H*)-quinolinone (**10**), and 13α,14β,17α-lanosta-7,24-diene-3β,11β,16α-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**,
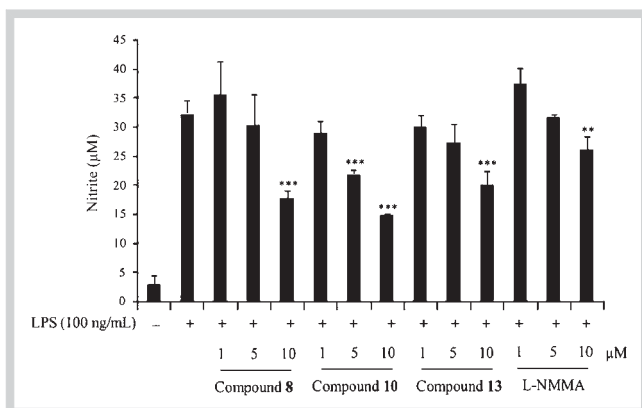
Fig. 4 Compounds **8, 10**, and **13** inhibited NO production in LPS-activated BV-2 cells. Nitrite was measured using Griess reagent at 24 h after treatment with LPS in the presence or absence of samples. Data are presented as the mean ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}p < 0.001$ 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.

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