Antiinflammatory Constituents of *Celastrus orbiculatus* Inhibit the NF-κB Activation and NO Production

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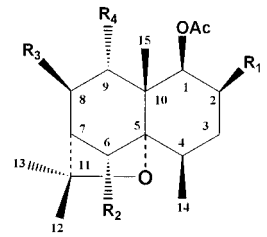
Two new sesquiterpene esters, 1β,8β-diacetoxyl-6α,9α-difuroyloxydihydro-β-agarofuran (**1**) and 1β-acetoxyl-2β,6α,9α-trifuroyloxydihydro-β-agarofuran (**2**), together with four known sesquiterpene esters (**3**−**6**), celastrol (**7**), and celaphanol A (**8**) were isolated from the roots of *Celastrus orbiculatus* in a search for inhibitors of NF-κB activation and nitric oxide production. Compound **7** was the most active, while compounds **1**, **2**, **4**, and **8** showed moderate inhibition in both NF-κB activation and nitric oxide production.

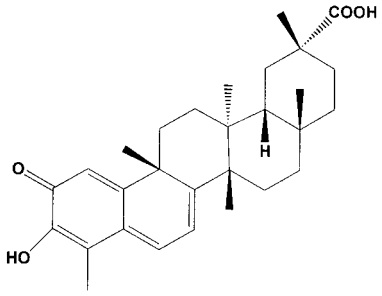
The nuclear factor kappa-B (NF-κB), a central mediator of the human immune response, is activated by many proinflammatory stimuli including cytokines, phorbol esters, bacterial or viral products, phosphatase inhibitors, oxidants, and ultraviolet radiation.[1] The activation of NF-κB results in the expression of numerous target genes encoding cytokines, cell adhesion molecules, and inflammatory enzymes such as inducible nitric oxide synthase (iNOS).[1] Therefore, it was suggested that NF-κB-targeted therapeutics might be effective in inflammatory diseases.[2]

In our search for inhibitors of NF-κB activation from natural products, the methanol extract of the roots of *Celastrus orbiculatus* (Celastraceae) showed potent inhibition of NF-κB activation in RAW264.7 cells transfected with NF-κB reporter construct. Members of the family Celastraceae produce various sesquiterpene polyol esters and alkaloids, some of which have exhibited insect antifeedant effects,[3,4] antitumor activitiy,[5,6] and multidrug resistance reversing activity.[7,8] The Celastraceae family also produces di- and triterpene compounds such as celaphanol and celastrol.[9,10] Celastrol has been reported to have antitiumor activity, antitiumor promoting activity, and inhibitory activity on IL-1 release in the LPS-stimulated human peripheral mononuclear cells.[10,11] *C. orbiculatus* extracts have been used to treat rheumatoid arthritis and bacterial infection in folk medicine.[12] Bioactivity-guided fractionation using the NF-κB reporter gene assay has led to isolation of two new sesquiterpene esters, orbiculin H (**1**) and orbiculin I (**2**), and six known compounds including orbiculin A (**3**), orbiculin D (**4**), orbiculin E (**5**), orbiculin F (**6**), celastrol (**7**), and celaphanol A (**8**) from the methanol extract of the roots of *C. orbiculatus*. We here describe the structure elucidation of **1** and **2** and the effect of compounds **1**−**8** on LPS-induced NF-κB activation in murine macrophage RAW264.7 cells transfected with NF-κB-mediated reporter gene construct and on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells.

Compound **1** had the molecular formula $C_{29}H_{34}O_{11}$ (HRFABMS). The ^{1}H NMR spectrum indicated the presence of two acetyl groups (δ 1.74 and 2.24), two sets of furoyl groups (δ 6.74, 7.46, 8.02 and 6.75, 7.42, 8.04), three tertiary methyl groups (δ 1.43, 1.48, and 1.49), and one secondary methyl group (δ 1.04). The signals observed at



	R1	R2	R3	R4
1	H	OFu	OAc	OFu
2	OFu	OFu	H	OFu
3	OAc	OBz	H	OBz
4	H	OFu	H	OFu
5	OAc	OFu	H	OBz
6	OFu	OFu	H	OBz



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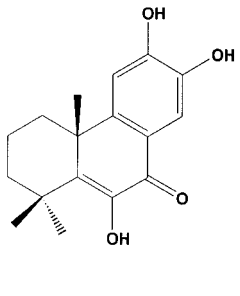

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δ 5.37 (1H, dd, J = 11.9, 4.3 Hz), 5.87 (1H, s), 5.27 (1H, d, J = 3.3 Hz), and 4.95 (1H, s) were assigned to the four protons attached to carbons bearing secondary ester groups. The ^{13}C NMR and DEPT spectra of **1** indicated the presence of six methyl carbons, two methylene carbons, six methine carbons, four ester carbons, three quaternary carbons, and two sets of furoyl carbons (δ 147.7, 144.2, 119.3, 109.6; 148.7, 143.8, 118.4, 109.7). These data suggested that **1** was a tetrasubstituted dihydro-β-agarofuran.[7,8] The two acetate carbonyl carbon signals at δ 170.1 and 169.4 showed long-range correlation (HMBC) with proton signals at δ 5.37 (H-1) and 5.27 (H-8), respectively, and two furoate carbonyl carbon signals at δ 161.9 and 161.3 correlated with proton signals δ 5.87 (H-6) and 4.95 (H-9), respectively, indicating that the acetate groups were located at C-1 and C-8, and the furoate groups were located at C-6 and C-9, respectively.

The stereochemistry of **1** was determined from the NOESY spectrum. The signal at δ 5.37 (H-1) was correlated with that of H-3ax, the signal at δ 5.87 (H-6) was correlated with those of H-14/H-15, the signal at δ 5.27 (H-8) was correlated with those of δ 2.60 (H-7) and 4.95 (H-9), and the signal at δ 4.95 (H-9) was correlated with that of H-15, respectively, in the NOESY spectrum. Generally, in this class of compounds, H-1 and H-6 are axial.[13] The weak coupling constant ($J_{8,9}$ = 0 Hz) between H-8 and H-9

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suggested that both H-8 and H-9 have an equatorial orientation, with a near 90° dihedral angle between H-8 and H-9.[4,14] Therefore, H-1, H-6, H-8, and H-9 were assigned as α-axial, *β*-axial, α-equatorial, and *β*-equatorial, respectively. Thus, **1** was elucidated as 1*β*,8*β*-diacetoxyl-6α,9α-difuroyloxydihydro-*β*-agarofuran and named orbiculin H.

Compound **2** analyzed for molecular formular $C_{32}H_{34}O_{12}$ by positive HRFABMS. The ^{1}H NMR and ^{13}C NMR spectra of **2** indicated that it contained one acetyl and three furoyl ester groups. The main difference of **2** from **1** was the presence of one more *β*-substituted furoyl group and one less acetyl group. The HMBC spectrum demonstrated that one acetate group was attached to C-1, and three furoyl groups were attached to C-2, C-6, and C-9, respectively. In the ^{1}H NMR spectra of **2**, the coupling constant ($J_{1,2}$ = 3.5 Hz) between H-1 and H-2 indicated their *cis*-relationship. In the NOESY spectrum, the signal at *δ* 5.61 (H-1) was correlated with that of H-3ax and the signal at *δ* 4.91 (H-9) was correlated with that of H-15. The signal at *δ* 5.60 (H-6) was correlated with H-14/H-15. From these facts, the structure of **2** was elucidated as 1*β*-acetoxyl-2*β*,6α,9α-trifuroxyloxydihydro-*β*-agarofuran and named orbiculin I. Compounds **3**−**8** were identified by comparison of their physical and spectral data with those of previous reports.[7−10]

Compounds **1**−**8** were examined for their dose−response effect on the LPS-induced NF-*κ*B activation using the NF-*κ*B mediated reporter gene assay system. RAW264.7 cells, which were stably transfected with a NF-*κ*B-mediated reporter gene construct, were stimulated with LPS in the presence of various concentrations of compounds **1**−**8**, and then the expression of reporter gene (secreted alkaline phosphatase gene) in the culture medium was measured.[15] Celastrol (**7**) showed the most potent inhibitory activity in the reporter gene expression, with an IC_{50} value of 0.27 *μ*M, and celaphanol (**8**) was also active with an IC_{50} value of 18.2 *μ*M. Among six dihydro-*β*-agarofuran sesquiterpenes, compounds **1**, **2**, and **4** (having two furoyloxy groups at C-6 and C-9) exhibited moderate inhibitory activities, with IC_{50} values of 33.5, 61.5, and 36.7 *μ*M, respectively; however, compounds **3**, **5**, and **6** (having benzoyloxy group at C-9) showed very weak activity with IC_{50} values of >300 *μ*M. These results suggest that the furoyloxy groups at C-6 and C-9 are important structural factors of dihydro-*β*-agarofuran sesquiterpenes in the modulation of NF-*κ*B activity.

NF-*κ*B regulates the expression of numerous target genes encoding inflammatory factors, notably the iNOS gene.[1] The excessive production of NO, which is produced by iNOS in macrophages and endothelial cells, is responsible for the inflammatory response and implicated in the pathogenesis of several inflammatory diseases such as septic shock, rheumatoid arthritis, graft rejection, and diabetes.[16] The effect of compounds **1**−**8** was tested on the NO production in LPS-stimulated RAW264.7 cells with respect to aminoguanidine, an iNOS inhibitor. Compounds **1**, **2**, **4**, **7**, and **8** inhibited LPS-induced NO production in the RAW264.7 cells dose-dependently with IC_{50} values of 50.4, 51.2, 43.6, 0.23, and 32.6 *μ*M, respectively. These data are comparable to that of aminoguanidine (IC_{50} 16.3 *μ*M) and to those for the NF-*κ*B activation (Table 1). The cell viability measured by MTT assay showed that all the compounds had no significant cytotoxicity to the RAW264.7 cells at their effective concentration for the inhibition of NF-*κ*B activation and NO production (data not shown).

Table 1. IC_{50} Values (*μ*M)[a] of Compounds **1**−**8** in the NF-*κ*B Activation and NO Production

compound	NF-*κ*B activation	NO production
1	33.5 ± 1.1	50.4 ± 0.8
2	61.5 ± 1.4	51.2 ± 1.3
3	>300	>300
4	36.7 ± 1.5	43.6 ± 1.2
5	>300	>300
6	>300	>300
7	0.27 ± 0.01	0.23 ± 0.02
8	18.2 ± 0.97	32.6 ± 1.4
AG		16.3 ± 0.4

[a] Data are mean ±SD from two separate experiments. AG: aminoguanidine.

Experimental Section

General Experimental Procedures. Melting points were measured on an Electrothermal 9100 instrument without correction. UV spectra were obtained on a Milton Roy 3000 spectrometer. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR spectra were determined on a JASCO Report-100 spectrometer (KBr plate). ^{1}H NMR, ^{13}C NMR, DEPT, NOESY, and HMBC spectra were recorded on Bruker DMX 600 NMR spectrometer with $CDCl_3$ as a solvent. HRFABMS was obtained on JEOL HX 110 mass spectrometer. Preparative HPLC was carried out on J'sphere ODS-H80 (150 × 20 mm, YMC, Japan). Fetal bovine serum, media, and supplement materials for cell culture were purchased from GIBCO-BRL (Gaithersberg, MD).

Plant Material. The roots of *C. orbiculatus* (no. 99101) were collected at Cheongju, Chungbuk Province, Korea, in October 1999, and identified by Dr. Kyong Soon Lee, a plant taxonomist at Chungbuk National University. A voucher specimen is deposited in our institute.

Extraction and Isolation. The dried roots of C. *orbiculatus* (1.5 kg) were extracted with MeOH at room temperature. The MeOH extract (145 g) was partitioned between H_2O and CH_2Cl_2. The CH_2Cl_2 fraction (55 g), exhibiting potent inhibitory effect on the NF-*κ*B activation, was chromatographed on a silica gel column (6 × 40 cm) eluting with a step gradient of CH_2Cl_2−MeOH (50:1, 30:1, 10:1, 5:1, 1:1, 100% MeOH each 1.5 L) to give seven fractions. Active fraction 4 (4.2 g) was subjected to Sephadex LH-20 column (3.5 × 100 cm) chromatography eluted with MeOH to give six fractions. Subfraction 4-3 (3.1 g) was rechromatographed on a silica gel column eluting with a step gradient of hexane−EtOAc (20:1, 10:1, 5:1, 2:1, 100% EtOAc) to obtain 10 fractions. Subfraction 4-3-9 was subjected to preparative HPLC (ODS-H80, 150 × 20 mm, YMC, Japan, MeOH−H_2O, 8:2) to afford compounds **1** (70 mg), **3** (37 mg), **5** (15 mg), and **6** (40 mg). Another subfraction 4-3-8 was also subjected to preparative HPLC (ODS-H80, 150 × 20 mm, YMC, Japan, MeOH−H_2O, 8:2) to afford compounds **2** (15 mg) and **4** (86 mg). Active fraction 5 (4 g) was subjected to a Sephadex LH-20 column (3.5 × 100 cm) eluting with CH_2Cl_2−MeOH (1:1) to afford compounds **7** (2 g) and **8** (70 mg).

Orbiculin H (1): white amorphous powder; mp 111−113 °C; $[\alpha]^{25}_D$ −19.5° (*c* 1.00, MeOH); UV (MeOH) λ_{max} (log ϵ) 207 (4.26), 241 (3.94) nm; IR (KBr) ν_{max} 2965, 2940, 1730, 1715, 1578, 1504, 1372, 1308, 1227, 1161, 1078, 1022, 970, 878, 762 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) *δ* 5.37 (1H, dd, *J* = 11.9, 4.3 Hz, H-1), 1.62 (1H, m, H-2a), 1.87 (1H, m, H-2b), 1.60 (1H, m, H-3ax), 2.21 (1H, m, H-3eq), 2.33 (1H, m, H-4), 5.87 (1H, s, H-6), 2.60 (1H, d, *J* = 3.3 Hz, H-7), 5.27 (1H, d, *J* = 3.3 Hz, H-8), 4.95 (1H, s, H-9), 1.43 (3H, s, Me-12), 1.49 (3H, s, Me-13), 1.04 (3H, d, *J* = 7.4 Hz, Me-14), 1.48 (3H, s, Me-15), 2 × OAc [1.74 (3H, s), 2.24 (3H, s)], 2 × OFu [8.02 (1H, d, *J* = 0.5 Hz), 7.46 (1H, t, *J* = 1.6 Hz), 6.74 (1H, d, *J* = 1.6 Hz); 8.04 (1H, d, *J* = 0.5 Hz), 7.42 (1H, t, *J* = 1.6 Hz), 6.75 (1H, d, *J* = 1.6 Hz)]; ^{13}C NMR (150 MHz, $CDCl_3$) *δ* 73.3 (C-1), 21.3 (C-2), 26.7 (C-3), 34.1 (C-4), 90.4 (C-5), 75.8 (C-6), 53.1 (C-7), 76.0 (C-8), 76.4 (C-9), 49.8 (C-10), 81.7 (C-11), 31.0 (C-12), 25.4 (C-13), 17.3 (C-14), 18.6 (C-15), 2 × OAc [21.1, 20.9; 170.1,

169.4 (2 × $-CO_2-$)], 2 × OFu [147.7, 144.2, 119.3, 109.6; 148.7, 143.8, 118.4, 109.7; 161.9, 161.3, (2 × $-CO_2-$)]; ESI-MS *m*/*z* 581.4 $[M + Na]^+$; HRFABMS *m*/*z* 559.2186 $[M + H]^+$ (calcd for $C_{29}H_{35}O_{11}$, 559.2179).

Orbiculin I (2): white needle crystal; mp 253−255 °C; $[\alpha]^{25}_D$ +39.2° (*c* 0.63, MeOH); UV (MeOH) λ_{max} (log ϵ) 209 (4.72), 241 (4.28) nm; IR (KBr) ν_{max} 2952, 2928, 1747, 1718, 1578, 1504, 1382, 1305, 1232, 1160, 1080, 1008, 964, 866, 760 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 5.61 (1H, d, J = 3.5 Hz, H-1), 5.79 (1H, dd, J = 6.4, 3.5 Hz, H-2), 1.92 (1H, dd, J = 15.0, 1.9 Hz, H-3ax), 2.52 (1H, m, H-3eq), 2.45 (1H, m, H-4), 5.60 (1H, s, H-6), 2.52 (1H, m, H-7), 2.37 (1H, m, H-8ax), 2.20 (1H, dd, J = 16.5, 3.1 Hz, H-8eq), 4.91 (1H, d, J = 6.9 Hz, H-9), 1.44 (3H, s, Me-12), 1.44 (3H, s, Me-13), 1.28 (3H, d, J = 7.6 Hz, Me-14), 1.56 (3H, s, Me-15), OAc 1.73 (3H, s), 3 × OFu [7.94 (1H, m), 7.42 (1H, t, J = 1.5 Hz), 6.69 (1H, d, J = 1.5 Hz); 8.04 (1H, m), 7.43 (1H, t, J = 1.5 Hz), 6.78 (1H, d, J = 1.5 Hz); 8.05 (1H, m), 7.48 (1H, t, J = 1.5 Hz), 6.75 (1H, d, J = 1.5 Hz)]; ^{13}C NMR (150 MHz, $CDCl_3$) δ 71.0 (C-1), 70.1 (C-2), 31.1 (C-3), 33.9 (C-4), 89.6 (C-5), 79.2 (C-6), 48.9 (C-7), 31.6 (C-8), 72.4 (C-9), 49.7 (C-10), 82.9 (C-11), 25.9 (C-12), 30.8 (C-13), 18.8 (C-14), 20.5 (C-15), OAc [20.5; 169.9 ($-CO_2-$)], 3 × OFu [147.4, 143.9, 119.5, 109.7; 147.8, 144.2, 119.3, 109.6; 148.6, 143.2, 118.7, 109.8; 162.3, 162.1, 162.0 (3 × $-CO_2-$)]; ESI-MS *m*/*z* 633.0 $[M + Na]^+$; HRFABMS *m*/*z* 633.1942 $[M + Na]^+$ (calcd for $C_{32}H_{34}O_{12}Na$, 633.1948).

Determination of Nitric Oxide Production. RAW264.7 cells were transferred in 96-well plates at a density of 1×10^5 cells/well. After 3 h incubation, the cells were stimulated with LPS (1 μg/mL) for 24 h in the absence or presence of the compounds tested. As a parameter of NO synthesis, nitrite concentration was measured in the supernatant of RAW264.7 cells by the Griess reaction.[17] Briefly, 100 μL of cell culture supernatant was reacted with 100 μL of Griess reagent [1:1 mixture of 0.1% *N*-(1-naphthyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate, and absorbance was read with a microplate reader (Molecular Devices Co., Menlo park, CA) at 570 nm. The nitrite concentration in the supernatants was calculated by comparison with a sodium nitrite standard curve.

NF-κB Activity Assay. RAW264.7 cells were transfected with a plasmid containing eight copies of κB elements linked to the SEAP (secreted alkaline phosphatase) gene.[15] Transfected cells were seeded in a 96-well plate at a density of 5×10^4 cells/well. After 3 h incubation at 37 °C, the cells were treated with various concentrations of compounds tested and LPS (1 μg/mL) for 24 h. Then 100 μL of each culture supernatant was transferred to a new 96-well plate and heated at 65 °C for 5 min. Additional 100 μL of 2× SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM l-homoarginine) was added to each well and incubated at 37 °C for 10 min. The reaction was initiated by the addition of 20 μL of 120 mM *p*-nitrophenyl phosphate dissolved in 1× SEAP assay buffer and incubated at 37 °C.[18] The absorbance of the reaction mixture was measured at 405 nm with a microplate reader (Molecular Devices Co., Menlo Park, CA).

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