Lignans from *Arctium lappa* and Their Inhibition of LPS-Induced Nitric Oxide Production

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A new butyrolactone sesquilignan, isolappaol C (1), together with four known lignans, lappaol C (2), lappaol D (3), lappaol F (4), and diarctigenin (5), were isolated from the methanolic extract of the seeds from the *Arctium lappa* plant. The structure of isolappaol C (1) was determined by spectral analysis including 1D- and 2D-NMR. All the isolates were evaluated for their inhibitory effects on the LPS-induced nitric oxide production using murine macrophage RAW264.7 cells. Lappaol F (4) and diarctigenin (5) strongly inhibited NO production in the LPS-stimulated RAW264.7 cells with IC_{50} values of 9.5 and 9.6 μM, respectively.

Key words *Arctium lappa*; Compositae; lignan; isolappaol C; nitric oxide production inhibitor

Arctium lappa L. (Compositae), also known as "burdock," is a perennial herb that has been cultivated as a vegetable in many countries for a long time. The roots are widely used as a food, whereas the seeds are used in traditional Korean medicine as a diuretic, anti-inflammatory or detoxifying agent.[1] The genus *Arctium* is a rich source of dibenzylbutyrolactone lignans, some of which have antiproliferative and apoptotic effects,[2] inhibit the binding of platelet-activating factor to rabbit platelets,[3] and induce differentiation against mouse myeloid leukemia cells.[4,5]

The nitric oxide (NO) production inhibitory activities of three lignans, arctiin, arctigenin, and lappaol B, from the seeds of *A. lappa* were reported as a part of a larger study searching for anti-inflammatory substances from natural products.[6] Further bioactivity-guided fractionation of this plant led to the isolation of a new sesquilignan, isolappaol C (**1**), along with four known sesqui- and dilignans, lappaol C (**2**), lappaol D (**3**), lappaol F (**4**), and diarctigenin (**5**).

This paper reports the isolation and structure elucidation of a new butyrolactone sesquilignan, isolappaol C, using spectroscopic method including 1D- and 2D-NMR as well as the inhibitory effects of compounds **1**—**5** on the NO production in LPS-stimulated murine macrophage RAW264.7 cells.

Compound **1** was isolated as a white amorphous powder. The molecular formula was established as $C_{30}H_{34}O_{10}$ from the HR-FAB-MS data at *m*/*z* 577.2047 $[M+Na]^+$ ($C_{30}H_{34}O_{10}Na$, Calcd for 577.2050). Its IR absorption of 1761 cm^{-1} indicated the presence of a γ-lactone. Both the ^1H- and ^{13}C-NMR spectra of **1** were almost identical to those of lappaol D (**3**), and suggest compound **1** is a butyrolactone sesquilignan derivative.[4] The ^1H-NMR spectrum of compound **1** showed the characteristic signals of the two sets of 1,3,4-trisubstituted aromatic ring protons at δ 6.69 (1H, d, J=1.8 Hz), 6.72 (1H, d, J=9.6 Hz), 6.55 (1H, dd, J=9.6, 1.8 Hz), 6.82 (1H, d, J=1.8 Hz), 6.67 (1H, d, J=9.6 Hz), and 6.75 (1H, dd, J=9.6, 1.8 Hz), a 1,3,4,5-tetrasubstituted aromatic ring protons at δ 6.65 (1H, d, J=1.8 Hz) and 6.53 (1H, d, J=1.8 Hz), an oxygenated methylene proton at δ 3.99 (1H, dd, J=9.0, 7.5 Hz) and 3.84 (1H, dd, J=9.0, 6.0 Hz), and three methoxyl groups at δ 3.68 (3″-OCH_3), 3.74 (3-OCH_3), and 3.79 (3′-OCH_3). In addition, an oxygenated methine at δ 5.53 (1H, d, J=5.6 Hz), a methine proton at δ 3.44 (1H, dd, J=6.8, 5.6 Hz), and an oxygenated methylene group at δ 3.72 and 4.02 were assigned to H-7″, H-8″, and H-9″ of the phenylpropanoid moiety, respectively. The ^{13}C-NMR spectrum of compound **1**, including the DEPT measurements, showed a carbonyl carbon (δ 179.6), three methoxyl carbons (δ 56.6, 56.8, 56.9), two oxygen bearing methylene carbons (δ 63.6, 72.1), two methylene carbons (δ 35.7, 38.9), one oxymethine carbon (δ 74.6), and three methine carbons (δ 42.1, 47.6, 52.7) as well as the carbon signals of three aromatic rings. The only difference between compound **1** from the lappaol D (**3**) is the absence of one methoxyl group. The location of the three methoxyl groups were assigned to C-3″, C-3, and C-3′ based on the observed HMBC correlations from 3″-OCH_3 (δ 3.68), 3-OCH_3 (δ 3.74), and 3′-OCH_3 (δ 3.79) to C-3″ (δ 148.2), C-3 (δ 149.1), and C-3′ (δ 148.8), respectively (Fig. 2). These data indicated that compound **1** is a demethylated derivative of lappaol D (**3**) at the position of C-4′. The absolute configuration at C-8 and C-8′ of compound **1** were confirmed to be 8(*R*) and 8′(*R*) from the negative cotton effect at 234 nm in the circular dichroism (CD) spectrum of this compound.[5] The relative stereochemistry of C-7″ and C-8″ was determined to be *erythro* by a comparison of the coupling constant between H-7″ and H-8″ (J=5.6 Hz) with those of the *erythro* and *threo* isomers.[7,8]

Therefore, the structure of compound **1** was determined to be a new sesquilignan, isolappaol C. However, the absolute configurations of the other chiral centers at C-7″ and C-8″ remain to be determined.

Two known sesquilignans, lappaol C (**2**) and lappaol D (**3**), and two dilignans, lappaol F (**4**) and diarctigenin (**5**), were also isolated and identified by a comparison of the observed $[\alpha]_D$, UV, IR, ^1H-NMR, ^{13}C-NMR, DEPT, HMQC, HMBC, and FAB-MS data with the literature values.[4,9—12]

Nitric oxide is an inorganic gaseous molecule that is synthesized by the oxidation of L-arginine catalyzed by nitric oxide synthase (NOS) and is involved in a number of physiological and pathological processes in mammals.[13] In the NOS family, iNOS is expressed in a variety of cells including macrophages, endothelial cells, and smooth muscle cells in response to pro-inflammatory stimuli such as IL-1β, TNF-α,

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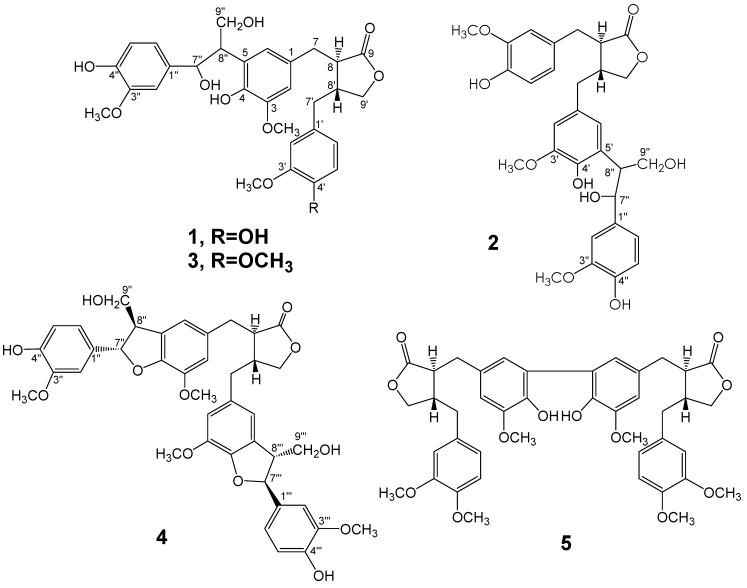

Fig. 1. Chemical Structures of Compounds **1**—**5** Isolated from *A. lappa*

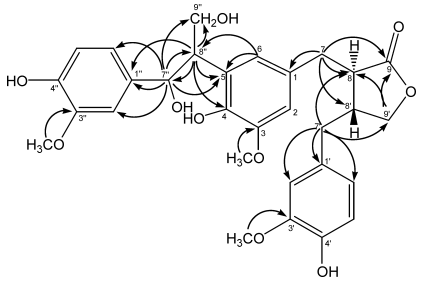

Fig. 2. Selected HMBC Correlations of Compound **1**

and LPS. The excess production of NO by iNOS is involved in the various inflammatory diseases including rheumatoid arthritis, autoimmune disease, chronic inflammation, and atherosclerosis. Therefore, inhibitors of NO production in macrophages are important target in the treatment of certain inflammatory diseases.[14,15]

All isolates were examined for their inhibitory effects on the production of NO in LPS-stimulated RAW264.7 cells. Of these compounds, lappaol F (**4**) and diarctigenin (**5**) showed potent inhibitory activity against NO production with IC_{50} values of 9.5 and 9.6 μM, respectively, which is stronger than that of aminoguanidine used as a positive control (IC_{50}= 28.1 μM). The cell viability measured by the MTT assay showed that these compounds had no significant cytotoxicity to the RAW264.7 cells at their effective concentration for the inhibition of NO production (data not shown). However, the other three compounds, isolappaol C (**1**), lappaol C (**2**) and lappaol D (**3**), were inactive (IC_{50}: >30 μM).

It is previously reported that arctigenin and lappaol B dose-dependently inhibited LPS-induced NO production in RAW264.7 cells with IC_{50} values of 12.5 and 25.9 μM, respectively, while arctiin, a glycoside of arctigenin, was inactive (IC_{50}: >30 μM). These results showed that glycoside and the open dihydrobenzofuran ring derivatives were all inactive (IC_{50} value: >30 μM). A recent study demonstrated that arctigenin inhibited the production of TNF-α, NO, and iNOS expression by inhibiting NF-κB activation.[16,17]

In conclusion, some lignans isolated from *A. lappa* might be valuable candidates for treating various inflammatory diseases.

Experimental

General Experimental Procedures The melting points were measured on Büchi model B-540 without correction. The optical rotations were determined on JASCO DIP-370 polarimeter. The UV and IR spectra were obtained on a JASCO UV-550 and Perkin-Elmer model LE599 spectrometer, respectively. The ^{1}H-NMR, ^{13}C-NMR, DEPT, HMQC, and HMBC spectra were recorded on a Bruker DRX-600 MHz NMR spectrometer. The FAB-MS was obtained on a JMS-HX 110/110A mass spectrometer. Column chromatography was carried out on silica gel 60 (230—400 mesh, Merck) and Diaion HP-20P (Mitsubishi Chemical Co., Japan). Thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} (0.25 mm layer thickness, Merck). A UV lamp and vanillin-sulfuric acid reagent were used to visualize the TLC plates. All other chemicals and reagents were analytical grade. The fetal bovine serum, media, and supplement materials for cell culture were purchased from GIBCO-BRL.

Plant Material The dried seeds of *A. lappa* were purchased from herbal drug store at Cheongju, Korea, in April, 2003 and identified by emeritus professor Kyong Soon Lee, a plant taxonomist at Chungbuk National University. The voucher specimens (CBNU 03021) were deposited at the Herbarium of the College of Pharmacy, Chungbuk National University.

Extraction and Isolation The dried seeds of *A. lappa* (3 kg) were extracted with MeOH (3×5 l) at room temperature. After filtration and subsequent evaporation of the solvent, the resulting extract was diluted with H_2O and extracted with CH_2Cl_2 and BuOH (each 3×3 l). The CH_2Cl_2 soluble fraction (150 g) exhibiting 70.6% inhibitory effect on the production of NO at the concentration of 100 μg/ml was subjected to column chromatography on silica gel (7.5×25 cm) eluted with a CH_2Cl_2–MeOH gradient system to obtain five fractions (AF1—AF5). The inhibition percentages of NO production of the five fractions (AF1—AF5) were 76.5, 48.2, 32.6, 25.4, and 20.9% at the concentration of 50 μg/ml, respectively. Accordingly, AF1 (45 g) was applied to a silica gel column and eluted with a step gradient solvent of CH_2Cl_2–acetone (20 : 1, 10 : 1, 5 : 1, 100% acetone) to give four fractions (AF11—AF14). AF-12 (5 g, 89.0% inhibition of NO production at 50 μg/ml) was further purified through a Diaion HP-20P column and eluted with MeOH–H_2O (20 : 80, 50 : 50, 80 : 20, 100 : 0) to afford four fractions (AF-121—AF-124). AF-121 (300 mg, 95.2% inhibition of NO production at 50 μg/ml) was subjected to preparative HPLC (ODS-H80, 150×20 mm, YMC, Japan, aetonitrile–H_2O, 28 : 72) to afford compounds **1** (15 mg, t_R=21.2 min), **2** (210.3 mg, t_R=23.2 min), **3** (62 mg, t_R=28.6 min), and **4** (35 mg, t_R=36.1 min). AF-123 (500 mg, 94.7% inhibition of NO production at 50 μg/ml) was also subjected to preparative HPLC (ODS-H80, 150×20 mm, YMC, Japan, aetonitrile–H_2O, 40 : 60) to afford compound **5** (40 mg, t_R=20.1 min).

Table 1. ^{1}H- and ^{13}C-NMR Data of Compound **1** (Acetone-d_6)[a,b]

Position	**1**	
	δ_C	δ_H
1	129.6	—
2	125.8	6.65 d (1.8)
3	149.1	—
4	145.2	—
5	127.4	—
6	112.5	6.53 d (1.8)
7	35.7	2.74 dd (14.2, 6.5)
		2.76 dd (14.2, 5.5)
8	47.6	2.53 m
9	179.6	—
1′	131.5	—
2′	113.7	6.69 d (1.8)
3′	148.8	—
4′	146.5	—
5′	116.2	6.72 d (9.6)
6′	122.8	6.55 dd (9.6, 1.8)
7′	38.9	2.48 dd (15.0, 9.6)
		2.59 dd (15.0, 7.8)
8′	42.1	2.42 m
9′	72.1	3.84 dd (9.0, 6.0)
		3.99 dd (9.0, 7.5)
1″	136.9	—
2″	111.5	6.82 d (1.8)
3″	148.2	—
4″	146.7	—
5″	115.5	6.67 d (9.6)
6″	120.4	6.75 dd (9.6, 1.8)
7″	74.6	5.53 d (5.6)
8″	52.7	3.44 dd (6.8, 5.6)
9″	63.6	3.72 dd (10.5, 6.8)
		4.02 dd (10.5, 6.8)
3″-OMe	56.8	3.68
3-OMe	56.9	3.74
3′-OMe	56.6	3.79

a) Assignments were confirmed by DEPT, HMQC and HMBC spectra. *b*) *J* values (in parenthesis) are reported in Hz.

Isolappaol C (**1**): White amorphous powder; $[\alpha]_D^{25}$ −43.6° (c=0.1, MeOH); UV λ_{max} MeOH nm (log ε): 230 (4.4), 281 (4.0); CD (c=4.84×10^{-4}, MeOH) $\Delta\varepsilon$ nm: −14.0 (234), −2.6 (288); IR ν_{max} cm^{-1}: 3405 (OH), 1761 (γ-lactone), 1600 (aromatic C=C); HR-FAB-MS *m*/*z*: 577.2047 $[M+Na]^+$ ($C_{30}H_{34}O_{10}Na$, Calcd for 577.2050); ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) data, see Table 1.

Lappaol C (**2**): White amorphous powder; $[\alpha]_D^{25}$ −54.6° (c=0.8, MeOH); UV λ_{max} MeOH nm (log ε): 230 (4.4), 281 (4.1); IR ν_{max} cm^{-1}: 3432 (OH), 1764 (γ-lactone), 1610 (aromatic C=C); HR-FAB-MS *m*/*z*: 577.2045 $[M+Na]^+$ ($C_{30}H_{34}O_{10}Na$, Calcd for 577.2050); ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) data: consistent with published values.[4,9]

Lappaol D (**3**): White amorphous powder; $[\alpha]_D^{25}$ −50.4° (c=0.8, MeOH); UV λ_{max} MeOH nm (log ε): 230 (4.3), 282 (4.0); IR ν_{max} cm^{-1}: 3432 (OH), 1763 (γ-lactone), 1607 (aromatic C=C); FAB-MS *m*/*z*: 591.1 $[M+Na]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) data: consistent with published values.[9]

Lappaol F (**4**): White amorphous powder; $[\alpha]_D^{25}$ +13.6° (c=1.0, MeOH); UV λ_{max} MeOH nm (log ε): 229 (4.2), 282 (4.0); IR ν_{max} cm^{-1}: 3450 (OH), 1760 (γ-lactone), 1604 (aromatic C=C); FAB-MS *m*/*z*: 737.1 $[M+Na]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) data: consistent with published values.[4,9]

Diarctigenin (**5**): White amorphous powder; $[\alpha]_D^{25}$ −12.5° (c=0.1, $CHCl_3$); UV λ_{max} MeOH nm (log ε): 235 (4.4), 282 (4.2); IR ν_{max} cm^{-1}: 3405 (OH), 1760 (γ-lactone), 1604 (aromatic C=C); FAB-MS *m*/*z*: 765.0 $[M+Na]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) data: consistent with published values.[12]

Cell Lines and Cell Culture The murine macrophage RAW264.7 cells were cultured in Dulbecco's modified Essential Medium (Gibco/BRL, Gaithersburg, MD, U.S.A.) supplemented with penicillin (100 units/ml)–streptomycin (100 μg/ml) (Gibco/BRL), and 10% heat-inactivated fetal bovine serum (Gibco/BRL). The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere and used for the experiments between passages 5 and 20.

Measurement of NO Production The Raw264.7 cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 2 h at 37 °C. The cells were incubated for 24 h with or without 1 μg/ml of LPS (Sigma Chemical Co., St. Louis, MO, U.S.A.) in the absence or presence of the test compounds. As a parameter of NO synthesis, the nitrite concentration was measured by the Griess reaction using the supernatant of the RAW264.7 cells as previously described.[18] Briefly, 100 μl of the cell culture supernatant were reacted with 100 μl of Griess reagent [1 : 1 mixture of 0.1% *N*-(1-naphtyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96 well plate and the absorbance was read with a microplate reader (Molecular Devices Co., Menlo park, CA, U.S.A.) at 570 nm.

Determination of Cell Viability The cell viability was assessed using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemical Co., St. Louis, MO, U.S.A.]-based colorimetric assay, as previously described.[19] After sampling the supernatant for the NO assay, 50 μl of fresh medium containing 0.5 mg/ml of MTT was added to each well and incubated for 2 h at 37 °C. The medium was then removed and the violet formazan crystals in the viable cells were dissolved in dimethyl sulfoxide. The absorbance of each well was then read at a wavelength of 570 nm using a microplate reader (Molecular Devices Co., Menlo park, CA, U.S.A.).

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