Melampolides from the Leaves of *Smallanthus sonchifolius* and Their Inhibitory Activity of LPS-Induced Nitric Oxide Production

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Two new melampolide-type sesquiterpene lactones, 8β-epoxyangeloyloxy-9α-ethoxy-14-oxo-acanthospermolide (1) and 8β-angeloyloxy-9α-ethoxy-14-oxo-acanthospermolide (2), were isolated from the leaves of yacon [*Smallanthus sonchifolia* (Poepp. *et* Endl.) H. Robinson] along with eleven known melampolides, *allo*-schkuhriolide (3), enhydrin (4), polymatin A (5), fluctuanin (6), 8β-angeloyloxy-9α-acetoxy-14-oxo-acanthospermolide (7), 8β-angeloyloxy-14-oxo-acanthospermolide (8), 8β-methacryloyloxymelampolid-14-oic acid methyl ester (9), uvedalin (10), polymatin B (11), 8β-tigloyloxymelampolid-14-oic acid methyl ester (12), and sonchifolin (13). Their structures were established on the basis of spectroscopic evidence including 1D- and 2D-NMR experiments. All isolates were evaluated for inhibition of LPS-induced nitric oxide production in murine macrophage RAW 264.7 cells.

Key words *Smallanthus sonchifolius*; Asteraceae; yacon; melampolide; sesquiterpene lactone; nitric oxide production inhibitor

Yacon [*Smallanthus sonchifolius* (Poepp. *et* Endl.) H. Robinson; Asteraceae; syn. *Polymnia sonchifolia*] is a vigorous and herbaceous perennial plant originally cultivated in the Andean highlands of South America.[1,2] Extracts and chemical constituents of the tubers and dried leaves of yacon have antimicrobial,[3] antifungal,[4] antihyperglycemic,[5] and antioxidative activity.[6,7] The tubers of yacon contain fructooligosaccharide and phenolic compounds,[1,8,9] and the leaves have several kaurene diterpenoids, acetophenone-type phytoalexins, and melampolide-type sesquiterpene lactones.[1,3,4] Enhydrin, a melampolide-type sesquiterpene lactone, has been shown to possess anti-inflammatory activity and inhibitory effects of the transcriptional factor NF-κB.[10,11]

In our search for natural products that inhibit nitric oxide (NO) production, we have identified two melampolide-type sesquiterpene lactones, 8β-epoxyangeloyloxy-9α-ethoxy-14-oxo-acanthospermolide (**1**) and 8β-angeloyloxy-9α-ethoxy-14-oxo-acanthospermolide (**2**), along with eleven known melampolides from the leaves of yacon. NO is involved in physiological and pathological process, such as vasodilation and chronic or acute inflammation.[12] Therefore, inhibitors of NO production could have therapeutic potential for treating inflammatory diseases. Here, we report the isolation, structure determination, and inhibitory effects of these melampolide-type sesquiterpene lactones on NO production in murine macrophage RAW 26.4 cells *in vitro*.

The CH_2Cl_2-soluble fraction of yacon leaves was successively subjected to silica gel and RP-18 column chromatography, as well as preparative HPLC, to produce two new (**1** and **2**) and eleven known melampolides.

Compound **1** was obtained as a pale yellow gum, and its molecular formula was determined as $C_{22}H_{28}O_7$ with nine degrees of unsaturation from HR-FAB-MS (*m*/*z* 427.1733 $[M+Na]^+$; Calcd 427.1733). Its IR spectrum revealed the presence of a γ-lactone (1766 cm^{-1}), an unsaturated ester (1720 cm^{-1}), and an unsaturated aldehyde moiety (1687 cm^{-1}). The ^{1}H-NMR spectrum of **1** exhibited an aldehyde [δ 9.47 (1H, d, J=2.0 Hz)], two olefinic methines [δ 6.77 (1H, dd, J=10.0, 7.5 Hz) and 4.91 (1H, br d, J=10.5 Hz)], five other methines [δ 6.61 (1H, br d, J=8.3 Hz), 5.05 (1H, t, J=10.0 Hz), 3.68 (1H, dd, J=8.3, 1.8 Hz), 3.03 (1H, q, J=5.3 Hz), 2.61 (1H, m)], an *exo*-methylene [δ 6.27 (1H, d, J=3.2 Hz) and 5.82 (1H, d, J=3.2 Hz)], another methylene [δ 3.35 (1H, dt, J=7.0, 1.3 Hz) and 3.08 (1H, dt, J=7.0, 1.3 Hz)], and four methyl groups at δ 1.94 (3H, s), 1.53 (3H, s), 1.25 (3H, d, J=5.2), and 1.10 (3H, t, J=7.0). The ^{13}C-NMR spectrum of **1** showed 22 carbon signals, which were confirmed by DEPT NMR experiment, consisting one conjugated aldehyde (δ 193.9), two carbonyl carbons (δ 169.3, 168.4), four methylene carbons (δ 122.3, 64.2, 36.9, 26.4), two olefinic methine carbons (δ 156.1, 127.3), five methine carbons (including three oxygenated sig-

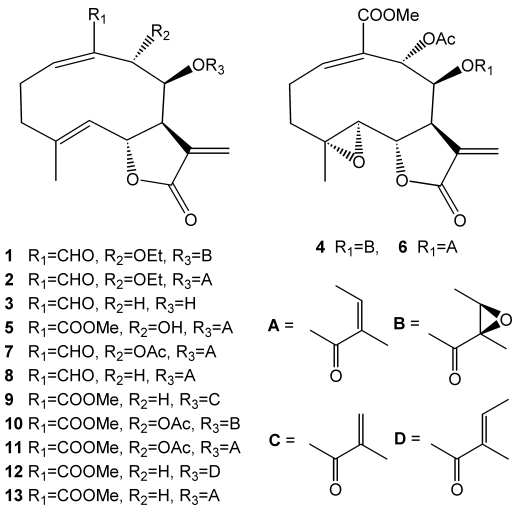

Chart 1

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nals at δ 76.3, 74.9, 70.6), four methyl carbons, and four quaternary carbons (including three olefinic signals at δ 141.4, 137.1, 134.1). Typical ^{1}H-NMR proton signals at δ 3.03 (1H, q, J=5.3 Hz, H-3′), 1.25 (3H, d, J=5.2 Hz, H-4′), and 1.53 (3H, s, H-5′) and ^{13}C-NMR carbon signals at δ 168.4 (C-1′), 59.6 (H-2′), 59.8 (H-3′), 13.8 (H-4′), and 19.3 (H-5′) are characteristic of an 2*R*,3*R*-epoxyangeloyloxy group.[4,13] The presence of the α-methylene-γ-lactone moiety was deduced from the ^{1}H-NMR signals at δ 6.27 (d, J=3.4 Hz, H-13a) and 5.82 (d, J=3.2 Hz, H-13b) and ^{13}C-NMR signals at δ 134.1 (C-11), 169.3 (C-12), and 122.3 (C-13). The aliphatic region of the spectrum of **1** showed the typical pattern of an ethoxy group. The above observations indicated that compound **1** has a melampolide-type sesquiterpene skeleton with an epoxyangeloyloxy group and an ethoxy group. The location of these substituents were assigned by the observed HMBC correlations from H-8 (δ 6.61, br d, J=8.3 Hz) to C-6 (δ 74.9), C-7 (δ 50.8), C-9 (δ 76.3) and epoxyangeloyloxyl carbonyl carbon (δ 168.4), and from H-9 (δ 3.68, dd, J=8.3, 1.8 Hz) to C-1 (δ 156.1), C-14 (δ 193.9) and oxygenated methylene (δ 64.2) (Fig. 1). According to the above correlations in the HMBC spectrum of **1**, an epoxyangeloyloxy group and an ethoxy group were placed at C-8 and C-9, respectively. The relative configurations of the oxy-substituents and the geometry of the two double bonds were established by a NOESY experiment (Fig. 2). The *E*-configuration of the C(1)=C(10) and C(4)=C(5) double bonds were assigned on the basis of the NOESY correlations of H-1 with H-14, H-6β with H-15, and H-5 with H-3α.[3] The oxy-substituents at C-8 and C-9 were in the β- and α-orientations, respectively, as deduced from the NOESY correlations of H-8α with H-7α and H-13, H-9β with H-2β and H-15, as well as H-5 with H-3α and H-7α. Moreover, an α-orientation of H-8 is consistent with the coupling constant $J_{8\alpha,9\beta}$=8.3 Hz.[14] Furthermore, we determined the absolute configuration of compound **1** on the basis of CD spectral correlations with polymatin A and melampodin A of which absolute configuration was determined by X-ray diffraction analysis.[15—17] The CD data indicated the ring system consisted of melampolide with anti-arrangement of C-14 and C-15 and the lactone ring is *trans*-fused. Consequently, the structure of compound **1** was elucidated as 8β-epoxyangeloyloxy-9α-ethoxy-14-oxo-acanthospermolide.

Compound **2** was obtained a pale yellow gum. The molecular formula $C_{22}H_{28}O_6$ with nine degrees of unsaturation was determined from HR-FAB-MS (*m/z* 411.1790 $[M+Na]^+$; Calcd 411.1784). The IR, ^{1}H- and ^{13}C-NMR spectral data of **2** were very similar to those of **1**, except for the epoxyangeloyloxy group. The ^{1}H-NMR spectrum of **2** showed a characteristic signals of an angeloyloxy group at δ 6.04 (1H, dq, J=7.2, 1.4 Hz, H-3′), 1.96 (3H, dd, J=7.2, 1.5 Hz, H-4′), and 1.87 (3H, t, J=1.4 Hz, H-5′) and ^{13}C-NMR signals at δ 166.4 (C-1′), 127.5 (H-2′), 137.9 (H-3′), 15.7 (H-4′), and 20.5 (H-5′).[4] These results indicated that compound **2** has an angeloyloxy moiety instead of the epoxyangeloyloxy moiety seen in compound **1**. The position of the angeloyloxy moiety was deduced to be at C-8 by the observed HMBC correlations from H-8 (δ 6.65, dd, J=8.4, 1.5 Hz) to the angeloyloxyl carbonyl carbon (δ 166.4) (Fig. 1). The relative stereochemistry and the geometry of the two double bonds were further substantiated by NOESY experiments (Fig. 2), which

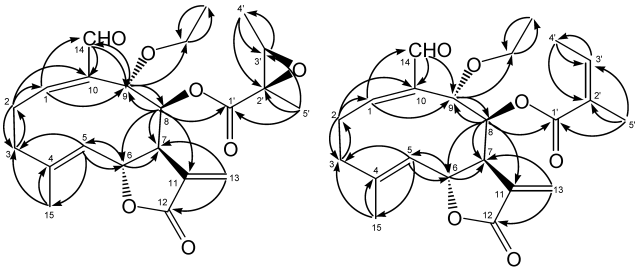

Fig. 1. Key HMBC Correlation of Compounds **1** and **2**

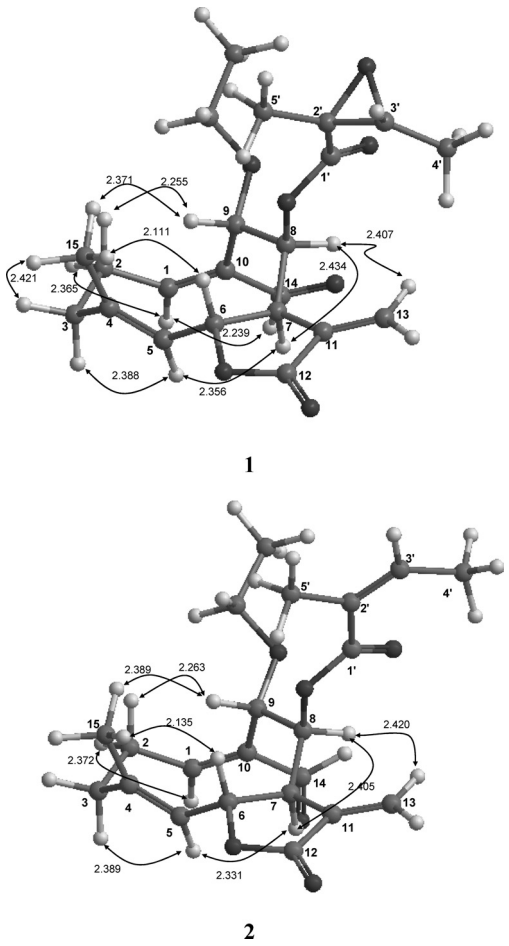

Fig. 2. Key NOESY Correlations and Corresponding Inter-atomic Distances (Å) of Compounds **1** and **2**

Computer modeled 3D structures of **1** and **2** were generated by using the molecular modeling program CS Chem 3D Ultra Version 9.0, using MM2 force field calculations for energy minimization.

showed the same correlations as in compound **1**. Furthermore, we determined the absolute configuration of compound **2** on the basis of CD data,[15—17] which indicated the ring system consisted of melampolide with anti-arrangement of C-14 and C-15 and the lactone ring is *trans*-fused. Thus, the structure of compound **2** was elucidated as 8β-angeloyloxy-9α-ethoxy-14-oxo-acanthospermolide.

Eleven known sesquiterpenoids were identified as *allo*-schkuhriolide (**3**),[18,19] enhydrin (**4**),[4] polymatin A (**5**)[17], fluctuanin (**6**),[3] 8β-angeloyloxy-9α-acetoxy-14-oxo-acanthospermolide (**7**),[20] 8β-angeloyloxy-14-oxo-acanthospermolide (**8**),[21] 8β-methacryloyloxymelampolid-14-oic acid

Table 1. ^{1}H-NMR Spectral Data for Compounds **1** and **2** in $CDCl_3$ (500 MHz, δ in ppm)

No.	**1**	**2**
1	6.77, 1H, dd (10.0, 7.5)	6.73, 1H, dd (10.0, 7.5)
2α	2.69, 1H, dddd (12.4, 12.4, 10.0, 2.1)	2.68, 1H, dddd (12.4, 12.4, 10.0, 2.1)
2β	2.36, 1H, dddd (12.4, 7.5, 5.9, 2.0)	2.35, 1H, dddd (12.4, 7.5, 5.6, 1.8)
3α	2.09, 1H, br t (12.4)	2.08, 1H, br t (12.4)
3β	2.44, 1H, ddd (12.4, 5.9, 2.1)	2.43, 1H, ddd (12.4, 5.6, 2.1)
5	4.91, 1H, br d (10.5)	4.92, 1H, br d (10.5)
6β	5.05, 1H, t (10.0)	5.04, 1H, t (10.0)
7α	2.61, 1H, m	2.62, 1H, m
8α	6.61, 1H, br d (8.3)	6.65, 1H, dd (8.4, 1.5)
9β	3.68, 1H, dd (8.3, 1.8)	3.68, 1H, dd (8.4, 2.0)
13a	6.27, 1H, d (3.2)	6.28, 1H, d (3.2)
13b	5.82, 1H, d (3.2)	5.89, 1H, d (3.2)
14	9.47, 1H, d (2.0)	9.49, 1H, d (2.1)
15	1.94, 3H, s	1.92, 3H, s
3′	3.03, 1H, q (5.3)	6.04, 1H, dq (7.2, 1.4)
4′	1.25, 3H, d (5.2)	1.96, 3H, dd (7.2, 1.5)
5′	1.53, 3H, s	1.87, 3H, t (1.4)
OEt	3.35, 1H, dt (7.0, 1.3)	3.40, 1H, dt (7.0, 1.8)
	3.08, 1H, dt (7.0, 1.3)	3.10, 1H, dt (7.0, 1.8)
	1.10, 3H, t (7.0)	1.08, 3H, t (7.0)

Table 2. ^{13}C-NMR Data for Compounds **1**, **2**, **5**, and **8** in $CDCl_3$ (125 MHz, δ in ppm)

No.	**1**	**2**	**5**	**8**
1	156.1	155.4	144.3	153.8
2	26.4	26.4	25.9	26.2
3	36.9	37.0	36.8	37.2
4	137.1	136.9	133.9	137.4
5	127.3	127.5	126.6	126.4
6	74.9	75.2	75.7	75.6
7	50.8	50.9	51.6	49.6
8	70.6	69.0	71.8	65.6
9	76.3	76.4	71.2	29.1
10	141.4	141.8	134.9	143.0
11	134.1	134.1	137.7	135.1
12	169.3	169.5	169.4	169.5
13	122.3	122.4	121.3	121.0
14	193.9	193.9	167.8	195.3
15	17.0	17.0	16.9	17.1
1′	168.4	166.4	167.0	166.4
2′	59.6	127.5	127.0	127.1
3′	59.8	137.9	139.6	139.3
4′	13.8	15.7	15.9	15.9
5′	19.3	20.5	20.5	20.6
OMe			52.3	
OEt	64.2	64.5		
	15.1	15.0		

methyl ester (**9**),[3] uvedalin (**10**),[4] polymatin B (**11**),[4,17] 8β-tigloyloxymelampolid-14-oic acid methyl ester (**12**),[3] and sonchifolin (**13**)[3,4] respectively, by comparing their spectral data with those reported in literatures. The complete ^{13}C-NMR data assignments based on 2D NMR spectroscopic correlations are presented here for the first time (Table 2). Among these isolates, compounds **3**, **5**, **7** and **8** were found in *Smallanthus* species for the first time.

Compounds **1**—**13** dose-dependently inhibited the production of NO in LPS-stimulated RAW264.7 cells (Table 3). The cell viability measured by a CCK-assay indicated that no compounds were cytotoxic at concentrations necessary to inhibit NO production (data not shown). All active compounds have the α-methylene-γ-lactone moiety in the molecule, which might be a common functional group. In the previous studies on structure–activity relationship of the sesquiterpene lactones revealed that the α-methylene-γ-lactone moiety is the most important group for the inhibition of NO production.[22]

Table 3. Inhibition of NO Production by Isolated Compounds

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	19.6±0.32	**8**	12.0±0.11
2	31.5±0.28	**9**	5.6±0.21
3	10.3±0.13	**10**	0.9±0.24
4	2.0±0.15	**11**	4.3±0.05
5	7.8±0.09	**12**	7.3±0.16
6	2.5±0.12	**13**	7.4±0.13
7	10.5±0.17	AG	17.5±0.34

Data are mean±S.D. from three separate experiments. AG: Positive control for NO production (Aminoguanidine).

Compounds **4**, **6**, **10**, and **11**, which have an acetoxy group at the C-9 position, exhibited strong inhibition of NO production, with IC_{50} values ranging 0.9—4.3 μM. Therefore, the acetoxy group seems necessary for strong inhibition. However, compound **7**, where a methyl ester group at the C-10 position was substituted with an aldehyde group, showed weak inhibition compared to compound **11**. Moreover, compounds **1**—**3** and **8**, which also have an aldehyde group, showed weaker inhibitory activity than that of the methyl ester derivatives at the C-10 position. In conclusion, two new and eleven known melampolide-type sesquiterpene lactones were isolated from the leaves of yacon and shown to inhibit NO production in LPS-stimulated murine macrophage RAW 264.7 cells.

Experimental

General Experimental Procedures The optical rotations were measured with a JASCO DIP-1000 polarimeter. The UV and IR spectra were obtained on a JASCO UV-550 and Perkin-Elmer model LE599 spectrometer, respectively. The ^{1}H-, ^{13}C-NMR and 2D-NMR spectra were recorded on a Bruker AMX 500 MHz NMR spectrometer. High-resolution fast atom bombardment (HR-FAB) mass spectra were obtained on a JMS-HX110/110A mass spectrometer. Preparative HPLC was carried out on a Waters system (two Waters 515 pumps with a 2996 photodiode array detector) and a YMC J'sphere ODS-H80 column (4 μm, 150×20 mm i.d.), using a mixed solvent system of ACN–H_2O at a flow rate of 6.0 ml/min. Open column chromatography was performed using a silica gel (Kieselgel 60, 70—230 mesh, Merck), Lichroprep RP-18 (40—63 μM, Merck), and thin layer chromatography (TLC) using a pre-coated silica gel 60 F_{254} (0.25 mm, Merck).

Plant Material The leaves of yacon (*Smallanthus sonchifolius*) were collected from Bonghwa, Gyeongbuk, Korea in September 2005. The plant material was identified by Emeritus Professor Kyong Soon Lee at Chungbuk National University. A voucher specimen of this plant was deposited at the Herbarium of College of Pharmacy, Chungbuk National University, Korea (CBNU0309SS).

Extraction and Isolation The air-dried leaves of yacon (500 g) were pulverized and extracted with 70% MeOH (3×5 l) at room temperature. The extract was filtered and concentrated, *in vacuo*, and diluted with water, then partitioned with CH_2Cl_2 (3×1.0 l) and EtOAc (3×1.0 l). The CH_2Cl_2 extract (2.1 g) was subjected to column chromatography on a silica gel (3×20 cm, 70—230 mesh) eluting with CH_2Cl_2–MeOH (100 : 1, 50 : 1, 30 : 1, 20 : 1, 10 : 1, 5 : 1, 2 : 1, 0 : 1), to yield five fractions (YLCA—YLCE). Fraction YLCB was subjected to vacuum liquid chromatography on RP-18 developing with ACN–H_2O (20 : 80, 30 : 70, 40 : 60, 50 : 50) to give nine fractions (YLCB01—YLCB09). Compound **3** (6.1 mg) was obtained from fraction

YLCB03 by preparative HPLC (ACN–H_2O=55 : 45). Compounds **1** (14.6 mg), **4** (42.9 mg) and **5** (3.5 mg) were isolated from fraction YLCB06 by preparative HPLC (ACN–H_2O=50 : 50). Fraction YLCB07 was further purified over silica gel column with CH_2Cl_2–MeOH (100 : 1, 70 : 1, 50 : 1, 20 : 1), then by preparative HPLC with ACN–H_2O (45 : 55), to yield compound **10** (62.8 mg). Fraction YLCB08 was further purified by preparative HPLC eluting with ACN–H_2O (55 : 45) to yield compounds **2** (1.6 mg), **6** (3.6 mg), **7** (2.4 mg), **8** (2.3 mg) and **9** (2.9 mg). Compounds **11** (3.1 mg), **12** (2.4 mg) and **13** (6.0 mg) were obtained from fraction YLCB09 by preparative HPLC with ACN–H_2O (65 : 45).

8β-Epoxyangeloyloxy-9α-ethoxy-14-oxo-acanthospermolide (**1**): Pale yellow gum; $[\alpha]_D^{20}$ −22° (c=0.04, CH_2Cl_2); UV λ_{max} (MeOH) nm (log ε): 221.3 (3.83); IR (KBr) cm^{-1}: 2929, 1766, 1720, 1687, 1259, 1147, 981; HR-FAB-MS m/z: 427.1733 $[M+Na]^+$ (Calcd for $C_{22}H_{28}O_7Na$: 427.1733); CD (MeOH) $\Delta\varepsilon$ nm: −1.1 (270), +0.2 (246), −46.5 (213); ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

8β-Angeloyloxy-9α-ethoxy-14-oxo-acanthospermolide (**2**): Pale yellow gum; $[\alpha]_D^{18}$ −15° (c=0.05, CH_2Cl_2); UV λ_{max} (MeOH) nm (log ε): 219.0 (3.51); IR (KBr) cm^{-1}: 2923, 1765, 1737, 1644, 1382, 1147, 983; HR-FAB-MS m/z: 411.1790 $[M+Na]^+$ (Calcd for $C_{22}H_{28}O_6Na$: 411.1784); CD (MeOH) $\Delta\varepsilon$ nm: −0.8 (273), +3.1 (248), −39.0 (217); ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

Determination of NO Production and Cell Viability Nitric oxide production was determined by measuring the amount of nitrite from cell culture supernatant as previously described.[23] In brief, RAW264.7 cells (1×10^5 cells/well) were stimulated for 24 h with or without 1 μg/ml of LPS (Sigma Chemical Co., St. Louis, MO, U.S.A.) in the absence of presence of the compounds tested. A 100 μl of cell culture supernatant was reacted with 100 μl of Griess reagent. The remaining cells after Griess assay were used for viability with the CCK (Cell Counting Kit, Dojindo, Tokyo, Japan).

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