A Novel NO-Production-Inhibiting Triterpene and Cytotoxicity of Known Alkaloids from *Euonymus laxiflorus*

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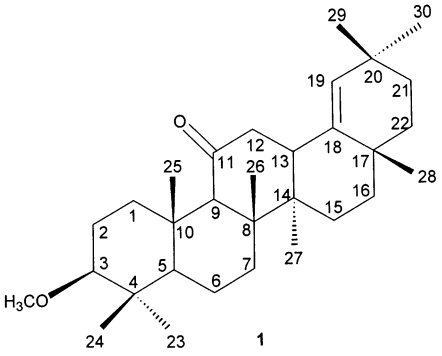
A new triterpene, laxifolone A (**1**), four known sesquiterpene alkaloids, ebenifoline E-II (**2**), carigorinine E (**3**), euojaponine C (**4**), and emarginatine E (**5**), and six triterpenoids, 3-hydroxyolean-12-en-22,29-γ-lactone, 3,11-dioxo-β-amyrene, 3β,22α-dihydroxyolean-12-en-29-oic acid, 28,29-dihydroxyfriedelan-3-one, 29-hydroxy-3-oxo-D:A-friedooleanan-28-oic acid, and putranjivadione, were isolated from the stems and leaves of *Euonymus laxiflorus*. Structural elucidations of these compounds were established by spectral analysis. Compound **1** displayed significant nitric oxide (NO) inhibitory effect.

In the search for potential antitumor agents from the plant family Celastraceae, we reported recently on the isolation and structural elucidation of several new sesquiterpene polyol esters with β-dihydroagarofuran skeletons[1−3] and triterpenes[4] from *Maytenus emarginata*, *M. diversifolia*, and *Celastrus hindsii* Benth. Some of these novel isolates exhibited cytotoxic effects. It is now well recognized that effective strategies for limiting tumor growth include not only inhibiting DNA replication but also interfering with the ability of growth factors and cytokines to regulate processes required for proliferation and survival. A potential target has been realized based on the finding that tumor growth is dependent upon a switch to an angiogenic phenotype and the subsequent formation of new vasculature.[5,6] Thus, inhibition of tumor blood supply has been realized as a unique approach to stop tumor growth.[7,8]

A number of independent lines of evidence indicate that nitric oxide (NO), synthesized by the enzyme family NO synthase (NOS), plays an important role in tumor growth, invasion, and angiogenesis.[9,10] In various tumor cell lines and solid tumors, the expression of inducible NOS (iNOS) has been recently documented.[11,12] It is also suggested that the enzyme activity in tumor tissue correlated well with its tumor grade and cell differentiation. Because NO appears to be involved in many of these processes, interfering with its production in tumors may be an important target for antitumor therapy. After a preliminary assay, we found that the Celastraceae plant *Euonymus laxiflorus* Champ. could inhibit nitric oxide (NO) production. The EtOH extract of the titled plant was partitioned between *n*-hexane and H_2O, then between $CHCl_3$ and H_2O, to give the *n*-hexane- and $CHCl_3$-soluble layers. These two solubles were respectively subjected to open column chromatography on Si gel and HPLC chromatography to yield a new triterpene lactone, laxifolone A (**1**), four sesquiterpene alkaloids, ebenifoline E-II (**2**), mayteine (**3**), euojaponine C (**4**), and emarginatine-E (**5**), and six triterpenoids, 3-hydroxyolean-12-en-22,29-γ-lactone, 3,11-dioxo-β-amyrene, 3β,22α-dihydroxyolean-12-en-29-oic acid, 28,29-dihydroxyfriedelan-3-one, 29-hydroxy-3-oxo-D:A-friedooleanan-28-oic acid, and putranjivadione.



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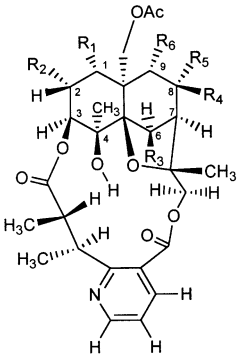

	R_1	R_2	R_3	R_4	R_5	R_6
2	OBz	OAc	OBz	H	OAc	OAc
3	OBz	OAc	OAc	H	OAc	OAc
4	OBz	OH	OBz	H	OAc	OAc
5	OH	pyridone	OAc	OAc	H	OH

The structure of compound **1** was established by 2D NMR spectra, including $^1H-^1H$ COSY, HMQC, HMBC, and NOE studies.

Compound **1** was obtained as colorless needles, mp 279−280 °C. The molecular formula, $C_{31}H_{50}O_2$, was determined

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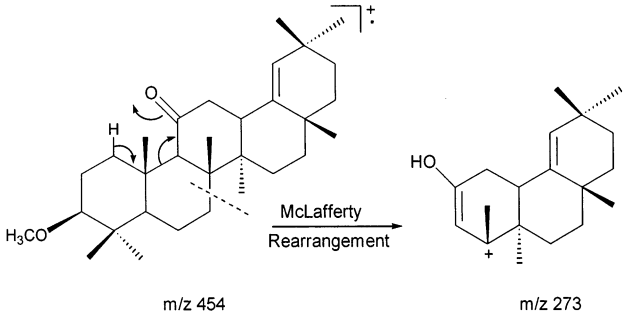
Table 1. ^{1}H and ^{13}C NMR Data[a] ($CDCl_3$) for Compound **1**

	δ_H	δ_C	^{1}H−^{13}C correlations (HMBC)[b]
1	0.80, 2.26 m (2H)	38.2	
2	1.47, 1.72 m (2H)	21.9	H-1
3	2.61 m (1H)	88.5	H-23, 24, OCH_3
4		38.8	H-5, 23, 24
5	0.55 m (1H)	55.4	H-6, 23, 24, 25
6	1.44 m (2H)	17.9	
7	1.49 m (2H)	33.6	H-26
8		46.0	H-6, 9, 15, 26
9	2.25 s (1H)	63.0	H-25, 26
10		36.5	H-1, 5, 9, 25
11		212.5	H-9, 12
12	2.05, 2.12 m (2H)	44.6	H-13
13	2.65 m (1H)	40.4	H-12, 19
14		43.3	H-15, 26, 27
15	1.38, 1.46 m (2H)	27.3	
16	1.56 m (2H)	37.2	H-28
17		34.7	H-13, 16, 19, 28
18		140.7	H-28
19	4.74 s (1H)	130.3	H-13, 29
20		32.4	H-21, 22
21	1.11, 1.38 m (2H)	33.2	
22	1.40 m (2H)	37.3	H-28
23	0.94 s	28.0	H-24
24	0.75 s	16.3	H-23
25	1.23 s	16.7	H-1, 9
26	1.02 s	17.6	
27	0.96 s	14.3	
28	0.98 s	25.1	
29	0.90 s	31.1	H-29, 21, 30
30	0.92 s	29.1	H-29
OCH_3	3.32 s	57.5	

[a] All assignments are based on 1D and 2D NMR experiments, including COSY 90, HETCOR, and HMBC spectra. [b] ^{1}H−^{13}C long-range correlation (HMBC) corresponded to two of three bond connectivities.

by HREIMS. Signals of ^{13}C NMR and DEPT spectra revealed that **1** was a triterpenoidal compound containing eight methyl, one methoxy, nine methylene, five methine, seven quaternary, and one carbonyl carbon (Table 1). The ^{1}H NMR spectrum of **1** showed singlets of a vinyl proton at δ 4.74 (1H, H-19) and a methoxy group [δ 3.32 (3H, s)] and eight tertiary methyl groups (δ 0.75, 0.90, 0.92, 0.94, 0.96, 0.98, 1.02, 1.23), which suggested the presence of an oleane type triterpene. Like those of the other olean-18-ene analogues, the unsaturated carbon signals at δ_C 140.7, 130.3 were assigned to C-18 and C-19.[13] Further evidence from HMQC and HMBC spectra supported that **1** had an olean-18-ene triterpene structure. Observing the HMBC spectrum, the cross-peak of a vinyl proton (δ_H 4.74, s) and a tertiary carbon was found, together with the correlations between this singlet proton and C-13 (δ_C 40.4), C-17 (δ_C 34.7), and C-29 (δ_C 31.1), confirming that the olefinic carbon at C-18 and -19. Moreover, owing to the long-range coupling with H-12, H-13, and H-9, a ketone group (δ_C 212.5) was assigned at C-11. As a result, the chemical shift of C-9 was confirmed by using the HMQC spectrum and sequentially assigned the two methyl groups at C-25 and C-26, respectively, due to the HMBC spectrum. Furthermore, the mass spectral fragments at *m*/*z* 273 due to the McLafferty rearrangement are also consistent with the assignment of structure **1** (Scheme 1). Inspection of the HMBC spectrum revealed correlation between OCH_3 (δ_H 3.32) and C-3 (δ_C 88.8), indicating that the methoxy group was located at C-3. Further confirmation of the stereochemistry of **1** was achieved by a NOE spectrum displaying the correlation between 5α-H and 3α-H. The NOESY spectrum of **1** also supported the methoxy group at C-3 in a β configuration,

Scheme 1. Proposed Mass Fragment Ion of **1**



as well as the stereochemistry of the other chiral centers in **1** as shown. From the above evidence, together with the HREIMS, which exhibited the molecular ion at *m*/*z* 454.3810, the structure of **1** was completely established as 3β-methoxyolean-11-oxo-18-ene and tentatively named laxifolone A.

The other known isolated compounds including four sesquiterpene polyol esters alkaloids (**2**−**5**) and six triterpenes (**6**−**11**) were confirmed by the spectral (^{1}H and ^{13}C NMR, IR, UV, MS) comparison with reference data or authentic samples. Thus **2**−**5** were verified as ebenifoline E-II (**2**),[14] mayteine (**3**),[15] euojaponine C (**4**),[14] and emarginatine-E (**5**),[16] and **6**−**11** were identified as 3-hydroxy-olean-12-en-22,29-γ-lactone (**6**),[17,18] 3,11-dioxo-β-amyrene (**7**),[19] 3β,22α-dihydroxyolean-12-en-29-oic acid (**8**),[17,20] 28,-29-dihydroxyfriedelan-3-one (**9**),[21,22] 29-hydroxy-3-oxo-D:A-friedooleanan-28-oic acid (**10**),[23] and putranjivadione (**11**),[24] respectively.

The sesquiterpene polyol esters alkaloids (**2**−**5**) and triterpenes (**6**−**11**) were assayed for cytotoxicity against four cancer cell lines: nasopharynx carcinoma (KB), colon carcinoma (COLO-205), hepatoma (Hepa-3B), and cervical carcinoma (Hela) cells. Only the *trans* configuration between H-8 and -9 in emarginatine-E (**5**) exhibited cytotoxicity against KB (ED_{50} = 1.7 μg/mL) and COLO-205 (ED_{50} = 4.1 μg/mL) cancer cells, whereas the other compounds were inactive, with ED_{50} values > 20 μg/mL. These results are consistent with our previous studies that showed that the H-8α epimer in the sesquiterpene agarofuran skeleton with pyridonyl ester and evoninate moieties is important for the cytotoxicity.[16,25]

To obtain further insight into the biological effects of laxifolone-A (**1**), in the present study we have investigated whether **1** affects NO production in the virus-transformed mouse macrophage-like cell line, RAW 264.7, stimulated with lipopolysaccharide (LPS) plus interferon-γ (IFN-γ). As shown in Figure 1, unstimulated macrophages produced basal levels of nitrite (6.2 ± 2.1 μM). Stimulating the cells with LPS/IFN-γ for 24 h induced a 10-fold increase in nitrite production from the basal level to 62.4 ± 1.1 μM. Compound **1** suppressed LPS/IFN-γ-induced nitrite accumulation in a concentration-dependent manner. The IC_{50} of **1** for inhibition of nitrite production was 0.12 ± 0.03 mg/mL. The compound's effects were significantly distinguished from those of the vehicle (data not shown). (Stock solution of **1** was dissolved in DMSO; thus, vehicle was defined as various concentrations of DMSO ranging from 0.02 to 0.5%, respectively.) Significant inhibition by **1** was observed at 0.05 mg/mL, and greater than 80% inhibition was noted at concentrations ≥0.2 mg/mL. On the basis of MTT reduction experiments, this level of **1** was not toxic to cells since cell viability was still greater than 95% when compared with control basal groups. Further studies on the

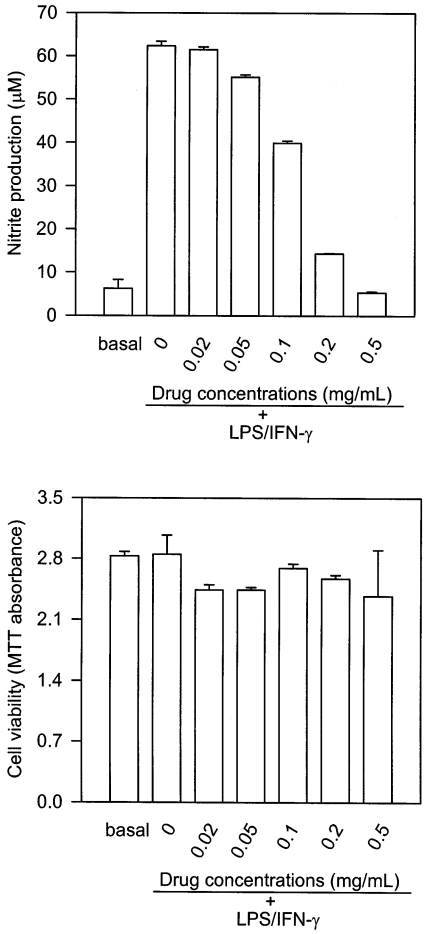


Figure 1. Effect of KELH5 on LPS/IFN-γ-induced nitrite formation and on cell viability by RAW 264.7 macrophages. RAW 264.7 macrophages cultured in medium were stimulated with LPS (1 μg mL^{-1}) plus IFN-γ (50 U mL^{-1}) at 37 °C for 24 h in a 96-well plate in the absence and presence of indicated concentrations of KELH5. Data are expressed as mean ±SEM of six individual experiments (triplicate in each experiment). *P < 0.05 and **P < 0.01 indicate the statistical significance as compared with the group without KELH 5 treatment. (LPS, lipopolysaccharide; IFN-γ, interferon-γ.)

anti-NO activity mechanism as well as a detailed structure−activity relationship remain to be explored.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ACP-300 spectrometer, and 2D NMR are measured on a Varian INOVA 500 MHz spectrometer. Heteronuclear long-range correlation (HMBC) spectra were performed by using coupling constants of 8 Hz. Samples for IR spectral measurements were prepared as KBr disks. EIMS were performed in the electron impact mode (20 eV).

Plant Material. The stems of *Euonymus laxiflorus* Champ. were collected in July 1997 in Taipei County, Taiwan. A voucher specimen was deposited at National Research Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and Isolation. The dried stems of titled plant (12 kg) were extracted exhaustively with ethanol. The crude ethanol syrup was extracted five times with hexane. The ethanol layer was partitioned with *n*-hexane−H_2O (1:1) three times to give *n*-hexane and H_2O layers. After the *n*-hexane layer was evaporated in vacuo, the residue (70 g) was chromatographed on a Si gel column with *n*-hexane−EtOAc (8:1, 6:1, 5:1, 4:1, 2:1, 1:1, EtOAc) to give 12 fractions, fr. 1−12. Fractions 5 (*n*-hexane−EtOAc, 5:1) and 7 (*n*-hexane−EtOAc, 4:1) were recrystallized from MeOH−$CHCl_3$ to yield **1** (6.5 mg) and **7** (10.2 mg), respectively. Fraction 12 was further separated by column chromatography on Si gel eluting with CH_2Cl_2−acetone (10:1, 6:1, 5:1, 4:1, 1:1) to yield 10 fractions, fr. 12-1−12-10. Fraction 12-5 was further chromatographed using HPLC [Si gel, 250 × 10 mm, with *n*-hexane−EtOAc (4:1) v/v] as the eluent to yield compounds **7** (2.5 mg) and **8** (3.1 mg). Moreover, the H_2O layer was partitioned with $CHCl_3$−H_2O (2:1) three times to give $CHCl_3$ and H_2O layers. The condensed $CHCl_3$-soluble layer (92 g) was subjected to column chromatography on Si gel with $CHCl_3$−acetone (7:1, 6:1, 5:1, 4:1, 3:1), and 12 fractions were obtained. Fraction 5 ($CHCl_3$−acetone, 5:1) was further chromatographed using HPLC [Si gel, 250 × 10 mm, with *n*-hexane−EtOAc (3:1) v/v] as the eluent to afford **2** (1.3 mg), **3** (1.5 mg), **4** (2.1 mg), and **5** (4.2 mg). Like **2**−**5**, compounds **10** (3.2 mg) and **11** (2.7 mg) were obtained from fraction 7 ($CHCl_3$−acetone, 4:1) by using HPLC with conditions similar to those above. Fraction 9 was chromatographed on Si gel, eluted with $CHCl_3$−MeOH (20:1) and gradually increasing MeOH to furnished 13 fractions, fr. 9-1−9-13. Furthermore, **6** (15 mg), **7** (1.2 mg), and **8** (4.5 mg) were purified from fraction 9-6 ($CHCl_3$−MeOH, 10:1), fraction 9-7 ($CHCl_3$−MeOH, 9:1), and fraction 9-10 ($CHCl_3$−MeOH, 8:1) by washing with MeOH, respectively.

Laxifolone-A (1): colorless fine crystals from MeOH−$CHCl_3$; mp 279−281 °C; $[\alpha]_D$ +15.0° (*c* 0.5, $CHCl_3$); IR (KBr) cm^{-1} 1710 (CO), 1650; HREI-MS *m*/*z* 454.3810 (calcd for $C_{31}H_{50}O_2$, 454.3813); EI-MS *m*/*z* 454 [M]$^+$, 407, 379, 300, 299, 286, 273, 189, 175; ^{1}H and ^{13}C NMR, see Table 1.

Cytotoxicity Assay. The in vitro cytotoxicity assay were performed as previously described.[26]

Cell Culture and NO Measurement. RAW 264.7 cells were cultured in DMEM containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and grown at 37 °C with 5% CO_2 in fully humidified air. To induce iNOS, fresh culture medium containing LPS (1 μg/mL) plus IFN-γ (50 U/mL) was added. To assay the effect on nitrite production, drugs were added together with LPS/IFN-γ stimulation. Nitrite was measured by adding Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). Then the optical density at 550 nm (OD_{550}) was measured with a microplate reader. Concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium.[27]

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