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Megastigmane and Phenolic Components from *Laurus nobilis* L. Leaves and Their Inhibitory Effects on Nitric Oxide Production

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Laurus nobilis L. leaves are widely used in cooking and in folk medicine. Five new megastigmane glucosides (**2**−**4**, **7**, and **9**) named laurosides A−E and a new phenolic glucoside **12** were isolated from the methanolic extract of *L. nobili*s L. leaves, along with 10 known components: megastigmane (**5**), megastigmane glucosides (**1**, **6**, **8**, **10**, and **11**), aromatic compounds (**13** and **14**), and flavonoids (**15** and **16**). The structures and relative stereochemistry have been elucidated by one- and two-dimensional nuclear magnetic resonance experiments (^{1}H and ^{13}C NMR, DEPT, correlation spectroscopy, heteronuclear multiple quantum correlation, heteronuclear multiple bond correlation, and nuclear Overhauser enhancement spectroscopy) and by chemical derivatization. The effect of isolated compounds on nitric oxide production in lipopolysaccharide-activated murine macrophages were examined.

KEYWORDS: ***Laurus nobilis*** **L.; Lauraceae; lauroside; megastigmane; phenolic glucosides; nitric oxide**

INTRODUCTION

Laurus nobilis L. (fam. Lauraceae) is a very representative plant of the Mediterranean bush. It is also known as laurel, bay laurel, and sweet bay and is the tree laurel of Greek and Roman mythology (*1*). During the Middle Ages, bay leaves were used for medicinal purposes in Europe to treat dyspepsia, colds, and viral infections, and next, leaves and berries, as an herbal medicine, have also been employed (*2*) against rheumatism and as a carminative, diaphoretic, and antiseptic. Leaves are often used in cooking for flavoring and as a spice in marinating and pickling foods (*3*). Previous phytochemical investigations on *L. nobilis* leaves and fruits have resulted in the isolation of sesquiterpene lactones (*4*), alkaloids (*5*), glycosylated flavonoids (*6*), and monoterpene and germacrane alcohols (*7*, *8*).

Roots and leaves are a source of sesquiterpene lactones, and two distinct chemical races were found containing, respectively, laurenobiolide (*9*) and costunolide (*10*) as major compounds. From the essential oil, also called laurel leaf oil, various volatile components were identified (*11*−*13*) with antimicrobial activities against bacteria, yeasts, and some molds (*12*). The in vitro fungistatic effect was also assayed against the fungus *Ascosphaera apis*, the causal agent of chalk brood disease of honey bees (*14*). Volatile components can also be used for the control of stored products and cut flower pests (*15*). The laurel oil is still present in some pharmaceutical preparations, but its topical use has occasionally caused severe contact dermatitis and has been related to the presence of sesquiterpene lactones (*16*, *17*). This oil was reported to be used in the preparation of hair lotion for its antidandruff activity and for the external treatment of psoriasis (*18*). The fresh leaves contain strong antimutagens against Trp-P-2, the heterocyclic amine that exerts genotoxicity (*19*).

Sesquiterpene lactones (*20*) have been isolated from plant sources, and many of them were shown to possess a range of biological and pharmacological activities including antimicrobial (*21*, *22*) and inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages (*23*, *24*). More recently, some of them have aroused particular interest since they showed immunomodulating activities (*24*) and exhibited a potent cytotoxicity against selected human cancer cell lines (*25*).

In the course of our studies on the bioactive constituents of medicinal foodstuffs, we carried out systematic chemical analyses of the methanolic extract of *L. nobilis* L. leaves and the isolated compounds were further tested on the inhibitory effects on NO production in LPS-activated murine macrophages (J774).

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Table 1. ^{1}H and ^{13}C NMR (CD_3OD, 500 and 125 MHz) Data for Compounds **2**–**4**, **7**, and **9**

	2		**3**		**4**		**7**		**9**	
C	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C
1		43.5		43.3		43.4		40.6		37.3
2	2.87 ax d (13.6)	52.0	2.93 d (13.5)	51.8	2.93 d (13.5)	52.0	1.65 t ax (12.0)	45.9	2.57 d eq (16.9)	48.1
	1.82 eq d (13.6)		1.84 d (13.5)		1.84 d (13.5)		1.40 d eq		2.02 d ax (16.9)	
3		214.3		214.3		214.3	3.78 m	67.5		198.9
4	2.46 ax t (13.2)	45.6	2.79 t (13.8)	40.5	2.81 t (13.8)	40.3	1.67 d[b] eq	39.9	6.04 s	124.7
	2.12 eq dd (13.2, 2.2)		2.28		2.25		1.39 t[b] ax			
5	2.30 m	37.8	2.19	42.7	2.21	42.6	1.98 m	35.4		152.6
6		77.7		78.0		78.1		78.0	1.95 m	51.8
7	5.80 d (15.7)	136.5	5.84 d (15.6)	132.8	5.91d (15.5)	135.9	5.68 d (15.7)	136.4	1.70–1.40[b]	27.2
8	5.70 dd (15.7, 7.6)	133.3	6.00 dd (15.6, 6.2)	134.6	5.82 dd (15.5, 7.6)	133.1	5.55 dd (15.7, 8.1)	133.1	1.70–1.40[b]	37.0
9	4.54 quint	74.8	4.47 quint	77.0	4.57 quint	74.0	4.50 quint	74.7	3.84 m	75.5
10	1.31 d (6.4)	21.9	1.34 d (6.4)	20.5	1.34 d (6.4)	21.0	1.29 d (6.4)	21.9	1.18 d (6.8)	19.6
11	0.96 s	24.6	0.95 s	23.7	0.92 s	23.9	0.95 s	25.2	1.01 s	28.8
12	0.91 s	24.7	0.92 s	24.1	0.91 s	24.2	0.87 s	26.2	1.11 s	27.4
13	0.97 d (6.6)	16.4	3.77 dd (10.7, 4.9)	63.9	3.84[b]	64.0	0.88 d (6.7)	16.7	4.35 d (18.0)	65.6
			3.58 dd (10.7, 2.8)		3.62[b]				4.18 d (18.0)	
1′	4.35 d (7.7)	100.4	4.37 d (7.7)	102.2	4.36 d (7.8)	101.0	4.35 d (7.8)	102.4	4.32 d (7.8)	100.8
2′	3.21[b]	74.8	3.19	74.7	3.21[b]	74.9	3.21[b]	75.2	3.21[b]	74.8
3′	3.27[b]	78.0	3.34	77.9	3.32[b]	77.8	3.34[b]	78.1	3.36[b]	77.7
4′	3.32[b]	71.2	3.28	71.1	3.28[b]	71.2	3.26[b]	71.5	3.26[b]	71.2
5′	3.19[b]	77.6	3.22	77.5	3.22[b]	77.6	3.20[b]	78.0	3.20[b]	77.6
6′	3.87 dd (11.9, 2.3)	62.5	3.84	62.5	3.85[b]	62.5	3.84[b]	62.6	3.87[b]	62.4
	3.64 dd (11.9, 6.1)		3.66 dd (11.9, 5.4)		3.66[b]		3.66[b]		3.66[b]	

[a] *J* are given in Hz. [b] Signals overlapped with other signals.

MATERIALS AND METHODS

General Methods. Fast atom bombardment mass spectrometry (FAB-MS), electron ionization mass spectrometry (EI-MS), high-resolution (HR) FAB-MS, and HR-EI-MS were recorded on a Fisons VG Prospec instrument. Optical rotations were determined on a Perkin-Elmer 141 polarimeter.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 500.13 and 125.76 MHz, respectively, on a Bruker AMX-500 spectrometer equipped with a Bruker ×32 computer, using the UXNMR software package. NMR spectra were measured in CD_3OD and $CDCl_3$. Chemical shifts were referenced to the residual solvent signal (CD_3OD, δ_H 3.31 and δ_C 49.0; $CDCl_3$, δ_H 7.26 and δ_C 77.0). The multiplicities of ^{13}C resonances were determined by DEPT experiments. The ^{1}H-detected one-bond and multiple-bond ^{13}C multiple quantum coherence experiments (HMQC and HMBC, respectively) were recorded on a 5 mm probe with reverse geometry and no sample spinning. The magnitude of delay for optimizing one-bond correlation in the HMQC spectrum and suppressing them in the HMBC spectrum was set to 3.5 ms, and the evolution delay for long-range couplings in the latter was set to 60 ms. Droplet counter-current chromatography (DCCC) was performed on a DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 250 tubes. High-performance liquid chromatography (HPLC) was performed by a Waters 510 pump equipped with a Waters U6K injector and a differential Waters 401 refractometer, using (30 cm × 3.9 mm i.d.) Nucleosil 100-5 (ET 200/4 Macherey-Nagel) and C_{18} μ-Bondapak (Waters) columns.

Plant Material. The leaves of *L. nobilis* L. were collected in the hills of Campania (Avellino, Italy) during the summertime of 1999 and identified at the Dipartimento di Scienze Animali, Vegetali e dell'Ambiente (University of Molise). A voucher specimen (LN73-99) is preserved at the Dipartimento di Scienze e Tecnologie per l'Ambiente e il Territorio dell'Università del Molise (Isernia, Italy).

Extraction and Isolation. The fresh leaves of *L. nobilis* L. (404 g) were chopped and soaked in MeOH at room temperature (11.5 L). Evaporation of MeOH extracts afforded 32.5 g of a glassy material, which was then subjected to a modified Kupchan's partitioning methodology (*26*) to give four extracts: *n*-hexane (6.3 g), CCl_4 (0.3 g), $CHCl_3$ (5.7 g), and *n*-BuOH (5.2 g). The $CHCl_3$ extract (5.7 g) was separated by medium-pressure liquid chromatography on silica gel (150 g) using *n*-hexane containing increasing amounts of EtOAc as the eluent. The fractions were monitored by thin-layer chromatography (TLC) on SiO_2 using *n*-hexane/EtOAc (1:1 and 4:6) and $CHCl_3$/MeOH (1:1) as eluents. Fractions eluted with *n*-hexanes−EtOAc (85:15) gave 1.17 g of costunolide. Further separation of the above fractions was obtained by HPLC on a Nucleosil 100-5 column with $CHCl_3$ as the eluent, to give the pure sesquiterpene lactones santamarine and reynosin along with **15** and **16**.

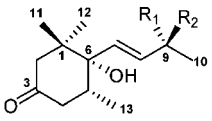
The *n*-BuOH extract (5.2 g) was chromatographed by DCCC using *n*-BuOH/Me_2CO/H_2O (3:1:5) in the descending mode (the upper phase was the stationary phase) and a flow rate of 18 mL/h; 6 mL fractions were collected and monitored by TLC on SiO_2 with *n*-BuOH/HOAc/H_2O (12:3:5) and $CHCl_3$/MeOH/H_2O (80:18:2) as eluents. Six major fractions (A−F) were obtained; fractions A−E were then purified by HPLC on a C_{18} μ-Bondapak column with MeOH/H_2O (2:8), while for fraction F the eluent was MeOH/H_2O (25:75). Fraction A gave compounds **3** and **13**; fraction B contained mainly **4**, **8**, and **12**; fraction C yielded **6** and **11**; fraction D gave **1**, **7**, and **9**; fraction E was composed mainly of **5**, **10**, and **14**; fraction F contained **2** and **14**.

Cell Cultures. The murine monocyte/macrophages cell line J774 was from ECACC (European Collection of Cell Cultures). J774 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker) and cultured at 37 °C in humidified 5% CO_2/95% air. The cells were plated in 24 well culture plates (Falcon) at a density of 2.5 × 10^6 cells/mL/well and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium and cells were activated by LPS (0.1 μg/mL) from *Escherichia coli* (Fluka). Thirty minutes before LPS, test compounds were added to cells at various concentrations ranging from 0.003 up to 30 μg/mL. After 24 h, the culture medium was removed and centrifuged and the supernatant was used for the determination of nitrite (NO_2^-) production. Cell viability (>95%) was determined with the MTT assay (*27*).

NO_2^- Assay. NO_2^- levels in culture media from J774 macrophages were measured 24 h after LPS challenge with the Griess reaction as previously described (*28*). Results are expressed as nmol/mL of NO_2^- and represent the means ± standard errors of the means (SEM) of three experiments run in triplicate (**Figure 3**). Comparisons were calculated by one-way analysis of variance and Bonferroni-corrected *P* value for multiple comparisons. The level of statistically significant differences was defined as $P < 0.05$.

Ampelopsisionoside (1). Yield, 3.0 mg; $[\alpha]_D^{25}$ −4.8° (c 0.2, MeOH). FAB-MS *m/z* 387 $[M - H]^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H (ppm) 5.91 (1H, dd, J = 15.7, 6.7 Hz, H-8), 5.73 (1H, d, J = 15.7 Hz, H-7),

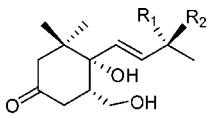
4.44 (1H, quint, H-9), 4.35 (1H, d, J = 7.6 Hz, H-1′), 3.84 (1H, dd, J = 11.9, 2.3 Hz, H-6′), 3.64 (1H, dd, J = 11.9, 6.1 Hz, H-6″), 3.35 (1H, overlapped, H-3′), 3.31 (1H, overlapped, H-4′), 3.22−3.18 (2H, overlapped, H-5′ and H-2′), 2.87 (1H, d, J = 13.6 Hz, H-2 ax), 2.43 (1H, t, J = 13.2 Hz, H-4 ax), 2.28 (1H, m, H-5), 2.11 (1H, dd, J = 13.2, 2.2 Hz, H-4 eq), 1.82 (1H, d, J = 13.6 Hz, H-2 eq), 1.32 (3H, d, J = 6.4 Hz, H-10), 0.99 (3H, s, H_3-11), 0.93 (3H, s, H_3-12), 0.90 (3H, d, J = 6.6 Hz, H_3-13). ^{13}C NMR (125 MHz, CD_3OD): δ_C (ppm) 214.9 (C-3), 134.8 (C-8), 133.7 (C-7), 77.8 (C-6), 77.6 (C-9), 52.4 (C-2), 45.9 (C-4), 43.9 (C-1), 37.7 (C-5), 24.8 (C-11), 25.2 (C-12), 21.4 (C-10), 16.3 (C-13). δ_C glucose: 102.5 (C-1′), 77.8 (C-3′), 77.7 (C-5′), 75.1 (C-2′), 71.3 (C-4′), 62.5 (C-6′).



1: R_1= O-Gluc R_2= H
2: R_1= H R_2= O-Gluc

Lauroside A (2). Yield, 2.5 mg; $[\alpha]_D^{25}$ −30.6° (c 0.25, MeOH). FAB-MS m/z 389 $[M + H]^+$. HR-FAB-MS m/z 389.2260 $[M + H]^+$ (calcd for $C_{19}H_{33}O_8$, 389.2175). ^{1}H and ^{13}C NMR in **Table 1**.

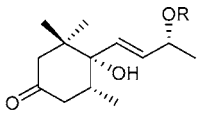
Lauroside B (3). Yield, 1.5 mg; $[\alpha]_D^{25}$ −93.7° (c 0.1, MeOH). HR-FAB-MS m/z 405.2254 $[M + H]^+$ (calcd for $C_{19}H_{33}O_8$, 405.2125). ^{1}H and ^{13}C NMR in **Table 1**.



3: R_1= O-Gluc R_2= H
4: R_1= H R_2= O-Gluc

Lauroside C (4). Yield, 0.9 mg; $[\alpha]_D^{25}$ −18.8° (c 0.09, MeOH). FAB-MS m/z 405 $[M + H]^+$. HR-FAB-MS m/z 405.2270 $[M + H]^+$ (calcd for $C_{19}H_{33}O_9$, 405.2125). ^{1}H and ^{13}C NMR in **Table 1**.

4,5-Dihydroblumenol A (5). Yield, 5.3 mg; $[\alpha]_D^{25}$ −1.32° (c 0.4, MeOH). EI-MS m/z 226 $[M]^+$. ^{1}H NMR (500 MHz, CD_3OD): δ_H (ppm) 5.83 (1H, dd, J = 15.8, 5.9 Hz, H-8), 5.66 (1H, d, J = 15.8 Hz, H-7), 4.34 (1H, quint, H-9), 2.87 (1H, d, J = 13.4 Hz, H-2 ax), 2.45 (1H, t, J = 13.4 Hz, H-4 ax), 2.27 (1H, m, H-5), 2.13 (1H, dd, J = 13.4, 2.1, H-4 eq), 1.82 (1H, dd, J = 13.4, 2.0 Hz, H-2 eq), 1.27 (3H, d, J = 6.4 Hz, H_3-10), 0.98 (3H, s, H_3-11), 0.92 (3H, s, H_3-12), 0.90 (3H, d, J = 6.6 Hz, H_3-13). ^{13}C NMR (125 MHz, CD_3OD): δ_C (ppm) 214.6 (C-3), 135.3 (C-8), 133.8 (C-7), 78.0 (C-6), 69.4 (C-9), 52.2 (C-2), 45.9 (C-4), 43.8 (C-1), 37.5 (C-5), 25.9 (C-11), 25.2 (C-12), 24.2 (C-10), 16.3 (C-13).



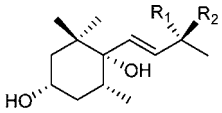
Compound **5**: R= H
Compound **5a**: R= (*R*)-MTPA
Compound **5b**: R= (*S*)-MTPA

Preparation of (*R*)- and (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)-phenylacetyl Chloride (MTPA) Esters (5a,b) from 5. A solution of **5** (1 mg) in 1 mL of dry CH_2Cl_2 was reacted with MTPA-Cl (5 μL) in the presence of triethylamine (10 μL) and a catalytic amount of 4-*N*,*N*′-(dimethylamino)pyridine (DMAP), and the mixture was stirred at 25 °C for 30 min. After the solvent was removed, the mixture was purified by Si gel column chromatography (a Pasteur pipet filled with a slurry of Si gel) using $CHCl_3$ as the eluent to afford **5a**. Compound **5b** was then prepared through a similar procedure from **5** (1 mg) using (−)-MTPA-Cl (5 μL), tetraethylammonium (10 μL), and 4-DMAP.

4,5-Dihydroblumenol A-9-(*R*)-MTPA Ester (5a). EI-MS m/z 442 $[M]^+$. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H (ppm) 7.30−7.58 (5H, aromatic protons), 5.831 (1H, d, J = 16.0 Hz, H-7), 5.822 (1H, dd, J = 16.0, 6.0 Hz, H-8), 5.645 (1H, quint., J = 6.0 Hz, H-9), 3.53 (3H, s, -OCH_3), 2.807 (1H, d, J = 13.4 Hz, H-2 ax), 2.394 (1H, t, J = 13.4 Hz, H-4 ax), 2.231 (1H, m, H-5), 1.913 (1H, d, J = 13.4 Hz, H-2 eq), 1.420 (1H, d, J = 6.5 Hz, H-10), 0.902 (3H, s, H_3-12), 0.915 (3H, s, H_3-11), 0.855 (3H, d, J = 6.5 Hz, H_3-13).

4,5-Dihydroblumenol A-9-(*S*)-MTPA Ester (5b). EI-MS m/z 442 $[M]^+$. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H (ppm) 7.32−7.58 (5H, aromatic protons), 5.734 (1H, d, J = 15.8, H-7), 5.660 (1H, dd, J = 15.8, 5.6, H-8), 5.666 (1H, quint, H-9), 3.53 (3H, s, -OCH_3), 2.788 (1H, d, J = 13.2 Hz, H-2 ax), 2.392 (1H, t, J = 13.2 Hz, H-4 ax), 2.250 (1H, m, H-5), 1.895 (1H, d, J = 13.2 Hz, H-2 eq), 1.463 (3H, d, J = 6.5 Hz, H_3-10), 0.874 (3H, s, H_3-11), 0.853 (3H, s, H_3-12), 0.821 (3H, d, J = 6.8 Hz, H_3-13).

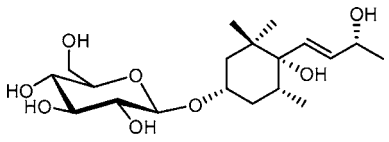
Alangioside A (6). Yield, 2.2 mg; $[\alpha]_D^{25}$ −9.5° (c 0.22, MeOH). FAB-MS m/z 389 $[M - H]^-$. ^{1}H NMR (500 MHz, CD_3OD): δ_H (ppm) 5.79 (1H, dd, J = 15.8, 7.2 Hz, H-8), 5.61 (1H, d, J = 15.8 Hz, H-7), 4.40 (1H, quint, H-9), 4.35 (1H, d, J = 7.8 Hz, H-1′), 3.82 (1H, dd, J = 12.0, 2.8 Hz, H-6″), 3.77 (1H, H-3), 3.65 (1H, dd, J = 12.0, 5.0 Hz, H-6′), 3.32 (1H, t, H-3′), 3.20 (1H, t, H-5′), 3.17 (1H, dd, overlapped, H-2′), 1.94 (1H, m, H-5), 1.67 (1H, t, H-4 eq), 1.65 (1H, t, H-2 ax), 1.40 (1H, H-2 eq), 1.39 (1H, H-4 ax), 1.30 (3H, d, J = 6.2 Hz, H_3-10), 0.98 (3H, s, H_3-11), 0.89 (3H, s, H_3-12), 0.81 (3H, d, J = 6.8 Hz, H_3-13). ^{13}C NMR (125 MHz, CD_3OD): δ_C (ppm) 135.6 (C-8), 133.7 (C-7), 78.5 (C-6), 78.0 (C-9), 67.5 (C-3), 46.0 (C-2), 40.8 (C-1), 40.0 (C-4), 35.6 (C-5), 26.3 (C-12), 25.4 (C-11), 21.5 (C-10), 16.6 (C-13). δ_C glucose: 102.3 (C-1′), 78.0 (C-3′), 77.8 (C-5′), 75.1 (C-2′), 71.3 (C-4′), 62.6 (C-6′).



6: R_1= O-Gluc R_2= H
7: R_1= H R_2= O-Gluc

Lauroside D (7). Yield, 1.4 mg; $[\alpha]_D^{25}$ −10.2° (c 0.1, MeOH). FAB-MS m/z 391 $[M + H]^+$. HR-FAB-MS (calcd for $C_{19}H_{35}O_8$, 391.2332). ^{1}H and ^{13}C NMR in **Table 1**.

Dendranthemoside A (8). Yield, 1.8 mg; $[\alpha]_D^{25}$ −40.2° (c 0.18, MeOH). EI-MS m/z 390 $[M]^+$.



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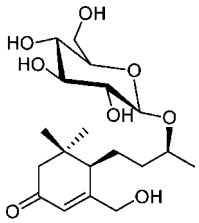
Lauroside E (9). Yield, 1.8 mg; $[\alpha]_D^{25}$ +20.0° (c 0.18, MeOH). ^{1}H and ^{13}C NMR in **Table 1**.



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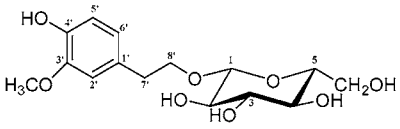

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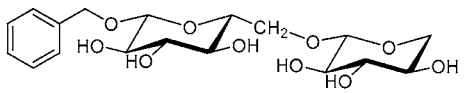

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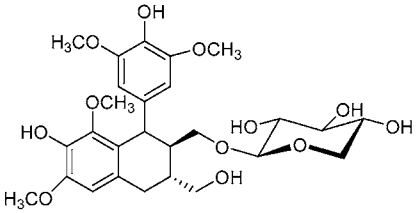

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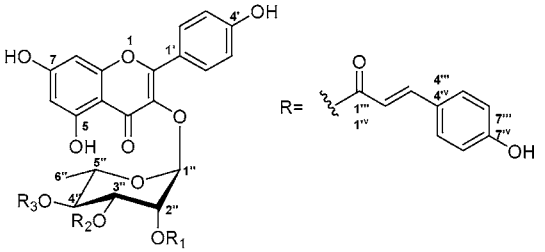
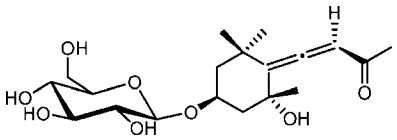


15: R_1 = H, R_2 = R_3 = E-*p*-coumaroyl
16: R_1 = E-*p*-coumaroyl, R_2 = R_3 = H

Figure 1. Bioactive compounds isolated from *L. nobilis* L. leaves.

Icariside B1 (10). Yield, 1.4 mg; $[\alpha]_D^{25}$ −50.0° (c 0.14, MeOH). FAB-MS *m/z* 385 $[M - H]^-$.



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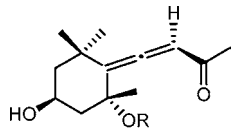
Citroside A (11). Yield, 1.5 mg; $[\alpha]_D^{25}$ −66.2° (c 0.15, MeOH). FAB-MS *m/z* 385 $[M - H]^-$.



11: R= glucose

Compound 12. Yield, 1.3 mg; $[\alpha]_D^{25}$ −12.5° (c 0.13, MeOH). HR-FAB-MS *m/z* 331.1480 $[M + H]^+$ (calcd for $C_{15}H_{23}O_8$, 331.1393). ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.86 (1H, d, J = 2.0 Hz, H-2′), 6.68 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 6.70 (1H, d, J = 8.0 Hz, H-5′), 4.31 (1H, d, J = 7.8 Hz, H-1), 4.06 (1H, m, H-8′a), 3.87 (1H, dd, J = 11.8, 2.1 Hz, H-6a), 3.83 (3H, s, -OCH_3), 3.73 (1H, m, H-8′b), 3.66 (1H, dd, J = 11.8, 6.0 Hz, H-6b), 3.35 (1H, overlapped, H-3), 3.28 (1H, overlapped, H-4), 3.27 (1H, overlapped, H-5), 3.20 (1H, t, J = 7.8 Hz, H-2), 2.86 (2H, br t, H2−7′). ^{13}C NMR (125 MHz, CD_3OD): δ_C (ppm) 148.9 (C-3′), 146.0 (C-4′), 131.8 (C-1′), 122.5 (C-6′), 116.2 (C-5′), 114.0 (C-2′), 104.4 (C-1), 78.2 (C-3), 78.0 (C-5), 75.2 (C-2), 72.0 (C-8′), 71.8 (C-4), 62.9 (C-6), 56.6 (-OCH_3), 36.8 (C-7′).

Compound (13). Yield, 1.5 mg; $[\alpha]_D^{25}$ −58.2° (c 0.15, MeOH). FAB-MS *m/z* 403 $[M + H]^+$.

Lyoniside (14). Yield, 0.9 mg; $[\alpha]_D^{25}$ +13.3° (c 0.09, MeOH). FAB-MS *m/z* 553 $[M + H]^+$.

Kaempferol-3-*O*-α-L-(3″,4″-di-E-*p*-coumaroyl)rhamnoside (15). Yield, 1.1 mg. FAB-MS *m/z* 725 $[M + H]^+$.

Kaempferol-3-*O*-α-L-(2″-E-*p*-coumaroyl)rhamnoside (16). Yield, 0.9 mg. FAB-MS *m/z* 579 $[M + H]^+$.

RESULTS AND DISCUSSION

The 1-butanol soluble portion of the methanol extract of the *L. nobilis* L. leaves was subjected to DCCC and HPLC purification to furnish the new megastigmane glucosides **2**−**4**, **7**, and **9**, named laurosides A−E, together with **1** (*29*, *30*), **5** (*31*), **6** (*32*, *33*), **8** (*34*, *35*), **10** (*36*), and **11** (*37*). Aromatic components were also isolated from butanol extract as a new 2-(4-hydroxy-3-methoxyphenyl)-ethyl-*O*-β-D-glucopyranoside (**12**) (*38*), and by spectroscopic evidence, benzyl alcohol xylopyranosyl(1→6)glucopyranoside (**13**) (*39*) and lyoniside, a lignan glycoside (**14**) (*40*), previously isolated from other plant species (**Figure 1**), were identified. C_{13} norisoprenoids are important aroma constituents in many essential oils. Recent studies suggested that many of those compounds are present in plants as glycosides, from which they can be released during fruit maturation, storage, or processing by enzyme action, and they play an important role as flavor precursors (*41*, *42*).

Purification of the chloroform soluble fraction led to the isolation of **15** (*43*) and **16** (*43*, *44*). Sesquiterpene lactones were also isolated from $CHCl_3$ and *n*-hexane extracts (unpublished data).

The structure of **1** was elucidated by correlation spectroscopy (COSY), HMQC, and HMBC experiments. The NMR spectra indicated the presence of a megastigmane skeleton and a glucopyranose unit and match well with data previously reported (*29*, *33*) but are quite different in some values from another report (*30*) (see the Materials and Methods).

Compound **2** showed a molecular formula of $C_{19}H_{32}O_8$ and was found to be the isomer of **1** at C-9. A comparative analysis of ^{1}H and ^{13}C NMR and ^{1}H−^{1}H COSY spectra of **2** with those of **1** suggested the presence of a megastigmane skeleton with a carbonyl group at C-3, hydroxyl functional groups at C-6 and C-9 positions, and a disubstituted double bond. The 5β-H orientation was assigned by analysis of the nuclear Overhauser enhancement spectroscopy (NOESY) experiment. Correlations of H-5 with H-7 and H-5 with CH_3-11 suggested that CH_3-13 had an equatorial orientation. The axial protons at the 2- and 4-position were shifted downfield due to the 1,3-diaxial interaction of the hydroxyl group at the C-6 position. Therefore, the 6-position must have the *S* configuration. In the ^{13}C NMR spectra of **1** and **2** (**Table 1**), the signals due to the carbohydrate moiety were identical and assigned to a glucose unit, in the β-configuration, linked at C-9. However, ^{1}H NMR analysis of glycosides **1** and **2** revealed several significant chemical shift differences of signals near to the C-9 asymmetric center (positions 7−10) and relative to CH_3-13 suggesting that **1** and **2** might be diastereoisomers. One of the most relevant differences between the two glycosides consisted of the inversion of the chemical shift of the olefinic protons 7 and 8. We also observed in **2** a downfield shift of the CH_3-13 signal (δ_H 0.97) with respect to data for the glycoside **1** (δ_H 0.90). It is known that in a β-D-glucosylation of a secondary hydroxyl group, different upfield shifts are observed for adjacent carbon atoms

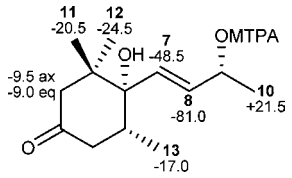


Figure 2. Results of the modified Mosher's method for **5**. The $\Delta\delta$ values are in Hz (δS-δR, 500 MHz).

depending on the chirality of the hydroxyl-bearing carbon (*45*). Pabst et al. (*46*) reported ^{13}C NMR chemical shifts of the β-D-glucopyranoside of (9*R*)- and (9*S*)-3-oxo-α-ionol to be δ_C 77.0 and 74.7, respectively. In **2**, the C-9 chemical shift was observed at δ_C 74.8. This finding suggested that the structure of **2** was (5*R*,6*S*,9*S*,7*E*)-megastigman-3-one-7-en-9,6-diol 9-*O*-β-D-glucopyranoside and was given the trivial name lauroside A.

Compound **3** was isolated as an amorphous powder, and its elemental composition, $C_{19}H_{32}O_9$, was deduced from ^{13}C NMR data and by HR-FAB-MS. It was found that the spectroscopic data (^{1}H and ^{13}C NMR) of **3** were similar to those of **1** except that the NMR signal for the CH_3-13 in **1** was missing and instead the resonance for a hydroxymethylene was observed in the ^{1}H NMR (δ_H 3.58 dd and 3.77 dd) and in the ^{13}C NMR (δ_C 63.7). On the basis of ^{1}H−^{1}H COSY, HMQC, and HMBC experiments, the connectivities of **3** could be established, and thus, the β-D-glucopyranose was located at the C-9 position. The relative stereochemistry was further determined on the basis of the results of a NOESY experiment. Key NOE correlations for **3** showed NOE interactions between H-5 and H-7 and H-5 with CH_3-11 revealing the α-orientation of the hydroxymethylene group. The absolute configuration at C-9 was expected to be *R* from the β-D-glucosylation-induced shift trend in the ^{13}C NMR spectrum (*46*) (**Table 1**).

Compound **4** showed a molecular formula, $C_{19}H_{32}O_9$, identical to that of **3** indicating that **3** and **4** are isomers. The six-membered ring skeleton and the β-D-glucose unit, elucidated by one- and two-dimensional NMR spectroscopy, were identical in both compounds. However, spectroscopic data (^{1}H and ^{13}C NMR) of the side chain, from C-7 to C-10, were shifted from **3**, and also, the chemical shift of oxymethylene protons at C-13 (δ_H 3.62 dd and 3.84 dd) differs from the data for **3** (**Table 1**). These values indicated that **3** and **4** could have a different absolute configuration at the C-9 position. Furthermore, the 9*S* configuration was assigned from the ^{13}C NMR chemical shifts according to Pabst et al. (*46*) and comparison with data observed for **2**.

The ^{1}H and ^{13}C NMR spectra of compound **5** were identical to those of the aglycone of **1**, previously isolated from *Perrottetia multiflora* as 4,5-dihydroblumenol A (*47*). ^{1}H NMR signals for 7−10 and 13-positions are closer to the data of **1** than the data for the isomer **2**. However, for an isolated chiral center at C-9, it is extremely difficult to determine the absolute configuration by conventional spectroscopic methods. The absolute configuration of C-9 was determined to be *R* by a modified Mosher's method after esterification with MTPA (*48*) as shown in **Figure 2** ($\Delta\delta S$-δR values are in parentheses). Recently, megastigmane glycosides with a 9*S* configuration have been reported from leaves of *Glochidion zeylanicum* (*49*) and elucidated by a modified Mosher's method.

Compound **7** showed in the HR-FAB-MS an intense ion peak at *m*/*z* 391.2452 $[M + H]^+$, which, supported by NMR data, suggested the empirical formula to be $C_{19}H_{34}O_8$, identical to that of **6** (*32*). The NMR spectra disclosed signals typical of a megastigmane glucoside, and the chemical shifts due to a six-membered ring were consistent with the 3*S*,5*R*,6*S* stereochemistry observed in alangioside A (*32*) and deduced from NMR data. The structures of the sugar moiety proved to be identical to those of compound **1** and **2**. The remaining chiral center at C-9 was elucidated to be the *S* configuration by comparison to the ^{13}C NMR chemical shift of C-9 (δ_C 74.7) with those of **6** (δ_C 78.0), which is known to possess the 9*R* configuration (*32*, *33*).

The elemental composition of **9** was determined as $C_{19}H_{32}O_8$ based on the positive FAB mass spectrum, which indicated a peak at *m*/*z* 411 $[M + Na]^+$. The ^{1}H and ^{13}C NMR spectroscopic data indicated it to be a megastigmane glucoside very similar to the cyclohexenone glucoside previously isolated from barley roots (*50*) and from mycorrhizal roots of tobacco and tomato (*51*). The chemical shifts for the signals attributed to the ring system and for the β-glucose were coincident with those reported

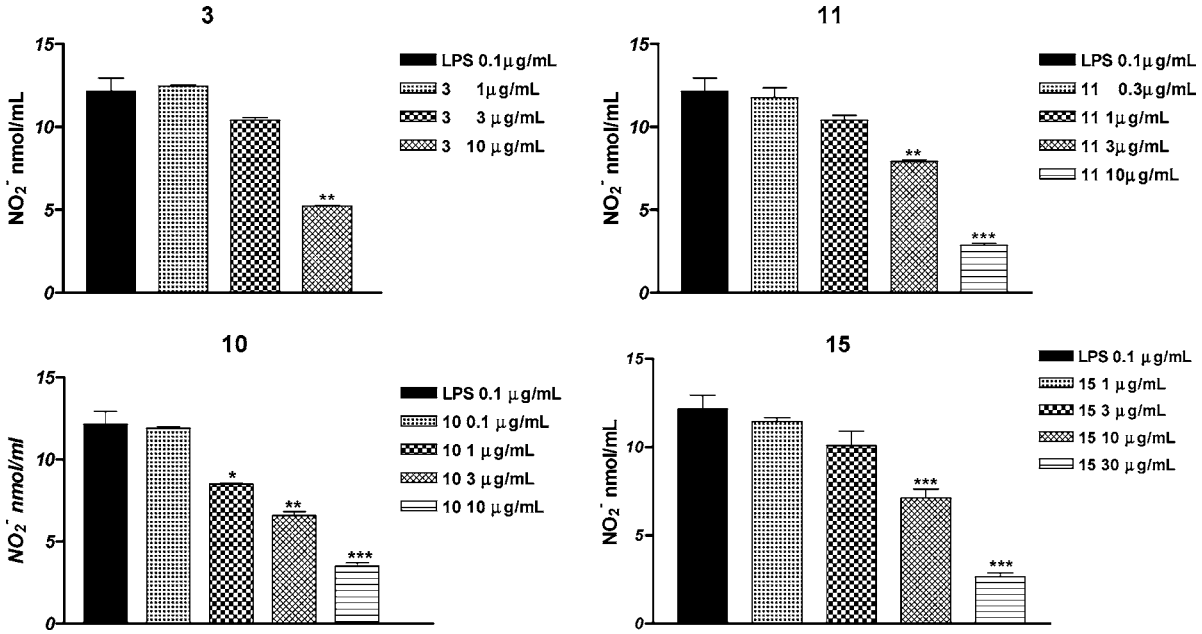


Figure 3. Effects of test compounds on NO_2^- release by LPS-stimulated J774 cell lines. Cells were treated as described in the Materials and Methods. Results are expressed as means ± SEM of three experiments run in triplicate. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 vs LPS.

by Peipp et al. (*50*), deduced from ^{1}H and ^{13}C NMR data. However, significant differences were detected for the resonances due to C-9 (δ_C 75.5) and to C-10 (δ_C 19.6), which are consistent with the 9*S* stereochemistry as observed for the byzantionoside B (*52*). The corresponding signals were observed at δ_C 77.5 and δ_C 21.9, respectively, in blumenol C glucoside (*46*, *52*, *53*), which has the *R* configuration at C-9.

Compound **12** showed in its mass spectrum (FAB-MS) a molecular ion peak at *m*/*z* 331 $[M + H]^+$. The ^{1}H and ^{13}C NMR data supported the presence of a β-glucose unit and a phenyl ethanoid system quite similar to those reported for a component of the bark of *Prunus grayana* (*38*). In **12**, the phenolic -OH at C-3 was missing and replaced by a -OCH_3 group (δ_H 3.83; δ_C 56.6). The ^{1}H NMR spectrum exhibited the aromatic protons H′-2, H′-5, and H′-6 (δ_H 6.86, 6.70, and 6.68, respectively) and two methylene protons attributable to H_2′-7 (δ_H 2.85 and δ_H 2.83) and H_2′-8 (δ_H 4.06 dd and 3.73 dd). Further analysis of ^{1}H−^{1}H COSY, HMQC, and HMBC experiments led us to locate the β-D-glucose at C-8 (72.0 ppm).

NO_2^- Production by J774.2 Macrophages. As a part of our characterization studies on the bioactive components of natural medicine, we report here a screening for the effect of the isolate constituents from *L. nobilis* L. on NO production from LPS-activated murine macrophages. The results are summarized in **Figure 3**.

NO, a short-lived mediator, is synthesized by a family of enzymes termed NO synthase (NOS) (*54*). Two types of NOS are recognized as follows: constitutive isoforms (endothelial NOS and neuronal NOS) and an inducible isoform for which mRNA translation and protein synthesis are required (*54*, *55*). Inducible NOS (iNOS) is regulated by inflammatory mediators (LPS and cytokines) (*56*), and the excessive production of NO by iNOS has been implicated in the pathogenesis of the inflammatory response (*57*−*59*). We measured the production of NO_2^- (stable metabolites of NO) as a parameter of macrophages activation and iNOS induction. Unstimulated J774 cells generated undetectable (<5 nmol/mL) amounts of NO_2^-. Stimulation of the cells with LPS (0.1 μg/mL) produced a release of NO_2^- (12.1 ± 0.8 nmol/mL, $n = 25$). When the cells were incubated with compounds **3**, **10**, **11**, and **15**, a significant inhibition of NO_2^- release was observed (**Figure 3**). None of the concentrations tested resulted in cytotoxicity (data not shown). All of the other compounds tested (**1**, **8**, **9**, **12**, and **13**) resulted in inactive cytotoxicities. Further study is needed to investigate the molecular mechanisms underlying the activity of these compounds.

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