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New Isoflavonoid Glycosides and Related Constituents from Astragali Radix (*Astragalus membranaceus*) and Their Inhibitory Activity on Nitric Oxide Production

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Supporting Information

ABSTRACT: Twenty-four secondary metabolites, including 16 isoflavonoids, 7 astragalasides, and 1 benzoquinone, have been isolated from the roots of *Astragalus membranaceus* (Astragali radix). Among these isolated isoflavonoids, (−)-methylinissolin 3-*O*-*β*-D-(6′-acetyl)-glucoside (**1**), (−)-methylinissolin 3-*O*-*β*-D-{6′-[(*E*)-but-2-enoyl]}-glucoside (**2**), and calycosin 7-*O*-*β*-D-(6″-acetyl)-glucoside (**3**) have been identified as new compounds on the basis of spectroscopic analysis; (−)-methylinissolin 3-*O*-*β*-D-glucoside (**4**) was isolated from the natural products for the first time. The nitric oxide (NO) production inhibitory activity of the major compounds has been assessed in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. To identify *A. membranaceus*, a fingerprint method was developed by using a high-performance liquid chromatography−evaporative light scattering detector (HPLC-ELSD) method. Furthermore, characteristic peaks for the 11 major compounds in the chromatogram were unambiguously confirmed.

KEYWORDS: Astragali radix, nitric oxide production, isoflavonoids, astragalasides

INTRODUCTION

Astragali radix (Huang-qi) is the root of *Astragalus membranaceus* Bunge, which belongs to the family Leguminosae, and is one of the most frequently used crude drugs for oriental medicine in China, Taiwan, Japan, Korea, and other Asian areas. It was first reported in the ancient Chinese medical text, *Ben Cao Gang Mu*, and mainly used as a tonic, especially for strengthening the spleen and lung functions.[1] Pharmacological investigations of *A. membranaceus* crude extract have demonstrated its promising improvements on the human immune system, sperm motility, chronic fatigue syndrome (CFS), kidney disease, diabetes, and high blood pressure.[2−5] The U.S. Dietary Supplement Health and Education Act (DSHEA) of 1994 (103rd Congress, 1994) has classified *A. membranaceus* as a dietary supplement; thus, Astragali tea and capsules are being sold as over-the-counter dietary supplements in the U.S. health food market.

Recently, most of the constituents including triterpene saponins (as astragalosides), isoflavonoids, and polysaccharides were isolated from *A. membranaceaus*, and their bioactive effects were also investigated. The astragalosides (glycosides of 20,24-epoxy- and 20,25-epoxycycloartanes) showed a remarkable IL-2 inducing activity, which might be involved in the mechanism for the immunomodulatory and anticancer effects.[6] Isoflavonoids from *A. mongholicus* protected PC12 cells from toxicity induced by L-glutamate,[7] and they are currently receiving much attention due to their various health benefits. It has been suggested that these beneficial effects of isoflavonoids might be, at least in part, mediated by their antioxidant activity. *A. membranaceus* could exhibit both in vitro and in vivo antitumor effects, which might be achieved through activating the antitumor immune mechanism of the host.[8] In addition, the aqueous extract of Astragali radix could reduce the suppression of macrophage cell proliferation induced by MTX and induce IL-1α, IL-1β, and IL-6 mRNA expressions in RAW 264.7 macrophage cells; it also inhibited NO production in LPS-stimulated RAW 264.7 macrophage cells, and the anti-NO production may be associated with the inhibition of iNOS mRNA expression.[9] As far as we know, there is no report concerning the anti-NO components from *A. membranaceus*.

The above findings motivated us to investigate the biological constituents from the 95% EtOH extracts of *A. membranaceus*. Bioassay-directed fractionation led to the isolation and characterization of 3 new isoflavonoid glycosides as (−)-methylinissolin 7-*O*-*β*-D-(6′-acetyl)-glucoside (**1**), (−)-methylinissolin 7-*O*-*β*-D-{6′-[(*E*)-but-2-enoyl]}-glucoside (**2**), and calycosin 7-*O*-*β*-D-(6″-acetyl)-glucoside (**3**), respectively, along with 7 astragalasides

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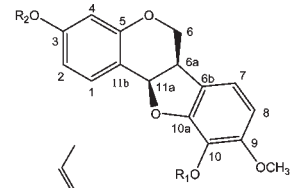
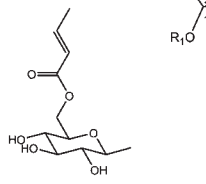
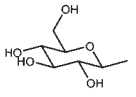
Chart 1



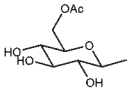
compound	R_1	R_2
1	CH_3	β-D-(6-acetyl)-glucose
2	CH_3	β-D-[6-(*E*)-but-enoyl]-glucose
4	CH_3	β-D-glucose
17	H	β-D-glucose
22	H	H
24	CH_3	CH_3



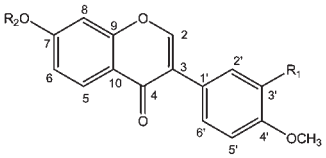
β-D-[6-(*E*)-but-2-enoyl]-glucose



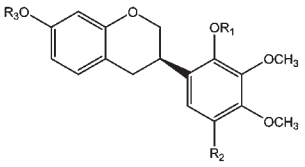
β-D-glucose



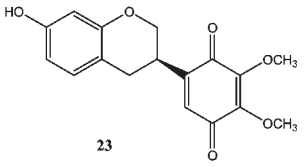
β-D-(6-acetyl)-glucose



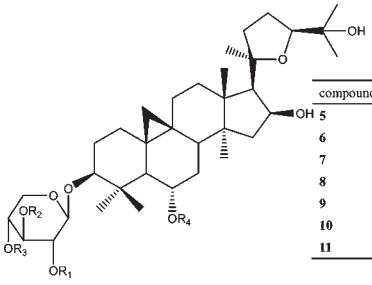
compound	R_1	R_2
3	OH	β-D-(6-acetyl)-glucose
12	H	β-D-glucose
13	H	β-D-(6-acetyl)-glucose
14	H	β-D-[6-(*E*)-but-enoyl]-glucose
15	OH	β-D-glucose
16	OH	β-D-[6-(*E*)-but-enoyl]-glucose
21	OH	H



Compound	R_1	R_2	R_3
18	CH_3	OH	H
19	H	H	β-D-glucose
20	H	H	H



23



compound	R_1	R_2	R_3	R_4
5	Ac	Ac	H	β-D-glucose
6	Ac	H	Ac	β-D-glucose
7	Ac	H	H	β-D-glucose
8	H	Ac	H	β-D-glucose
9	β-D-glucose	H	H	H
10	H	H	H	β-D-glucose
11	H	H	Ac	β-D-glucose

(**5**−**11**), 13 isoflavonoids (**4**, **12**−**22**, and **24**), and 1 benzoquinone (**23**). Isoflavonoid (−)-methylinissolin 7-*O*-β-D-glucoside (**4**) was isolated from the natural source for the first time. The astragalasides (**5**−**11**) and 9 isoflavonoids (**1**−**4** and **12**−**16**; structures of all compounds are given in Chart 1) were tested for their inhibitory effects on NO production in LPS-stimulated RAW 264.7 macrophage cells. The HPLC-ELSD fingerprint profile of bioactive *A. membranaceus* fraction was also established.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were obtained on a JASCO P-1020 polarimeter. CD spectra were measured with a JASCO J-715 spectropolarimeter. UV spectra were measured with a GBC 918 spectrophotometer. IR spectra were recorded as KBr disks, using an IR-FT Mattson Genesis II spectrometer (Thermo Nicolet, Madison, WI). NMR spectra were recorded using a Bruker UltraShield 400 MHz spectrometer. Electrospray ionization mass spectrometry data were obtained on an ESI trap tandem mass spectrometer (Thermo Finnigan LCQ-Duo, San Jose, CA). High-resolution ESIMS were determined using a Shimadzu IT-TOF HR mass spectrometer (Shimadzu, Kyoto, Japan). For column chromatography, silica gel 60 (230−400 mesh, Merck, Darmstadt, Germany), Diaion HP-20 (particle size = 200−600 μm, Mitsubishi Chemical Co., Tokyo, Japan), and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) were used. Precoated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected under UV lamps (254 nm) and by spraying with an anisaldehyde/sulfuric acid solution and then heating. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with an SPD-10A UV detector, equipped with a 250 × 10 mm i.d. preparative Cosmosil 5C_{18} AR-II column (Nacalai Tesque, Inc., Kyoto, Japan).

Plant Material. The roots of *A. membranaceus* (Huang-qi), in dried form, were purchased from Sun Ten Pharmaceutical Co., Ltd., in Taiwan, in 2007.

Isolation and Identification of Astragali Radix Metabolites. The dried roots of *A. membracaceus* (1.0 kg) were extracted three times with 95% EtOH (10 L) for 48 h at 50 °C. After evaporation of EtOH in vacuo at 45 °C, the residue (semisolid 200 g) was suspended in 10% aqueous EtOH and then loaded on the Diaion HP-20 (1500 mL, 9.0 × 34 cm), which was prewet with 3 L of deionized water and successively washed with 100% H_2O (4.0 L, fraction 1), 40% EtOH (5.0 L, fraction 2), 70% EtOH (6.0 L, fraction 3, 14 g), and 95% EtOH (6.0 L, fraction 4), to yield four fractions. The 70% EtOH fraction (fraction 3, 5 g) was subjected to an LH-20 column (4.5 × 80 cm) eluting with 100% MeOH, to get 10 fractions (fractions 3-1−3-10). The astragalosides were isolated from fraction 3-2. Fraction 3-2 (1.9 g) was chromatographed over silica gel (230−400 mesh, 4.0 × 28 cm), eluting with a $CHCl_3$/MeOH (100% $CHCl_3$ 500 mL, 15/1 480 mL, 12/1 780 mL, 10/1 1650 mL, 9/1 1000 mL, 8/1 500 mL, v/v) system in gradient to give 10 subfractions (fractions 3-2-1−3-2-10). Compound **5** (369.6 mg) was yielded from fraction 3-2-2. Fraction 3-2-3 was further purified by HPLC (5C18-AR-II, 10 × 250 mm, Cosmosil, 45% MeCN in H_2O, 2.5 mL/min, UV 210 nm) to give compound **6** (45.9 mg). The other subfractions were purified by HPLC in similar conditions: compound **8** (40.5 mg) from fraction 3-2-4; compounds **7** (148.3 mg) and **11** (3.1 mg) from fraction 3-2-6; and compounds **9** (11.1 mg) and **10** (15.5 mg) from fraction 3-2-10. The isoflavonoids and their glycosides were isolated from fractions 3-6, 3-8, and 3-9. Fraction 3-6 was purified over the HPLC [5C18-AR-II, 250 × 10 mm, Cosmosil, mobile phase: H_2O (A), MeCN (B), in 0−30 min, 25−32% (B), in 30−60 min, 32−50% (B), in 60−70 min, 50−80% (B), 3 mL/min, UV 210 nm] to give compounds **1** (6.8 mg), **2** (4.2 mg), **4** (92.7 mg), **12** (97.5 mg), **13** (7.7 mg), **14** (3.3 mg), and **15** (7.4 mg). Fraction 3-8 was purified also by HPLC [5C18-AR-II, 250 × 10 mm, Cosmosil, mobile phase: H_2O (A), MeCN (B), in 0−20 min, 25−35% (B), in 20−40 min, 35−50% (B), in 40−60 min, 50−80% (B), in 60−65 min, 80−100% (B), 3 mL/min, UV 210 nm] to give compounds **3** (6.0 mg), **16** (28.2 mg), **17** (7.2 mg), **18** (6.5 mg), and **19** (35.4 mg). Compounds **20** (48.6 mg), **21** (33.6 mg), **22** (3.9 mg), **23** (3.2 mg), and **24** (2.3 mg) were obtained from fraction 3-9 [5C18-AR-II, 250 × 10 mm, Cosmosil, mobile phase: H_2O (A), MeCN (B), in 0−10 min, 28−32% (B), in 10−50 min, 32−50% (B), in 50−60 min, 50−80% (B), in 60−65 min, 80−100% (B), 3 mL/min, UV 210 nm].

*(−)-Methylinissolin 3-O-β-D-(6′-acetyl)-glucoside (**1**):* pale orange amorphous powder, $[\alpha]25_D$ −116.2° (*c* 0.68, MeOH); CD $[\theta]_{231}$ −20383.6, $[\theta]_{285}$ 12072.2 (*c* 1.0 × 10^{-4} M, MeOH); UV (MeOH) λ_{max} 230 (sh), 284 nm; IR (KBr) ν_{max} 3373, 2938, 1737, 1620, 1590, 1499, 1461, 1380, 1269, 1084 cm^{-1}; ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectral data, Tables 1 and 2, respectively (see also the Supporting Information); ESIMS *m*/*z* 527.2 $[M + Na]^+$; HRESIMS *m*/*z* 527.1538 $[M + Na]^+$ (calcd for $C_{25}H_{28}O_{11}Na$, 527.1529).

*(−)-Methylinissolin 3-O-β-D-{6′-[(E)-but-2-enoyl]}-glucoside (**2**):* pale yellow amorphous powder, $[\alpha]25_D$ −114.3° (*c* 0.42, MeOH); CD $[\theta]_{231}$

Table 1. ^{1}H NMR (400 MHz) Data of Compounds 1, 2, and 4 (δ in Parts per Million, J in Hertz)

position	1[a]	2[a]	2[b]	4[b]
1	7.43 (d, 8.4)	7.42 (d, 8.8)	7.54 (overlapped)	7.48 (d, 8.8)
2	6.76 (dd, 8.4, 2.4)	6.76 (dd, 8.4, 2.4)	7.00 (overlapped)	7.02 (dd, 8.4, 2.4)
4	6.62 (d, 2.4)	6.63 (d, 2.4)	7.05 (br s)	7.01 (d, 2.4)
6	4.25 (dd, 8.8, 2.0)	4.26 (dd, 8.8, 2.8)	4.30 (overlapped)	4.27 (dd, 10.0, 2.4)
	3.59 (overlapped)	3.59 (overlapped)	3.59 (t, 10.0)	3.69 (t, 10.4)
6a	3.59 (overlapped)	3.59 (overlapped)	3.55 (m)	3.55 (m)
7	6.94 (d, 8.4)	6.95 (d, 8.4)	6.95 (d, 8.8)	6.94 (d, 8.0)
8	6.52 (d, 8.4)	6.52 (d, 8.4)	6.53 (d, 8.4)	6.51 (d, 8.0)
11a	5.54 (d, 6.4)	5.54 (d, 6.4)	5.58 (d, 7.2)	5.55 (7.2)
9-OCH_3	3.79 (3H, s)	3.79 (3H, s)	3.71 (3H, s)	3.70 (3H, s)
10-OCH_3	3.84 (3H, s)	3.81 (3H, s)	3.93 (3H, s)	3.92 (3H, s)
1′	4.86 (d, 7.6)	4.86 (d, 7.2)	5.56 (d, 7.2)	5.60 (d, 7.6)
2′	3.45 (overlapped)	3.45 (overlapped)	4.30 (overlapped)	4.24 (t, 6.4)
3′	3.45 (overlapped)	3.45 (overlapped)	4.33 (overlapped)	4.33 (overlapped)
4′	3.34 (t, 8.4)	3.34 (t, 8.4)	4.14 (t, 8.4)	4.30 (overlapped)
5′	3.64 (m)	3.64 (m)	4.18 (m)	4.05 (m)
6′	4.39 (dd, 12.0, 2.0)	4.48 (dd, 12.0, 2.0)	5.03 (overlapped)	4.45 (dd, 12.0, 2.0)
	4.21 (dd, 12.0, 6.8)	4.21 (dd, 12.0, 7.2)	4.76 (dd, 11.6, 6.8)	4.34 (dd, 12.0, 6.4)
2″	2.02 (3H, s)	5.88 (dd, 15.6, 1.6)	5.90 (br d, 15.6)	
3″		7.00 (dq, 15.6, 7.2)	6.99 (overlapped)	
4″		1.84 (dd, 6.8, 1.6)	1.59 (d, 6.8)	

[a] NMR D-solvent was MeOD-d_4. [b] NMR D-solvent was pyridine-d_5.

Table 2. ^{13}C NMR (100 MHz) Data of Compounds 1, 2, and 4 (δ in Parts per Million)

position	1[a]	2[a]	2[b]	4[b]	position	1[a]	2[a]	2[b]	4[b]
1	133.1	133.1	132.6	132.5	11b	115.7	115.8	114.8	114.4
2	111.9	112.0	111.3	111.0	9-OCH_3	56.9	56.9	56.4	56.2
3	160.1	160.2	159.8	159.7	10-OCH_3	60.9	60.9	60.4	60.3
4	105.7	105.8	105.4	104.9	1′	101.9	102.1	102.3	104.9
5	157.8	157.9	157.1	157.0	2′	74.8	74.8	74.7	74.7
6	67.4	67.6	66.7	66.5	3′	77.9	77.9	78.3	78.2
6a	41.2	41.2	40.3	40.1	4′	71.7	71.8	71.3	70.9
6b	123.3	123.2	122.3	122.1	5′	75.4	75.5	75.5	78.7
7	119.8	119.9	118.9	118.9	6′	64.7	64.7	64.3	62.0
8	106.4	106.4	105.8	105.5	1″	172.7	167.9	166.2	
9	152.5	152.6	152.1	151.9	2″	20.7	123.2	122.2	
10	135.2	135.2	134.9	134.6	3″		146.9	144.9	
10a	154.4	154.4	153.9	153.7	4″		18.2	17.8	
11a	80.3	80.3	79.2	79.1					

[a] NMR D-solvent was MeOD-d_4. [b] NMR D-solvent was pyridine-d_5.

−13451.9, $[\theta]_{285}$ 6875.7 (c 1.0 × 10^{-4} M, MeOH); UV (MeOH) λ_{max} 230 (sh), 284 nm; IR (KBr) ν_{max} 3370, 2937, 1711, 1620, 1590, 1499, 1461, 1269, 1084 cm^{-1}; ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data, Tables 1 and 2, respectively (see also the Supporting Information); ESIMS m/z 553.2 $[M + Na]^+$; HRESIMS m/z 553.1706 $[M + Na]^+$ (calcd for $C_{27}H_{30}O_{11}Na$, 553.1686).

Calycosin 7-O-β-D-(6″-acetyl)-glucoside (**3**): ivory-white amorphous powder, $[\alpha]25_D$ −60.0° (c 0.6, MeOH); UV (MeOH) λ_{max} 260, 286, 325 (sh); IR (KBr) ν_{max} 3373, 2939, 1728, 1625, 1590, 1513, 1460, 1382, 1444, 1382, 1266, 1199, 1075 cm^{-1}; ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data, Table 3 (see also the Supporting Information); ESIMS m/z 511.1 $[M + Na]^+$; HRESIMS m/z 511.1248 $[M + Na]^+$ (calcd for $C_{24}H_{24}O_{11}Na$, 511.1216).

(−)-Methylinissolin 3-O-β-D-glucoside (**4**): pale yellow amorphous powder, $[\alpha]25_D$ −175° (c 0.12, MeOH) ; CD $[\theta]_{228}$ −89238.0, $[\theta]_{285}$ 52371.5 (c 2.0 × 10^{-5} M, MeOH); UV (MeOH) λ_{max} 205, 230 (sh), 283 nm; ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data, Tables 1 and 2, respectively (see also the Supporting Information); ESIMS m/z 551.1 $[M + Na]^+$; HRFABMS m/z 528.1627 $[M]^+$ (calcd for $C_{27}H_{28}O_{11}$ 528.1631).

In addition, the known compounds, astragaloside I (**5**),[10] isoastragaloside I (**6**),[11] astragaloside II (**7**),[11] isoastragaloside II (**8**),[12] astragaloside III (**9**),[10] astragaloside IV (**10**),[10] cyclocephaloside II (**11**),[13] ononin (**12**),[14] 6″-acetylononin (**13**),[15] ammopiptanoside A (**14**),[16] calycosin 7-*O*-β-D-glucoside (**15**),[17] calycosin 7-*O*-β-D-{6″-[(*E*)-but-2-enoyl]}-glucoside (**16**),[18] licoagroside D (**17**),[19] (*R*)-3-(5-hydroxy-2,3,4-trimethoxyphenyl)-chroman-7-ol (**18**),[20] isomucronulatol 7-*O*-glucoside (**19**),[21] isomucronulatol

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Table 3. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Data of Compound 3 (in MeOD-d_4, δ in Parts per Million, J in Hertz)

position	^{1}H	^{13}C	position	^{1}H	^{13}C
2	8.18 (s)	155.2	4′		149.2
3		125.9	5′	6.97 (br s)	112.6
4		177.9	6′	6.97 (br s)	121.6
5	8.12 (d, 8.8)	128.3	4′-OCH_3	3.87 (3H, s)	56.4
6	7.17 (br d, 8.4)	117.0	1″	5.09 (d, 6.4)	101.6
7		163.3	2″	3.41 (m)	74.7
8	7.19 (br s)	104.9	3″	3.52 (m)	77.7
9		159.1	4″	3.52 (m)	71.5
10		120.3	5″	3.76 (t, 8.0)	75.6
1′		126.0	6″	4.45 (br d, 11.6), 4.23, (dd, 11.6, 6.4)	64.7
2′	7.05 (br s)	117.3	1‴		172.7
3′		147.4	2‴	2.07 (3H, s)	20.7

(**20**),[21] calycosin (**21**),[22] vesticarpan (**22**),[23] pendulone (**23**),[24] and (−)-methylinissolin (**24**)[25] were also isolated and identified by comparing their physical and spectroscopic data with those of authentic samples and references.

HPLC Analysis. *Materials and Reagents.* The 70% EtOH fraction (fraction 3) was prepared as above-mentioned. The reference compounds astragalosides I, II, III, and IV, isoastragalosides I and II, ononin, (−)-methylnissolin-3-*O*-glucoside, isomucromulatol-7-*O*-glucoside, isomucromulatol, and calycosin were isolated from the 70% EtOH fraction (fraction 3). Acetonitrile and methanol (LC grade) were purchased from Merck. Milli-Q ultrapure water (Millipore, Q-gard 1/Quantum EX) was used throughout the study.

Apparatus and Conditions. The HPLC profile was performed on a Shimadzu 10A series system equipped with two pumps (Shimadzu, LC-6AD), an evaporative light scattering detector (ELSD, Varian, 380-LC), a four-channel vacuum degasser (Biotech, model 2003), a 7715i (Shimadzu) manual injector with a 200 μL sample loop, an SCL-10AVP system controller, and SISC software (Scentific Information Service Corp.) for data analysis. A separation column (Cosmosil 5C18-AR-II, 5 μm, 250 × 4.6 mm i.d.) was employed eluting at a rate of 0.8 mL/min under room temperature. The mobile phase consisted of water (A) and acetonitrile (B) using a gradient program of 25−32% (B) in 0−20 min, 32−50% (B) in 20−50 min, and 50−80% (B) in 50−60 min.

ELSD Conditions. The flow rate of nebulizer gas (N_2) was maintained at 1.5 L/min, the nebulizer temperature was set at 50 °C, and the drift tube temperature was set at 70 °C.

Preparation of Standards Solution. Each reference compound was accurately weighed and dissolved in MeOH; the terminate concentration was ca. 1 mg/mL.

Preparation of Sample Solution. Fraction 3 was dried under vacuum; then about 100 mg was accurately weighed and dissolved in MeOH, in a 10 mL volumetric flask. The sample solutions were all filtered through a 0.45 μm filter (Millipore) before use, and the injected volume was 20 μL.

Cell Culture and NO Measurement. The macrophage cell line RAW 264.7 was obtained from ATCC (Rockville, MD) and 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. Cells were plated at a density of 2 × 10^5 cells/well in 96-well culture plate and stimulated with LPS (100 ng/mL) in the presence or absence of different concentrations of tested compounds (1−30 μM) for 18 h simultaneously. All compounds were dissolved in DMSO and further diluted with sterile PBS and sterilized via a 0.2 μm filter. Nitrite (NO_2^-) accumulation in the medium was used as an indicator of NO production, which was measured by adding Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 550 nm.[26] All experiments were performed in triplicate. NO production by LPS stimulation was designated as 100% for each experiment. A well-known iNOS inhibitor, aminoguanidine (AMG), was employed as a positive control.

Cytotoxicity Assay. Cells (3 × 10^3 per well) were seeded in 180 μL of MEM in 96-well plates. After 4 h, 20 μL of test agents was dissolved in PBS solution and added at final concentrations of 1, 3, 10, and 30 μM under 37 °C incubator with 5% CO_2. After 3 days, 20 μL of MTT solution (2 mg/mL) was added to each well and incubated for 4 h to make cellular conversion of a tetrazolium salt into a formazan product. Then the supernatant was removed, and 200 μL of DMSO was added to dissolve the formazan. Finally, the formazan could be detected by ELISA reader in the absorbance at 570 nm and provided a relative estimate of cell proliferation.

Acid Hydrolysis of Compounds 1, 3, and 4. A solution of each compound (3 mg) dissolved in 1 N HCl (dioxane/H_2O, 1:1, 2 mL) was refluxed for 3 h. After each reaction solution had been partitioned with $CHCl_3$ and H_2O, the water layer was neutralized with $NaHCO_3$, and the aqueous solution was concentrated to dryness. Each residue was dissolved in dry pyridine (1.0 mL), and acetic anhydride (0.6 mL) was added; then each mixture was reacted at 60 °C for 3 h. Each reaction mixture was evaporated to dryness, and each dried reactant was partitioned with *n*-hexane/EtOAc (1:1) and water. The *n*-hexane/EtOAc fraction was subjected to gas chromatography (GC column, Varian capillary column CP-chirasil-L-val for optical isomers, 25 m × 0.25 mm, 0.12 μm; column temperature, 50−150 °C, 30 °C/min, 150−180 °C, 0.8 °C/min; injector temperature, 200 °C; He carrier gas, 2.0 kg/cm^3; mass detector, Thermo DSQ2; electron energy, 70 eV). Under these conditions, the sugars of each reactants were identified by comparison with authentic samples: t_R (min) 30.60 (D-glucose), 30.22 (L-glucose). All of the isolated glucoses from the titled plant were identified as D-form.

RESULTS AND DISCUSSION

Structural Elucidation. Compound **1** was obtained as a pale yellow powder. Its quasi-molecular ion $[M + Na]^+$ at m/z 553.1706 in the HRESIMS revealed the formula $C_{25}H_{28}O_{11}$ [larger by 42 mass units than that of (−)-methylinissolin 3-*O*-β-D-glucoside (**4**)].[27] The IR spectrum showed characteristic signals for OH group (3373 cm^{-1}), ketone (1737 cm^{-1}), and aromatic ring (1590, 1499, 1461 cm^{-1}). The ^{1}H NMR spectrum showed characteristic signals at δ_H 6.62 (d, J = 2.4 Hz), 6.76 (dd, J = 8.4, 2.4 Hz), and 7.43 (d, J = 8.4 Hz), corresponding to aromatic protons of an ABX-type system, two aromatic doublets

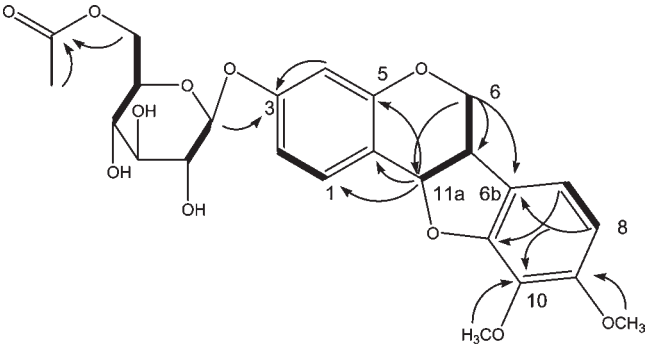

Figure 1. Key HMBC (→) and $^1H-^1H$ COSY (—) correlations of compound **1**.

at δ_H 6.52 (d, J = 8.4 Hz), 6.94 (d, J = 8.4 Hz) of an AB system, and two aromatic methoxy groups at δ_H 3.79, 3.84 (each 3H, s). The above data, together with 12 degrees of unsaturation for **1** and the sequentially coupled proton signals at δ_H 3.59 (m, 2H, H-6a, H_{ax}-6), 4.25 (dd, J = 8.8, 2.0 Hz, H_{eq}-6), and 5.54 (d, J = 6.4 Hz, H-11a) in the $^1H-^1H$ COSY spectrum, suggested that the skeleton of **1** possessed a diphenyl moiety connected by a fused pyran−furan ring, like the key feature of pterocarpan.[19] In addition, proton signals for an anomeric proton (δ_H 4.86, d, J = 7.6 Hz) of a β-glucose and the acetylic methyl protons (δ_H 2.02, 3H, s) were also observed. The ^{13}C NMR spectra exhibited signal for 25 carbons, corresponding to 1 oxygenated methylene carbon at δ_C 67.4 (C-6), 1 conjugated neighbored carbon at δ_C 41.2 (C-6a), and 1 oxygenated carbon at δ_C 80.3 (C-11a) and 12 aromatic carbons assignable to two isolated aromatic rings, as well as 2 methoxy, a glucose, and an acetyl group. Five of the aromatic carbons were oxygenated, as shown by their deshielded carbon chemical shifts. In the HMBC spectrum of **1**, long-range correlations between H-8 and C-9, C-10 and between $OCH_3 \times 2$ (δ_H 3.79, 3.84) and C-9, C-10, respectively, revealed that two methoxy groups are located at C-9 and C-10, respectively. Moreover, correlations between anomeric proton (H-1′) and C-3 and between H_2-6′ of glucose and C-1″ (δ_C 172.7) decided the locations of the acetyl group and glucose at C-6′ and C-3, respectively. The glucose was further confirmed to be β glucose by comparing the NMR data with the reported literature,[28] and the D-form was identified by acid hydrolysis of compound **1**. Thus, the planar structure of **1** was determined as shown, which was similar to that of **4** except for an acetylic group signals in **1**.

With regard to the stereochemistry of **1**, the coupling constant of 6.4 Hz between H-6a and H-11a indicated their *cis*-configuration.[29] The absolute configuration was further determined as 6a-*R* and 11a-*R*, due to CD spectrum analysis: a negative Cotton effect at 228 nm and a positive Cotton effect at 285 nm.[19]. Accordingly, compound **1** was deduced as (−)-methylinissolin 3-*O*-β-D-(6′-acetyl)-glucoside (see Figure 1).

Compound **2** was obtained as a pale yellow powder, with the molecular formula of $C_{27}H_{30}O_{11}$, based on the $[M + Na]^+$ peak at m/z 553.1706 in the HRESIMS, and confirmed by ^1H and ^{13}C NMR experiments (Table 1). The IR spectrum was very similar to that of compound **1**. By comparison of the ^1H and ^{13}C NMR data of **2** with those of **1**, similar data for the aglycone and glucose moieties were observed. The difference between **1** and **2** contained the carbon signals for an acyl moiety containing four C-atoms (appearing at δ_C 167.9, 146.9, 123.2, and 18.2) in **2**, rather than an acetylic group at Glc in **1**. The carbon signal at

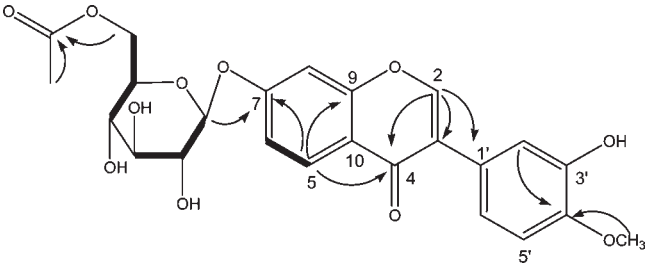

Figure 2. Key HMBC (→) and $^1H-^1H$ COSY (—) correlations of compound **3**.

167.9 could be assigned to an α,β-unsaturated CO ester group. Two vinyl carbon signals at δ_C 146.9 and 123.2 (C-3″, and C-2″, respectively) corresponding to proton signals at δ_H 7.00 (dq, 15.6, 7.2) and 5.88 (dd, 15.2, 1.6) indicated an *E*-configured double-bond moiety. The above data, together with the signal for a methyl group at δ_H 1.84 (dd, 6.8, 1.6 Hz), indicated the substituent to be a (2*E*)-but-2-enoxy group (MeCH═CHC=O). The CD spectrum of **2** was also similar to **1**, indicating that they possess the same configuration. According to the biological relations, the glucose was also assigned as D-form. On the basis of the above evidence, compound **2** was characterized to be (−)-methylinissolin 3-*O*-β-D-{6′-[(*E*)-but-2-enoyl]}-glucoside.

Compound **3** was obtained as an ivory-white amorphous powder, and its HRESIMS spectrum indicated a molecular formula of $C_{24}H_{24}O_{11}$, as determined by sodiated ion at m/z 511.1248 $[M + Na]^+$. The IR spectrum showed absorption bands for an OH group (3373 cm^{-1}), a ketone (1737 cm^{-1}), an α,β-unsaturated ketone (1625 cm^{-1}), and an aromatic ring (1590, 1516, 1460 cm^{-1}). The UV spectrum exhibited maximum presence at 260 (band II) and 325 (sh) nm, indicating the absorptions of an isoflavone. The ^1H NMR spectrum of **3** showed the characteristic signal for H-2 at δ_H 8.27 (1H, s), together with an typical ABX-type aromatic proton system at δ_H 7.17 (br d, J = 8.4 Hz), 7.19 (br s), and 8.12 (d, J = 8.8 Hz) in the A-ring and untypical ABX coupled protons at δ_H 6.97 (2H, br s) and 7.05 (1H, br s) in the B-ring, suggesting an isoflavone skeleton. The remaining signals contained an aromatic methoxy group at δ_H 3.87 (each 3H, s) and an anomeric proton (δ_H 5.09, d, J = 6.4 Hz) of a β-glucose, as well as the acetylic methyl protons (δ_H 2.07, 3H, s). The ^{13}C NMR spectrum of **3** exhibited 24 carbon signals, including the characteristic signals of isoflavone, such as two oxygenated aromatic carbons at δ_C 163.3 (C-7) and 159.1 (C-9) in the A-ring and two *ortho* oxygenated aromatic carbons at δ_C 147.4 (C-3′) and 149.2 (C-4′) in the B-ring, as well as an oxygenated vinyl carbon at δ_C 155.2 (C-2), a vinyl quaternary carbon at 125.9 (C-3), and a carbonyl carbon at 177.9 (C-4) in the C-ring. The positions of substituted functional groups were further determined from the HMBC spectrum as shown in Figure 2. Long-range correlations between H-2′ and C-3′ and C-4′, between the methoxy protons and C-4′, between the anomeric proton (H-1 of Glc) and C-7, and between H_2-6 (δ_H 4.45, br d, J = 11.6 Hz; 4.23, dd, J = 11.6, 6.4 Hz) of Glc and C-1‴ (δ_C 172.7) revealed that the methoxy was located at C-4′ and the acetyl group at C-6″ of glucose, which was located at C-7 of the A-ring. Like **1**, the glucose was determined as β-D-glucose. Therefore, the structure of **3** was elucidated as calycosin 3-*O*-β-D-(6″-acetyl)-glucoside.

HPLC Analysis. We studied the HPLC profile of the active part of the 70% EtOH fraction (fractioni 3) of *A. membranaceus*

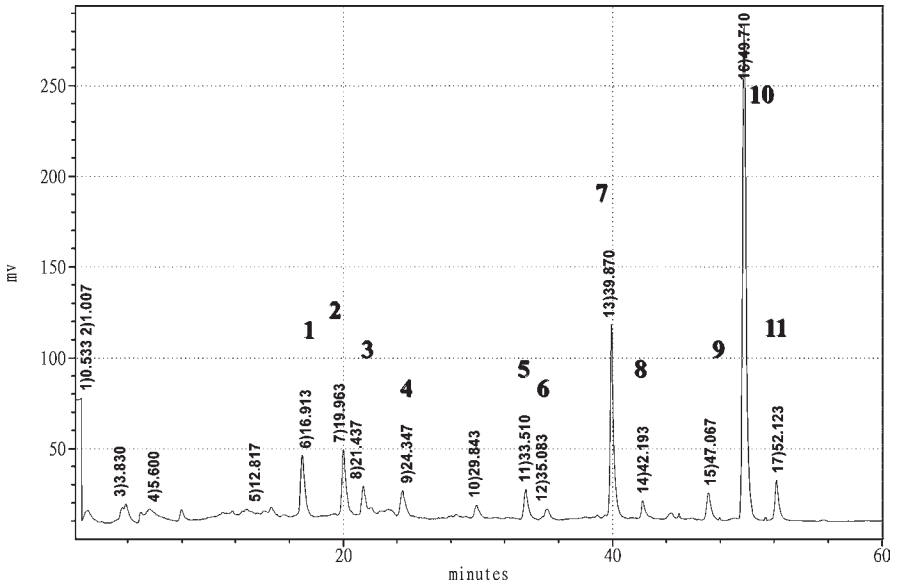

Figure 3. HPLC profile of the active part 70% EtOH fraction of *A. membranaceus*.

Table 4. Effects of Compounds 1−16 on Lipopolysaccharide-Induced Nitric Oxide (NO) Production in RAW 264.7 Macrophages[a]

compound	EC_{50} (μM)	compound	EC_{50} (μM)
1	28.7 ± 3.5	**9**	−
2	19.2 ± 0.6	**10**	−
3	4.5 ± 1.2	**11**	22.3 ± 0.9
4	14.7 ± 0.9	**12**	17.1 ± 1.1
5	−[b]	**13**	12.6 ± 0.9
6	−	**14**	10.5 ± 1.6
7	−	**15**	4.1 ± 0.1
8	−	**16**	15.2 ± 2.0
aminoguanidine	32.6 ± 3.9		

[a] All data were expressed as mean ± SEM. EC_{50} represented the 50% effective concentration to inhibit NO production. [b] EC_{50} > 50 μM.

and identified the 11 main peaks by comparing the retention times with the reference compounds isolated from the title plant. As shown in Figure 3, the fingerprint chromatogram showed the relative retention times of ononin (**12**) (peak 1, t_R 16.91 min), methylnissolin-3-*O*-glucoside (**4**) (peak 2, t_R 19.96 min), isomucronulatol 7-*O*-glucoside (**19**) (peak 3, t_R 21.44 min), calycosin (**21**) (peak 4, t_R 24.35 min), astragaloside IV (**11**) (peak 5, t_R 33.51 min), astragaloside III (**10**) (peak 6, t_R 35.08 min), astragaloside II (**7**) (peak 7, t_R 39.87 min), isoastragaloside II (**8**) (peak 8, t_R 42.19 min), isomucromulatol (**20**) (peak 9, t_R 47.07 min), astragaloside I (**5**) (peak 10, t_R 49.71 min), and isoastragaloside I (**6**) (peak 11, t_R 52.12 min), respectively. The HPLC profile (Figure 3) revealed that astragalosides I and II were the main components in fraction 3 of the 95% EtOH extract.

Anti-NO Activities. The effects of tested compounds (**1**−**16**) on the LPS-induced NO production in RAW 264.7 macrophages was evaluated (Table 4). The 50% effective concentration (EC_{50}) for inhibiting the production of NO was calculated on the basis of nitrite released into the culture media. As shown in Table 4, except for **5**−**10** (EC_{50} > 50 μM), other isolates displayed more potent effects on inhibiting NO production than the positive control, aminoguanidine. Compounds **3** and **15** strongly inhibited NO production with EC_{50} values of 4.1 ± 0.1 and 4.5 ± 1.2 μM, respectively. The drug's effects were significantly distinguished from those of the vehicle (data not shown). Cell viability of most tested isolates still exceeded 95% when they were measured by MTT assay (data not shown), indicating that the anti-NO production by these compounds was not resulting in cell death. Some conclusions could be deduced from the struture−activity relationship (SAR) discussion. First, the astragalosides **5**−**10** were not effective in the anti-NO production induced by LPS on RAW 264.7, except for **11**; second, the nine isoflavonoids, **1**−**4** and **12**−**16**, showed more potent effects on inhibiting NO production than astragalosides. Moreover, the isoflavonoids isolated from *A. membranaceus* may be divided into three parts: the first group **1**, **2**, and **4** (pterocarpan type), the second group **12**, **13**, and **14** (3′-methoxy isoflavone 7-*O*-β-glucoside), and the third group **3**, **15**, and **16** (3′-methoxy-4′-hydroxy isoflavone 7-*O*-β-glucoside). With a detailed inspection of anti-NO data in Table 4, these three isoflavonoid groups displayed different inhibitory effects on the NO production in the sequence third group > second group > first group. The biological evaluation revealed that the pterocarpan type isoflavonoids possessed weaker anti-NO activity than normal isoflavonoids, and the 4′-hydroxy-containing isoflavonoids could enhance the anti-NO ability. Although it was reported that the aqueous extract of Astragali radix could inhibit NO production in LPS-stimulated RAW 264.7 macrophage cells,[9] the anti-NO component was not found. In this study, we demonstrated that isoflavonoids were the main anti-NO components in *A. membranaceaus*.

■ ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR spectra of compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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REFERENCES

(1) Li, Sh. Zh. (Ming Dynasty) *Ben Cao Gang Mu*; National Research Institute of Chinese Medicine: Taipei, Taiwan, 1994; Vol. 3, pp 403−405.

(2) Lau, B. H. S.; Ong, P.; Tosk, J. Macrophage chemiluminescence modulated by Chinese medicinal herbs *Astragalus membranaceus* and *Ligustrum luidum*. *Phytother. Res.* **1989**, *3*, 148–153.

(3) Zhao, K. S.; Mancini, C.; Doria, G. Enhancement of the immune response in mice by *Astragalus membranaceus* extracts. *Immunopharmacology* **1990**, *20*, 225–233.

(4) Hong, C. Y.; Ku, J.; Wu, P. *Astragalus membranaceus* stimulates human sperm motility in vitro. *Am. J. Chinese Med.* **1992**, *20*, 289–294.

(5) Jiangsu College of New Medicine. *A Dictionary of Traditional Chinese Medicines*; Shanghai Scientific and Technology Press: Shanghai, China, 2004; Vol. 2, pp 2036−2040.

(6) Yesilada, E.; Bedir, E.; Çalış, İ.; Takaishic, Y.; Ohmotod, Y. Effects of triterpene saponins from *Astragalus* species on in vitro cytokine release. *J. Ethnopharmacol.* **2005**, *96*, 71–77.

(7) Yu, D. H.; Duan, Y. L.; Bao, Y. M.; Wei, C. L.; An, L. J. Isoflavonoids from *Astragalus mongholicus* protect PC12 cells from toxicity induced by L-glutamate. *J. Ethnopharmacol.* **2005**, *98*, 89–94.

(8) Cho, W. C. S.; Leung, K. N. In vitro and in vivo anti-tumor effects of *Astragalus membranaceus*. *Cancer Lett.* **2007**, *252*, 43–54.

(9) Lee, Y. S.; Han, O. K.; Park, C. W.; Yang, C. H.; Jeon, T. W.; Yo, W. K.; Kimf, S. H.; Kim, H. J. Pro-inflammatory cytokine gene expression and nitric oxide regulation of aqueous extracted *Astragali* radix in RAW 264.7 macrophage cells. *J. Ethnopharmacol.* **2005**, *100*, 289–294.

(10) Hirotani, M.; Zhou, Y.; Lui, H.; Furuya, T. Astragalosides from hairy root cultures of *Astragalus membranaceus*. *Phytochemistry* **1994**, *36*, 665–670.

(11) Hirotani, M.; Zhou, Y.; Rui., H.; Furuya, T. Cycloartane triterpene glycosides from the hairy root cultures of *Astragalus membranaceus*. *Phytochemistry* **1994**, *37*, 1403–1407.

(12) Kitagawa, I.; Wang, H. K.; Saito, M.; Tagaki, A.; Yoshikawa Saponin and sapogenol. XXXV. Chemical constituents of Astragali Radix, the root of *Astragalus membranaceus* Bunge. (2). Astragalosides I, II, and IV, acetylastragaloside I, and isoastragalosides I, and II. *Chem. Pharm. Bull.* **1983**, *31*, 698–708.

(13) Bedir, E.; Çalis, I.; Aquino, R.; Piacente, S.; Pizza, C. Cycloartane triterpene glycosides from the roots of *Astragalus brachypterus* and *Astragalus microcephalus*. *J. Nat. Prod.* **1998**, *61*, 1469–1472.

(14) Lee, H. J.; Lee, O. K.; Kwon, Y. H.; Choi, D. H.; Kang, H. Y.; Lee, H. Y.; Paik, K. H.; Lee, H. J. Isoflavone glycosides from the bark of *Amorpha fruticosa*. *Chem. Nat. Compd.* **2006**, *42*, 415–418.

(15) Nechepurenko, I. V.; Polovinka, M. P.; Komarova, N. I.; Korchagina, D. V.; Salakhutdinov, N. F.; Nechepurenko, S. B. Low-molecular-weight phenolic compounds from *Hedysarum theinum* roots. *Chem. Nat. Compd.* **2008**, *44*, 31–34.

(16) Tian, X. M.; Chen, S. Z.; Tang, L.; Tu, P. F. Three new isoflavonoids from the aerial parts of *Ammopiptanthus mongolicus*. *Helv. Chim. Acta* **2008**, *91*, 1015–1022.

(17) Du, X. G.; Bai, Y. J.; Liang, H.; Wang, Z. Y.; Zhao, Y. Y.; Zhang, Q. Y.; Huang, L. Q. Solvent effect in ^{1}H NMR spectra of 3′-hydroxy-4′-methoxy isoflavonoids from *Astragalus membranaceus* var. *mongholicus*. *Magn. Reson. Chem.* **2006**, *44*, 708–712.

(18) Pei, Y. H.; Li, R. F.; Fu, H. W.; Wang, J.; Zhou, Y. Z. A new isoflavone glucoside from *Astragalus membranaceus* var. *mongholicus*. *Fitoterapia* **2007**, *78*, 602–604.

(19) Li, W.; Koike, K.; Asada, Y.; Hirotani, M.; Rui, H.; Yoshikawa, T.; Nikaido, T. Flavonoids from *Glycyrrhiza pallidiflora* hairy root cultures. *Phytochemistry* **2002**, *60*, 351–355.

(20) Spencer, G. F.; Jones, B. E.; Plattner, R. D.; Barnekow, D. E.; Brinen, L. S.; Clardy, J. A pterocarpan and two isoflavans from alfalfa. *Phytochemistry* **1991**, *30*, 4147–4149.

(21) Subarnas, A.; Oshima, Y.; Hikino, H. Isoflavans and a pterocarpan from *Astragalus mongholicus*. *Phytochemistry* **1991**, *30*, 2777–2780.

(22) Kamnaing, P.; Fanso Free, S. N. Y.; Nkengfack, A. E.; Folefoc, G.; Fomum, Z. T. An isoflavan-quinone and a flavonol from *Millettia laurentii*. *Phytochemistry* **1999**, *51*, 829–832.

(23) Piccinelli, A. L.; Fernandez, M. C.; Cuesta-Rubio, O.; Hernández, I. M.; Simone, F. D.; Rastrelli, L. Isoflavonoids osolated from Cuban *propolis*. *J. Agric. Food Chem.* **2005**, *53*, 9010–9016.

(24) Takahashi, M.; Fuchino, H.; Sekita, S.; Satake, M.; Kiuchi, F. Vitro leishmanicidal constituents of *Millettia pendula*. *Chem. Pharm. Bull.* **2006**, *54*, 915–917.

(25) Spencer, G. F.; Jones, B. E.; Plattner, R. D.; Barnekow, D. E.; Brinen, L. S.; Clardy, J. A pterocarpan and two isoflavans from alfalfa. *Phytochemistry* **1991**, *30*, 4147–4149.

(26) Chiou, W. F.; Lin, J. J.; Chen, C. F. Andrographolide suppresses the expression of inducible nitric oxide synthase in macrophage and restores the vasoconstriction in rat aorta treated with lipopolysaccharide. *Br. J. Pharmacol.* **1998**, *125*, 327–334.

(27) Yang, D.; Cai, S.; Liu, H.; Guo, X.; Li, C.; Shang, M.; Wang, X.; Zhao, Y. On-line identification of the constituents of Buyang Huanwu decoction in pig serum using combined HPLC−DAD−MS techniques. *J. Chromatogr., B* **2006**, *831*, 288–302.

(28) Yu, D. Q.; Yang, J. S. *Analytical Chemistry Manual (VII), Nuclear Magnetic Resonance Spectra*; Chemical Industry Press: Beijing, China, 1999; Vol. 2, p 901.

(29) Pachler, K.; Underwood, W. A proton magnetic resonance study of some pterocarpan derivatives. The conformation of the 6a,11a-dihydro-6*H*-benzofuro[3,2-*c*][1]benzopynan ring system. *Tetrahedron* **1967**, *23*, 1817–1826.