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NF-κB Inhibitory Activity of Sucrose Fatty Acid Esters and Related Constituents from *Astragalus membranaceus*

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Supporting Information

ABSTRACT: Twelve compounds, including six sucrose fatty acid esters (**1**−**6**), four galactosyl acylglycerols (**7**−**10**), and two sphingolipids (**11** and **12**), were isolated from the roots of *Astragalus membranaceus*. Their structures were identified on the basis of spectroscopic analysis. Among the isolated sucrose fatty acid esters, 6′-*O*-linoleyl sucrose (**1**) was identified as a new compound, and 6′-*O*-palmitoyl sucrose (**2**) and 6-*O*-palmitoyl sucrose (**3**) were isolated from nature for the first time. This is the first report on sucrose fatty acid ester components from *A. membranaceus*. The nuclear factor-κB (NF-κB) inhibitory activity of isolated compounds was measured in HepG2 cells stimulated with TNF-α using a luciferase reporter system. Among them, compounds **1**−**6** exhibited significant inhibition of NF-κB activation in a dose-dependent manner, with IC_{50} values ranging from 4.4 to 24.7 μM. Compounds **1**−**6** also exhibited inhibition of TNF-α-induced expression of iNOS and ICAM-1 mRNA and dose-dependent inhibition of iNOS promoter activity, with IC_{50} values ranging from 3.3 to 5.0 μM. These data demonstrate the potential of sucrose fatty acid esters from *A. membranaceus* to prevent and treat inflammatory diseases.

KEYWORDS: *Astragalus membranaceus, Leguminosae, sucrose fatty acid ester, NF-κB inhibitory activity*

INTRODUCTION

Astragalus membranaceus Bunge is an important adaptogenic plant belonging to the Leguminosae family. It has been widely used as a Chinese medicinal herb for tonifying the spleen and blood and for vital energy. Today it is used as an antiperspirant, a diuretic, or a tonic in Oriental medicines in many Asian countries. Moreover, it is popularly used in functional foods and nutraceuticals in Western countries. Pharmacological studies and clinical practice have demonstrated that *A. membranaceus* extract has cardiotonic, immunostimulatory, antioxidant, antiviral, and anti-inflammatory activities.[1−3]

The main constituents of *A. membranaceus* including triterpene saponins, flavonoids, polysaccharides, amino acids, and trace elements have been reported, and their bioactive effects are well-known.[3,4] However, no study had described the sucrose fatty acid ester components from *A. membranaceus*. Previous pharmacological studies on *A. membranaceus* have determined the bioactive components such as flavonoids and triterpene saponins can significantly inhibit membrane lipid peroxidation by superoxide (O_2^-), hydrogen peroxide (H_2O_2), and ultraviolet rays, whereas the polysaccharides express weaker protective activity.[5] Triterpene saponins containing astragalosides I−VII were found to have a positive effect on heart function by inhibiting the formation of lipid peroxides in the myocardium and by decreasing blood coagulation.[6] *Astragalus* polysaccharides have been shown to potentiate the immune-mediated antitumor activity of interleukin-2 (IL-2) as well as that of monocytes, improve the lymphocyte response in normal subjects and patients with cancer, and enhance natural killer (NK) cell activity of normal subjects and patients with systemic lupus erythematosis.[7,8] In addition, *A. membranaceus* has various documented anti-inflammatory effects. Recent studies showed that the aqueous extract of *A. membranaceus* could reduce the suppression of macrophage cell proliferation induced by MTX and induce IL-1α, IL-1β, and IL-6 mRNA expressions; the isoflavonoids could inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells.[9,10] However, the active NF-κB inhibitory components of *A. membranaceus* had not been reported.

NF-κB represents a family of Rel domain-containing proteins including five NF-κB units that can form 15 transcription factors through homo- and heterodimerization. NF-κB plays an important role in the transcriptional regulation of numerous cytokines and adhesion molecules. It is the most extensively studied transcription factor in the immune system. Known inducers of NF-κB activity are highly variable and include reactive oxygen species (ROS), tumor necrosis factor alpha (TNF-α), interleukin IL-1β, bacterial LPS, isoproterenol, cocaine, and ionizing radiation. The activation of NF-κB causes transcription at the κB site, which is involved in many diseases, including inflammatory disorders and cancer. Hence, the inhibition of NF-κB signaling has become a therapeutic target for the treatment of inflammatory diseases and cancer.[11−13] In this study, the effects of compounds **1**−**12** from *A. membranaceus* on TNF-α-induced NF-κB transcriptional activity in human hepatocarcinoma (HepG2) cells were evaluated using an NF-κB-luciferase assay. To confirm the

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Table 1. ^{1}H NMR Spectroscopic Data of Compounds 1−5[a]

	1	2	3	4	5
1	6.19 d (3.4)	6.21 d (3.4)	6.14 d (3.4)	6.19 d (3.4)	6.17 d (3.4)
2	4.17 dd (3.4, 9.8)	4.17 dd (3.4, 9.8)	4.83 dd (3.4, 9.8)	4.17 dd (3.4, 9.8)	4.83 dd (3.4, 9.8)
3	4.76 t (9.5)	4.75 t (9.5)	4.76 t (9.5)	4.76 t (9.5)	4.80 t (9.5)
4	4.20 t (9.0)	4.20 t (9.0)	4.04 t (9.0)	4.20 t (9.0)	4.10 t (9.0)
5	4.68 m	4.70 m	4.68 m	4.65 m	4.72 m
6	4.33 d (10.0)	4.35 d (10.0)	4.74 m	4.35 d (10.0)	4.74 m
	4.50 d (10.0)	4.52 d (10.0)	4.84 m	4.52 d (10.0)	4. 91 m
1′	4.34 br s	4.34 br s	4.43 br s	4.34 br s	4.50 br s
3′	4.99 d (8.0)	4.99 d (8.0)	4.93 m	4.99 d (8.0)	4.99 m
4′	4.94 t (9.0)	4.95 t (9.0)	4.92 t (8.0)	4.95 t (9.0)	4.95 t (8.0)
5′	4.63 m	4.59 m	4.55 m	4.59 m	4.55 m
6′	4.94 m	4.97 m	4.30 m	4.97 m	4.41 m
	5.02 m	5.04 m		5.04 m	
2″	2.31 t (7.5)	2.34 m	2.31 m	2.31 t (7.5)	2.38 t (7.5)
3″	1.57 m	1.17−1.27 m	1.13−1.23 m	1.60 m	1.57 m
4″	1.19−1.32 m	1.17−1.27 m	1.13−1.23 m	1.17−1.30 m	1.18−1.31 m
5″	1.19−1.32 m	1.17−1.27 m	1.13−1.23 m	1.17−1.30 m	1.18−1.31 m
6″	1.19−1.32 m	1.17−1.27 m	1.13−1.23 m	1.17−1.30 m	1.18−1.31 m
7″	1.19−1.32 m	1.17−1.27 m	1.13−1.23 m	1.17−1.30 m	1.18−1.31 m
8″	2.06 m	1.17−1.27 m	1.13−1.23 m	2.08 m	2.10 m
9″	5.45 m	1.17−1.27 m	1.13−1.23 m	5.46 m	5.47 m
10″	5.47 m	1.17−1.27 m	1.13−1.23 m	5.47 m	5.48 m
11″	2.93 m	1.17−1.27 m	1.13−1.23 m	2.95 m	2.93 m
12″	5.49 m	1.17−1.27 m	1.13−1.23 m	5.50 m	5.50 m
13″	5.49 m	1.17−1.27 m	1.13−1.23 m	5.50 m	5.50 m
14″	2.06 m	1.57 m	1.54 m	2.94 m	2.09 m
15″	1.19−1.32 m	1.17−1.27 m	1.13−1.23 m	5.48 m	1.18−1.31 m
16″	1.19−1.32 m	0.84 t (7.5)	0.81 t (7.5)	5.45 m	1.18−1.31 m
17″	1.19−1.32 m			2.07 m	1.18−1.31 m
18″	0.93 t (7.5)			0.95 t (7.5)	0.98 t (7.5)

[a]Assignments were done by HMQC and HMBC experiments; *J* values (Hz) are in parentheses. Measured in pyridine-d_5, 600 MHz.

inhibitory effects of the compounds on NF-κB transcriptional activity, the effects of the isolated compounds on the up-regulation of the pro-inflammatory proteins inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) were evaluated in TNF-α-stimulated HepG2 cells by reverse transcriptase polymerase chain reaction (RT-PCR), as were their iNOS promoter activities.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were determined using a Jasco DIP-370 automatic polarimeter. UV spectra were recorded using a Beckman Du-650 UV−vis recording spectrometer. The FT-IR spectra were measured using a Jasco Report-100 infrared spectrometer. The NMR spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz). The LCQ Advantage trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was equipped with an electrospray ionization (ESI) source, and high-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC-MS system. GC-MS data were obtained with an Clarus 600 GC equipped with a 600T mass selective detector and a 30 m (0.25 mm i.d., 0.25 μm film) HP-5 ms capillary column (Agilent, Wilmington, DE, USA). Helium was used as carrier gas with a flow rate of 30 cm/s. A sample of 0.02 μL of essential oil was analyzed in the GC-MS system with a cooled on-column injection. The column temperature was held at 50 °C for 2 min and then programmed to 270 °C at 3 °C/min. The mass selective detector was operated in positive EI mode with a mass scan range from *m*/*z* 30 to 350 at 70 eV. GC-MS data processing was achieved using the following software programs: automated mass spectral deconvolution and identification system (AMDIS, version 2.65, National Institute of Standards and Technology, Gaithersburg, MD, USA) and an in-house Microsoft Excel-based data analysis program for GC-MS data processing, which identifies GC peaks on the basis of mass spectrum and retention index match. Column chromatography was performed using a silica gel (Kieselgel 60, 70−230, and 230−400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using precoated silica-gel 60 F_{254} and RP-18 F_{254S} plates (both 0.25 mm, Merck).

Plant Material. Dried roots of *A. membranaceus* were purchased from a herbal company, Naemome Dah, Ulsan, Korea, in March 2010. Its scientific name was identified by Prof. Young Ho Kim. A voucher specimen (CNU 10110) was deposited at the Herbarium of College of Pharmacy, Chungnam National University.

Extraction and Isolation. Dried roots (2.0 kg) of *A. membranaceus* were extracted with MeOH (7 L × 3) under reflux. The MeOH extract (250.0 g) of *A. membranaceus* was suspended in water and partitioned with EtOAc and *n*-BuOH. The EtOAc fraction (56.0 g) was subjected to silica gel (5.0 × 30 cm) column chromatography with a gradient of $CHCl_3$/MeOH (1:0, 50:1, 20:1,10: 1, 1:1; 2 L for each step) to give seven fractions (1A−1G). Fraction 1B was separated using YMC (3.0 × 80 cm) column chromatography with a MeOH/acetone/H_2O (1:0.9:1, 1.45:0.95:1; 550 mL for each step) elution solvent to give compounds **11** (18.0 mg; 0.0072%) and **12** (40.0 mg; 0.016%). Fraction 1E was subjected to silica gel (2.5 × 60 cm) column chromatography with a gradient of $CHCl_3$/MeOH/H_2O (7:1:0, 6.5:1:0, 5.5:1:0.1, 4:1:0.1, 1:1:0.2; 2 L for each step) to give five subfractions (1E-1−1E-5). Fraction 1E-4 was separated using YMC (1.0 × 80 cm) column chromatography with a MeOH/H_2O (2.5:1, 2.7:1; 700 mL for each step) elution solvent to give compounds **3** (16.0 mg; 0.0064%) and **6** (7.0 mg; 0.0028%).

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Fraction 1E-5 was separated using YMC (1.0 × 80 cm) column chromatography with a MeOH/acetone/H_2O (0.85:0.85:1, 1:1:1; 650 mL for each step) elution solvent to give compounds **1** (32.0 mg; 0.0128%), **2** (17.0 mg; 0.0068%), **4** (13.0 mg; 0.0052%), and **5** (11.0 mg; 0.0044%). Fraction 1F was separated using silica gel (1.8 × 80 cm) column chromatography with $CHCl_3$/MeOH/H_2O (7:1:0.1, 6.3:1:0.1, 4.5:1:0.1; 1.5 L for each step) to give compounds **7** (15.0 mg; 0.006%), **8** (6.0 mg; 0.0024%), and **9** (16.0 mg; 0.0064%). Fraction 1G was separated using YMC (1 × 80 cm) column chromatography with a MeOH/acetone/H_2O (0.9:1:1; 850 mL) elution solvent to give compound **10** (12.0 mg; 0.0048%).

*6′-O-Linoleylsucrose (**1**):* colorless oil; $[\alpha]_D^{25}$, +46.8 (*c* 0.3, MeOH); UV (MeOH), 230, 203 nm; IR (KBr), ν_{max} 3419, 2975, 1731, 1069 cm^{-1}; ^1H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR data (pyridine-d_5, 150 MHz), see Tables 1 and 2; HR-ESI-MS, *m*/*z* 627.3284 $[M + Na]^+$ (calcd for 627.3351); ESI-MS/MS, *m*/*z* 605 $[M + H]^+$, 443 ($[M + H]^+ - 162$), and 425 ($[M + H]^+ - 162 - H_2O$).

Table 2. ^{13}C NMR Spectroscopic Data of Compounds 1−5[a]

	1	2	3	4	5
1	93.1	93.1	93.2	93.1	93.2
2	73.3	73.3	71.6	73.3	71.6
3	74.5	74.5	74.7	74.5	74.7
4	71.9	71.9	71.6	71.9	71.4
5	74.9	75.0	73.1	75.0	73.1
6	62.5	62.5	64.5	62.5	64.5
1′	64.2	64.2	63.3	64.2	63.3
2′	105.8	105.8	105.5	105.8	105.5
3′	79.2	79.2	79.5	79.2	79.5
4′	76.5	76.5	75.6	76.5	75.6
5′	80.6	80.7	84.3	80.7	84.3
6′	66.5	66.5	64.2	66.5	64.2
1″	173.4	173.4	173.6	173.4	173.5
2″	34.0	34.1	34.0	34.0	34.0
3″	25.0	25.0	25.0	25.0	25.0
4″	29.1	29.2−29.7	29.2−29.7	29.1	29.1
5″	29.1	29.2−29.7	29.2−29.7	29.1	29.1
6″	29.2	29.2−29.7	29.2−29.7	29.3	29.3
7″	29.4	29.2−29.7	29.2−29.7	29.6	29.4
8″	27.3	29.2−29.7	29.2−29.7	27.3	27.3
9″	128.2	29.2−29.7	29.2−29.7	127.3	128.2
10″	128.2	29.2−29.7	29.2−29.7	127.9	128.2
11″	25.8	29.2−29.7	29.2−29.7	25.7	25.8
12″	130.2	29.2−29.7	29.2−29.7	128.4	130.2
13″	130.3	29.2−29.7	29.2−29.7	128.4	130.2
14″	27.2	31.9	31.9	25.8	27.2
15″	29.7	22.7	22.7	130.4	29.7
16″	31.5	14.1	14.1	131.9	31.5
17″	22.6			20.6	22.6
18″	14.0			14.2	14.0

[a]Assignments were done by HMQC and HMBC experiments; measured in pyridine-d_5, 150 MHz.

*6′-O-Palmitoylsucrose (**2**):* colorless oil; $[\alpha]_D^{25}$, +52.9 (*c* 0.3, MeOH); IR (KBr), ν_{max} 3365, 2857, 1071 cm^{-1}; ^1H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR data (pyridine-d_5, 150 MHz), see Tables 1 and 2; HR-ESI-MS, *m*/*z* 603.3263 $[M + Na]^+$ (calcd for 603.3351); ESI-MS/MS, *m*/*z* 581 $[M + H]^+$, 419 ($[M + H]^+ - 162$), and 401 ($[M + H]^+ - 162 - H_2O$).

*6-O-Palmitoylsucrose (**3**):* colorless oil; $[\alpha]_D^{25}$, +55.2 (*c* 0.3, MeOH); IR (KBr), ν_{max} 3362, 2875, 1056 cm^{-1}; ^1H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR data (pyridine-d_5, 150 MHz), see Tables 1 and 2; HR-ESI-MS, *m*/*z* 603.3259 $[M + Na]^+$ (calcd for 603.3351); ESI-MS/MS, *m*/*z* 581 $[M + H]^+$, 419 ($[M + H]^+ - 162$), and 401 ($[M + H]^+ - 162 - H_2O$).

Alkaline Hydrolysis. Compounds **1**−**3** (3.0 mg each) were treated with 1 N NaOH (2 mL) at 60 °C for 40 min followed by neutralization of the solution. The mixture was neutralized with 1 N HCl and extracted with $CHCl_3$. The H_2O layer was analyzed by silica gel thin-layer chromatography (TLC). The solvent system was CH_2Cl_2/MeOH/H_2O (2:1:0.2), and spots were visualized by spraying with 90% EtOH/H_2SO_4 (9:1, v/v) and heated at 180 °C for 2 min. D-Glucose (R_f = 0.42) and D-fructose (R_f = 0.50) were confirmed by comparison with authentic samples. The $CHCl_3$ layer was analyzed by GC-MS, and linoleic acid (t_R = 30.37 min) was confirmed by comparison with authentic sample (see the Supporting Information).

Cell Culture and Reagents. Human hepatocarcinoma HepG2 cells were maintained in Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37 °C and 5% CO_2.

Cell Toxicity Assay. A Cell-Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) was used to analyze the effect of compounds on cell toxicity according to the manufacturer's instructions. Cells were cultured overnight in a 96-well plate (∼1 × 10^4 cells/well). Cell toxicity was assessed after the addition of compounds in a dose-dependent manner. After 24 h of treatment, 10 μL of the CCK-8 solution was added to triplicate wells and incubated for 1 h. Absorbance was measured at 450 nm to determine viable cell numbers in wells.

NF-κB and iNOS-Luciferase Assay. Human hepatocarcinoma HepG2 cells were maintained in DMEM (Invitrogen) containing 10% heat-inactivated FBS, 100 units/mL penicillin, and 10 μg/mL streptomycin at 37 °C and 5% CO_2. The luciferase vector was first transfected into HepG2 cells. After a limited amount of time, the cells were lysed, and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg^{2+} and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2 × 10^5 cells per well in a 12-well plate and grown. After 24 h, cells were transfected with inducible NF-κB or iNOS firefly luciferase reporter constitutively expressing Renilla reporter. After 24 h of transfection, medium was changed to assay medium (Opti-MEM + 0.5% FBS + 0.1 mM NEAA + 1 mM sodium pyruvate +100 units/mL penicillin + 10 μg/mL streptomycin), and cells were pretreated for 1 h with either vehicle (1% DMSO in water) and compounds, followed by 1 h of treatment with 10 ng/mL TNF-α for 23 h. Unstimulated HepG2 cells were used as a negative control (−), and PDTC was used as a positive control. Dual luciferase assay was performed 48 h after transfection, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization.[14]

RNA Preparation and RT-PCR. Total RNA was extracted using Easy-blue reagent (Intron Biotechnology, Seoul, Korea). Approximately 2 μg of total RNA was subjected to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo-dT primers (Promega, Madison, WI, USA) for 1 h at 42 °C. PCR for synthetic cDNA was performed using a Taq polymerase premixture (TaKaRa, Japan). The PCR products were separated by electrophoresis on 1% agarose gels and stained with EtBr. PCR was conducted with the following primer pairs: iNOS sense 5′-TCATCC-GCTATGCTGGCTAC-3′, iNOS antisense 5′-CTCAGGGTCACG-GCCATTG-3′, ICAM-1 sense 5′-CTGCAGACAGTGACCATC-3′, ICAM-1 antisense 5′-GTCCAGTTTCCCGGACAA-3′, β-actin sense 5′-TCACCCACACTGTGCCCATCTACG-3′, and β-actin antisense 5′-CAGCGGAACCGCTCATTGCCAATG-3′. The specificity of products generated by each set of primers was examined using gel electrophoresis and further confirmed by a melting curve analysis. HepG2 cells were pretreated in the absence and presence of compounds for 1 h and then exposed to 10 ng/mL TNF-α for 6 h. Total mRNA was prepared from the cell pellets using Easy-blue. The levels of mRNA were assessed by RT-PCR.[15]

Statistical Analysis. All data represent the mean ± standard deviation (SD) of at least three independent experiments performed in triplicate. Statistical significance is indicated as * ($p < 0.05$) and ** ($p < 0.01$) as determined by Dunnett's multiple-comparison test.

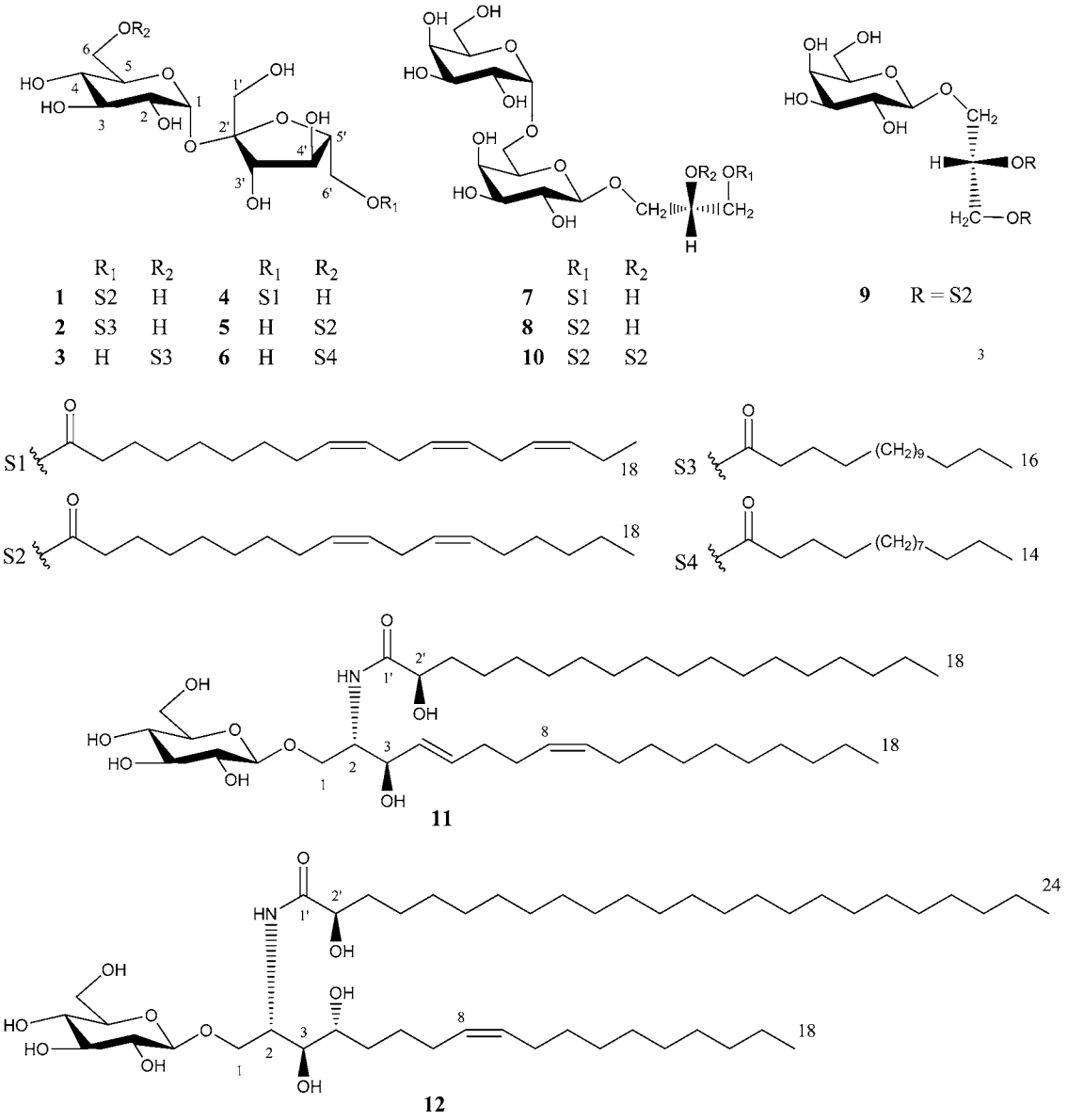

Figure 1. Structures of compounds **1**−**12** from the roots of *A. membranaceus*.

RESULTS AND DISCUSSION

Identification of Compounds 1−12. Twelve compounds, including six sucrose fatty acid esters (**1**−**6**), four galactosyl acylglycerols (**7**−**10**), and two sphingolipids (**11** and **12**), were isolated from the EtOAc fraction of *A. membranaceus*. Their structures were identified as 6′-*O*-linoleylsucrose (**1**), 6′-*O*-palmitoylsucrose (**2**), 6-*O*-palmitoylsucrose (**3**), 6′-*O*-linolenoylsucrose (**4**),[16] 6-*O*-linoleylsucrose (**5**),[17] 6-*O*-myristoylsucrose (**6**),[18] gingerglycolipid A (**7**),[19] gingerglycolipid B (**8**),[19] monogalactosyl dilinoleylglycerol (**9**),[20] digalactosyl dilinoleylglycerol (**10**),[21] 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*)-hydroxyoctadecanoyl]-4,8-sphingadienine (**11**),[22] and 1-*O*-*β*-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxytetracosanoyl]-4-hydroxy-8-sphingenine (**12**).[23] 6′-*O*-Linoleylsucrose (**1**) was identified as a new compound; 6′-*O*-palmitoylsucrose (**2**) and 6-*O*-palmitoylsucrose (**3**) were isolated from nature for the first time. Note that all of the compounds were isolated for the first time from *Astragalus* species (Figure 1).

Compound **1** was isolated as a colorless oil. The molecular formula was established as $C_{30}H_{52}O_{12}$ by HR-ESI-MS (*m*/*z* 627.3284 ([M + Na]$^+$); calcd 627.3351), corresponding to five degrees of unsaturation. The IR absorption bands at 3419 and 1731 cm^{-1} implied the presence of hydroxy and ester groups, respectively. The ^{1}H NMR spectrum of **1** (Table 1) revealed four olefinic proton signals at δ_H 5.45−5.49 (H-9″, -10″, -12″, -13″), five methylene group signals at δ_H 1.57−2.93 (H-2″, -3″, -8″, -11″, -14″), the overlap signals at δ_H 1.19−1.32 belonging to seven methylene groups (H-4″, -5″, -6″, -7″, -15″, -16″, -17″), and one terminal methyl group at δ_H 0.93 (t, *J* = 7.5 Hz, H-18″). Correspondingly, the ^{13}C NMR spectrum (Table 2) showed the signals of 4 olefinic carbons at δ_C 128.2, 130.2, and 130.3 (C-9″, -10″, -12″, -13″), 12 methylene groups at δ_C 22.6−34.0 (C-2″−8″, -11″, -14″−17″), a methyl group signal at δ_C 14.0 (C-18″), and a carbonyl group at δ_C 173.4 (C-1″). Because the signals of the allylic carbons were shifted upfield at δ_C 27.2 and 27.3, the geometry of the double bonds in **1** was determined as (*Z*).[24,25] Combined with the ^{1}H−^{1}H COSY spectrum, these data suggest that **1** possessed a linoleic acid

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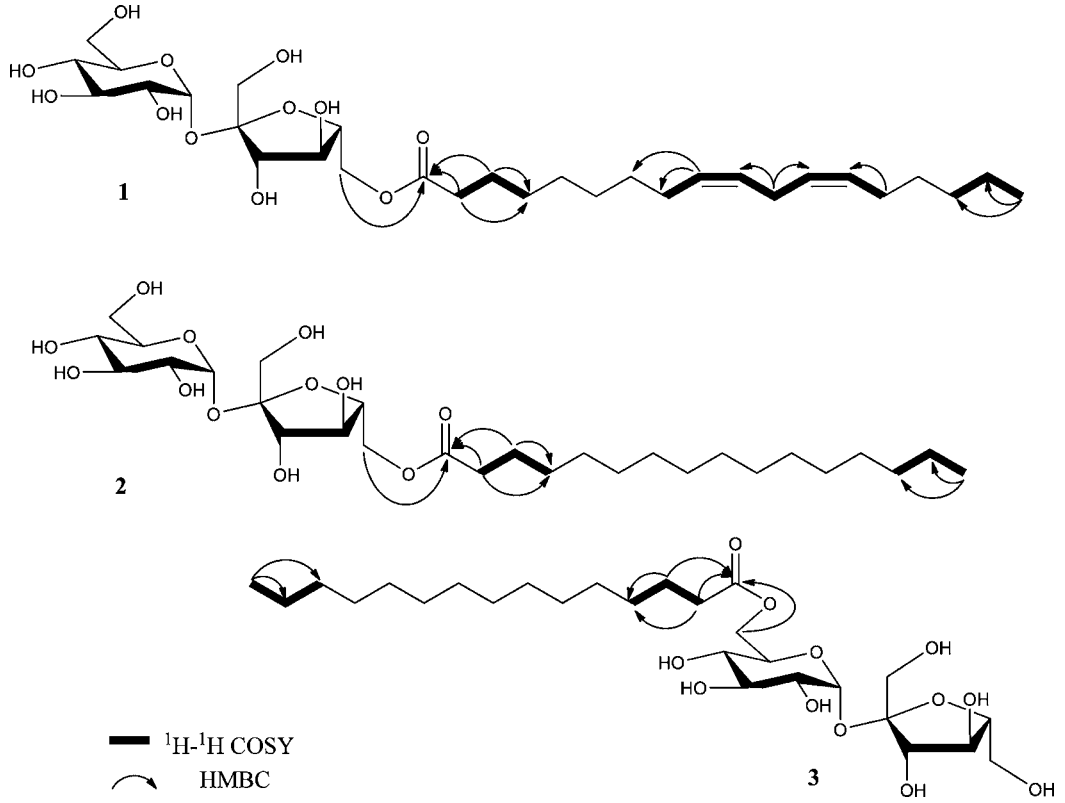

Figure 2. ^{1}H−^{1}H COSY and HMBC correlations of compounds **1**−**3**.

residue; this was confirmed by comparison of the spectroscopic data with those reported in the literature.[26] The other signals in the NMR spectrum of **1** suggested that the remainder of the molecule was a disaccharide, based on the 12 carbon signals at δ_C 62.5−105.8 (Table 2). NMR analysis and alkaline hydrolysis supported this conclusion. Detailed analysis of the NMR spectra provided evidence that the disaccharide was sucrose. Signals for three methyleneoxy carbons at δ_C 62.5, 64.2, and 66.5 and two anomeric carbons at δ_C 93.1 and 105.8 also suggested that the disaccharide was sucrose. Alkaline hydrolysis of **1** with 0.5% NaOH yielded linoleic acid, D-glucose, and D-fructose. The ESI-MS/MS spectrum showed peaks at m/z 443 ($[M + H]^+ - 162$) and 425 ($[M + H]^+ - 162 - H_2O$), which indicated a fragment of monosaccharide. The HMBC spectrum revealed a key correlation between H-6′ (δ_H 4.94, 5.02) and C-1″ (δ_C 173.4), suggesting that the linoleic acid residue was attached to C-6′ of sucrose (Figure 2). On the basis of these data, compound **1** was characterized as 6′-*O*-[(9*Z*,12*Z*)-octadeca-9,12-dienoyl]-*β*-D-fructofuranosyl *α*-D-glucopyranoside and named 6′-*O*-linoleylsucrose.

Compound **2** was obtained as a colorless oil. The molecular formula was determined to be $C_{28}H_{52}O_{12}$ from a pseudomolecular ion peak $[M + Na]^+$ at m/z 603.3263 (calcd 603.3351) in the HR-ESI-MS spectrum. The ^{1}H and ^{13}C NMR spectra revealed that the disaccharide in **2** was the same as the disaccharide in **1**. The two compounds differed in their fatty acid chains. The ^{1}H NMR spectrum of **2** (Table 1) showed 14 methylene groups at δ_H 1.17−2.34 (H-2″−15″) and one terminal methyl group at δ_H 0.84 (t, J = 7.5 Hz, H-16″). The ^{13}C NMR spectrum (Table 2) showed 14 methylene groups at δ_C 22.7−34.1 (C-2″−15″), a methyl group at δ_C 14.1 (C-16″), and a carbonyl group at δ_C 173.4 (C-1″). All of the signals suggested that **2** possessed a palmitic acid residue. Detailed analysis of the ^{1}H−^{1}H COSY and HMBC spectra showed that the palmitic acid residue was attached to C-6′ of sucrose, the same as in **1** (Figure 2). Accordingly, the structure of compound **2** was determined to be 6′-*O*-palmitoylsucrose.

Compound **3** was obtained as a colorless oil. The molecular formula was established as $C_{28}H_{52}O_{12}$ by HR-ESI-MS (m/z 603.3259 ($[M + Na]^+$); calcd 603.3351). A comparison of the spectroscopic data with those of **2** revealed an overall similarity. The difference between them was determined by the HMBC spectrum; the correlation between H-6 (δ_H 4.74, 4.84) and C-1″ (δ_C 173.6) suggested the palmitic acid residue was attached to C-6 of sucrose (Figure 2). Consequently, the chemical structure of compound **3** was determined to be 6-*O*-palmitoylsucrose.

Compounds **2** and **3** were previously synthesized by the transesterification of sucrose with aliphatic esters.[18] However, to the best of our knowledge, **2** and **3** have not been isolated from nature. Compounds **2** and **3** were further characterized by MeOH extraction and subfraction analysis using TLC (RP_{18}, acetone/MeOH/H_2O = 1:1:1, R_f = 0.18, 0.22). The presence of identical spots indicated that **2** and **3** were present in the roots of *A. membranaceus*. On the basis of the data, we concluded that compounds **2** and **3** were isolated for the first time from nature.

NF-κB Inhibitory Activity of Isolated Compounds. The NF-*κ*B inhibitory activity of compounds **1**−**12** was evaluated through the inhibition of TNF-*α*-induced NF-*κ*B luciferase reporter. Compounds **1**−**6** were evaluated by the attenuation of TNF-*α*-induced pro-inflammatory gene expression of iNOS and ICAM-1 in HepG2 cells.[27] Cell viability was measured using a Cell-Counting Kit (CCK)-8. The results showed that compounds **1**−**12** did not exhibit significant cytotoxicity in HepG2 cells at the tested concentrations (data not shown).

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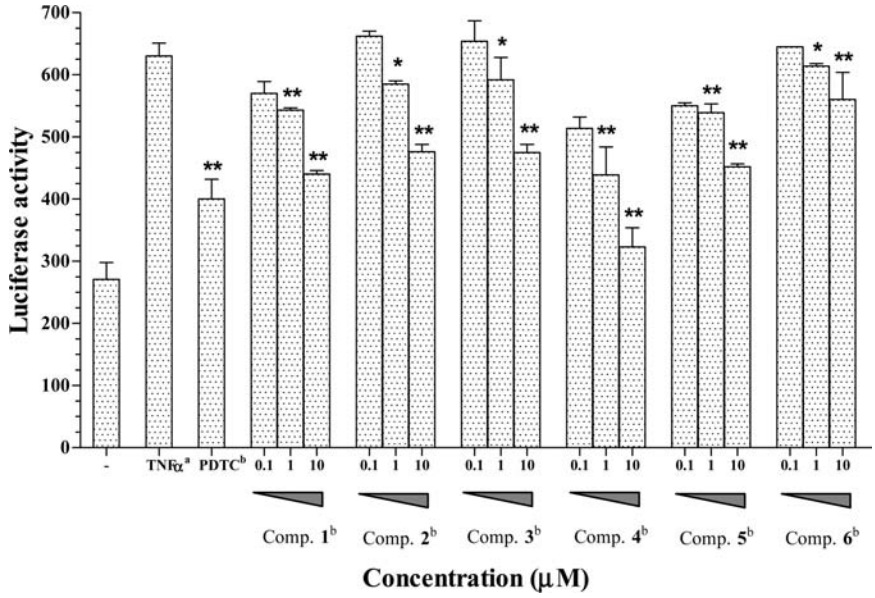

Figure 3. Effects of compounds **1**−**6** on the TNF-α-induced NF-κB luciferase reporter activity in HepG2 cells. The values are means ± SDs (n = 3). [a] Stimulated with TNF-α. [b] Stimulated with TNF-α in the presence of **1**−**6** (0.1, 1, and 10 μM) and pyrrolidine dithiocarbamate (PTDC). PDTC, positive control (10 μM). Statistical significance is indicated as * ($p < 0.05$) and ** ($p < 0.01$) as determined by Dunnett's multiple-comparison test.

HepG2 cells were treated with 10 ng/mL TNF-α and showed increased transcriptional activity compared to untreated cells. The compounds were pretreated with transfected HepG2 cells at various concentrations (0.1, 1, and 10 μM), followed by stimulation with TNF-α. Pyrrolidine dithiocarbamate (PDTC) was used as a positive control (Figure 3). The results showed that compounds **1**−**6** significantly inhibited TNF-α-induced NF-κB transcriptional activity in a dose-dependent manner, with IC_{50} values ranging from 4.4 to 24.7 μM (Table 3). However, other compounds (**7**−**12**) were inactive at the indicated concentrations (IC_{50} > 100 μM, data not shown). Upon examination of the structure−activity relationship of the sucrose fatty acid esters (**1**−**6**), these compounds have similar structures. Compound **4** exhibited the strongest inhibitory effect on TNF-α-induced NF-κB transcriptional activity, which has three olefinic bonds on the aliphatic moiety; compounds **1** and **5** also had significant inhibitory effects and have two olefinic bonds on the aliphatic moiety. The inhibition increased significantly when the sugar chain was linked to an unsaturated aliphatic moiety, and the observed activity was proportional to the number of olefinic bonds on each of the aliphatic moieties. Compounds **2**, **3**, and **6**, with no unsaturated aliphatic chains, did not effectively inhibit NF-κB transcriptional activity, in contrast to compounds **1**, **4**, and **5**. Therefore, olefinic bonds at the aliphatic moiety seem to be key functional elements. Compounds **7**−**10**, which also have olefinic bonds at the aliphatic moiety, did not show inhibitory effects. These results showed that glycerol derivatives were inactive in this bioassay system. Moreover, a comparison of compounds **2**, **3**, and **6** indicated that the length of the fatty acid moieties also plays an important role.

Table 3. Inhibitory Effects of Compounds 1−6 on the TNF-α-Induced NF-κB Transcriptional and iNOS Promoter Activity

	IC_{50}[a] (μM)	
compd	NF-κB	iNOS
1	9.0 ± 0.39	3.6 ± 0.51
2	11.3 ± 0.04	4.2 ± 0.23
3	11.3 ± 1.07	5.0 ± 0.85
4	4.4 ± 0.91	3.4 ± 0.29
5	9.7 ± 0.67	4.0 ± 0.69
6	24.7 ± 1.12	6.1 ± 0.32
PDTC[b]	8.3 ± 0.89	3.3 ± 0.21

[a]Values are means ± SDs (n = 3). [b]Positive control.

The activation of NF-κB is involved in immunity, inflammation, cell proliferation, and negative feedback of the NF-κB signal. NF-κB targets the expression of genes such as iNOS and ICAM-1, which play important roles in inflammatory response.[28−30] Compounds **1**−**6** were investigated for their transcriptional inhibitory effects on iNOS and ICAM-1 gene expression (Figure 4). Compounds **1**−**5** significantly inhibited the induction of iNOS and ICAM-1 mRNA, indicating that these compounds reduced the transcription of these genes. Among them, compounds **3** and **4** inhibited the induction of ICAM-1 mRNA, and compounds **1**, **2**, and **4** significantly inhibited the induction of iNOS mRNA in a concentration-dependent manner. The housekeeping protein, β-actin was unchanged by the presence of compounds **1**−**6** at the same concentration. Moreover, compounds **1**−**6** also decreased TNF-α-induced iNOS promoter activity in a dose-dependent manner, with IC_{50} values ranging from 3.3 to 5.0 μM (Figure 5). These data suggest that the sucrose fatty acid ester compounds isolated from *A. membranaceus* suppress TNF-α-induced NF-κB transcriptional activity via inhibition of iNOS gene transcription.

In this study, six sucrose fatty acid esters (**1**−**6**), four galactosyl acylglycerols (**7**−**10**), and two sphingolipids (**11** and **12**) were isolated from *A. membranaceus*. 6′-*O*-Linoleylsucrose (**1**) is a new compound, 6′-*O*-palmitoylsucrose (**2**) and 6-*O*-palmitoylsucrose (**3**) were isolated from nature for the first time, and other compounds were isolated for the first time from *Astragalus*. To our knowledge, this is the first report on the sucrose fatty acid ester components of *A. membranaceus* and

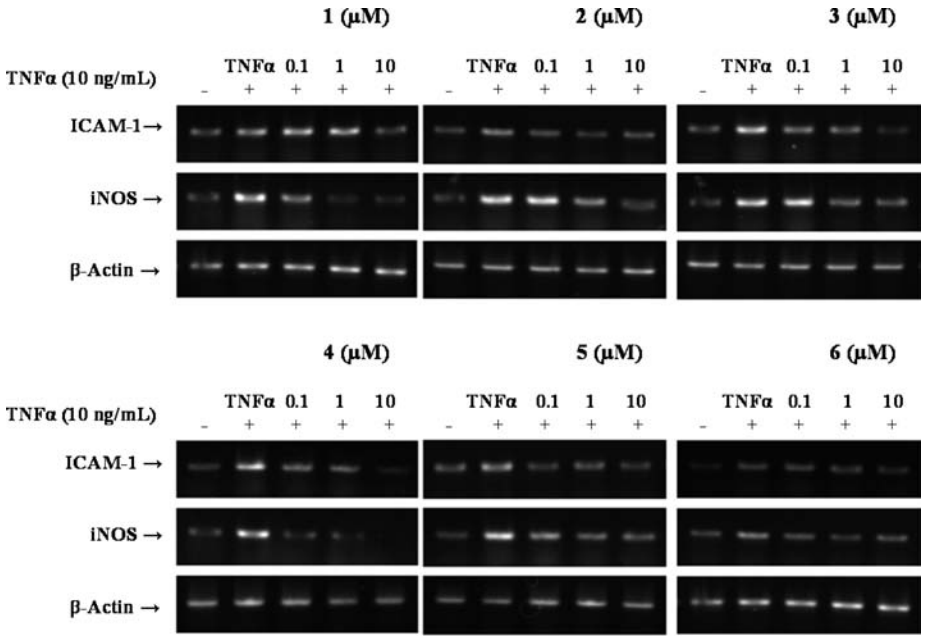

Figure 4. Effects of compounds **1**–**6** on iNOS and ICAM-1 mRNA expression in HepG2 cells: −, cells were treated without 10 μg/mL TNF-α and compounds; +, cells were treated with 10 μg/mL TNF-α only; + 0.1, 1, 10, cells were treated with 10 μg/mL TNF-α and compounds.

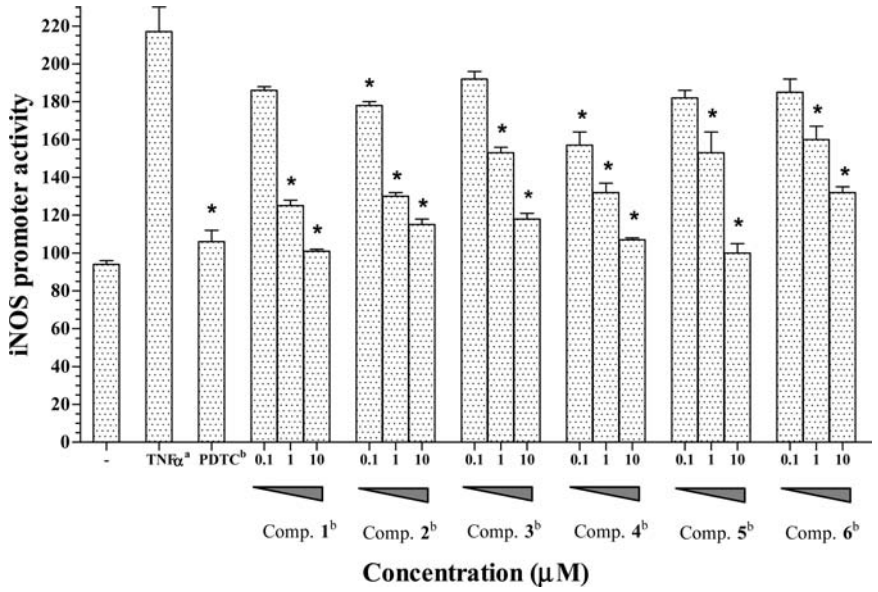

Figure 5. Effect of compounds **1**–**6** on iNOS transcription in HepG2 cells. HepG2 cells were transfected with pGL2-iNOSLuc and pRL-TK. Values are means ± SDs (n = 3). [a] Stimulated with TNF-α. [b] Stimulated with TNF-α in the presence of **1**–**6** (0.1, 1, and 10 μM) and pyrrolidine dithiocarbamate (PTDC). PDTC, positive control (10 μM). Statistical significance is indicated as * ($p < 0.05$) as determined by Dunnett's multiple-comparison test.

their effects on NF-κB inhibition. Notably, these results suggest that sucrose fatty acid esters are new bioactive components of this plant. These results may provide a scientific basis for the complement of the active components in *A. membranaceus*, as well as for the development of novel anti-inflammatory agents.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR, HMQC, HMBC, COSY, HR-ESI-MS, and ESI-MS/MS spectra of compounds **1**–**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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