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Isolation, Characterization, and NO Inhibitory Activities of Sesquiterpenes from *Blumea balsamifera*

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Supporting Information

ABSTRACT: *Blumea balsamifera* belongs to the family Compositae, and its leaves have been used as a flavoring ingredient and a tea. A phytochemical investigation of the aerial parts of *B. balsamifera* led to the isolation of 10 new (**1**−**10**) and 1 known (**11**) sesquiterpenes. Their structures were elucidated on the basis of extensive one- and two-dimensional nuclear magnetic resonance (heteronuclear multiple-quantum coherence, heteronuclear multiple-bond correlation, $^{1}H-^{1}H$ correlation spectroscopy, and nuclear Overhauser effect spectrometry) spectroscopic data analyses, and the structure of compound **1** was confirmed by X-ray crystallography. The inhibitory activities on lipopolysaccharide-induced NO production in murine microglial BV-2 cells of these sesquiterpenes were evaluated, and all of the compounds showed inhibitory effects.

KEYWORDS: *Blumea balsamifera, sesquiterpenes, flavoring ingredient, NO inhibitory activities*

INTRODUCTION

Blumea balsamifera (L.) DC, belonging to the family Compositae, is a perennial herbaceous or subshrub plant with strong aromatic properties, distributed in southeast Asia.[1] Its leaves have been used as a flavoring ingredient and a tea for cough and gas pain.[2−4] The roots and leaves of *B. balsamifera* have also been used as a folk medicine for the treatment of arthritis, rheumatism, and gynecologic diseases.[2,3] Previous phytochemical investigations on *B. balsamifera* revealed the main presence of sesquiterpenoids and flavnoids in this plant,[3−6] which showed the plasmin inhibitory, free-radical-scavenging, and NO inhibitory activities.[3,5,6] Although some chemical constituents from *B. balsamifera* and their bioactivities have been reported, much attention was still given to this aromatic plant *B. balsamifera* because of its various functions.[3] In our continuous survey on the chemical composition of traditional folk medicines or medicinal food,[7−10] we investigated the chemical constituents of the aerial parts of *B. balsamifera*, whose leaves, used a flavoring ingredient and a tea,[2,3] evoked our great interest.[2−4] As a result, 10 new sesquieterpenes, named balsamiferines A−J (**1**−**10**), and 1 known sesquiterpene (**11**) (Figure 1), were isolated from the aerial parts of *B. balsamifera*. Their structures were elucidated on the basis of extensive one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) [heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), $^{1}H-^{1}H$ correlation spectroscopy (COSY), and nuclear Overhauser effect spectrometry (NOESY)] spectroscopic data analyses, and the structure of compound **1** was confirmed by X-ray crystallography. The inhibitory activities on lipopolysaccharide (LPS)-induced NO production in murine microglial BV-2 cells of these sesquiterpenes were evaluated, and all of the compounds showed inhibitory effects. This paper herein describes the isolation and characterization of these sesquiterpenes and their inhibitory effects on LPS-induced NO production in murine microglial BV-2 cells.

MATERIALS AND METHODS

General Procedures. Melting points were determined with an XT-4 microscopic thermometer. The optical rotations were measured in CH_2Cl_2 using a Rudolph Autopol IV automatic polarimeter. The infrared (IR) spectra were taken on a Bruker Tensor 27 Fourier transform infrared (FTIR) spectrometer with KBr discs. The electrospray ionization mass spectrometry (ESIMS) spectra were obtained on a LCQ-Advantage mass spectrometer (Finnigan Co., Ltd., San Jose, CA). High resolution (HR)-ESIMS spectra were recorded by IonSpec 7.0 T FTICR MS (IonSpec Co., Ltd., Lake Forest, CA) or Agilent 6520 Q-TOF LC/MS (Agilent, Santa Clara, CA). The 1D and 2D NMR spectra were recorded on a Bruker AV 400 instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C) with tetramethylsilane (TMS) as an internal standard. High-performance liquid chromatography (HPLC) separations were performed on a CXTH system, equipped with a UV3000 detector at 210 nm (Beijing Chuangxintongheng Instruments Co., Ltd., China), and a YMC-pack ODS-AM (250 × 20 mm) column (YMC Co., Ltd., Japan). X-ray crystallographic analysis was carried out on a Rigaku Saturn 944 charge-coupled device (CCD) diffractometer equipped with a multilayer monochromator and Mo Kα radiation (λ = 0.710 75 Å) (Rigaku Co., Ltd., Japan). The structure was solved by direct methods (SHELXL-97), expanded using Fourier techniques, and refined with full-matrix least-squares on F^2 (SHELXL-97). Silica gel was used for column chromatography (200−300 mesh, Qingdao Marine Chemical Group Co., Ltd., China). Chemical reagents for isolation were of analytical grade and purchased from Tianjin Yuanli Co., Ltd., China. Biological reagents were from Sigma Company. The murine microglial BV-2 cell line was from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (China).

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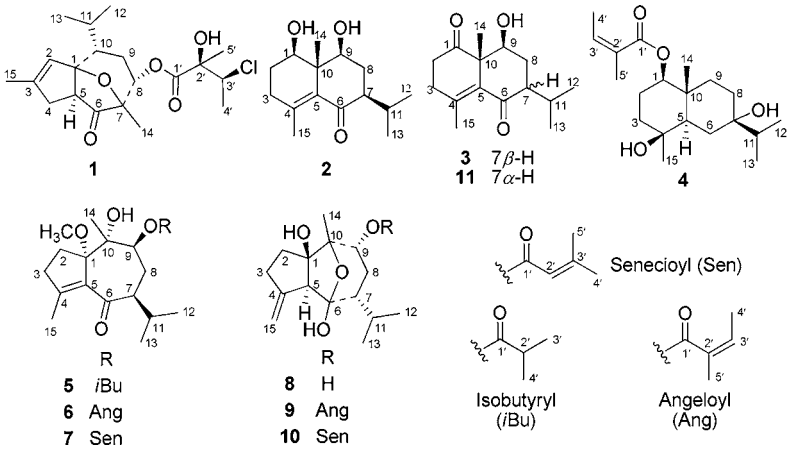

Figure 1. Structures of compounds **1**−**11** from *B. balsamifera*.

Table 1. ^{13}C NMR Data (δ_C) of Compounds 1−10 ($CDCl_3$, 100 MHz)[a]

position	1	2	3	4	5	6	7	8	9	10
1	97.1	70.2	216.8	80.8	96.3	96.5	96.3	92.8	92.3	92.4
2	125.6	24.9	34.9	23.1	28.2	28.5	28.4	38.5	38.1	38.2
3	147.1	27.8	32.6	39.0	37.3	37.5	37.3	33.7	33.7	33.8
4	37.2	139.3	139.9	70.7	162.7	162.8	162.5	150.2	150.4	150.6
5	51.2	135.3	135.2	44.5	132.9	133.0	132.9	59.2	58.6	58.9
6	214.1	205.9	206.2	28.5	201.1	201.3	201.1	103.0	103.0	103.0
7	83.8	53.6	56.0	73.1	51.6	51.8	51.6	48.8	48.6	48.6
8	74.4	28.3	29.0	28.2	27.1	27.2	27.0	30.1	26.7	26.8
9	27.2	69.3	66.7	34.0	76.7	77.1	76.1	76.6	76.9	76.3
10	44.9	45.0	52.2	37.5	79.0	79.0	78.8	79.3	79.3	79.4
11	28.4	25.2	28.2	38.7	26.7	26.8	26.7	27.7	27.7	27.7
12	18.4	17.5	19.8	16.5	18.1	18.2	18.0	18.8	18.8	18.8
13	22.4	20.4	21.4	16.6	20.7	20.6	20.7	22.9	22.9	23.0
14	16.7	18.2	18.0	12.5	15.4	15.8	15.5	18.7	18.2	18.3
15	17.1	20.8	20.5	29.2	17.7	17.8	17.6	111.0	110.7	110.8
1′	173.6			167.4	176.8	167.7	166.2		165.9	164.6
2′	77.0			127.9	34.1	128.1	116.2		126.7	115.1
3′	62.2			137.0	18.7	137.8	156.6		140.0	159.3
4′	17.7			15.4	18.9	15.8	27.2		15.7	27.5
5′	22.6			20.3		20.8	20.0		20.6	20.4
OCH_3					49.3	49.5	49.3			

[a]The assignments of ^{13}C NMR data are based on distortionless enhancement by polarization transfer (DEPT), $^1H-^1H$ COSY, HMQC, and HMBC experiments.

Plant Material. The aerial parts of *B. balsamifera* native to Guangxi province, China, were purchased in July 2008 from Bozhou Materia Medica Market, Anhui province, China. A voucher specimen (20080710) was identified by Dr. Yuanqiang Guo (College of Pharmacy, Nankai University, China) and deposited at the laboratory of the Research Department of Natural Medicine, College of Pharmacy, Nankai University, China.

Extraction and Isolation. The air-dried aerial parts of *B. balsamifera* (6.0 kg) were powdered and extracted with MeOH (3 × 42 L) under reflux. The organic solvent was evaporated to obtain a crude extract (800 g). The extract was suspended in H_2O (0.8 L) and partitioned with EtOAc (3 × 0.8 L). The EtOAc-soluble part (220.0 g) was subjected to silica gel column chromatography, using a gradient of acetone in petroleum ether (1−40%), to give seven fractions (F_1−F_7) based on thin-layer chromatography (TLC) analyses. F_5 was fractionated by medium-pressure liquid chromatography (MPLC) over ODS eluting with a step gradient from 60 to 90% MeOH in H_2O to give four subfractions ($F_{5\text{-}1}$−$F_{5\text{-}4}$). $F_{5\text{-}3}$ was purified by preparative HPLC (YMC-pack ODS-AM, 20 × 250 mm, 75% MeOH in H_2O) to afford compound **1** (t_R = 39 min, 29.9 mg). The further purification of $F_{5\text{-}2}$ with the same HPLC system using 72% MeOH in H_2O resulted in the isolation of compounds **3** (t_R = 36 min, 15.6 mg) and **11** (t_R = 32 min, 12.5 mg). Fraction F_4, using the same MPLC (60−90% MeOH in H_2O), provided three subfractions $F_{4\text{-}1}$−$F_{4\text{-}3}$, and the further purification of $F_{4\text{-}2}$ by the above HPLC system (77% MeOH in H_2O) afforded compound **2** (t_R = 32 min, 11.9 mg). Fraction F_3 was subjected to the same MPLC (60−90% MeOH in H_2O) to obtain three subfractions $F_{3\text{-}1}$−$F_{3\text{-}3}$, and the following purification of $F_{3\text{-}2}$ by the same HPLC system (79% MeOH in H_2O) yielded compounds **4** (t_R = 34 min, 14.7 mg), **6** (t_R = 45 min, 12.5 mg), and **8** (t_R = 28 min, 16.8 mg). Compounds **5** (t_R = 27 min, 14.7 mg) and **7** (t_R = 33 min, 12.5 mg) were isolated from $F_{2\text{-}3}$ (88% MeOH in H_2O), which was obtained from F_2 by the above MPLC. Using the same protocols for the above fractions and subfractions, fraction F_6 yielded three subfractions $F_{6\text{-}1}$−$F_{6\text{-}3}$, compound **9** (t_R = 32 min, 8.9 mg) was isolated from $F_{6\text{-}3}$ (70% MeOH in H_2O), and compound **10** (t_R = 37 min, 12.7 mg) was obtained from $F_{6\text{-}2}$ (67% MeOH in H_2O).

Balsamiferine A (1). Colorless small quadrate crystals (MeOH). Melting point (mp) = 132−134 °C. $[\alpha]_D^{25}$: +44.0 (*c* 0.10, CH_2Cl_2). IR (KBr) ν_{max}: 3480, 2959, 2930, 1750, 1652, 1455, 1378, 1266, and 746

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Table 2. ^{1}H NMR Data (δ_H) of Compounds 1−5 ($CDCl_3$, 400 MHz)[a]

position	1	2	3	4	5
1		3.70 br s		4.48 d (11.2)	
2	5.51 s	2.20 m	2.79 m	1.53 m	1.72 m
		1.68 m	2.40 m	1.50 m	1.36 m
3		2.16 m	2.60 m	1.58 m	2.42 m
		1.86 m	2.54 m	1.46 m	2.31 m
4	2.44 dd (17.4, 6.0)				
	2.66 dd (17.4, 10.6)				
5	3.06 dd (10.6, 6.0)			1.45 m	
6				1.24 m	
				1.18 m	
7		2.04 m	2.10 m		2.47 m
8	4.86 dd (10.8, 6.3)	1.91 m	2.13 m	1.43 m	2.32 m
		1.58 m	1.86 m	1.37 m	1.82 m
9	2.31 m	4.28 dd (11.4, 3.8)	4.29 dd (11.8, 4.0)	1.39 m	5.45 dd (11.8, 3.5)
	2.25 m			1.25 m	
10	2.21 m				
11	1.70 m	1.67 m	1.90 m	1.43 m	2.20 m
12	0.79 d (6.8)	0.74 d (6.7)	0.99 d (6.6)	0.78 d (6.0)	0.80 d (6.8)
13	0.95 d (6.8)	0.80 d (6.7)	0.87 d (6.6)	0.78 d (6.0)	0.87 d (6.8)
14	1.82 s	0.79 s	1.27 s	0.96 s	0.99 s
15	1.24 s	1.60 s	1.89 s	1.02 s	2.09 s
2′					2.52 q (5.6)
3′	4.26 q (6.8)			5.89 q (7.0)	1.12 d (5.6)
4′	1.54 d (6.8)			1.82 d (7.0)	1.14 d (5.6)
5′	1.43 s			1.72 s	
OH			3.99 br s		
OCH_3					3.03 s

[a]The assignments of ^{1}H NMR data are based on ^{1}H−^{1}H COSY, HMQC, and HMBC experiments.

Table 3. ^{1}H NMR Data (δ_H) of Compounds 6−10 ($CDCl_3$, 400 MHz)[a]

position	6	7	8	9	10
2	1.28 m	1.32 m	2.13 m	2.02 m	2.03 m
	0.86 m	0.84 m	1.70 m	1.76 m	1.78 m
3	2.47 m	2.35 m	2.48 m	2.58 m	2.54 m
	2.39 m	2.31 m	2.28 m	2.35 m	2.34 m
5			3.07 s	3.11 s	3.10 s
7	2.54 m	2.47 m	1.91 m	2.03 m	2.03 m
8	2.40 m	2.18 m	2.10 m	2.24 m	2.20 m
	2.25 m	1.75 m	1.72 m	1.72 m	1.71 m
9	5.61 d (11.7)	5.48 dd (11.6, 2.0)	3.61 dd (10.6, 6.1)	5.00 dd (11.2, 6.2)	4.94 dd (11.2, 6.2)
11	1.87 m	2.10 m	1.88 m	1.89 m	1.92 m
12	0.86 d (6.9)	0.77 d (6.8)	0.91 d (6.5)	0.92 d (6.8)	0.90 d (6.8)
13	0.94 d (6.9)	0.85 d (6.8)	1.00 d (6.5)	1.03 d (7.8)	1.02 d (6.8)
14	1.07 s	0.97 s	1.33 s	1.25 s	1.24 s
15	2.15 s	2.07 s	4.90 s	4.92 s	4.92 s
			5.29 s	5.32 s	5.31 s
2′		5.64 s			5.66 s
3′	6.06 q (7.1)			6.15 q (7.3)	
4′	1.99 d (7.1)	1.81 s		2.03 d (7.3)	1.92 s
5′	1.91 s	2.09 s		1.90 s	2.20 s
OCH_3	3.09 s	3.01 s			

[a]The assignments of ^{1}H NMR data are based on ^{1}H−^{1}H COSY, HMQC, and HMBC experiments.

cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^{1}H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 2. ESIMS m/z: 402 $[M + NH_4]^+$. HR-ESIMS m/z: 402.2047 $[M + NH_4]^+$, calcd for $C_{20}H_{33}ClNO_5$, 402.2047.

X-ray Crystal Data of Balsamiferine A (1).[11] $C_{20}H_{29}ClO_5$, M_r = 384.88, orthorhombic, space group $P2(1)2(1)2(1)$, a = 9.9580 (12) Å, b = 11.1160 (16) Å, c = 18.302 (2) Å, V = 2025.9 (4) Å^3, Z = 4, D_{calcd} = 1.262 g/cm^3, and crystal dimensions of 0.24 × 0.22 × 0.20 mm were used for measurements. The total number of reflections measured was 25 455, of which 4811 were unique and 4141 were observed, $I > 2\sigma(I)$. Final indices: R_1 = 0.0275 and wR_2 = 0.0666 for observed reflections and R_1 = 0.0313 and wR_2 = 0.0674 for all reflections.

Balsamiferine B (2). Colorless oil. $[\alpha]_D^{25}$: +49.6 (c 0.56, CH_2Cl_2). IR (KBr) ν_{max}: 3442, 2958, 2875, 1667, 1637, 1459, 1376, 1265, and

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748 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 2. ESIMS m/z: 275 $[M + Na]^+$. HR-ESIMS m/z: 275.1614 $[M + Na]^+$, calcd for $C_{15}H_{24}NaO_3$, 275.1623.

Balsamiferine C (3). Colorless oil. $[\alpha]_D^{25}$: −21.5 (c 0.13, CH_2Cl_2). IR (KBr) ν_{max}: 3481, 2959, 2929, 1685, 1457, 1377, 1266, and 744 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 2. ESIMS m/z: 273 $[M + Na]^+$. HR-ESIMS m/z: 273.1459 $[M + Na]^+$, calcd for $C_{15}H_{22}NaO_3$, 273.1467.

Balsamiferine D (4). Colorless oil. $[\alpha]_D^{25}$: −15.7 (c 0.94, CH_2Cl_2). IR (KBr) ν_{max}: 3484, 2960, 2933, 1699, 1649, 1458, 1384, 1262, and 749 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 2. ESIMS m/z: 337 $[M - H]^-$. HR-ESIMS m/z: 337.2365 $[M - H]^-$, calcd for $C_{20}H_{33}O_4$, 337.2379.

Balsamiferine E (5). Colorless oil. $[\alpha]_D^{25}$: −51.3 (c 0.16, CH_2Cl_2). IR (KBr) ν_{max}: 3474, 2960, 2928, 1729, 1674, 1639, 1469, 1260, and 750 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 2. ESIMS m/z: 375 $[M + Na]^+$. HR-ESIMS m/z: 375.2146 $[M + Na]^+$, calcd for $C_{20}H_{32}NaO_5$, 375.2147.

Balsamiferine F (6). Colorless oil. $[\alpha]_D^{25}$: −21.7 (c 0.12, CH_2Cl_2). IR (KBr) ν_{max}: 3486, 2958, 2930, 1716, 1677, 1457, 1265, and 744 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 3. ESIMS m/z: 387 $[M + Na]^+$. HR-ESIMS m/z: 387.2139 $[M + Na]^+$, calcd for $C_{21}H_{32}NaO_5$, 387.2147.

Balsamiferine G (7). Colorless oil. $[\alpha]_D^{25}$: −36.2 (c 0.26, CH_2Cl_2). IR (KBr) ν_{max}: 3468, 2957, 2931, 1714, 1675, 1650, 1444, 1229, 1149, and 750 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 3. ESIMS m/z: 387 $[M + Na]^+$. HR-ESIMS m/z: 387.2145 $[M + Na]^+$, calcd for $C_{21}H_{32}NaO_5$, 387.2147.

Balsamiferine H (8). Colorless oil. $[\alpha]_D^{25}$: +1.5 (c 0.14, CH_2Cl_2). IR (KBr) ν_{max}: 3464, 2958, 2930, 1649, 1458, 1265, and 746 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 3. ESIMS m/z: 291 $[M + Na]^+$. HR-ESIMS m/z: 291.1569 $[M + Na]^+$, calcd for $C_{15}H_{24}NaO_4$, 291.1572.

Balsamiferine I (9). Colorless oil. $[\alpha]_D^{25}$: +2.9 (c 0.14, CH_2Cl_2). IR (KBr) ν_{max}: 3458, 2961, 2931, 1645, 1456, 1265, and 747 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 3. ESIMS m/z: 373 $[M + Na]^+$. HR-ESIMS m/z: 373.1985 $[M + Na]^+$, calcd for $C_{20}H_{30}NaO_5$, 373.1991.

Balsamiferine J (10). Colorless oil. $[\alpha]_D^{25}$: +14.3 (c 0.14, CH_2Cl_2). IR (KBr) ν_{max}: 3461, 2959, 2929, 1698, 1457, 1265, and 744 cm^{-1}. ^{13}C NMR (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data: see Tables 1 and 3. ESIMS m/z: 373 $[M + Na]^+$. HR-ESIMS m/z: 373.1978 $[M + Na]^+$, calcd for $C_{20}H_{30}NaO_5$, 373.1991.

Bioassay for NO Production. Murine microglial BV-2 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) inactivated fetal bovine serum and 100 units/mL penicillin/streptomycin under a water-saturated atmosphere of 95% air and 5% CO_2. The cells were seeded in 96-well culture plates (5×10^4 cells/well) and allowed to adhere for 24 h at 37 °C. The cells were incubated for 20 h with or without 0.4 μg/mL LPS (Sigma Chemical Co., St. Louis, MO) in the absence or presence of the test compounds. 2-Methyl-2-thiopseudourea, sulfate (SMT) was used as a positive control. As a parameter of NO synthesis, the nitrite concentration was measured by the Griess reaction using the supernatant of the BV-2 cells. Briefly, 50 μL of the cell culture supernatant were reacted with 50 μL of the Griess reagent [1:1 mixture of 0.1% *N*-(1-naphtyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate. and the absorbance was read with a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA) at 550 nm. The experiment was performed 3 times, and the IC_{50} values for the inhibition of NO production were determined on the basis of linear or nonlinear regression analysis of the concentration−response data curves.

RESULTS AND DISCUSSION

The ethyl acetate-soluble part of the methanol extract of the aerial parts of *B. balsamifera* was fractionated by column chromatography and purified by HPLC to obtain 10 new (**1**−**10**) and 1 known (**11**) compounds. The known compound was identified by a comparison of spectroscopic data to those reported in the literature as samboginone (**11**).[4]

Compound **1** was obtained as colorless small quadrate crystals. Its HR-ESIMS provided the molecular formula, $C_{20}H_{29}ClO_5$, through the presence of a peak at m/z 402.2047 $[M + NH_4]^+$ (calcd for $C_{20}H_{33}ClNO_5$, 402.2047). The ^1H NMR spectrum of compound **1** exhibited six methyl signals [δ_H 0.79 (3H, d, J = 6.8 Hz, H_3-12), 0.95 (3H, d, J = 6.8 Hz, H_3-13), 1.24 (3H, s, H_3-15), 1.43 (3H, s, H_3-5′), 1.54 (3H, d, J = 6.8 Hz, H_3-4′), and 1.82 (3H, s, H_3-14)] (Table 2), one olefinic proton [δ_H 5.51 (1H, s, H-2)] and one oxygenated methine [δ_H 4.86 (1H, dd, J = 10.8, 6.3 Hz, H-8)]. The ^{13}C NMR spectrum of compound **1** showed 20 carbon resonances. From the ^1H and ^{13}C NMR spectra, one isopropyl moiety was evident from the proton signals [δ_H 1.70 (1H, m, H-11), 0.79 (3H, d, J = 6.8 Hz, H_3-12), and 0.95 (3H, d, J = 6.8 Hz, H_3-13)] and corresponding carbon resonances [δ_C 28.4 (C-11), 18.4 (C-12), and 22.4 (C-13)]. Additionally, the carbonyl carbon signal at δ_C 173.6 (C-1′) suggested the presence of a side chain of acyl moiety, which was further deduced and defined as 2-hydroxy-2-methyl-3-chlorobutyryl based on those sesquiterpenes with acyl groups and the analyses of the HMQC, HMBC, and HR-ESIMS data.[3,4] Apart from the above eight signals for the acyl group and the isopropyl moiety, there are additional 12 resonances exhibited for the parent skeleton in the ^{13}C NMR spectrum, which comprised 2 methyls (C-14 and C-15), 2 methylenes (C-4 and C-9), 4 methines (C-2, C-5, C-8, and C-10), and 4 quaternary carbons (C-1, C-3, C-6, and C-7) based on the DEPT and HMQC spectra. The 12 carbon resonances and the 3 carbon resonances for the isopropyl moiety suggested that compound **1** should be a sesquiterpene with an acyloxy group (2-hydroxy-2-methyl-3-chlorobutyryloxy). The following interpretation of HMQC and HMBC spectra led to the elucidation of the skeleton. In the HMBC spectrum, the long-range correlations of proton H-2 with the carbons C-1, C-3, C-4, and C-5 revealed the presence of a five-membered ring, which was fused with the seven-membered ring via C-1 and C-5 units based on the cross-peaks of H-2 to C-10, C-1, and C-5, H-5 to C-4, C-1, C-2, C-3, C-6, and C-7, and H-10 to C-8, C-9, C-1, C-2, and C-5. The long-range couplings of H-11 to C-12, C-13, C-10, C-9, and C-1, H-14 to C-6, C-7, and C-8, and H-8 to C-9, C-10, C-6, and C-7 led to the assignments of carbon signals at δ_C 214.1 (C-6), 83.8 (C-7), 74.4 (C-8), 27.2 (C-9), and 44.9 (C-10). The HMBC correlation of the proton signal at δ_H 4.86 (H-8) with the carbonyl carbon at δ_C 173.6 (C-1′) suggested that the acyloxy group was located at C-8. After further analysis of the HMQC, HMBC, and $^1H-^1H$ COSY spectra (Figure 2), all of the proton and carbon signals were assigned unambiguously, which resulted in the establishment of the planar structure for compound **1**. However, the molecular formula based on the deduced structure for compound **1** was not compatible with the HR-ESIMS data, which implied the presence of another ring based on the total unsaturation degrees. According to the NMR data of compound **1**, an oxygen bridge was further assumed, which could only be 1,7-epoxy. Thus, the planar structure of compound **1** was determined.

The relative configuration of compound **1** was elucidated on the basis of the NOESY spectrum and Chem3D modeling. The NOESY correlations observed for H-5/H-11, H-5/H-9α, H-9α/H-11, H-8/H-10, H-10/H-2, and H-9β/H_3-12 (Figure 3), and the Chem3D modeling suggested a conformation for

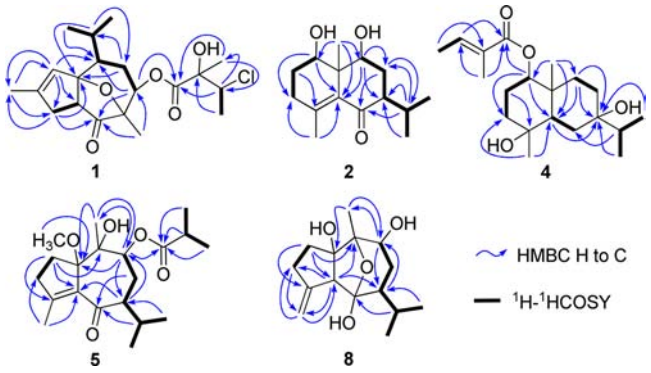

Figure 2. Selected HMBC and $^1H-^1H$ COSY correlations of compounds **1**, **2**, **4**, **5**, and **8**.

compound **1** as depicted in Figure 3, where H-5 was in an α position and H-8 and H-10 were in β positions. However, it is difficult to determine the relative configuration of the acyl moiety. Ultimately, to confirm the above assignments and the configuration, a single-crystal X-ray crystallographic analysis using anomalous scattering of Mo Kα radiation was carried out.[11] A drawing of thermal ellipsoid representation, with the atom numbering indicated, is shown in Figure 4. All of the above evidence confirmed the structure of compound **1** as depicted in Figure 1, which has been named balsamiferine A.

Compound **2** possessed a molecular formula $C_{15}H_{24}O_3$ as determined by the HR-ESIMS (m/z 275.1614 $[M + Na]^+$, calcd for $C_{15}H_{24}NaO_3$, 275.1623). The ^1H NMR spectrum of compound **2** exhibited four methyls [δ_H 0.74 (3H, d, J = 6.7 Hz, H_3-12), 0.79 (3H, s, H_3-14), 0.80 (3H, d, J = 6.7 Hz, H_3-13), and 1.60 (3H, s, H_3-15)] and two oxygenated methines [δ_H 3.70 (1H, br s, H-1) and 4.28 (1H, dd, J = 11.4, 3.8 Hz, H-

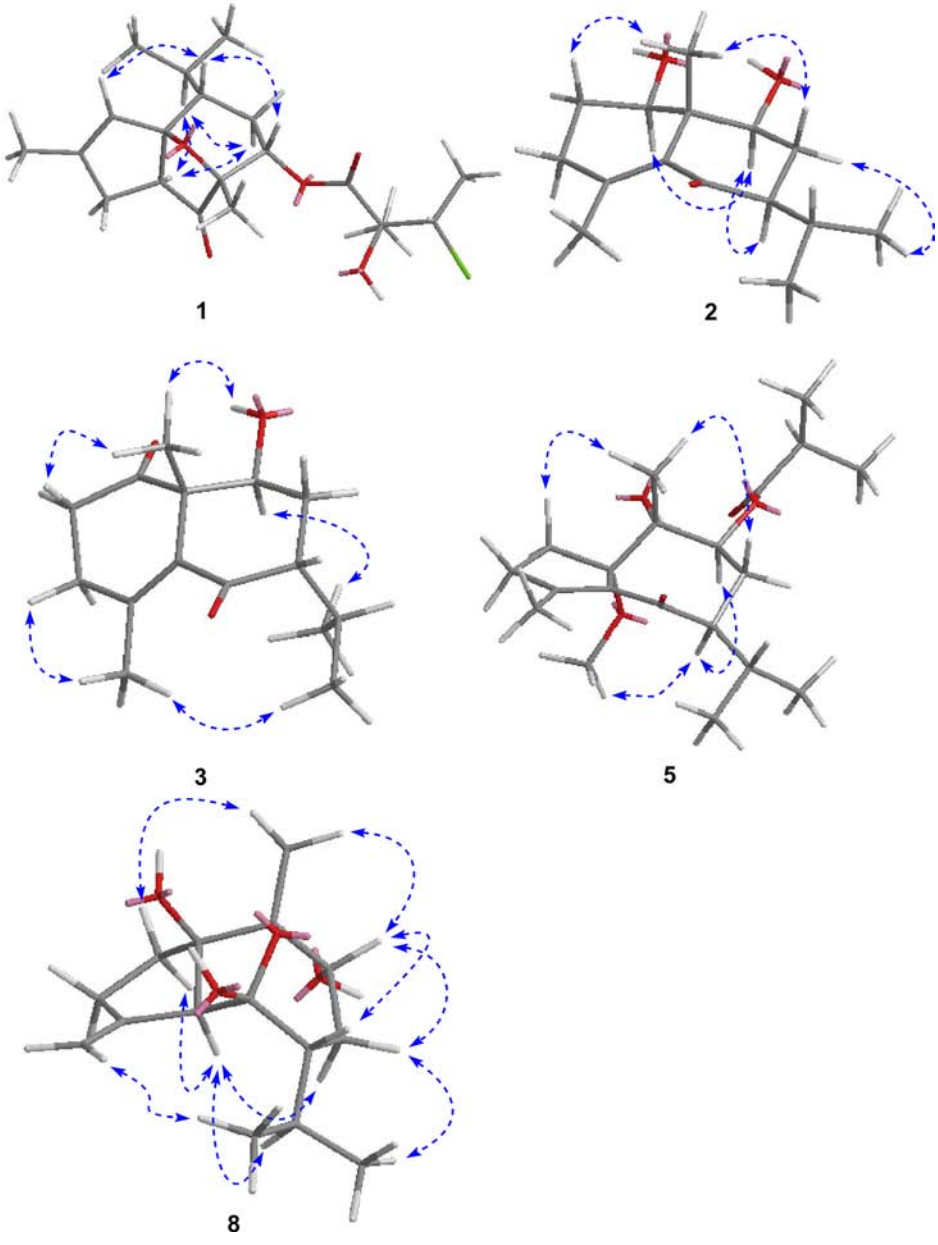

Figure 3. Key NOESY correlations of compounds **1**–**3**, **5**, and **8**.

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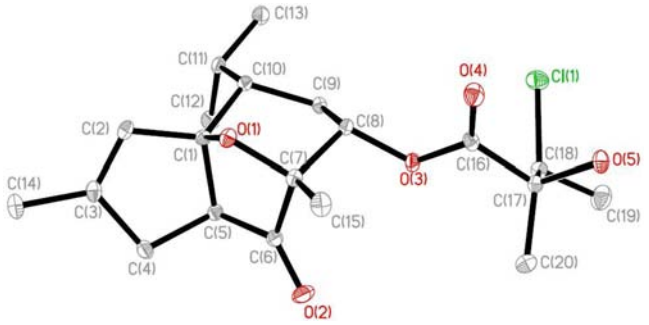

Figure 4. Thermal ellipsoid representation of compound **1**.

9)] (Table 2). The ^{13}C NMR spectrum of compound **2** showed 15 carbon signals, which were classified into 4 methyls (C-12, C-13, C-14, and C-15), 3 methylenes (C-2, C-3, and C-8), 4 methines (C-1, C-7, C-9, and C-11), and 4 quaternary carbons (C-4, C-5, C-6, and C-10), including two olefinic carbons (C-4 and C-5) and one carbonyl carbon (C-6) based on the DEPT and HMQC spectra. The same isopropyl moiety as in the case of compound **1** was confirmed from the ^{1}H and ^{13}C NMR spectra. On the basis of the aforementioned spectroscopic features and the reported sesquiterpenenes from *B. balsamifera*,[4,5] compound **2** might be a eudesmane-type sesquiterpene. To corroborate the above assumption, a HMBC experiment was performed. In the HMBC spectrum, the long-range correlations of H_3-15 to C-3, C-4, and C-5, H_3-14 to C-1, C-10, C-9 and C-5, and H-11 to C-6, C-7, C-8, C-12, and C-13 supported a eudesmane skeleton for compound **2**. Consequently, the carbonyl carbon conjugated with the double bond (C-4 and C-5) at δ_C 205.9 and the two oxygenated carbons at δ_C 70.2 and 69.3 were ascribed to C-6, C-1, and C-9, respectively, by the corresponding HMBC correlations. The detailed analyses of 2D NMR spectra led to the assignments of all of the protons and carbons. Thus, the planar structure of a eudesmane-type sesquiterpene for compound **2** was established.

The relative configuration of compound **2** was deduced on the basis of the NOESY spectrum and Chem3D modeling. The molecular conformation for compound **2** was depicted as in Figure 3, which was supported by the NOESY correlations observed for H-1/H-9, H-9/H-7, H_3-14/H-8β, and H-8α/H_3-12. The structure of compound **2** was therefore characterized and named balsamiferine B.

The HR-ESIMS provided a molecular formula $C_{15}H_{22}O_3$ for compound **3**. The ^{1}H NMR spectrum of compound **3** displayed four methyl groups [δ_H 0.87 (3H, d, J = 6.6 Hz, H_3-13), 0.99 (3H, d, J = 6.6 Hz, H_3-12), 1.27 (3H, s, H_3-14), and 1.89 (3H, s, H_3-15)] and one oxygenated methine proton [δ_H 4.29 (1H, dd, J = 11.8, 4.0 Hz, H-9)] (Table 1). The ^{13}C NMR spectrum showed 15 carbon signals (Table 1), which implied that compound **3** should also be a sesquiterpene. The same isopropyl moiety and carbonyl carbon [δ_C 206.2 (C-6)] conjugated with a double bond [δ_C 139.9 (C-4) and 135.2 (C-5)] in **3** as those in compound **2** were revealed by the ^{1}H and ^{13}C NMR spectra. Upon comparison of the ^{13}C NMR spectroscopic data of compound **3** to those of compound **2**, the main difference is that one oxygenated carbon [δ_C 70.2 (C-1)] in compound **2** was replaced by a carbonyl carbon [δ_C 216.8 (C-1)] in compound **3**. To assign the protons and carbons unambiguously, the following HMQC, HMBC, and ^{1}H−^{1}H COSY experiments were performed. From analysis of the 2D NMR spectra, the other carbonyl carbon at δ_C 216.8 and the oxygenated carbon at δ_C 66.7 were attributed to C-1 and C-9, respectively. The planar structure for compound **3** was therefore established on the basis of the above deductions, which was the same as that of the known compound, samboginone (**11**).[4] However, the NMR spectroscopic data for two compounds seem a little different, which implied that compound **3** should be an isomer of the known compound. From the Chem3D modeling and the NOESY correlations of H_3-14/OH-9, H_3-14/H-2β, H_3-12/H-9, and H_3-13/H_3-15, the conformation of compound **3** was elucidated, where C-9 hydroxy and H-7 were both in β positions. Thus, compound **3** was determined as the C-7 isomer of the known compound, samboginone (**11**), which has been named balsamiferine C.

Compound **4** was isolated as a colorless oil. The molecular formula was determined as $C_{20}H_{34}O_4$ according to the HR-ESIMS, which showed a quasi-molecular ion peak at m/z 337.2365 $[M - H]^-$ (calcd for $C_{20}H_{33}O_4$, 337.2379). The ^{1}H NMR spectrum of compound **4** exhibited six methyl groups [δ_H 0.78 (6H, d, J = 6.0 Hz, H_3-12, and H_3-13), 0.96 (3H, s, H_3-14), 1.02 (3H, s, H_3-15), 1.72 (3H, s, H_3-5′), and 1.82 (3H, d, J = 7.0 Hz, H_3-4′)] (Table 2). The ^{13}C NMR spectrum of compound **4** showed 20 carbon resonances. On the basis of the ^{1}H and ^{13}C NMR spectra, an angeloyloxy group was deduced from the observation of the proton signals [δ_H 1.72 (3H, s, H_3-5′), 1.82 (3H, d, J = 7.0 Hz, H_3-4′), and 5.89 (1H, q, J = 7.0 Hz, H-3′)] and the corresponding carbons [δ_C 167.4 (C-1′), 127.9 (C-2′), 137.0 (C-3′), 15.4 (C-4′), and 20.3 (C-5′)]. Apart from the above 5 carbon signals for the angeloyloxy group, the remaining 15 carbons in the ^{13}C NMR spectrum, comprising 4 methyls (C-12, C-13, C-14, and C-15), 5 methylenes (C-2, C-3, C-6, C-8, and C-9), 3 methines (C-1, C-5, and C-11), and 3 quaternary carbons (C-4, C-7, and C-10), constituted a characteristic eudesmane-type sesquiterpene.[12,13] To confirm the above eudesmane-type skeleton and determine the position of the angeloyloxy group, the further HMQC and HMBC spectra were recorded. From the interpretation of 1D and 2D NMR spectra, the eudesmane-type sesquiterpene skeleton was defined (Figure 1), where the oxygenated carbon signals at δ_C 80.8, 70.7, and 73.1 were assigned to C-1, C-4, and C-7, respectively. The HMBC correlation of H-1 (δ_H 4.48) with the carbonyl carbon at δ_C 167.4 demonstrated that the angeloyloxy group was attached to C-1. There is no additional acyloxy group in compound **4**; the substituent groups at C-4 and C-7 needed to be hydroxy groups, which was supported by the HR-ESIMS data. Thus, the planar structure of compound **4** was determined, which was a derivative of the known compound lβ,4β,7α-trihydroxyeudesmane.[12] The coincidence of chemical shifts for the skeletal carbons of compound **4** and the known compound implied that compound **4** had the same relative configuration as lβ,4β,7α-trihydroxyeudesmane, which was also supported by the NOESY spectrum. The structure of compound **4** was therefore characterized, which has been named balsamiferine D.

Compound **5** was obtained as a colorless oil. Its HR-ESIMS provided the molecular formula, $C_{20}H_{32}O_5$, through the presence of a peak at m/z 375.2146 $[M + Na]^+$ (calcd for $C_{20}H_{32}NaO_5$, 375.2147). From the ^{1}H NMR spectrum of compound **5**, six methyl groups [δ_H 0.80 (3H, d, J = 6.8 Hz, H_3-12), 0.87 (3H, d, J = 6.8 Hz, H_3-13), 0.99 (3H, s, H_3-14), 1.12 (3H, d, J = 5.6 Hz, H_3-3′), 1.14 (3H, d, J = 5.6 Hz, H_3-4′), and 2.09 (3H, s, H_3-15)] and one methxoy group (δ_H 3.03 s; δ_C 49.3) were observed. The ^{13}C NMR spectrum of compound **5**

showed 20 carbon resonances. On the basis of the ^{1}H and ^{13}C NMR spectra of compound **5**, one isobutyryl group was deduced and defined from the corresponding proton and carbon signals (δ_H 2.52 q, 1.12 d, 1.14 d; δ_C 176.8, 34.1, 18.7, and 18.9) (Tables 1 and 2). In addition to the above 4 carbon signals for the isobytyryloxy group and 1 methoxy signal, the residual 15 carbons exhibited in the ^{13}C NMR spectrum, comprising 4 methyls (C-12, C-13, C-14, and C-15), 3 methylenes (C-2, C-3, and C-8), 3 methines (C-7, C-9, and C-11), and 5 quaternary carbons (C-1, C-4, C-5, C-6, and C-10), formed a typical 1,9,10-trihydroxyguaian-4-en-6-one-type sesquiterpene.[4] The following HMQC and HMBC spectra were recorded to confirm the skeleton and determine the positions of the methoxy group and the isobutyryloxy group. From the interpretation of HMQC and HMBC spectra, the assignments of the skeletal prontons and carbons were accomplished, which supported a 1,9,10-trihydroxyguaian-4-en-6-one sesquiterpene skeleton for compound **5**. The HMBC correlation of the proton signal at δ_H 5.45 (H-9) with the carbonyl signal at δ_C 176.8 (CO of the isobutyryloxy), demonstrated that the isobutyryloxy group was attached at C-9. The methoxy group was attributed to C-1 by the HMBC correlation of the methoxy protons to C-1. Thus, the planar structure for compound **5** was established. The NOESY correlations of H_3-14/H-2β, H_3-14/H-8β, H-9/H-7, and H-7/OCH_3-1 but not for H_3-14/OCH_3-1 and H_3-14/H-9 revealed the H_3-14, H-2β, and H-8β were in β positions on the same side and H-7, H-9, and 1-OCH_3 were in α positions on the opposite side.[4] Therefore, compound **5** was determined as depicted in Figure 1 and named balsamiferine E.

The ^{1}H and ^{13}C NMR spectra of compounds **6** and **7** were similar to each other. Analyses of the ^{13}C and ^{1}H NMR data (Tables 1 and 3) of the two compounds revealed that compounds **6** and **7** had the same 1,9,10-trihydroxyguaian-4-en-6-one-type sesquiterpene skeleton as compound **5**.[3,4] The difference of compounds **5**−**7** is that they have different acyl groups based on their ^{1}H and ^{13}C NMR spectra. For compound **6**, in addition to the methoxy group (δ_H 3.09 s; δ_C 49.5), an angeloyloxy group (δ_H 6.06 q, 1.99 d, and 1.91 s; δ_C 167.7, 128.1, 137.8, 15.8, and 20.8) was deduced and determined according to its ^{13}C and ^{1}H NMR spectra. Using the same HMBC and NOESY experiments as for compound **5**, the positions of the substituent groups (one angeloyloxy group and one methoxy group) and the configuration of compound **6** were determined, where the methoxy group was attached at C-1 and existed in an α position, the angeloyloxy group was at C-9 and existed in a β position, and C-10 hydroxy and H-7 were in α positions.[4] For compound **7**, besides the same methoxy group as those that appeared in compounds **5** and **6**, a senecioyloxy group was deduced and defined on the basis of its ^{13}C and ^{1}H NMR spectra, which was located at C-9, replacing the angeloyloxy group in compound **6** or the isobutyryloxy group in compound **5**. The same relative configuration to be inferred for compounds **6** and **7** was revealed by the careful comparison of the NOESY spectra of compounds **6** and **7**, which was also supported by their identical laeotropic optical rotation. Therefore, the structures of compounds **6** and **7** were elucidated and named balsamiferines E and F, respectively.

Analyses of the ^{13}C and ^{1}H NMR spectra of compounds **8**−**10** (Tables 1 and 3) revealed that the three compounds had the same characteristic 1,6,9-trihydroxyguaian-6,10-epoxy-4(15)-en-type sesquiterpene skeleton as blumeaenes H−J.[3] The main difference between compounds **8**−**10** and the known blumeaenes H−J is that they possessed different substituent groups. For compound **8**, there were no acyloxy groups, which implied the presence of three hydroxy groups located at C-1, C-6, and C-9. To confirm the 1,6,9-trihydroxyguaian-6,10-epoxy-4(15)-en structure for compound **8**, the following HMQC, HMBC, ^{1}H−^{1}H COSY, and NOESY experiments were performed. From the interpretation of the 2D NMR spectra, the protons and carbons of 1,6,9-trihydroxyguaian-6,10-epoxy-4(15)-en for compound **8** were assigned, where the double bond was ascribed to C-4 (δ_C 150.2) and C-15 (δ_C 111.0) and the oxygenated carbons at δ_C 92.8, 103.0, 76.6, and 79.3 were attributed to C-1, C-6, C-9, and C-10, respectively. The Chem3D modeling and the NOESY correlations observed for H_3-14/H-2β, H_3-14/H-9, H-9/H-7, H-9/H-8β, H-8α/H-5, H-5/H-11, and H-5/H-2α implied a conformation for compound **8** as depicted in Figure 3, where H-5 was in an α position and H-7 and H-9 were in β positions. The only difference between compounds **9** and **8** was that the C-9 hydroxy group in compound **8** was replaced by an angeloyloxy group in compound **9**. Compound **10** possessed a senecioyloxy moiety at C-9 instead of the hydroxy moiety in compound **8**. The same relative configuration to be verified for compounds **8**−**10** was revealed by the careful comparison of the NOESY spectra of **8**−**10**, which was also confirmed by their identical dexiotropic optical rotation. Therefore, compounds **8**−**10** were elucidated and named balsamiferines H−J, respectively.

To explore the undiscovered and potential pharmacological activities of these sesquiterpenes isolated from the aerial parts of *B. balsamifera*, compounds **1**−**11** were evaluated for their inhibitory activities on LPS-induced NO production in murine microglial BV-2 cells by the Griess reaction as described.[10,14] SMT was used as a positive control (IC_{50} value of 5.2 μM). Compounds **1**−**3** and **11** exhibited weak inhibitory effects on LPS-induced NO production only at the high concentration of 100 μM. Compounds **4**−**10** inhibited LPS-induced NO production in BV-2 cells dose-dependently (IC_{50} values; see Table 4). The inhibitory effects of these active sesquiterpenes were shown in Figure 5. The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay indicated that all of the compounds had no significant cytotoxicity to the BV-2 cells at their effective concentration for the inhibition of NO production (data not shown).

Table 4. IC_{50} Values of Compounds 1−11 Inhibiting NO Production in BV-2 Cells

compound	IC_{50} (μM)	compound	IC_{50} (μM)
1	>100	**7**	5.4
2	>100	**8**	62.7
3	>100	**9**	61.3
4	22.7	**10**	38.0
5	54.7	**11**	>100
6	26.6	SMT[a]	5.2

[a]SMT was used as a positive control.

In summary, 10 new and 1 known sesquiterpenes were successfully isolated from the aerial parts of *B. balsamifera*. Their structures were elucidated by the 1D and 2D NMR spectra, Chem3D modeling, and X-ray crystallography. Biological studies disclosed that all of the isolates exhibited inhibitory effects on LPS-induced NO production and compound **7** exerted the most inhibition against NO production, which was comparable to the positive control

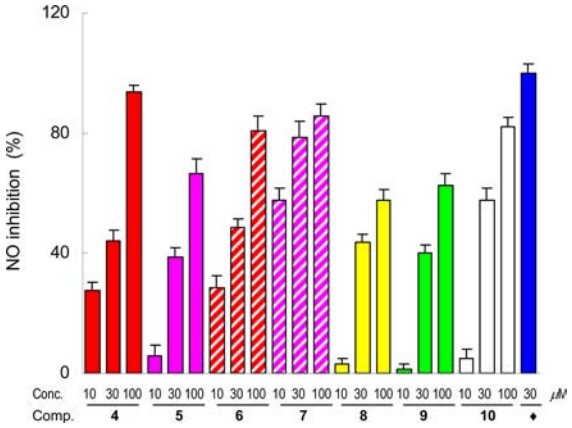

Figure 5. Inhibitory effects of compounds **4**–**10** on LPS-induced NO production in BV-2 cells. BV-2 cells were treated with LPS alone or together with each compound at the concentrations indicated. After 20 h of incubation, the supernatants were tested by the Griess assay and the NO inhibitory rates were calculated. The experiment was performed 3 times, and the data are expressed as the mean ± standard deviation (SD) values. The inhibitory rate on NO production was calculated as follows: inhibitory rate (%) = (1 − (LPS/sample − untreated)/(LPS − untreated)) × 100. (♦) Positive control, SMT.

(SMT). The results of our chemical investigation further revealed the chemical composition of *B. balsamifera*, and the biological screening of these isolates exhibited that the leaves of *B. balsamifera*, used as a flavoring ingredient and a tea, may be beneficial to human health. The current biological data suggest that these sesquiterpenes from *B. balsamifera*, especially compounds **4**, **6**, **7**, and **10** with strong NO inhibitory activities, may have the potential to be developed as anti-inflammatory agents for various inflammatory diseases. Further biological studies on these compounds are still underway by our group.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The 1D and 2D NMR and MS spectra of compounds **1**–**10** and the CIF file of the X-ray data of compound **1**. 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.

■ REFERENCES

(1) Perry, L. M. *Medicinal Plants of East and Southeast Asia*; MIT Press: Cambridge, MA, 1980; p 87.

(2) Burkill, I. H. *A Dictionary of the Economic Products of the Malay Peninsula*; Ministry of Agriculture and Cooperatives: Kuala Lumpur, Malaysia, 1966; Vol. *1*, p 337.

(3) Chen, M.; Qin, J. J.; Fu, J. J.; Hu, X. J.; Liu, X. H.; Zhang, W. D.; Jin, H. Z. Blumeaenes A-J, sesquiterpenoid esters from *Blumea balsamifera* with NO inhibitory activity. *Planta Med.* **2010**, *76*, 897–902.

(4) Shirota, O.; Oribello, J. M.; Sekita, S.; Satake, M. Sesquiterpenes from *Blumea balsamifera*. *J. Nat. Prod.* **2011**, *74*, 470–476.

(5) Osaki, N.; Koyano, T.; Kowithayakorn, T.; Hayashi, M.; Komiyama, K.; Ishibashi, M. Sesquiterpenoids and plasmin-inhibitory flavonoids from *Blumea balsamifera*. *J. Nat. Prod.* **2005**, *68*, 447–449.

(6) Nessa, F.; Ismail, Z.; Mohamed, N.; Mas Haris, M. R. H. Free radical-scavenging activity of organic extracts and of pure flavonoids of *Blumea balsamifera* DC leaves. *Food Chem.* **2004**, *88*, 243–252.

(7) Xu, J.; Guo, Y.; Xie, C.; Li, Y.; Gao, J.; Zhang, T.; Hou, W.; Fang, L.; Gui, L. Bioactive myrsinol diterpenoids from the roots of *Euphorbia prolifera*. *J. Nat. Prod.* **2011**, *74*, 2224–2230.

(8) Guo, P.; Li, Y.; Xu, J.; Liu, C.; Ma, Y.; Guo, Y. Bioactive *neo*-clerodane diterpenoids from the whole plants of *Ajuga ciliata* Bunge. *J. Nat. Prod.* **2011**, *74*, 1575–1583.

(9) Xu, J.; Guo, Y.; Li, Y.; Zhao, P.; Liu, C.; Ma, Y.; Gao, J.; Hou, W.; Zhang, T. Sesquiterpenoids from the resinous exudates of *Commiphora myrrha* and their neuroprotective effects. *Planta Med.* **2011**, *77*, 2023–2028.

(10) Xu, J.; Jin, D. Q.; Guo, Y.; Xie, C.; Ma, Y.; Yamakuni, T.; Ohizumi, Y. New myrsinol diterpenes from *Euphorbia prolifera* and their inhibitory activities on LPS-induced NO production. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3612–3618.

(11) Crystallographic data for compound **1** have been deposited in the Cambridge Crystallographic Data Centre (CCDC 885493). Copies of the data can be obtained, free of charge, on application to the director, CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K.

(12) Jung, K. Y.; Kim, D. S.; Oh, S. R.; Lee, I. S.; Lee, J. J.; Lee, H. K.; Shin, D. H.; Kim, E. H.; Cheong, C. J. Sesquiterpene components from the flower buds of *Magnolia fargesii*. *Arch. Pharm. Res.* **1997**, *20*, 363–367.

(13) Wang, Y. F.; Wang, X. Y.; Lai, G. F.; Lu, C. H.; Luo, S. D. Three new sesquiterpenoids from the aerial parts of *Homalomena occulta*. *Chem. Biodiversity* **2007**, *4*, 925–931.

(14) Schmidt, H. H. H. W.; Kelm, M. Determination of nitrite and nitrate by the Griess reaction. In *Methods in Nitric Oxide Research*; Feelisch, M., Stamler, J. S., Eds.; John Wiley and Sons: West Sussex, U.K., 1996; pp 491–497.