Sesquiterpenoids and Plasmin-Inhibitory Flavonoids from *Blumea balsamifera*

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Two new sesquiterpenoid esters (**1** and **2**) were isolated from the extract of *Blumea balsamifera*, a tropical Compositae plant. Compound **2** proved to be weakly cytotoxic when tested against Jurkat human T-cell leukemia cells. Nine known flavonoids, of which two showed plasmin-inhibitory activity, were also isolated.

During our search for bioactive natural products from tropical plants,[1] we investigated the chemical constituents of leaves of *Blumea balsamifera* collected in Thailand. *Blumea balsamifera* DC. (Compositae) is an aromatic hairy herb growing in Southeast Asia, and the leaves are commonly used as a medicinal herb, containing significant amounts of essential oils. Previous chemical investigations of this plant led to the isolation of several terpenoids and flavonoid glycosides.[2,3] Here we describe the isolation and structure elucidation of two new sesquiterpenoid esters, together with the isolation of known flavonoids with plasmin-inhibitory activities.

The leaves of *B. balsamifera* were extracted with MeOH, and the extract was separated by solvent partitions to give hexane-, EtOAc-, *n*-BuOH-, and water-soluble fractions. The hexane- and EtOAc-soluble fractions were combined and subjected to silica gel and ODS column chromatography, followed by final purification by reversed-phase HPLC on ODS to give two new sesquiterpenoid esters (**1** and **2**) together with nine known flavonoids (**3**−**11**), identified by comparison with reported spectroscopic data.[4]

Compound **1** was obtained as an amorphous solid, and its molecular formula was established as $C_{20}H_{30}O_5$ by HRFABMS data [*m/z* 351.2170]. The IR spectrum of **1** showed bands at 3477 and 1709 cm^{-1} characteristic of hydroxyl and carbonyl groups, respectively. The ^1H NMR spectrum of **1** (Table 1) showed signals due to a tertiary methyl (δ_H 1.42, 3H, s), two secondary methyls (δ_H 0.95 and 0.86, each 3H, d, $J = 6.8$ Hz), two olefinic methyls (δ_H 1.93, 3H, br s; δ_H 2.01, 3H, br d), a vinylic proton (δ_H 6.13, 1H, br q), and an exo-methylene group (δ_H 5.23 and 4.78, each 1H, s). The ^{13}C NMR spectrum of **1** showed signals assignable to one carbonyl, one ester carbonyl, four olefinic carbons, two oxygenated quaternary carbons, an oxymethine, three unoxygenated sp^3 methine carbons, three sp^3 methylenes, and five methyl carbon atoms. Since four out of six unsaturation equivalents were accounted for from the ^{13}C NMR data, **1** was inferred to have two rings. The presence of a 2-methylbutenoic acid was suggested by the $^1H-^1H$ COSY (H_3-19/H-18) and the HMBC (from H-18 to C-20; from H_3-19 to C-18 and C-17; from H_3-20 to C-16 and C-17) data, and the geometry of this unsaturated ester was assigned as *Z* (angelic acid) from the ^{13}C NMR chemical shift data of two vinylic methyl carbons (**1**, δ_C 20.6 and 15.9; angelic acid,[5] δ_C 20.8 and 16.0; tiglic acid,[6] δ_C 14.2 and 11.9). This angelic acid moiety was attached to the sesquiterpene nucleus at the C-9 position through an ester linkage, which was shown by the HMBC correlation from H-9 (δ_H 5.22) to C-16. The $^1H-^1H$ COSY data showed proton connectivities for H_2-2/H_2-3 (two vicinal methylene groups), H-9/H_2-8/H-7, and H_3-12/H-11/H_3-13 (isopropyl) moieties for the sesquiterpene skeleton part of **1**. The isopropyl group was shown to be connected to the C-7 methine by the HMBC correlation data (H_3-12/C-7, H_3-13/C-7, and H-7/C-11), although the $^1H-^1H$ COSY cross-peak was obscure between H-7 and H-11. Thus, the partial structure **a** was constructed. Analysis of the HMBC correlation data[7] gave rise to the following carbon connectivities (partial structure **b**): C-6 (ketone)−C-5 (methine)−C-4 (exomethylene)−C-3/C-2 (vicinal methylenes)−C-1 (quaternary carbon bearing tertiary hydroxyl group)−C-10 (quaternary carbon bearing tertiary methyl and tertiary hydroxyl groups). The partial structures **a** and **b** were then

Table 1. ^1H and ^{13}C NMR Data of Compounds **1** and **2** in $CDCl_3$

	1		**2**	
	δ_H/Hz	δ_C	δ_H/Hz	δ_C
1		85.9		213.5
2	(α) 1.97 m	35.1	(α) 3.58 ddd 14.8, 13.4, 2.4	34.4
	(*β*) 1.80 m		(*β*) 2.15 ddd 14.8, 5.5, 2.8	
3	2.36 (2H) m	30.5	(α) 2.24 ddd 11.9, 5.5, 2.4	36.7
			(*β*) 2.69 ddd 13.4, 11.9, 2.8	
4		146.8		149.2
5	3.71 br s	58.7	5.85 br s	127.5
6		209.6		204.6
7	2.47 dt 12.2, 4.5	55.0	1.88 m	55.3
8	(α) 1.97 m	28.0	(α) 1.80 dd 15.6, 13.1	28.8
	(*β*) 1.80 m		(*β*) 1.56 m	
9	5.22 dd 11.0, 1.5	76.9	4.77 d 7.9	74.0
10		77.9		82.5
11	2.22 hept d, 6.8, 4.5	31.8	1.88 m	31.0
12	0.95 (3H) d 6.8	20.5	0.88 (3H) d 6.6	20.5
13	0.86 (3H) d 6.8	18.4	0.88 (3H) d 6.6	19.5
14	1.42 (3H) s	17.1	1.31 (3H) s	17.0
15	(a) 5.23 br s	111.4	1.97 (3H) d 1.2	17.8
	(b) 4.78 br s			
16		167.9		171.1
17		127.5		60.0
18	6.13 br q 7.3	139.2	3.06 q 5.4	59.9
19	2.01 (3H) br d 7.3	15.9	1.32 (3H) d 5.4	14.0
20	1.93 (3H) br s	20.6	1.56 (3H) s	19.2

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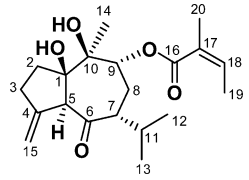
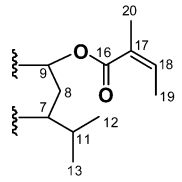
connected through the formation of C-7/C-6 and C-10/C-9 bonds by the HMBC correlations observed for H-7/C-6, H_2-8/C-6, H-9/C-10, H-9/C-14, and H_3-14/C-9. The remaining C-1 quaternary carbon and C-5 methine carbon had to be connected by a process of elimination to construct two rings (a five-membered and a seven-membered ring), leading to a guaiane sesquiterpene skeleton for **1**.

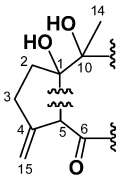
The NOEDIF experiments showed correlations for H-5/H_3-14, H-7/H-9, and H-9/OH-10; these observations along with stereomodel conclusions suggested the relative configuration of **1**, with H-5 and H_3-14 having the α-orientations and OH-1, H-7, H-9, and OH-10 having the *β*-orientations.



1

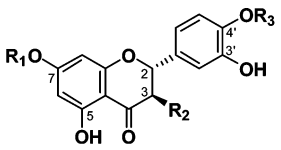


a

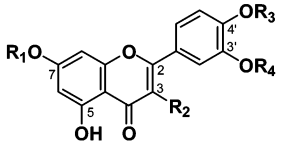


b

Compound **2**, $[\alpha]_D^{24}$ +32 (*c* 0.08, $CHCl_3$), had the molecular formula $C_{20}H_{30}O_6$ according to HRFABMS data [*m/z* 367.2097, $(M + H)^+$, Δ −2.4 mmu], containing one more oxygen atom than **1**. The UV spectrum of **2** showed an absorption maximum at 234 nm, indicating the presence of a conjugated system, and its IR spectrum was suggestive of the presence of three carbonyl groups (1733, 1712, and 1686 cm^{-1}). The ^1H NMR spectrum of **2** showed only one olefinic proton (δ_H 5.85, 1H, s) and six methyl groups [three secondary methyls (δ_H 0.88, 6H, d, J = 6.8 Hz; δ_H 1.32, 3H, d, J = 5.4 Hz) and three tertiary methyls (δ_H 1.56, 1.42, 1.31, each 3H, s)]. The ^{13}C NMR data of **2** revealed that compound **2** had two carbonyls (one nonconjugated and one conjugated), one ester carbonyl, an olefin, two oxygenated quaternary carbons, two oxymethines, two unoxygenated sp^3 methines, three sp^3 methylenes, and six methyls. The relatively high-field resonances of the two oxygenated C-17 and C-18 (δ_C 60.0 and 59.9) carbons implied the presence of an epoxide ring, and this epoxide moiety was inferred to be adjacent to two methyls and the ester carbonyl group from the HMBC correlation data (H-18/C-19, H_3-19/C-18, H_3-20/C-16, H_3-20/C-17, and H_3-20/C-18) to give a 2,3-dimethyloxirane-2-carboxylic acid moiety. This C-5 acid moiety was attached to C-9 oxymethine of the sesquiterpene part through an ester bond, indicated by the HMBC cross-peak for H-9/C-16. The proton and carbon connectivities for the sesquiterpene part of compound **2** were elucidated by the $^1H-^1H$ COSY and HMBC data[8] to reveal that **2** has a 10-membered ring (germacrane) skeleton with a carbonyl at C-1, an enone at C-4−C-6, and a tertiary hydroxyl group at C-10. The relative configuration was elucidated by interpretation of NOEDIF data (H-7/H-8*β*, H-8*β*/H-9, and H-9/OH-10) as well as stereomodel investigations to suggest that relative configurations at C-7, C-9, and C-10 were similar to those of compound **1**.[9] The *E*-configuration of the Δ^5-double bond was suggested by the ^{13}C NMR chemical shift (δ_C 17.8)[10] of the methyl carbon



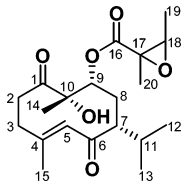
	R_1	R_2	R_3	IC_{50}
3	H	OH	Me	>300
4	Me	OH	Me	44.5
5	Me	H	H	30.5



	R_1	R_2	R_3	R_4	IC_{50}
6	H	OMe	Me	H	47.3
7	Me	OMe	H	H	1.5
8	Me	H	H	H	2.3
9	Me	OMe	Me	H	29.1
10	Me	OMe	H	Me	>300
11	Me	H	H	Me	56.4

Figure 1. Flavonoids from *B. balsamifera* and their plasmin-inhibitory activities (IC_{50} values, *μ*M).

on C-15, and the two methyl groups (C-19 and C-20) had *trans*-orientation, indicated by the NOEDIF data (H_3-19/H-18 and H-18/H_3-20).



2

Cytotoxicity tests against Jurkat human T-cell leukemia cells showed that compound **2** was marginally cytotoxic, with an IC_{50} value of 26.2 *μ*M, while compound **1** was inactive (IC_{50} > 70 *μ*M). These sesquiterpenoids were inactive in a plasmin-inhibitory test, while some of the known flavonoids from *B. balsamifera* were active (Figure 1). Particularly, the plasmin-inhibitory activity of two flavonoids (**7** and **8**, IC_{50} 1.5 and 2.3 *μ*M, respectively) with two free hydroxyl groups on both C-3′ and C-4′ positions was as strong as that of leupeptin (IC_{50} 1.2 *μ*M).

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter. UV spectra were obtained on a Hitachi U-3400 spectrometer. IR spectra were measured on a JASCO FT-IR 230 spectrophotometer using attenuated total reflectance (ATR) on a DuraScope, SensIR Technologies. NMR spectra were recorded on JEOL ECP 600 and A500 spectrometers. High-resolution fast atom bombardment (HRFAB) mass spectra were obtained on a JMS HX-110 mass spectrometer.

Plant Material. Leaves of *Blumea balsamifera* were collected in Khon Kaen, Thailand, in April 2001. A voucher specimen (6-364) is maintained at the Department of Horticulture, Faculty of Agriculture, Khon Kaen University.

Extraction and Isolation. The air-dried leaves (85 g) were extracted with MeOH, and the MeOH extract (10 g) was partitioned between hexane (450 mL × 2) and 10% aqueous MeOH (350 mL). The aqueous phase was further extracted

with EtOAc (200 mL × 3) and *n*-BuOH (200 mL × 2) to give four fractions (hexane phase, 3.3 g; EtOAc phase, 1.2 g; *n*-BuOH phase, 0.7 g; aqueous phase, 5.1 g). The hexane and EtOAc phases, which showed almost the same profiles by TLC examination, were combined and subjected to silica gel column chromatography (column A: 35 × 350 mm) eluted with gradient mixtures of 10−100% EtOAc in hexane. The fraction eluted with 40−50% EtOAc in hexane was further separated by an ODS column (column B: 15 × 300 mm) eluted with gradient mixtures of 60−100% MeOH in H_2O. The fraction (9.1 mg) of column B eluted with 75% MeOH was then purified with HPLC (Develosil C30-UG-5, 10 × 250 mm; eluent, 80% MeOH; flow rate, 2.0 mL/min) to afford compound **1** (2.7 mg, t_R 31.2 min). The fraction (10.3 mg) of column B eluted with 75% MeOH was purified with HPLC (Develosil C30-UG-5, 10 × 250 mm; eluent, 80% MeOH; flow rate, 2.0 mL/min) to afford compounds **9** (1.1 mg, t_R 33 min), **10** (2.7 mg, t_R 35 min), and **11** (2.4 mg, t_R 44 min). The fraction (23.4 mg) of column B eluted with 70% MeOH was purified with HPLC (Develosil ODS-UG-5, 10 × 250 mm; eluent, 65% MeOH; flow rate, 1.8 mL/min) to afford compounds **2** (1.6 mg, t_R 25 min) and **5** (11.5 mg, t_R 20 min). The fraction (730 mg) of column A eluted with 40−50% EtOAc in hexane was further separated by ODS column (column C: 30 × 250 mm, 40−100% MeOH in H_2O). A fraction of column C eluted with 60% MeOH afforded compound **3** (79.1 mg) after recrystallization from MeOH, while another fraction of column C eluted with 80% MeOH gave compound **4** (19.6 mg). The fraction (16 mg) of column C eluted with 80% MeOH was further purified with HPLC (Develosil ODS-UG-5, 10 × 250 mm; eluent, 70% MeOH; flow rate, 2.0 mL/min) to give compound **6** (2.4 mg, t_R 14 min) and a mixture of compounds **7** and **8** (7.8 mg, t_R 23 min), which was finally separated by HPLC (Develosil C30-UG-5, 10 × 250 mm; eluent, 90% MeOH; flow rate, 1.8 mL/min) to afford compound **7** (2.4 mg, t_R 12.6 min) and compound **8** (2.4 mg, t_R 14.7 min).

Compound 1: amorphous solid; $[\alpha]_D^{24}$ +35 (*c* 0.067, $CHCl_3$); CD (EtOH) λ_{ext} 290 nm ($\Delta\epsilon$ −1.9); IR (ATR) λ_{max} 3477 and 1709 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS *m/z* 351 $(M + H)^+$; HRFABMS *m/z* 351.2170 [calcd for $C_{20}H_{31}O_5$, $(M + H)^+$ 351.2172].

Compound 2: amorphous solid; $[\alpha]_D^{24}$ +32 (*c* 0.08, $CHCl_3$); UV (EtOH) λ_{max} 234 nm (ϵ 30 000); CD (EtOH) λ_{ext} 310 ($\Delta\epsilon$ +10.5) and 235 nm (+24.9); IR (ATR) λ_{max} 3487, 1733, 1712, and 1686 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS *m/z* 367 $(M + H)^+$ and 349 $(M - H_2O + H)^+$; HRFABMS *m/z* 367.2097 [calcd for $C_{20}H_{31}O_6$, $(M + H)^+$ 367.2121].

Plasmin Inhibition Test. Plasmin (EC 3.4.21.7), purchased from Calbiochem-Novabiochem Corporation, and substrate Boc-Val-Leu-Lys-MCA, purchased from Peptide Institute Inc., were used. Test samples (20 μL, dissolved in 50% MeOH) were added to a well of 96-well microtiter plates. Enzyme solution (80 μL, 3 mU/mL) was added to the sample solution and preincubated at 37 °C for 5 min. After preincubation, 100 μL of substrate solution (0.1 mM) was added to the mixture to begin the reaction. The fluorescence values were measured at an excitation of 390 nm and an emission of 460 nm after incubation at 37 °C for 1 h.

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(7) The following HMBC correlation data were observed for the partial structure **b**: H-5/C-6, H-5/C-4, H-15a/C-5, H-15a/C-3, H-15b/C-3, H_2-3/C-5, H_2-3/C-4, H_2-3/C-15, H_2-3/C-1, H_2-2/C-3, H_2-2/C-1, H_3-14/C-1, and H_3-14/C-10.

(8) The $^1H-^1H$ COSY correlations observed for sesquiterpene part of **2**: H_2-2/H_2-3, H-9/H_2-8/H-7, and H-11/H_3-12(H_3-13). The HMBC correlations observed for sesquiterpene part of **2**: H_2-2/C-1, H_2-2/C-4, H_2-3/C-4, H_3-15/C-3, H_3-15/C-4, H_3-15/C-5, H-5/C-15, H-5/C-6, H_2-8/C-6, H_2-8/C-7, H_2-8/C-9, H-9/C-7, H-9/C-8, H-9/C-10, H-9/C-14, H_3-14/C-9, H_3-14/C-10, and H_3-14/C-1.

(9) Stereomodel investigations of the 10-membered ring of compound **2** suggested that it predominantly adopted a conformation with H-9 being *syn* to OH-10 and *anti* to CH_3-14. It may be biogenetically conceivable that an aldol-type C−C bond formation between C-1 and C-5 of compound **2** affords the same carbon skeleton of compound **1**. This consideration may suggest that the C-7, C-9, and C-10 configurations of compound **2** are likely to be the same as that of **1**.

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