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Antiplatelet principles from the root of *Petasites formosanus*

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Abstract

Three bakkenolide-type compounds, bakkenolides-G, -H and deisobutyryl bakkenolide-H were isolated from the roots of *Petasites formosanus*. The structures were characterized by spectral methods. Bakkenolide-G and -H showed inhibitory activity against platelet activation factor (PAF), whereas deisobutyryl bakkenolide-H and ferulic acid showed inhibitory activity against arachidonic acid (AA) and collagen (Col)-induced aggregation of washed rabbit platelets. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Petasites formosanus*; Compositae; Bakkenolide-G; Bakkenolide-H; Deisobutyryl bakkenolide-H; Antiplatelet principle; Inhibitory activity

1. Introduction

Petasites formosanus Kitamura (Compositae) is a perennial shrub widely distributed at high altitude mountainous locations in Taiwan. It has been utilized in folk medicine as an antidote, analgesic, expectorant and for the treatment of hypertension and snake-bite (Hsu & Chiu, 1986). The components of several *Petasites* species have been studied and shown to contain petasine, eremophilenolides, and furoeremophilanes (Abe, Onoda, Ro & Kurihara, 1968; Naya, Kawai, Naito & Kasai, 1972; Shirahata, Abe, Kato & Kurihara, 1968; Naya, Haysshi, Takagi, Nakamura & Kobayashi, 1972; Yamada, Tatematsu, Hirata, Haga & Hirono, 1968; Sugama, Hayashi, Nakagawa, Mitsuhashi & Yoshida, 1983; Sugama, Hayashi & Mitsuhashi, 1985; Antonio & Francesco, 1982; Jamieson, Reid, Turner & Jamieson, 1976; Yaoita, Nagata, Suzuki & Kikuchi, 1992; Naya, Nakagawa, Hayashi, Tsuji & Naito, 1971; Appleton & Enzell, 1971; Pouchert, 1983). However, we could not find any description of *P. formosanus*. The isolation of bioactive compounds from this plant was attempted because its crude methanolic extract showed antiplatelet aggregation effects. We now report the isolation and characterization of some bioactive compounds whose inhibition of platelet aggregation is also discussed.

2. Results and discussion

The hot methanolic extract of *P. formosanus* was partitioned successively between H_2O and $CHCl_3$, and then *n*-BuOH. The two organic layers gave three new bakkenolide-type compounds, bakkenolide-G (**1**), -H (**2**) and deisobutyryl bakkenolide-H (**3**), together with three known compounds, bakkenolide-D (**4**) (Abe et al., 1968), lupeol (**5**) (Appleton & Enzell, 1971) and ferulic acid (**6**) (Pouchert, 1983) after chromatography.

Bakkenolide-G (**1**) was isolated as optically active colorless plates. High resolution mass spectrometry established the molecular formula as $C_{22}H_{32}O_6$. The similiarity of the UV, IR, ^1H and ^{13}C NMR spectra with those of known bakkenolides-B (**7**) and -D (**4**)

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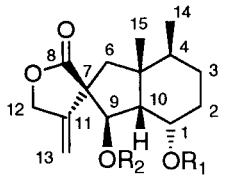
Table 1
^{1}H and ^{13}C NMR spectral data for **1**–**3** ($CDCl_3$, δ, multiplicity, J, Hz)

	1		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	5.03 *dt* (7.2, 4.6)	70.8	5.04 *dt* (11.8, 4.9)	70.5	4.59 *m*	76.2
2	1.72, 1.78 *m*	26.5	1.70, 1.78 *m*	25.6	1.76, 2.06 *m*	27.5
3	1.28, 1.64 *m*	29.5	1.28, 1.66 *m*	29.5	1.31, 1.62 *m*	29.6
4	1.52 *m*	35.3	1.56 *m*	35.4	1.51 *m*	35.1
5		43.2		43.5		43.4
6	1.93 *d* (14.8, 6α) 2.21 *d* (14.8, 6β)	46.4	1.93 *d* (14.2, 6α) 2.24 *d* (14.2, 6β)	46.2	1.92 *d* (14.8, 6α) 2.18 *d* (14.8, 6β)	45.8
7		54.6		54.9		54.9
8		177.8		177.7		178.5
9	5.77 *d* (11.2)	80.5	5.77 *d* (11.1)	80.6	5.72 *d* (11.2)	80.4
10	2.67 *dd* (11.2, 4.6)	51.8	2.65 *dd* (11.1, 4.9)	52.2	2.80 *dd* (11.2, 4.8)	52.4
11		147.8		147.9		147.7
12	4.65, 4.69 *dt* (13.2, 2.4)	70.5	4.62, 4.68 *dt* (14.8, 1.8)	70.5	4.63, 4.66 *d* (13.2)	70.7
13	5.17, 5.21 *t* (2.4)	108.3	5.17, 5.19 *t* (1.8)	108.4	5.14, 5.18 *s* (*br s*)	108.1
14	0.87 *d* (6.8)	15.5	0.89 *d* (6.6)	15.5	0.87 *d* (*d*, 6.8)	15.5
15	1.07 *s*	19.6	1.09 *s*	19.6	1.06 *s*	19.6
1′		169.9		175.8		
2′	1.91 *s*	22.3	2.28 *sept* (7.0)	33.9		
3′,4′			1.06, 108 *d* (7.0)	19.1, 19.3		
1″		171.8		176.5		176.8
2″	2.11, 2.19 *dd* (14.2, 6.8)	43.4	2.48 *sept* (7.0)	33.9	2.57 *sept* (7.2)	33.9
3″ or 3″,4″	2.06 *m*	25.2	1.13, 1.18 *d* (7.0)	18.1, 18.6	1.10, 1.12 *d* (7.2)	18.3, 18.8
4″,5″	0.93 *d* (6.4)	22.4, 22.5				

(Abe et al., 1968) suggested that compound **1** should contain a bakkenolide-type skeleton (Table 1). This kind of sesquiterpenoid bakkenolide nucleus exhibited a *cis*-fused 6/5 membered ring [δ 5.03 (*dt, J* = 7.2, 4.6 Hz) for H-1, 1.2–1.8 (*m*) for H-2–H-4, 1.93 and 2.21 (*d, J* = 14.8 Hz) for H-6, 5.77 (*d, J* = 11.2 Hz) for H-9, 2.67 (*dd, J* = 11.2, 4.6 Hz) for H-10], with a spiro-γ-lactone having a terminal methylene group [δ 4.65 and 4.69 (*dt, J* = 13.2, 2.4 Hz) for H-12, 5.17 and 5.21 (*t, J* = 2.4 Hz) for H-13]. It also has two methyls attached on C-4 [δ 0.87 (*d, J* = 6.8 Hz)] and C-5 [δ 1.07 (s)]. The downfield-shifted H-1 and H-9 indicated two extra substituents bearing oxygenated functionalities. An acetoxy group at δ 1.91 was deduced to be on C-1; an isovaleryloxy group at δ 0.93 (6H, *d, J* = 6.4 Hz, H-4″ and H-5″), 2.06 (1H, *m*, H-3″), 2.11 and 2.19 (each 1H, *dd, J* = 14.2, 6.8 Hz, H-2″) was assigned on C-9. The regiochemistry of these two acyl substituents was confirmed by the presence of the ^{1}H-^{13}C long range correlations of C-1″ (δ 171.8) with H-3″ (δ 2.06) and H-9 (δ 5.77) which suggested the isovaleryloxy group should be attached on C-9. In addition, the existence of NOE of H-2″ (δ 2.11 and 2.19) with H-6 (δ 2.21) referred a β-oriented H-6 with the chemical shift of δ 2.21. The full assignment of the ^{1}H and ^{13}C NMR signals was conducted by COSY, HMQC, HMBC and NOESY experiments (Figs. 1 and 2). Based on the analysis, bakkenolide-G possesses the structure as shown for **1**.

Bakkenolide-H(**2**), isolated as optically active colorless needles, has the molecular formula $C_{23}H_{34}O_6$. The UV, IR, MS and NMR spectra suggested that compound **2** was a bakkenolide derivative as in **1**. The non-bakkenolide peaks in the ^{1}H NMR spectrum could be assigned to two isobutyryloxy groups on C-1 and C-9 by a COSY experiment. One isobutyryloxy moiety at δ 1.13, 1.18 (each 3H, *d, J* = 2.0 Hz, H-3″ and H-4″) and 2.48 (1H, *sept, J* = 2.0 Hz, H-2″) was substituted on C-9 as shown by the presence of NOEs between the signal at δ 2.48 (H-2″) and the signal at δ 2.65 (H-10), and the HMBC of the ^{13}C signal at δ 176.5 (C-1″) with ^{1}H signals at δ 1.13 (CH_3) and 5.77 (H-9) (Figs. 1 and 2). Apparently, the other isobutyryloxy group at δ 1.06, 1.08 (each 3H, *d, J* = 2.0 Hz, H-3′ and H-4′) and 2.28 (1H, *sept, J* = 2.0 Hz, H-2′) was substituted on C-1. Therefore, structure **2** was established for bakkenolide-H.

Deisobutyryl bakkenolide-H (**3**) was also obtained as optically active colorless needles, with a molecular formula $C_{19}H_{28}O_5$. The IR, MS and NMR spectra were closely related to those of **2** (Table 1). The existence of only one set of isobutyryloxy resonances at δ 1.10, 1.12 (each 3H, *d, J* = 7.2 Hz, H-3″ and H-4″) and 2.57 (1H, *sept, J* = 7.2 Hz, H-2″) located on C-9 was supported by the HMBC of carbonyl carbon C-1″ (δ 176.8) with the methyl protons (δ 1.10, 1.12) and H-9 (δ 5.72) (Fig. 2). Consequently, the structure of **3** is deisobutyryl bakkenolide-H.



1: $R_1 = \overset{1'}{C}O\overset{2'}{C}H_3$, $R_2 = \overset{1''}{C}O\overset{2''}{C}H_2\overset{3''}{C}H(\overset{4'',5''}{C}H_3)_2$

2: $R_1 = COCH(CH_3)_2$, $R_2 = COCH(CH_3)_2$

3: $R_1 = H$, $R_2 = COCH(CH_3)_2$

4: $R_1 =$ *trans*-$COCH{=}CHSCH_3$, $R_2 = COCH_3$

7: $R_1 =$ *trans*-$COC(CH_3){=}CHCH_3$, $R_2 = COCH_3$

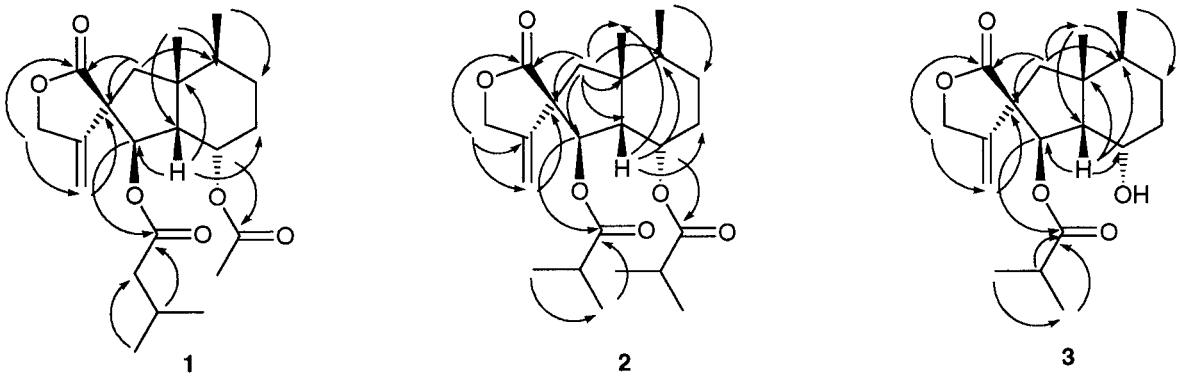


Fig. 1. The HMBC correlations of compounds 1–3.

Compounds **1**–**6** were evaluated for antiplatelet aggregation activity (Teng, Chen, Ko & Ouyang, 1987; O'Brien, 1962). The results are summarized in Table 2. Bakkenolide-G (**1**) and -H (**2**) showed inhibitory activity against platelet activation factor (PAF, 2 nM) in a rabbit platelet aggregation assay; in addition, deisobutyryl bakkenolide-H (**3**) and ferulic acid (**6**) showed inhibitory activity against arachidonic acid (AA, 100 μM) and collagen (Col, 10 μg/ml)

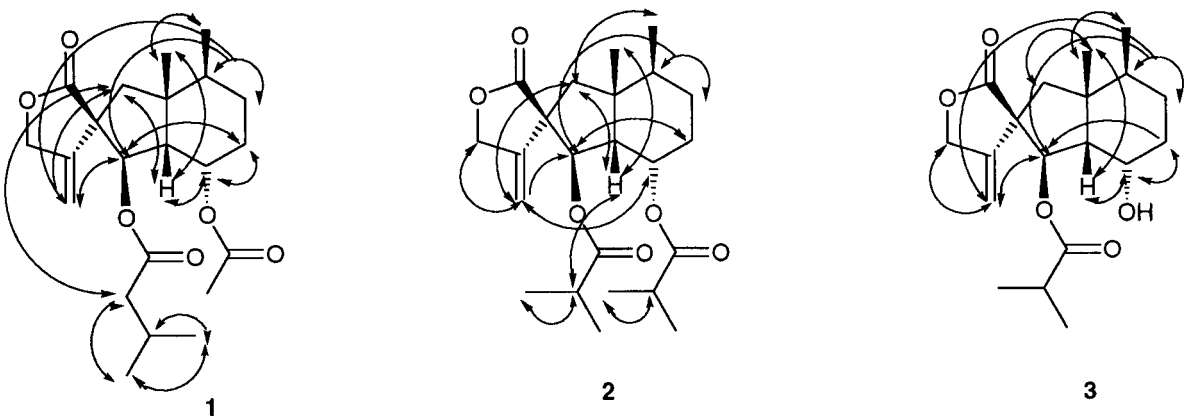


Fig. 2. The NOESY correlations of compounds 1–3.

Table 2
The effects of compounds 1–6 on the aggregation of washed rabbit platelets induced by thrombin (Thr), arachidonic acid (AA), collagen (Col) and platelet activation factor (PAF)[a]

		Aggregation inhibition						
Inducer	Concn (μg/ml)	Aspirin	**1**	**2**	**3**	**4**	**5**	**6**
AA (100 μM)	100		$8.7 \pm 1.7^{***}$	$12.2 \pm 4.5^{*}$	$91.7 \pm 6.8^{***}$	A[b]		$100.0 \pm 0.0^{***}$
	50	100.0 ± 0.0			$75.6 \pm 10.1^{***}$	A[b]		
	20				8.3 ± 4.2	3.4 ± 1.6	12.2 ± 4.5	
	10				4.4 ± 3.5			
	5				1.7 ± 2.3			$100.0 \pm 0.0^{***}$
	2							$52.3 \pm 13.6^{***}$
	1							$14.3 \pm 9.5^{*}$
Col (10 μ/ml)	100		$10.3 \pm 3.0^{***}$	$10.1 \pm 4.0^{*}$	$85.5 \pm 13.0^{***}$	20.6 ± 5.5		$93.5 \pm 1.3^{***}$
	50	12.2 ± 1.7			$78.2 \pm 14.6^{**}$	42.7 ± 11.5		
	20				$61.9 \pm 16.0^{**}$	$10.1 \pm 4.0^{*}$	$10.3 \pm 3.0^{***}$	
	10				$35.8 \pm 11.9^{**}$			
	5				$10.9 \pm 4.7^{*}$			
	2							
PAF (2 nM)	100		$100.0 \pm 0.0^{**}$	$91.6 \pm 6.8^{***}$	$21.0 \pm 1.7^{***}$			$16.0 \pm 1.3^{*}$
	50	9.6 ± 1.2						
	20					$26.7 \pm 13.2^{*}$	$3.1 \pm 2.1^{***}$	
	10		$100.0 \pm 0.0^{***}$					
	5		$97.6 \pm 2.0^{***}$					
	2		$39.9 \pm 2.0^{***}$					
	1		$23.6 \pm 4.7^{***}$					
	0.5		$13.0 \pm 3.2^{***}$					
Thr (0.1 μg/ml)	100		$10.9 \pm 1.5^{***}$	$18.7 \pm 4.5^{***}$	-1.1 ± 1.1	A[b]		7.2 ± 2.1
	50					A[b]		
	20					0.6 ± 1.7	0.5 ± 0.3	

[a] Values are means ± sem ($n = 3$–5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the respective control.
[b] A: platelet aggregation was promoted. Platelets were preincubated with **1**–**6** or DMSO (0.5%, control) at 37°C for 3 min; the inducer was then added.

3. Experimental

3.1. General

Melting points were not corrected. IR spectra were measured as solid dispersions in KBr. NMR spectra were recorded on 200 or 400 MHz for ^{1}H and 50 or 100 for ^{13}C; all chemical shifts are reported in ppm from tetramethylsilane as an internal standard. Mass spectra were performed in the EI or FAB mode.

3.2. Plant material

P. formosanus was collected from Al Li mountain, Taiwan, in August 1992 and verified by Prof. C. S. Kuoh. A specimen of this plant was deposited in the herbarium of National Cheng Kung University, Tainan, Taiwan.

3.3. Extraction and isolation

Dry roots (4.1 kg) of *P. formosanus* were extracted with hot MeOH (×8) and concentrated to give a deep brown syrup (280 g). This syrup was partitioned successively between H_2O and $CHCl_3$, and then *n*-BuOH. The $CHCl_3$ extract (60 g) was subjected to chromatography on silica gel eluting with C_6H_6–Me_2CO (25:1) to give nine fractions. The second fraction, the filtrate of the third fraction and the fourth to the eighth fractions, whose TLC results gave similar spots, were combined and chromatographed over silica gel (C_6H_{14}–EtOAc, 5:1) followed by HPLC (C-18 column, MeOH–H_2O, 8:2) and HPLC (C-8 column, MeOH–H_2O, 7:3) to yield **1** (0.54 g), **2** (0.84 g), **3** (1.24 g), **4** (1.20 g) and **5** (1.10 g). The *n*-BuOH layer (115 g) was subjected to chromatography on Dianion HP-20 eluting with a solvent gradient of H_2O and MeOH to give twenty-one fractions. The thirteenth through sixteenth fractions were combined and rechromatographed on silica gel eluting with $CHCl_3$–MeOH (10:1) to give **6** (22.7 mg).

3.4. Bakkenolide-G (*1*)

Colorless plates (MeOH), mp 135–137°C. $[\alpha]_D = -119^{\circ}$ (*c* 0.13, MeOH). HRMS: calcd for $C_{22}H_{32}O_6$, *m*/*z* 392.2198 $[M]^+$, found 392.2199. IR ν_{max} cm^{-1}: 1772, 1734, 1699. EIMS *m*/*z* (rel. int.): 392 (M^+, 1), 308

(36), 248 (86), 230 (50), 186 (44), 138 (46), 110 (36), 85 (100). CD (1.6×10^{-4} M, MeOH) $\Delta\epsilon_{203}$ 1.19, $\Delta\epsilon_{205}$ 0, $\Delta\epsilon_{212}$ −12.19, $\Delta\epsilon_{254}$ 0. ORD (1.6×10^{-4} M, MeOH) $[\phi]_{207}$ −16690, $[\phi]_{211}$ −19280, $[\phi]_{217}$ −14150, $[\phi]_{224}$ 17140, $[\phi]_{262}$ −4299.

3.5. Bakkenolide-H (**2**)

Colorless needles (MeOH), mp 116–118°C. $[\alpha]_D = -31.6°$ (*c* 0.0079, MeOH). HRMS: calcd for $C_{23}H_{34}O_6$, *m/z* 406.2355 $[M]^+$, found 406.2354. IR ν_{max} cm^{-1}: 1770, 1737, 1732. EIMS *m/z* (rel. int.): 406 (M^+, 8), 336 (19), 248 (100), 230 (84), 186 (50), 138 (15), 110 (10). CD (1.9×10^{-4} M, MeOH) $\Delta\epsilon_{205}$ 0.045, $\Delta\epsilon_{250}$ 0. ORD (1.9×10^{-4} M, MeOH) $[\phi]_{208}$ 0, $[\phi]_{227}$ −26770, $[\phi]_{263}$ −7831.

3.6. Deisobutyryl bakkenolide-H (**3**)

Colorless needles (MeOH), mp 178–180°C. $[\alpha]_D = -93.0°$ (*c* 0.365, MeOH). HRMS: calcd for $C_{19}H_{28}O_5$, *m/z* 336.1937 $[M]^+$, found 336.1939. IR ν_{max} cm^{-1}: 3627, 1770, 1732. EIMS m/z (rel. int.): 362 (M^+, 12), 248 (10), 230 (32), 186 (100), 138 (23), 110 (14). CD (1.1×10^{-4} M, MeOH) $\Delta\epsilon_{206}$ −28.98, $\Delta\epsilon_{280}$ 0. ORD (1.1×10^{-4} M, MeOH) $[\phi]_{208}$ 0, $[\phi]_{226}$ −28690, $[\phi]_{232}$ −26240, $[\phi]_{257}$ −11800, $[\phi]_{287}$ −7303.

3.7. Antiplatelet aggregation test

Washed rabbit platelets were obtained from EDTA-anticoagulated platelet-rich plasma according to the washing procedure described previously (Teng et al., 1987). The platelet pellets were suspended in Tyrode's solution of the following composition (mM): NaCl (136.8), KCl (2.8), $NaHCO_3$ (11.9), $MgCl_2$ (2.1), NaH_2PO_4 (0.33), $CaCl_2$ (1.0), and glucose (11.2), containing bovine serum albumin (0.35%). Platelet aggregation was measured by the turbidimetric method as described by O'Brien (1962). Percentages of aggregation were calculated using the absorbance of platelet suspension to represent 0% aggregation and the absorbance of Tyrode's solution as 100% aggregation.

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