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Orthosiphol A and B, Novel Diterpenoid Inhibitors of TPA (12-*O*-tetradecanoylphorbol-13-acetate)-Induced Inflammation, from *Orthosiphon stamineus*

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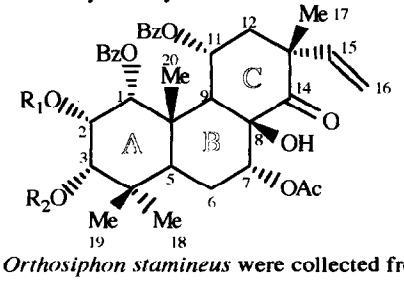
Abstract: Two novel highly oxygenated pimarane diterpenes, orthosiphol A (**1**) and B (**2**), have been isolated from the dry leaves of *Orthosiphon stamineus* Benth (Labiatae). Their structures were elucidated by spectroscopic and chemical methods. Orthosiphol A and B showed potent inhibitory activity against the inflammation induced by a tumor promoter, TPA (12-*O*-tetradecanoylphorbol-13-acetate), on mouse ears

For the chemo-prevention of cancer, anti-tumor-promoters have been investigated and isolated from natural source.[1] Yasukawa *et al.*[2] and Hirota *et al.*[3] suggested the inhibitory effect for TPA-induced inflammation roughly paralleled the anti-tumor-promoter activity *in vivo*. This inhibitory assay is thought to be useful for screening the anti-promoter as a first assay because of its simplicity. In the course of our studies for finding new anti-inflammatory compounds from medicinal plants, we have investigated the chemical constituents found in the leaves of *Orthosiphon stamineus*, which is well known in Southeast Asia to have not only diuretic activity but also potent anti-inflammatory activity.[4] From this plant, we isolated novel highly oxygenated pimarane diterpenes and found that they have potent anti-inflammatory activity against TPA-induced inflammation. This paper deals with the detailed structure determination of the diterpenes[5] and their inhibitory activity of TPA-induced inflammation.



1 R_1=Ac, R_2=H

2 R_1=H, R_2=Ac

The leaves of *Orthosiphon stamineus* were collected from the cultivated plant on Okinawa Island, south of Japan, and extracted with CH_2Cl_2 after air-drying. Orthosiphol A (**1**) and B (**2**) were isolated from the extract by repeated silica gel chromatography.[6]

Orthosiphol A (**1**), colorless plates, mp 210 °C, $[\alpha]_D$ -127 °. The molecular formula was determined to be $C_{38}H_{44}O_{11}$ on the basis of the elementary analyses and a pseudo-molecular ion peak at m/z 677 $[MH]^+$ in the SIMS of **1**. Four carbon signals [δ 164.0, 166.2, 168.9, and 170.1] in the ^{13}C NMR of **1** and an absorption band at 1723 cm^{-1} in the IR spectrum of **1** suggested **1** was an esterified compound. Six

Table 1. ^{13}C NMR Chemical Shift Data and C-H Long Range Coupled Protons in the COLOC Spectra of **1** and **2** ($CDCl_3$)

	1		**2**	
position	C	correlated proton	C	correlated proton
1	74.2	H-3, 20	78.9	H-20
2	67.8	H-1	66.2	
3	77.4	H-1, 18, 19	78.4	H-18, 19
4	38.3	H-5, 18, 19	37.2	H-18, 19
5	35.5	H-1, 3, 6, 7, 19, 20	36.7	H-18, 19
6	21.4		21.5	
7	70.6	H-6,	70.9	H-6
8	75.8	H-6, 7	75.5	H-6, 7
9	42.1	H-7, 20, 8-OH	41.2	H-20, 8-OH
10	43.7	H-6, 9, 20	44.0	H-6, 9, 20
11	68.8	H-9, 12β	68.8	H-12β
12	39.7	H-17	40.1	H-17
13	47.8	H-15, 16, 17	47.9	H-16, 17
14	208.6	H-12β, 17	208.5	H-12β, 17
15	142.0	H-12α, 12β, 16, 17	141.5	H-12β, 17
16	113.1		114.1	
17	26.6		25.9	
18	28.9	H-19	27.8	H-19
19	22.3	H-18	22.6	H-18
20	16.8	H-5, H-9	16.4	H-9
2-CH_3CO	170.1	H-2, 2-CH_3CO		
2-CH_3CO	20.9			
3-CH_3CO			170.6	H-3, 3-CH_3CO
3-CH_3CO			20.3	
7-CH_3CO	168.9	H-7, 7-CH_3CO	168.6	H-7, 7-CH_3CO
7-CH_3CO	21.0		21.0	
1-PhCO	164.0	H-1, 1-Bz(7.60 ppm)	167.7	1-Bz(7.84 ppm)
1-PhCO	132.9		133.5[c]	
	130.8[a]		130.3[d]	
	129.7[b]		130.0[e]	
	128.2		128.1	
11-PhCO	166.2	11-Bz(7.58 ppm)	166.2	11-Bz(7.59 ppm)
11-PhCO	132.2		132.8[c]	
	130.2[a]		130.1[d]	
	129.6[b]		129.6[e]	
	127.8		128.1	

a, b, c, d, e, These assignments are interchangable.

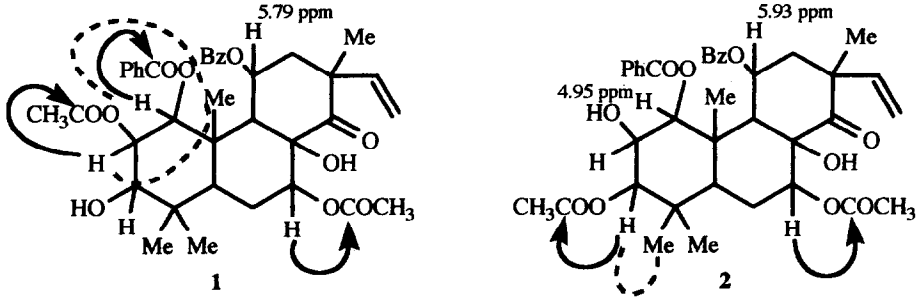


Fig. 1. The Substituted Positions of Ester Groups Confirmed by the COLOC Correlations (arrow), NOESY Correlations (dashed), and Proton Chemical Shifts.

oxygenated carbon signals [δ 67.8, 68.8, 70.6, 74.2, 75.8, and 77.4] indicated that **1** had a highly oxygenated gross structure. The oxygenated structure of **1** was determined by ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY, and COLOC spectra.[7] The ^{1}H-^{1}H COSY spectrum of **1** gave four proton coupling networks [C-1-C-2-C-3, C-5-C-6-C-7, C-9-C-11-C-12, and C-15-C-16] in **1** and the positions of oxygenated carbons [C-1 (δ 74.2), C-2 (δ 67.8), C-3 (δ 77.4), C-7 (δ 70.6), and C-11 (δ 68.8)] in the networks were determined by the ^{13}C-^{1}H COSY of **1**. In addition to the ^{1}H-^{1}H networks, four methyl signals [δ 1.04 (3H, s), 1.07 (3H, s), 1.14 (3H, s), and 1.49 (3H, s)] and five quaternary carbon signals [δ 38.3, 43.7, 47.8, 75.8 and 208.6], one of which was assignable to a carbonyl carbon, were observed in the ^{1}H NMR and ^{13}C NMR of **1**. These obtained segments of the gross structure of **1** were connected on the basis of the COLOC spectrum (Table 1). From these results, **1** has a highly oxygenated pimarane diterpenoid structure. Substituted ester groups and their substituted positions were determined as follows. In the ^{1}H NMR of **1**, signals assignable to two benzoyl groups [δ 7.11 (2H, t, *J*=7.3 Hz), 7.29 (2H, t, *J*=7.3 Hz), 7.41 (1H, t, *J*=7.3 Hz), 7.54 (1H, t, *J*=7.3 Hz), 7.58 (2H, d, *J*=7.3 Hz), and 7.60 (2H, d, *J*=7.3 Hz)] and two acetyl groups [δ 1.94 (3H, s) and 2.17 (3H, s)] were observed. The substituted positions of one of the benzoyl groups and the two acetyl groups were determined to be the 1-, 2-, and 7-positions, respectively, from the correlations of H-1 [δ 5.30 (1H, br d, *J*=3.0 Hz)] with a carbonyl signal [δ 164.0] of a benzoyl group, H-2 [δ 5.45 (1H, br t, *J*=3.0 Hz)] with a carbonyl signal [δ 170.1] of an acetyl group, and H-7 [δ 5.43 (1H, br t, *J*=3.0 Hz)]with a carbonyl signal [δ 168.9] of the other acetyl group in the COLOC spectrum of **1**. The substituted position of the other benzoyl group was also determined by the downfield chemical shift of H-11 [δ 5.79 (1H, m)] (Fig. 1). The stereochemistry of the A and B rings of **1** was determined on the basis of NOEs and coupling constants (Fig. 2). The observed NOEs in the A and B rings indicated that the A and B rings were *trans*-fused and H-2 [δ 5.45 (1H, br t, *J*=3.0 Hz)] was β-oriented. The axial-equatorial coupling constants (ca. 3 Hz, each) between H-1, H-2, and H-3 revealed that the stereochemistry of the 1-benzoyloxy, 2-acetoxy, and 3-hydroxy groups were all α-oriented. As for the stereochemistry of the C ring of **1**, no effective NOE was obtained except for a strong NOE between H-16 [δ 4.81 (1H, d, *J*=17.7 Hz)] and a methyl signal [δ 2.17 (3H, s)] of the 7-acetoxy group. The NOE indicated that the vinyl group at the 13-position was α-oriented and also that the C ring had a boat-like flexible conformation. The boat-like conformation of the C ring impeded the orientation determination of the 11-benzoyloxy group by the coupling constant between H-9 and H-11 (*J*=5.5 Hz). It was presumed that one of the reasons for the boat-like conformation of the C ring depended on the 2-alkylketone effect[8] caused by the 14-carbonyl group. **1** was reduced by an excess amount of $LiAlH_4$ and subsequently acetylated with Ac_2O in pyridine to give the 2,11-diacetate **3**. The observed diaxial coupling constant ($J_{H\text{-}9,H\text{-}11}$=11.0 Hz) between H-9 and H-11 in the ^{1}H NMR of **3** revealed that the 11-benzoyloxy group in **1** was α-oriented (Fig. 3). The absolute stereochemistry of **1** was judged by an exiton chirality method.[9] A positive cotton effect caused by the 1- and 11-benzoyloxy groups, observed in the CD spectrum of **1**, revealed that C-11 has an *R* configuration. Thus, the structure of orthosiphol A (**1**) was determined to have the structural formula **1**.

Orthosiphol B (**2**), colorless plates, mp 240 °C, $[\alpha]_D$ -82 °, showed the same pseudo-molecular ion peak at m/z 677 to that of **1** in SIMS and similar ^{1}H and ^{13}C NMR spectra to those of **1**. In the ^{1}H NMR of **2**, a methyl signal [δ 1.40 (3H, s)], which should be assigned to an acetyl group, was shifted upfield, suggesting the acetyl group was affected by anisotropy of a benzoyl group. These results strongly indicated **2** was an acetyl migrated product of **1** from the 2-position in **1** to the 3-position in **2**, which was supported by a derivation of **2** from **1** with an acid treatment. The acylated positions of **2** were further concluded by the COLOC spectrum and proton chemical shifts. In the COLOC spectrum of **2**, two acetyl carbonyl signals at 170.6 and 168.6 ppm were correlated with proton signals at 5.04 and 5.44 ppm, respectively, the positions of which were determined to be the 3- and 7-positions from NOESY and ^{1}H-^{1}H COSY spectra (Table 2). The two substituted benzoyl groups were also determined to be at the 1-*O*- and 11-*O*-positions by considering the chemical shifts of H-1 and H-11 [δ 4.95 (1H, br s) and 5.93 (1H, br t, *J*=6.4 Hz),

Table 2. ^{1}H NMR Chemical Shift Data and Correlated Protons in the NOESY Spectra of **1** and **2** ($CDCl_3$)

	1[a]		2[b]	
position	H	correlated proton	H	correlated proton
1	5.30, br d (3.0)	H-20	4.95, br s	H-11, 20
2	5.45, br t (3.0)	H-19, 20	4.41, br s	H-19, 20
3	3.49, m	H-18, 19	5.04, br d (3.1)	H-18, 19
5	2.45, dd (11.0, 4.9)	H-9	2.40, dd (10.4, 4.9)	H-9, 18
6α	1.99-2.12, m		2.01-2.15, m	
6β	1.99-2.12, m	H-20	2.01-2.15, m	H-20
7	5.43, br t (3.0)		5.44, br t (2.8)	
9	3.11, br d (5.5)	H-5	3.39, d (7.3)	H-5
11	5.79, m	H-1, 20	5.93, br t (6.4)	H-1, 20
12α	2.57, dd (16.0, 4.9)	H-9, 17	2.59, dd (15.9, 5.8)	
12β	1.96, dd (16.0, 1.7)	H-17	1.90, br d (15.9)	H-17
15	5.66, dd (17.7, 10.4)	H-17	5.82, dd (17.7, 11.0)	
16-*trans*	4.75, d (10.4)		4.87, d (11.0)	
16-*cis*	4.81, d (17.7)	7-CH_3CO	4.94, d (17.7)	7-CH_3CO
17	1.14, s	H-12α, 12β, 15	1.15, s	H-12β
18	1.04, s	H-3	0.87, s	H-3, 5, 19
19	1.07, s	H-3, 20	1.09, s	H-2, 3, 20
20	1.49, s	H-1, 2, 6β, 11, 19	1.52, s	H-1, 2, 6β, 11, 19
2-CH_3CO	1.94, s			
3-CH_3CO			1.40, s	
7-CH_3CO	2.17, s	H-16-*cis*	2.20, s	H-16-*cis*
1-PhCO	7.29, t (7.3)		7.48, t (7.3)	
	7.54, t (7.3)		7.64, t (7.3)	
	7.60, d (7.3)		7.84, d (7.3)	
11-PhCO	7.11, t (7.3)		7.16, t (7.3)	
	7.41, t (7.3)		7.41, t (7.3)	
	7.58, d (7.3)		7.59, d (7.3)	

Coupling constants (J in Hz) in parentheses. [a]3-OH [2.23, d (5.5)], 8-OH [2.80, br s]. [b]2-OH [4.01, br d (4.0)], 8-OH [2.95, br s].

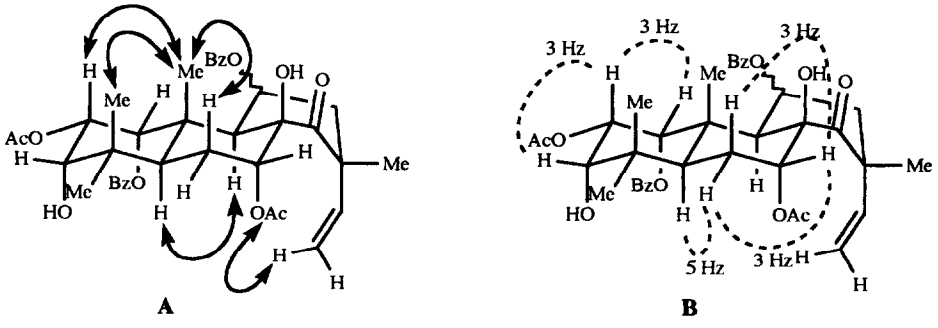


Fig. 2. Selected NOEs (A) and J values (B) of **1**

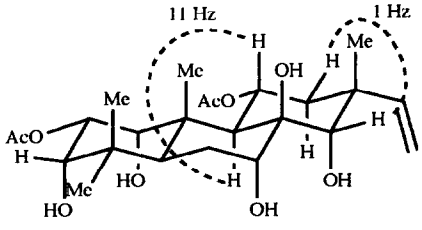


Fig. 3. Stereostructure of **3** and Selected J values in the C ring of **3**

respectively] (Fig. 1). Thus, the structure of orthosiphol B (**2**) was determined to have the structural formula **2**.

The anti-inflammatory activity of **1** and **2** was measured on mouse ears using a tumor promoter, TPA (12-*O*-tetradecanoylphorbol-13-acetate, 2 μg) as an inducer according to Gschwendt's procedure.[10] Each 200 μg (0.3 μmol) application of **1** and **2** showed potent inhibitory activity, the ratio of which was 42 % and 50 %, respectively. Yasukawa *et al.*[2] and Hirota *et al.*[3] reported the inhibitory activity of some triterpenoids having anti-tumor-promoter activity. The activity of **1** and **2** was equal to those of the triterpenoids. Orthosiphol A and B were first reported as diterpenoid inhibitors against TPA-induced inflammation and are expected to have anti-tumor-promoter activity.

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EXPERIMENTAL

General remarks

All melting points were measured on a Yanagimoto micro-meltingpoint apparatus and are uncorrected. Optical rotations were measured with a Union PM-101. Spectral analyses were carried out using the following instruments; UV, Hitachi 220A; IR, Perkin-Elmer FT-IR 1720; CD, Jasco J-500E; SIMS, Hitachi M-2000; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz), JEOL JNM GX-400.

Plant material

The leaves of *Orthosiphon stamineus* Benth were collected in a field of the Nakazen Pharmacy Co., Okinawa, Japan. A voucher specimen is maintained at our laboratory.

Animals

Male Jcl:ICR mice, 6 weeks of age, were obtained from CLER JAPAN INC., Tokyo, Japan, and used for anti-inflammatory assay (5 mice/group).

Extraction and isolation of 1 and 2

The dry leaves of *Orthosiphon stamineus* (1 kg) were extracted three times with CH_2Cl_2 (6 l) at room temperature. Evaporation of the CH_2Cl_2 solution gave a brownish residue (34 g). Twenty-four grams of the residue was separated into 10 fractions by silica gel column chromatography eluted with 10 % acetone in benzene. Fraction 3 (3.6 g) was re-chromatographed on silica gel with the same solvent system to give **1** (1.4 g, colorless plates from ether) and a fraction containing **2**. The fraction was filtered with CH_2Cl_2 and the filtrate was chromatographed on silica gel with EtOAc-benzene (1:5) to give **2** (0.6 g, colorless plates from ether). Orthosiphol A (**1**): mp 210 °C; $[\alpha]^{26}_D$ -127 ° (c 1.0, $CHCl_3$); SIMS (glycerin matrix) m/z 677 $[MH]^+$; Anal. calcd for $C_{38}H_{44}O_{11}$: C 67.44, H 6.55; found C 67.99, H 6.65; IR νmax (film) cm^{-1}: 3425, 2967, 1723, 1287, 1240, 756, 710; UV λmax (MeOH) nm: 230 (ε 22000); CD λmax (MeOH) nm: 215 (θ -6600), 222 (θ 0), 234 (θ +35000), 247 (θ 0); ^{13}C NMR: see Table 1; ^{1}H NMR: see Table 2. Orthosiphopl B (**2**): mp 240 °C; $[\alpha]^{11}_D$ -82 ° (c 1.0, $CHCl_3$); SIMS (glycerin matrix) m/z: 677 $[MH]^+$; IR νmax (film) cm^{-1}: 3420, 2970, 1717, 1289, 1240, 733, 711; ^{13}C NMR: see Table 1; ^{1}H NMR: see Table 2.

Derivation of 2 from 1

To a solution of **1** (4.2 mg) in toluene (0.4 mg) was added *p*-toluenesulfonic acid (1.4 mg) at 23 °C. After stirring at 80 °C for 30 min, the mixture was purified on silica gel TLC with EtOAc-hexane (1:2) as the eluent to give **2** (1 mg).

Synthesis of 3 from 1

To a solution of **1** (10 mg) in dry tetrahydrofuran (0.5 ml) was added $LiAlH_4$ (11 mg) under nitrogen. After stirring for 2 hr at 23 °C, excess $LiAlH_4$ was decomposed with EtOAc. The mixture was poured into 1N HCl, extracted 4 times with EtOAc, dried over anhyd. Na_2SO_4, and concentrated. To the residue solution in pyridine (1 ml) was added acetic anhydride (1 ml). After standing for 1 hr at 23 °C, the mixture was evaporated *in vacuo* and purified by silica gel column chromatography with 5 % MeOH in CH_2Cl_2 to give **3** (3 mg). **3**: mp 159-162 °C; SIMS (glycerin matrix) m/z: 471 $[MH]^+$; ^1H NMR ($CDCl_3$) δ: 0.95 (3H, s, H-18), 1.05 (3H, s, H-19), 1.19 (3H, s, H-17), 1.35 (3H, s, H-20), 1.58 (1H, br d, *J*=13.4 Hz, H-6α), 1.70 (1H, dd, *J*=11.0 and 4.3 Hz, H-12β), 1.93 (1H, br t, *J*=11.0 Hz, H-12α), 1.96 (1H, br t, *J*=13.4 Hz, H-6β), 2.01 (3H, s, Ac), 2.15 (3H, s, Ac), 2.42 (1H, br d, *J*=13.4 Hz, H-5), 2.72 (1H, d, *J*=11.0 Hz, H-9), 3.22 (1H, br s, H-14), 3.53 (1H, br s, H-1 or 3), 3.70 (1H, br s, H-7), 4.04 (1H, br s, H-1 or 3), 5.05 (1H, d, *J*=17.7 Hz, H-16), 5.20 (1H, br s, H-2), 5.21 (1H, d, *J*=10.4 Hz, H-16), 5.57 (1H, dt, *J*=11.0 and 4.3 Hz, H-11), 5.73 (1H, dd, *J*=17.7 and 10.4 Hz, H-15).

Anti-inflammatory assay

The assay was carried out using Gschwendt's method[10] with some modification. A sample (200 μg) in acetone (20 μg) and vehicle were applied to the inner part of the left ear and the right ear, respectively, of the same mouse. Thirty minutes after the sample application, TPA (2 μg) in acetone (20 μg) was applied to the same part of both ears. After 6.5 hr, the mouse was killed. Immediately after the killing, plugs of each ear were obtained with a punch (0.6 cm diameter) and weighed. The inhibitory effect [IE (%)] was determined using the following equation: {[weight of TPA applied ear]-[weight of sample+TPA applied ear]}x100 / {[weight of TPA applied ear]-[weight of vehicle applied ear]}. IE for **1**: 42 % [D±SE=6.8±0.9 mg (n=5)], IE for **2**: 50 % [D±SE=8.1±1.3 mg (n=5)].

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