Grayanane Diterpenoids from *Pieris formosa*†

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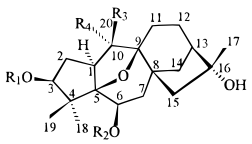
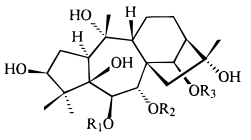
Two new diterpenoids, pierisformoside A (**1**) and pierisformosin D (**2**), and two known diterpenoids, asebotoxins VIII (**3**) and V (**4**), were isolated from leaves of *Pieris formosa*. Their structures were elucidated on the basis of spectral analysis, including $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, HMBC, and NOESY experiments.

Pieris formosa (Wall) D. Don (Ericaceae) is a well-known poisonous plant, distributed mainly in hilly and valley regions of south and southwest China. Poultry have been reported to go into coma after accidentally eating leaves or stems of this plant. In folk practice, the juice of the fresh leaves can be used as an insecticide or as a lotion for treatment of ring worm and scabies.[1] Recently, we found that EtOAc and n-BuOH fractions of ethanol extracts were toxic to brine shrimp. From the EtOAc fraction, we isolated several new and known grayanane diterpenoids.[2,3] Our continuing studies on the same fraction of the plant led to the isolation of two new grayanane diterpenoids, pierisformoside A (**1**) and pierisformosin D (**2**), together with two known diterpenoids, asebotoxins VIII (**3**) and V (**4**) (Chart 1). In this paper we describe the isolation and structural elucidation of these diterpenoids and the unambiguous assignment of NMR spectral data by a combination of NMR techniques, including $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, HMBC, and NOESY.

Chart 1



	R_1	R_2	R_3	R_4
1	H	glc	CH_3	H
5	glc	H	H	CH_3
6	H	H	H	CH_3



	R_1	R_2	R_3
2	H	CH_3CO	CH_3CH_2CO
3	CH_3CO	H	CH_3CH_2CO
4	CH_3CO	CH_3CH_2CO	H

Results and Discussion

An EtOAc fraction of the EtOH extract was subjected to repeated column chromatography on silica gel and Sephadex LH-20 to give diterpenoids **1**−**4**. Pierisformoside A (**1**), a viscous syrup, had a molecular formula $C_{26}H_{42}O_9$ from the FABMS and NMR data. It showed positive reaction in the Molish test and IR absorption for hydroxyl groups (3406 cm^{-1}). Acidic hydrolysis afforded glucose. In addition to the glucose unit, the ^1H NMR spectrum (Table 1) showed signals for four methyls (δ 1.11, d, J = 7.5 Hz, 1.30, 1.37, 1.75, each s), two oxygenated methines (δ 3.77, br d, J = 10.0 Hz; 4.25, d, J = 3.2 Hz) and a ABq methylene (δ 2.57, 2.80, each d, J = 15.5 Hz). Furthermore, the ^{13}C NMR data (Table 2) revealed the presence of one secondary and three tertiary methyls, six methylenes, five methines (two oxygenated), and five quaternary carbons (three oxygenated) in its aglycon, consistent with a grayanane diterpenoid skeleton. Five oxygenated carbons in the skeleton indicated that one oxygen must constitute an ether linkage since **1** had only four oxygens in the molecule. Through literature investigation we found that the spectra of **1** showed a close resemblance to two known grayanane diterpenoids, grayanoside D (**5**) and its aglycon (**6**).[4] Comparing the ^{13}C NMR data of **1** with that of **5** and **6**, we observed the following characteristics: (a) A resemblance of C-5 and C-9 data, δ 95.9, 88.9 in **1**, δ 93.4, 89.0 in **5**, and δ 95.9, 88.8 in **6**, respectively, indicating that **1** also had a C_5-O-C_9 linkage like **5** and **6**. (b) C-3 data of **1** (δ 85.7) were similar to that of **6** (δ 85.0) and different from that of **5** (δ 91.1) suggesting that the 3-hydroxyl group in **1** was free, not glycosylated. (c) C-6 signal of **1** was at lower field (δ 72.6) than that of **5** (δ 69.6, −3.0 ppm) and **6** (δ 68.6, −4.0 ppm), indicating that the 6-hydroxyl group in **1** was glucosylated. These assignments for **1** were further confirmed by an HMBC experiment (Table 3), in which the correlations between C-6 (δ 72.6)/H-1′ (δ 4.91) and C-1′ (δ 100.8)/H-6 (δ 4.25) were observed. The anomeric configuration of the glucose unit was deduced as β-orientation by $J_{1',2'}$ = 7.6 Hz in the ^1H NMR spectrum.

Unambiguous assignments of ^1H and ^{13}C NMR signals were made by comparison with literature values for known diterpenoids and were verified using various 2D-NMR techniques. The following fragments were observed in the $^1H-^1H$ COSY experiment: $-CH(OH)CH_2CHCHCH_3$ and $CH(OH)CH_2$, which indicated the arrangements from C_3 to C_{20} and C_6 to C_7, respectively. In the NOESY spectrum, the correlations between H-1/H-6, H-3/H-18, and H-6/H-18 indicated that H-1, H-3, H-6, and C-18 all have α-orientations. Most grayanane diterpenoids possess a 5/7/6/5 (trans, cis, cis) ring system with 1α-H and 10β-methyl.[5] Few of them have the difference in 5/7 ring conjunction (cis) and 10-methyl orientation (α-form). Drieding model studies of **1** indicated that the spatial distance between H-10α and H-14α was very small. Correlations between H-14α at δ 1.37 and H-10 at δ 2.65 in the NOESY spectrum indicated H-10 was to be the α-orientation. A slight difference of two C-10 enantiomers, $J_{H-10\alpha,H-20}$ = 8 Hz and $J_{H-10\beta,H-20}$ = 6 Hz, respectively, has been published.[6] Consequently, the H-20 has a J value of 7.5 Hz with H-10 in **1**, confirming the β-orientation for 10-methyl.

Only six grayanoid glycosides, each with a glucose moiety at C-3, have been previously reported from Ericaceae plants.[4,6−10] Compound **1** is the first sample with glycosylation at C-6.

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Table 1. ^{1}H NMR Data for Compounds **1**−**4**[a]

H	**1**[b]	H	**2**[c]	**3**[c]	**4**[c]
1	2.57 (m)	1	3.71 (dd, 9.8, 5.9)	3.58 (dd, 9.5, 5.2)	3.44 (dd, 11.6, 3.7)
2	2.03 (m)	2	2.62 (dd, 4.1, 9.2)	2.55 (m)	2.49 (m), 2.60 (dd, 15.0, 4.1)
3	3.77 (br d, 10.0)	3	3.93 (br s)	3.91 (br s)	3.88 (br s)
6	4.25 (d, 3.2)	6	4.18 (d, 9.6)	5.65 (d, 9.5)	6.16 (d, 9.9)
7α	1.74 (m)	7	5.71 (d, 9.6)	4.28 (d, 9.5)	6.09 (d, 9.9)
7*β*	2.01 (m)	9	2.25 (d, 6.4)	2.23 (d, 6.2)	2.26 (d, 6.0)
10	2.65 (m)	11α	2.06 (dd, 13.3, 6.1)	2.10 (m)	2.05 (m)
11	1.35 (m)	11*β*	1.61 (m)	1.63 (m)	1.67 (m)
12	1.62 (m)	12α	2.69 (m)	2.75 (m)	2.66 (m)
13	2.03 (m)	12*β*	1.63 (m)	1.61 (m)	1.67 (m)
14α	1.37 (m)	13	2.31 (br s)	2.55 (br s)	2.49 (br s)
14*β*	2.42 (dd, 10.5, 3.0)	14	6.43 (s)	6.43 (s)	5.24 (d, 2.9)
15α	2.80 (d, 15.5)	15α	2.55 (d, 15.3)	3.79 (d, 15.1)	2.48 (d, 15.1)
15*β*	2.57 (d, 15.5)	15*β*	1.98 (d, 15.3)	1.90 (d, 15.1)	1.94 (d, 15.1)
17	1.37 (s)	17	1.45 (s)	1.52 (s)	1.50 (s)
18	1.30 (s)	18	1.35 (s)	1.11 (s)	1.01 (s)
19	1.75 (s)	19	1.67 (s)	1.54 (s)	1.50 (s)
20	1.11 (s, 7.5)	20	1.87 (s)	1.84 (s)	1.85 (s)
glc-1′	4.91 (dd, 7.6)	acetyl			
2′	4.11 (t, 8.8, 7.6)	CH_3	2.21 (s)	2.13 (s)	2.08 (s)
3′	4.32 (t, 8.8)	propionyl			
4′	4.22 (t, 8.8)	CH_3	1.19 (t, 7.5)	1.18 (t, 7.2)	1.14 (t, 7.5)
5′	4.00 (ddd, 8.8, 5.7, 2.6)	CH_2	2.46 (q, 7.5)	2.45 (q, 7.2)	2.47 (q, 7.5)
6′	4.64 (dd, 11.4, 2.6)				
	4.39 (dd, 11.4, 5.7)				

[a] Spectra determined in C_5D_5N; data reported in *δ* (ppm). [b] 400 MHz. [c] 500 MHz.

Table 2. ^{13}C NMR Data for Compounds **1**−**6**[a]

C	**1**[b]	**5**[c]	**6**[c]	C	**2**[d]	**3**[d]	**4**[d]
1	49.5 d	48.0	48.7	1	50.2 d	51.9 d	51.5 d
2	32.4 t	31.7	32.3	2	35.7 t	35.6 t	35.5 t
3	85.7 d	91.1	85.0	3	82.7 d	82.7 d	82.7 d
4	48.8 s	47.8	48.1	4	52.3 s	51.8 s	52.0 s
5	95.9 s	93.4	95.9	5	83.6 s	82.9 s	83.1 s
6	72.6 d	69.6	68.6	6	77.2 d	80.5 d	78.8 d
7	33.7 t	31.7	40.7	7	80.3 d	74.9 d	77.6 d
8	46.7 s	46.7	46.7	8	56.2 s	56.2 s	56.6 s
9	88.9 s	89.0	88.8	9	54.5 d	55.2 d	54.6 d
10	37.5 d	36.4	37.3	10	77.6 s	77.5 s	77.6 s
11	26.0 t[e]	25.8	25.8[e]	11	22.3 t	22.7 t	22.6 t
12	25.9 t[e]	25.8	25.7[e]	12	27.1 t	27.3 t	27.0 t
13	46.6 d	46.2	46.2	13	55.1 d	55.3 d	56.5 d
14	40.8 t	40.6	40.3	14	82.0 d	83.2 d	80.1 d
15	51.1 t	51.9	51.6	15	53.5 t	52.1 t	51.8 t
16	79.8 s	79.9	79.7	16	78.7 s	78.5 s	79.5 s
17	24.2 q	24.3	24.3	17	23.3 q	24.0 q	24.0 q
18	22.9 q	24.8	23.6	18	23.0 q	23.0 q	23.1 q
19	19.8 q	19.8	19.5	19	20.3 q	19.7 q	19.7 q
20	15.5 q	14.3	15.2	20	28.2 q	28.6 q	28.5 q
glc-1′	100.8 d	105.8		acetyl			
2′	75.4 d	75.8		CH_3	21.7 q	21.7 q	21.6 q
3′	78.9 d	78.1		C=O	171.3 s	171.2 s	169.8 s
4′	72.6 d	71.9		propionyl			
5′	78.4 d	78.1		CH_3	9.4 q	9.1 q	9.2 q
6′	63.6 t	63.0		CH_2	28.6 t	28.2 t	28.3 t
				C=O	173.5 s	173.8 s	174.3 s

[a] Spectra determined in C_5D_5N; data reported in *δ* (ppm). [b] 400 MHz. [c] Literature value. [d] 500 MHz. [e] Chemical shifts are interchangeable.

Pierisformosin D (**2**) had a molecular formula of $C_{25}H_{40}O_9$ based on FABMS and NMR data. The IR spectrum showed characteristic absorptions for hydroxyl (3400−3500 cm^{-1}) and two ester carbonyl groups (1736 and 1720 cm^{-1}). The ^{1}H NMR spectrum of **2** (Table 1) contained signals of four singlet methyls (*δ* 1.35, 1.45, 1.67, 1.87), four oxygenated methines (*δ* 3.93, 4.18, 5.17, 6.43), one *O*-acetyl (*δ* 2.21, s, 3H), and one *O*-propionyl (*δ* 1.19, t, 3H, $J = 7.5$ Hz; 2.46, q, 2H, $J = 7.5$ Hz). Apart from two ester groups (*δ* 21.7, 171.3 and 9.4, 28.6, 173.5), 20 other carbon signals were observed in the ^{13}C NMR spectrum (Table 2), including four methyls, four methylenes, seven methines (four oxygenated), and five quaternary carbons (three oxygenated). The ^{1}H−^{1}H COSY revealed the following fragments: $CHCH_2$-CH(OH)−, CH(OR)CH(OR)−, and $CHCH_2CH_2CH$−, which were all connected to quaternary carbon atoms at one or both ends. These data confirmed the proposed structure of the grayanane diterpenoid.

According to the literature, the C-3, C-6, C-7, C-14, C-16, and C-20 positions in grayanoids can be linked with hydroxyl group(s). The C-6, C-7, and C-14 positions were found to be esterified and connected to one or two acyl group(s).[5] Meanwhile, the 14-OH or 14-OCOR was usually in the *β*-orientation, and in this case H-14α showed a singlet ^{1}H NMR signal due to a dihedral angle of ca. 90° between H-14α and H-13α. A singlet proton signal at *δ* 6.43 (1H, s) in **2** indicated an ester group at C-14 in the *β*-orientation. In ^{1}H NMR spectrum of **2** the signal of C*H*OH at *δ* 4.18 (d, $J = 9.6$ Hz) and signal of C*H*OCOR at *δ* 5.71 (d, $J = 9.6$ Hz) were in the ABq system, assigned to H-6 and H-7 or H-7 and H-6 reversely. The NOESY spectrum (Table 3) showed that the 18-methyl (*δ* 1.35) correlated with the proton signal at *δ* 4.18 in an ABq system. This indicated the presence of 6α-hydrogen and 6*β*-hydroxyl groups. In the HMBC spectrum (Table 3), the carbonyl signal at *δ* 171.3 (acetyl) correlated with the proton signal at *δ* 5.71, and the carbonyl signal at *δ* 173.5 (propionyl) correlated with the proton signal at *δ* 6.43, respectively, demonstrating that the C-7 hydroxyl was acetylated and C-14 was propionylated.

From the NOESY spectrum, it was concluded that H-1, H-3, H-6, H-14, and C-18 are all in the α-orientation and that H-7, H-9, C-17, and C-20 have the *β*-orientation. Further studies on the ^{1}H−^{1}H COSY, NOESY, and HMBC spectra, and comparison of NMR data with other grayanoids,[5] suggested the assignments of 3*β*, 5*β*, 6*β*, 16α, 20α-pentahydroxyl, 7α-*O*-acetyl, and 14*β*-*O*-propionyl groups.

In the course of our study, two isomers of **2** were isolated from the same plant and identified as asebotoxins VIII (**3**) and V (**4**) by a combination of one- and two-dimensional NMR techniques. Both **3** and **4** had the same molecular formula ($C_{25}H_{40}O_9$), same functional groups, and similar ^{1}H and ^{13}C NMR spectral features as **2**. The differences

Table 3. NOESY and HMBC Data of **1** and **2**[a]

1[b]			**2**[c]		
H	NOESY	HMBC(H-C)	H	NOESY	HMBC(H-C)
1	H-2, H-6, H-10	C-2, C-3, C-6, C-9	1	H-2, H-6, H-14, H-18	C-2
2	H-1, H-3	C-3, C-4	2	H-1, H-3	C-1
3	H-2, H-18, H-19		3	H-2, H-18, H-19	C-1, C-5
6	H-1, H-18	C-1′ C-8	6	H-1, H-18	C-7
7	H-1, H-14*β*	C-5, C-6, C-8, C-9	7	H-9	C-6, CH_3CO
10	H-1	C-20	9	H-7, H-11*β*, H-20	C-8, C-11
11		C-12	11α	H-11*β*	
12		C-11	11*β*	H-9, H-11α, H-17	
13	H-14*β*, H-17	C-15	12α	H-12*β*, H-13	
14α	H-10	C-8, C-15	12*β*	H-12α, H-13, H-17	
14*β*	H-7	C-9	13	H-12α, H-12*β*, H-17	C-8
15α	H-15*β*	C-8, C-9, C-13, C-16	14	H-1	C-7, C-15, C-16, CH_3CH_2CO
15*β*	H-15α	C8, C-9, C-13, C-14, C-17	15α	H-15*β*	C-16
17	H-13	C-13, 15, 16	15*β*	H-11*β*, H-15α,H-17	
18	H-3, H-6, H-19	C-3, C-4, C-5, C-19	17	H-11*β*, H-12*β*, H-13, H-15*β*	C-13, C-15, C-16
19	H-3, H-18	C-3, C-4, C-5, C-18	18	H-1, H-3, H-6, H-19	C-3, C-4, C-5, C-19
20		C-1, C-9, C-10	19	H-3, H-18	C-3, C-4, C-5, C-18
glc-1′	H-6, H-7*β*	C-6	20	H-9	C-1, C-9, C-10
2′		C-1′, C-3′			
3′		C-2′, C-4′			
4′		C-6′			
5′	H-6′				
6′	H-5′	C-5′			

[a] Spectra determined in C_5D_5N; data reported in δ (ppm). [b] 400 MHz. [c] 500 MHz.

between them were in the positions of acetyl and propionyl groups. The presence of 6-*O*-acetyl and 14-*O*-propionyl in **3** were deduced from the following: (a) Two oxygenated methine protons at δ 5.65 (d, J = 9.5 Hz) and 4.28 (d, J = 9.5 Hz) constituted an ABq system, and the former proton correlated with the 18-methyl in the NOESY spectrum, assigned as H-6α. (b) A singlet signal at δ 6.43 indicated the presence of a C-14*β* ester group. (c) Correlation between H-6α (δ 5.65)/C═O (δ 171.2) of an acetyl and H-14α (δ 6.43)/C═O (δ 173.8) of a propionyl was observed in the HMBC spectra. The presence of 6-*O*-acetyl and 7-*O*-propionyl groups in **4** was deduced in the same way as for **3** from various spectral evidences, including NOESY and HMBC spectra. Asebotoxin VIII was first isolated from *P. japonica*[11] and had identical spectral characters to that of our isolate **3**. However, the location of acetyl and propionyl groups in asebotoxin VIII was not defined at that time. Asebotoxin V was also isolated from *P. japonica*, and the position of acetyl and propionyl was elucidated only by partial hydrolysis.[12] This report is the first to give unambiguous NMR assignments of **3** and **4**. It is interesting that the three isomers, differing only in substituent positions, were obtained from the same plant.

Experimental Section

General Experimental Procedures. $[\alpha]_D$: JASCO, DIP-181, polarimeter. IR: Nicolet Magna FTIR-750. ^1H and ^{13}C NMR spectra: Bruker AM-400 and DRX 500. Chemical shifts are reported in ppm (δ) with solvent signal as internal standard. MS: MAT-95.

Plant Material. The leaves of *P. formosa* were collected from Kaihua County of Zhejiang Province in November 1996 and identified by Prof. Bing-Yang Ding of Department of Plant Sciences, Hangzhou University. A voucher specimen (No. SIMM 96111001) was deposited in the Herbarium of Shanghai Institute of Materia Medica.

Extraction and Isolation. The leaves of *P. formosa* (20 kg) were air-dried, ground, and extracted with 95% ethanol under reflux. After removal of the solvent by evaporation, the residue was adjusted to about 15% ethanol and stored in a refrigerator overnight to precipitate chlorophyll. The supernatant was extracted successively with $CHCl_3$, EtOAc, and n-BuOH. The EtOAc extract was evaporated to give a red mass (200 g), which was applied to a silica gel column, eluting with EtOAc containing increasing amounts of MeOH. Repeated column chromatography yielded **1** (10 mg), **2** (20 mg), **3** (8 mg), and **4** (20 mg).

Pierisformoside A (1): viscous syrup, $[\alpha]^{16}_D$ −16.39 (*c* 0.32, MeOH); IR (KBr) ν max 3406, 1647, 1446, 1078, 1040 cm^{-1}; FAB-MS *m/z* 499 $[M + H]^+$; ^1H NMR (C_5D_5N), see Table 1; ^{13}C NMR (C_5D_5N), see Table 2.

Pierisformosin D (2): amorphous powder. $[\alpha]^{16}_D$ 41.91 (*c* 0.36, MeOH); IR (KBr) ν max 3400−3500, 1736, 1720, 1373, 1182, 1117, 1043, 812 cm^{-1}; FAB-MS *m/z* 523 $[M + K]^+$; EIMS *m/z* 466 $[M - H_2O]^+$, 448, 430, 388, 370, 332, 314 (100), 296, 271, 222, 205, 191, 162, 153, 125, 109, 71; ^1H NMR (C_5D_5N), see Table 1; ^{13}C NMR (C_5D_5N), see Table 2.

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