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GRAYANOIDS FROM *PIERIS FORMOSA*†

LI-QUAN WANG BING-YANG DING‡ GUO-WEI QIN* GANG LIN and KIN-FAI CHENG*§

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China; ‡College of Life Science, Hangzhou University, Hangzhou 310012, China; §Department of Chemistry, The University of Hong Kong, Hong Kong, China

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Key Word Index—*Pieris formosa*; Ericaceae; grayanoids; pierisformosins B and C; asebotoxin IV.

Abstract—Two new grayanane diterpenoids named pierisformosins B and C, together with a known asebotoxin IV were isolated from leaves of *Pieris formosa*. Their structures were elucidated based on spectral analysis, including ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, HMBC and NOESY experiments. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Grayanane diterpenoids possess a 5/7/6/5 (*trans* or *cis*/*cis*/*cis*) ring system, formed probably by rearrangement of the kaurane skeleton. They have been found particularly in the genera *Pieris*, *Rhododendron*, *Lyonia* and *Leucothoe* of the Ericaceae family. Up to now nearly 60 grayanoids have been reported, of which the majority exhibited remarkable antifeedant and insecticidal activities [1]. In order to search for bioactive natural products, we have initiated chemical studies on grayanane diterpenoids from Chinese Ericaceae plants.

Pieris formosa (Wall) D. Don is an evergreen shrub or tree, growing mainly in hilly and valley regions of south and southwest China. It is a well-known poisonous plant as recorded in many Chinese monographs. It has been described that poultry would fall into coma after eating leaves or stems of the plant accidentally. Various symptoms, including dyspnoea, motion imbalance and spreading the four limbs would appear if mice were administrated with the chloroform extract. In folk medicine, the juice of fresh leaves can be used as an insecticide and as a lotion for treatment of tinea and scabies [2]. In the course of our study, we found that the ethyl acetate and n-butanol fractions of the ethanol extracts were active in brine shrimp tests and this encouraged us to undertake a chemical investigation of the plant. In a previous paper, we have reported a new natural grayanoid, named pierisformosin A, and two known grayanoids: grayanotoxin XVIII and grayanoside C [3]. Our continuing studies on the plant have led to the isolation of three additional grayanoids: pierisformosins B (**1**) and C (**2**), together with a known grayanoid asebotoxin IV (**3**). In this paper, we present the structural determination of the two new grayanane diterpenoids **1** and **2**, and the unambiguous assignment of their NMR spectral data by a combination of NMR techniques, including ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, HMBC and NOESY experiments.

RESULTS AND DISCUSSION

An ethyl acetate fraction of the ethanol extracts was subjected to repeated column chromatography on silica gel and Sephadex LH-20 to give pierisformosins B (**1**), C (**2**) and asebotoxin IV (**3**). Pierisformosin B (**1**) had a molecular formula $C_{23}H_{38}O_7$ from its FAB-mass spectrum and ^{1}H and ^{13}C NMR data. The IR spectrum showed absorption bands of hydroxyl (3400–3500 cm^{-1}) and ester carbonyl group (1718 cm^{-1}). The ^{1}H NMR spectrum of **1** showed the presence of four singlet methyls (δ 1.50, 1.30, 1.71, 1.89), three oxygenated methines (δ 3.93, 4.19, 6.26) and a propionyl group (δ 1.11, 3H, *t*, J = 7.6 Hz; 2.35, 2H, *q*, J = 7.6 Hz) (Table 1). In the ^{13}C NMR spectrum 23 carbon signals were observed, including four methyl, five

*Authors to whom correspondence should be addressed.
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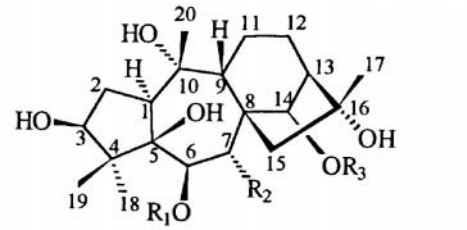

1	R_1=H	R_2=H	R_3=CH_3CH_2CO
2	R_1=H	R_2=OH	R_3=CH_3CH_2CO
3	R_1=CH_3CH_2CO	R_2=OH	R_3=H
4	R_1=H	R_2=H	R_3=CH_3CO

methylene, six methine (three oxygenated) and five quaternary carbons (three oxygenated) for the diterpene skeleton, together with three carbons for a propionyl group (δ_C173.8*s*, 28.4*q* and 9.6*t*) (Table 2). The ^{1}H–^{1}H COSY revealed the existence of the following fragments: CH–CH_2–CH(OH)–, CH (OH)–CH_2– and CH–CH_2–CH_2–CH–, each of which was connected to quaternary carbon atoms at one or both ends. The above data conformed to the structural requirements of a grayanane diterpenoid. The signal at δ 3.93 (1H, *br s*) was assigned as H-3α from correlation with two methyls at C-4 (δ 1.30, 1.71) in the NOESY spectrum and its coupling patterns in the ^{1}H NMR spectrum. The signal at δ 4.19 (1H, *dd*, J = 1.5, 7.2 Hz) coupled with vicinal proton pairs in ^{1}H–^{1}H COSY and correlated with the C-18 methyl, and thus it was assigned to H-6α. A literature survey determined that the oxygenated H-14α signal appeared at very low field in pyridine-d_5 solvent around δ 5.50 ppm with 14β-OH and δ 6.20 with 14β-OCOR. Considering all of the above, the signal at δ 6.26 was assigned to H-14α with 14β-OCOR. Further studies on its ^{1}H–^{1}H COSY, NOESY and HMBC spectra (Tables 3 and 4), and comparison of the NMR data with other grayanoids [1], suggested the assignments of 3β, 5β, 6β, 16α, 20α-pentahydroxyl and 14β-propionyl groups. Thus the structural features of **1** were very similar to those of grayanotoxin I (**4**), a known grayanoid isolated previously from *Leouthoe grayana*, except for the difference of their C-14 ester groups [4]. In fact, the ^{13}C NMR data of the grayanane skeleton of **1** was virtually identical with that of the literature value of **4** in the same solvent (Table 2). Considering the unambiguous stereochemistry of **4**, obtained from previous X-ray structure analysis [5], the configuration and conformation of **1** were deduced to be the same as that of **4** as shown in Fig. 1.

Hikino *et al*. [6] reported the isolation of a compound named asebotoxin I from *Pieris japonica*, and its structure was proposed to be the same as **1**. Later, Kaitai *et al*. [7] isolated the same compound again from the same species and determined its structure by comparison of ^{1}H NMR data with that of Ref. [6]. Since only one-dimensional NMR techniques were available at that time, for such complicated diterpenoids, only a few proton signal assignments, mainly from methyls and methines, were reported. In the present study, we observed large differences of characteristic ^{1}H NMR data between our compound and asebotoxin I. In pyridine-d_5, the methyl signals of asebotoxin I were reported to appear at δ 0.95, 1.15, 1.31, 1.37 (each 3H, *s*) and oxygenated methine signal at δ 5.66 (1H, *s*) [7], which differed remarkably from our observation (see Table 1), thus casting doubt on the structure proposed for asebotoxin I. However, the compound obtained in this work agreed with the proposed structure of **1** from all above evidence and the ^{1}H NMR and ^{13}C NMR data, which were

Table 1. ^{1}H NMR data for compound **1**–**3** in pyridine-d_5

H	**1**	**2**	**3**
1α	3.31(*dd*, 6.2, 10.1)	3.61(*t*, 8.8)	3.32(*dd*, 4.0, 11.8)
2	2.62*m*	2.63(*m*)	2.47(*dd*, 5.2, 12.6), 2.60(*dd*, 4.6, 14.7)
3α	3.93(*br s*)	3.94(*br s*)	3.91(*t*, 3.8)
6α	4.19(*br d*, 1.5, 7.2)	4.15(*d*, 9.4)	5.94(*d*, 9.8)
7α	2.62*m*		
7β	2.52*m*	4.10(*d*, 9.4)	4.27(*t*, 9.8)
9β	2.22(*d*, 6.8)	2.22(*d*, 6.7)	2.21(*d*, 6.5)
11α	2.07(*dd*, 5.4, 13.4)	2.10(*dd*, 6.4, 13.5)	2.08(*dd*, 6.1, 13.4)
11β	1.62*m*	1.74(*m*)	1.75(*m*)
12α	2.69*m*	2.75(*m*)	2.64(*m*)
12β	1.67*m*	1.71(*m*)	1.73(*m*)
13α	2.52(*br s*)	2.45(*br s*)	2.44(*br s*)
14α	6.26(*s*)	6.39(*s*)	5.35(*d*, 5.4)
15α	2.28(*d*, 14.8)	3.73(*d*, 14.5)	3.84(*d*, 14.5)
15β	2.19(*d*, 14.8)	1.92(*d*, 14.5)	1.90(*d*,14.5)
17	1.50(*s*)	1.54(*s*)	1.56(*s*)
18	1.30(*s*)	1.39(*s*)	1.04(*s*)
19	1.71(*s*)	1.76(*s*)	1.59(*s*)
20	1.89(*s*)	1.89(*s*)	1.85(*s*)
CH_3	1.11(*t*, 7.6)	1.08(*t*, 7.6)	1.23(*t*, 7.5)
CH_2	2.35(*q*, 7.6)	2.34(*q*, 7.6)	2.54(*q*, 7.5)

Table 2. ^{13}C NMR data for compound **1**–**4** in pyridine-d_5

C	1	2	3	4
1	51.3 *d*	50.9 *d*	52.4 *d*	51.3
2	35.8 t	35.9 t	35.6 t	35.8
3	82.6 *d*	82.9 *d*	82.7 *d*	82.5
4	51.8 *s*	52.3 *s*	52.0 *s*	51.7
5	84.5 *s*	83.6 *s*	83.1 *s*	84.4
6	73.8 *d*	78.7 *d*	81.7 *d*	73.6
7	44.1 *t*	77.3 *d*	77.0 *d*	44.1
8	51.1 *s*	56.2 *s*	56.3 *s*	51.0
9	55.7 *d*	54.9 *d*	53.9 *d*	55.6
10	78.0 *s*	77.7 *s*	77.7 *s*	77.9
11	22.5 *t*	22.4 *t*	22.7 *t*	22.4
12	27.4 *t*	27.3 *t*	26.6 *t*	27.3
13	55.1 *d*	55.5 *d*	56.8 *d*	55.0
14	82.6 *d*	82.9 *d*	81.5 *d*	82.8
15	61.1 *t*	52.8 *t*	52.2 *t*	61.1
16	78.6 *s*	78.6 *s*	79.6 *s*	78.5
17	23.9 *q*	23.5 *q*	23.8 *q*	23.9
18	23.4 *q*	23.5 *q*	23.1 *q*	23.4
19	19.8 *q*	20.2 *q*	19.9 *q*	19.8
20	28.3 *q*	28.4 *q*	28.6 *q*	28.3
CH_3	9.6 *q*	9.3 *q*	9.5 *q*	21.2
CH_2	28.4 *t*	28.6 *t*	28.6 *t*	
C=O	173.8 *s*	173.5 *s*	174.7 *s*	170.2

made by unambiguous assignments based on various 2D experiments.

Pierisformosin C (**2**) had a molecular formula $C_{23}H_{38}O_8$ from its HRFAB-mass spectrum and NMR data. The ^1H NMR, ^{13}C NMR and IR spectra showed the presence of *O*-propionyl group (1741 cm^{-1}; δ_H 1.08*t*, 2.34*q*; δ_C 173.5*s*, 28.6*q*, 9.3*t*). The ^{13}C NMR and DEPT experiments showed the 20 carbons were divided into Me × 4, CH_2×4, CH × 7 (4 oxygenated) and C × 5 (3 oxygenated), excluding 3 carbons for propionyl. All of the above data indicated a structural similarity with **1**, having the same grayanane skeleton, while the major difference was that **2** had an extra hydroxyl function.

Table 3. ^1H–^1H COSY and NOESY spectra of **1** and **2** in pyridine-d_5

H	^1H–^1H COSY		NOESY	
	1	2	1	2
1	H-2	H-2	H-2, 6, 14, 18	H-2, 6, 14, 18
2	H-3	H-3	H-1, 3	H-1, 3
3	H-2	H-2	H-2, 18, 19	H-2, 18, 19
6	H-7α, 7*β*	H-7*β*	H-1, 7α, 18	H-1, 18
7α	H-6, 7*β*	–	H-15α	–
7*β*	H-6, 7α	H-7α	H-9, 20	H-9
9	H-11α	H-11α	H-7*β*,11α	H-7*β*, 11α, 20
11α	H-9, 11*β*	H-9, 11*β*	H-11*β*, 12α	H-11*β*
11*β*	H-11α, 12α	H-11α, 12α	H-9, 11α, 17	H-9, 11α, 17
12α	H-11*β*, 12*β*	H-11*β*, 12*β*	H-12*β*	H-12α, 13
12*β*	H-12α, 13	H-12α, 13	H-12α, 13, 17	H-12α, 13, 17
13	H-12*β*	H-12*β*	H-12β, 17	H-12α, 12*β*, 17
14	–	–	H-1	H-1
15α	H-15*β*	H-15*β*	H-7α	–
15*β*	H-15α	H-15α	H-17	H-17
17	–	–	H-13, 12*β*, 15*β*	H-12*β*, 13, 15*β*
18	–	–	H-1, 3, 6, 19	H-1, 3, 6, 19
19	–	–	H-3, 18	H-3, 18
20	–	–	H-9	H-9

Table 4. HMBC data for **1** and **2** in pyridine-d_5

Carbon	1	2
C-1	H-9, 20	H-2, 20
C-2	H-1	H-1
C-3	H-18, 19	H-18, 19
C-4	H-18, 19	H-18, 19
C-5	H-2, 7α, 18, 19	H-3, 18, 19
C-6	H-1,7α, 7*β*	H-7
C-7	H-15α	H-6
C-8	H-7α, 7*β*, 11*β*, 15*β*	H-9, 13
C-9	H-15α, 15*β*, 20	H-20
C-10	H-1, 11α, 20	H-20
C-11	H-13	H-9
C-12	H-13	–
C-13	H-11*β*, 17	H-15α, 15*β*, 17
C-14	H-7, 13, 15	–
C-15	H-14, 17	H-13
C-16	H-14, 15α, 17	H-13, 14, 15α, 17
C-17	H-15*β*	–
C-18	H-19	H-19
C-19	H-18	H-18
C-20	H-1	–
C = O	H-14, CH_2, CH_3	H-14

Further study of the ^1H–^1H COSY, ^{13}C–^1H COSY, HMBC and NOESY spectra revealed that the NMR spectral characteristics of **2** were very similar to those of **1**. The location of the additional hydroxyl group in **2** was deduced to be at C-7 with an α-configuration because this extra oxygenated proton signal at δ 4.10 ppm correlated with another oxygenated proton signal at δ 4.15 in the ^1H–^1H COSY spectrum, and exhibited two doublets as an AB system (J = 9.4 Hz) in the ^1H NMR spectrum. The H-14α appeared at δ 6.39 as a singlet like that of **1** and correlated with the carbonyl signal at δ 173.5 ppm in the HMBC spectrum. Therefore, the *O*-propionyl moiety should be connected to C-14 as in **1**. In the ^{13}C NMR spectrum the C-1 chemical shift of **2** was almost the same as that of **1** suggesting that the H-1 in **2** should be symbol α-oriented as in **1**, otherwise chemical shift of the C-1 signal would differ remarkably according to literature reports [8]. In the NOESY spectrum the H-1 signal correlated with H-6, H-14 and H-18, and H-3 signal with H-6 and H-18, which indicated that H-1, H-3, H-6 and H-18 all take the α-orientation. In addition, H-7 correlated with H-9 and H_3-20 and H_3-17 correlated with H-11*β* and H-12*β*, which revealed that H-7, H-9, H_3-20 and C-17 methyl all

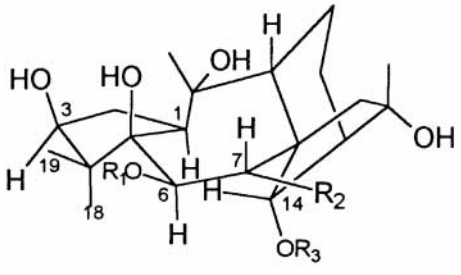

Fig. 1. The conformation of compounds **1** and **2**.

have the β-orientation. Consequently, we established the conformation of **2** was as shown in Fig. 1.

Compound **3** had a molecular formula $C_{23}H_{38}O_8$ the same as **2**. Its NMR spectral feature were very similar to **2**. The differences observed were the downfield shift of H-6α signal (δ 4.15 in **2** and δ 5.94 in **3**) and the upfield shift of H-14α (δ 6.39 in **2** and δ 5.35 in **3**). In the HMBC spectrum the H-6α was coupled to the ester carbonyl carbon, indicating that the *O*-propionyl was at C-6. On the basis of the spectral evidence and comparison of the physical data with the literature values, **3** was identified as the known asebotoxin IV, which was isolated from *Pieris japonica* [9]. Through ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, 2D NOESY and HMBC techniques, all of the ^{1}H and ^{13}C NMR data of **1**–**3** were unambiguously assigned.

EXPERIMENTAL

General

$[\alpha]_D$: JASCO, DIP-181, polarimeter. IR: Perkin-Elmer 599B spectrometer. ^{1}H and ^{13}C NMR spectra: Bruker DRX 500. Chemical shifts are reported in ppm with solvent signal as int. standards. MS: MAT-95.

Plant material

The leaves of the plant were collected from Kaihua county of Zhejiang Province in November, 1996 and identified by Professor 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% EtOH under reflux. After removal of the solvent by evaporation, the residue was adjusted to about 15% EtOH and stored in a refrigerator overnight to precipitate chlorophyll. The supernatant was extracted with $CHCl_3$, EtOAc and *n*-BuOH, successively. 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 CC led to **1** (15 mg), **2** (25 mg) and **3** (16 mg).

Pierisformosin B (**1**). Amorphous powder. $[\alpha]_D^{16}$ 16.84 (MeOH, *c* 0.02); IR ν_{max}^{KBr} cm^{-1}: 3400–3509, 1718, 1633, 1464, 1367, 1223, 1033. 935, 881; FAB-MS *m/z*: 449 ($[M + Na]^+$), EIMS, *m/z* 408 $[M - H_2O]^+$; ^{1}H NMR (pyridine-d_5): see Table 1. ^{13}C NMR (pyridine-d_5): see Table 2.

Pierisformosin C (**2**). Amorphous powder. $[\alpha]_D^{15}$ -4.27° (MeOH, *c* 0.08); IR ν_{max}^{KBr} cm^{-1}: 3400–3510, 1630, 1741, 1371, 1238, 1030; FAB-MS *m/z*: 465 $[M + Na]^+$, EIMS *m/z* 424 ($[M - H_2O]^+$, 424.2461), 406, 388, 370, 332, 314, 292, 265, 191 (100), 109, 93, 71, 57; ^{1}H NMR (pyridine-d_5): see Table 1. ^{13}C NMR (pyridine-d_5): see Table 2.

Asebotoxin IV (**3**). Amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3400–3510, 1745, 1633, 1373, 1170, 1041, 973; ^{1}H NMR (pyridine-d_5): see Table 1. ^{13}C NMR (pyridine-d_5): see Table 2.

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