Pierisformotoxins A–D, Polyesterified Grayanane Diterpenoids from *Pieris formosa* and Their cAMP-Decreasing Activities

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Four highly acylated diterpenoids, designated as pierisformotoxins A–D (**1**–**4**, resp.), along with 26 known compounds, were isolated from the flowers of *Pieris formosa*. Among them, pierisformotoxins A and B (**1** and **2**, resp.) were new highly acylated grayanane diterpenoids, of which the five-membered ring *A* has undergone an oxidative cleavage between C(3) and C(4), followed by lactonization, to give rise to a five-membered lactone ring between C(3) and C(5), differing from the previously reported grayanane diterpenoids with a 5/7/6/5 ring system. Results of the cAMP-regulation-activity assay showed that pierisformotoxin C (**3**) at 10 μm (inhibitory ratio (*IR*): 10.1%) or 2 μm (9.8%), and pierisformotoxin B (**2**) at 50 μm (13.9%) significantly decreased the cAMP level in N1E-115 neuroblastoma cells ($p < 0.05$).

Introduction. – *Pieris formosa* (Wall) D.Don (Ericaceae), a well-known poisonous plant, is distributed mainly in hilly and valley regions of south and southwest China. Poultry have been reported to fall into coma after accidentally eating leaves or stems of this plant [1]. In folk practice, the juice of the fresh leaves of *P. formosa* is used as an insecticide or lotion for the treatment of ring worm and scabies [1]. Previous studies revealed that the plant contains grayanane with a 5/7/6/5 ring system, leucothane type with 6/6/6/5 consecutive carbocyles, and 5,6-secokaurane diterpenoids and diphenylamine [2–6]. Recently, six novel highly acylated diterpenoids with a new 3,4-secograyanane skeleton have been reported from the flower buds of *Rhododendron molle* and the flowers of *P. formosa* by *Shi et al.*, *Li et al.*, and our groups [7–10]. Some of these diterpenoids have shown significant physiological properties, including potent acute toxicity in mammals [10][11] and antifeedant, growth-inhibitory, and insecticidal activities [12][13].

As part of a program to assess the chemical and biological diversity of the family Ericaceae [9–10][14–20], we investigated *P. formosa*. A new grayanol diterpenoid, grayanotoxin XXII, and a new phenolic glucoside, benzyl 2-hydroxy-4-*O*-[β-xylopyranosyl-(1″→6′)-β-glucopyranosyl]benzoate, were reported from this species in our previous paper [16]. In the present study, four highly acylated diterpenoids, designated as pierisformotoxins A–D (**1**–**4**, resp.; *Fig. 1*), along with 26 known compounds, were isolated from the flowers of *P. formosa*. Among them, pierisformotoxins A and B were new highly acylated grayanane diterpenoids, in which the five-membered ring *A* has

undergone an oxidative cleavage between C(3) and C(4), followed by lactonization, to give rise to a five-membered lactone ring between C(3) and C(5), differing from the previously reported grayanane type with a 5/7/6/5 ring system, and thus representing a new group of grayanotoxins. In addition, compounds **1–4** were tested for their cAMP-regulation activities. Herein, we report the isolation, structure elucidation, and biological activities of compounds **1–4**.
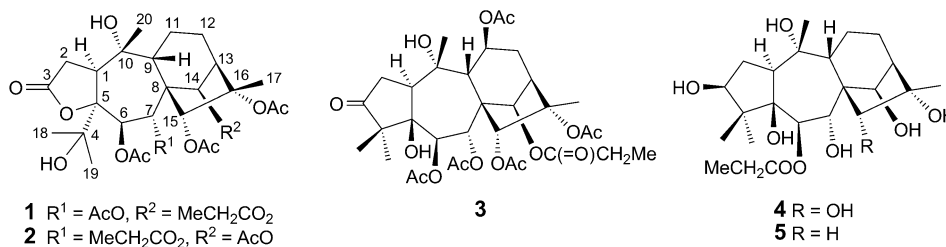


Fig. 1. *Structures of compounds* **1–5**

Results and Discussion. – Pierisformotoxin A (**1**), a white amorphous powder, had the molecular formula $C_{31}H_{44}O_{14}$ as established on the basis of HR-ESI-MS (m/z 639.2661 ([$M - H$]$^-$; calc. 639.2652)). The IR spectrum indicated the presence of OH (3441 cm^{-1}), five-membered lactone (1777 cm^{-1}), and ester C=O (1736 cm^{-1}) functionalities [7]. The ^{1}H-NMR spectrum of **1** showed resonances attributed to four Ac Me groups (δ(H) 2.13, 2.15, 2.16, and 2.31), as well as resonances due to a propanoyl unit (δ(H) 1.30 (t, $J=7.5$, 3 H), 2.36 and 2.59 (q, $J=7.5$, each 1 H)). In addition, it showed resonances due to four tertiary Me groups (δ(H) 1.43, 1.44, 1.62, and 1.64), together with four O-bearing CH groups (δ(H) 5.44 (s), 5.68 (d, $J=8.5$), 6.61 (d, $J=8.5$), and 6.81 (br. s)). The ^{13}C- and DEPT spectra displayed 31 C-atom resonances comprising those of four Ac groups, a propanoyl unit, four tertiary Me, three CH$_2$, and seven CH groups (including four O-bearing ones), seven quaternary C-atoms (including three O-bearing ones and a C=O group), and two exchangeable OH groups (*Table 1*). These C-atom-signal information, coupled with the molecular formula indicated that compound **1** was a highly acylated diterpenoid with four rings and two free OH groups.

The complete structure of **1** was elucidated by analyzing the 2D-NMR data and by comparing these results with the NMR data reported for secorhodomollolide A [7]. The close similarities of the NMR data for rings *B – D* with those of secorhodomollolide A suggested that they possess a similar 7/6/5 ring system, indicating that **1** is a grayanane-type diterpenoid (*Fig. 2*). The HMBC spectrum of **1** showed obvious correlations from Me(18) (δ(H) 1.64 (s)) to C(4), C(5), and C(19), and from Me(19) (δ(H) 1.62 (s)) to C(4), C(5), and C(18), which required Me(18) and Me(19) to be attached to the same O-bearing quaternary C-atom (δ(C) 76.0 (s, C(4))). In addition, a Me *singlet* at δ(H) 1.43 (Me(20)) showed HMBCs to C(1), C(9), and C(10). Other HMBCs were detected between H–C(2) (δ(H) 2.88 (dd, $J=18.5$, 10.5), 3.02 (dd, $J=19.0$, 9.6)), and C(1), C(3), C(5), and C(10), between H–C(6) (δ(H) 6.61 (d, $J=8.5$)) and C(4), and between a free OH (δ(H) 7.11 (s)), and C(1), C(9), C(10), and Me(20).

Table 1. ^{1}H- and ^{13}C-NMR (at 500 and 125 MHz, resp.) Data of Compounds **1** and **2**. In (D_5)pyridine; δ in ppm, J in Hz. Atom numbering as indicated in Fig. 1.

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1	4.00 (t, J = 10.0)	50.3 (d)	4.01 (t, J = 9.0)	50.2 (d)
2	3.02 (dd, J = 19.0, 9.6, H$_α$), 2.88 (dd, J = 18.5, 10.5, H$_β$)	34.0 (t)	3.02 (dd, J = 18.5, 11.0, H$_α$), 2.88 (dd, J = 19.0, 9.0, H$_β$)	34.0 (t)
3		175.6 (s)		175.5 (s)
4		76.0 (s)		76.0 (s)
5		93.5 (s)		93.5 (s)
6	6.61 (d, J = 8.5)	69.8 (d)	6.62 (d, J = 8.5)	69.8 (d)
7	5.68 (d, J = 8.5)	68.5 (d)	5.73 (d, J = 8.5)	68.1 (d)
8		55.8 (s)		55.7 (s)
9	2.68–2.76 (m)	48.4 (d)	2.71–2.80 (m)	48.3 (d)
10		76.5 (s)		76.5 (s)
11	2.07–2.14 (m, H$_α$), 1.66–1.73 (m, H$_β$)	20.2 (t)	2.08–2.14 (m, H$_α$), 1.66–1.76 (m, H$_β$)	20.1 (t)
12	2.12–2.20 (m, H$_α$), 1.61–1.65 (m, H$_β$)	25.3 (t)	2.13–2.22 (m, H$_α$), 1.69–1.72 (m, H$_β$)	25.3 (t)
13	3.53–3.60 (m)	45.2 (d)	3.60–3.68 (m)	44.8 (d)
14	6.81 (br. s)	79.0 (d)	6.77 (br. s)	79.4 (d)
15	5.44 (s)	87.1 (d)	5.45 (s)	87.2 (d)
16		88.3 (s)		88.2 (s)
17	1.44 (s)	19.0 (q)	1.44 (s)	18.9 (q)
18	1.64 (s)	25.8 (q)	1.64 (s)	25.8 (q)
19	1.62 (s)	28.1 (q)	1.61 (s)	28.1 (q)
20	1.43 (s)	34.0 (q)	1.44 (s)	34.0 (q)
HO–C(4)	7.02 (s)		7.02 (s)	
HO–C(10)	7.11 (s)		7.10 (s)	
AcO–C(6)	2.31 (s)	21.8 (q), 169.8 (s)	2.17 (s)	21.3 (q), 169.3 (s)
AcO–C(7)	2.13 (s)	22.9 (q), 169.8 (s)		
MeCH$_2$CO$_2$–C(7)			1.31 (t, J = 7.5), 2.67 (q, J = 7.5), 2.58 (q, J = 7.5)	9.2 (q), 28.3 (t), 173.0 (s)
AcO–C(14)			2.17 (s)	22.1 (q),
MeCH$_2$CO$_2$–C(14)	1.30 (t, J = 7.5), 2.59 (q, J = 7.5), 2.36 (q, J = 7.5)	9.3 (q), 28.4 (t), 174.1 (s)		
AcO–C(15)	2.16 (s)	20.8 (q), 172.0 (s)	2.13 (s)	20.9 (q), 172.1 (s)
AcO–C(16)	2.15 (s)	21.3 (q), 169.3 (s)	2.13 (s)	22.8 (q), 170.1 (s)

The above observed HMBCs indicated a cleavage between C(3) and C(4), and a OH group was determined at C(10). The propanoyloxy group was placed at C(14), and the three AcO groups were located at C(6), C(7), and C(15), respectively, as evident from the HMBC features of H–C(14), H–C(6), H–C(7), and H–C(15) with the corresponding C=O C-atoms. Commonly, the O-bearing C(16) signal of grayanane
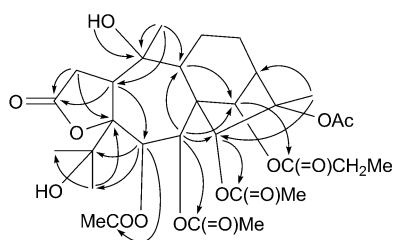
Fig. 2. *Selected HMBCs of compound* **1**

diterpenoids with a OH group at C(16), such as rhodomollein III, appeared at *ca.* δ(C) 79.0 ppm in (D$_5$)pyridine [21][22]. Considering the downfield shifts of C(16) (δ(C) 79.0 in rhodomollein III, while δ(C) 88.3 in **1**), HO–C(16) was also acetylated (*Table 1*). Considering the degrees of unsaturation of **1**, as well as the upfield shift of C(3) and the downfield shift of C(5) compared with secorhodomollolide A, it was thus concluded that **1** contained a five-membered lactone ring between C(3) and C(5). Furthermore, according to the O-bearing nature of C(4), and since both Me(18) and Me(19) appeared as *singlet*s, the remaining OH group should be located at C(4). This assignment was in accordance with the observation of similar chemical shifts of C(1), C(2), and C(3) of **1** and secorhodomollolide A. Accordingly, the constitution of compound **1** could be established.

The relative configuration of **1** was established mainly using information from ROESY spectrum (*Fig. 3*) and by comparison of its spectroscopic data with those of secorhodomollolide A [7]. In the ROESY spectrum, cross-peaks H–C(1)/H–C(6), H–C(6)/H–C(13) and H–C(14), and HO–C(10)/H–C(1) indicated that these H-atoms were in the same α-orientation. Meanwhile, H–C(7), H–C(9), H–C(15), Me(17), and
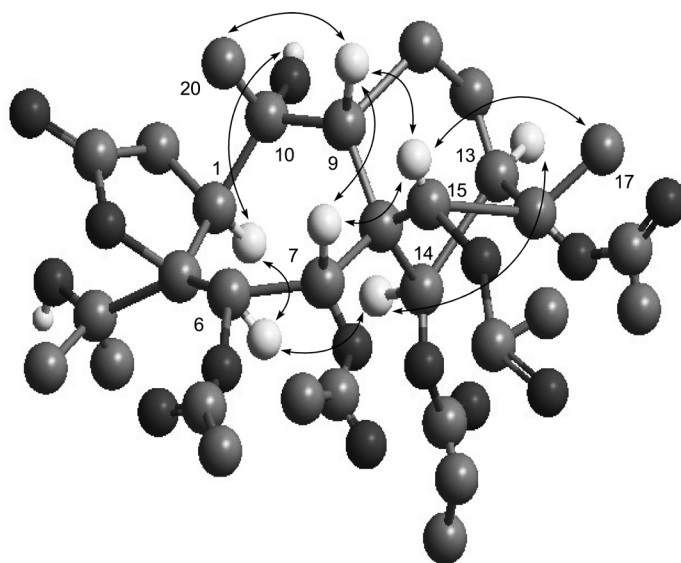
Fig. 3. *Key ROESY correlations of* **1**

Me(20) were established as β-oriented in the light of the NOE interactions of H–C(9) and H–C(15) with Me(20), of H–C(7) with H–C(9), and of H–C(15) with Me(17). H–C(7) was assigned to be opposite to H–C(6) also based on a coupling constant of 8.5 Hz for the vicinal H-atoms. Consequently, the structure of **1** was established as depicted in *Fig. 1* and named pierisformotoxin A.

Pierisformotoxin B (**2**) had the same molecular formula $C_{31}H_{44}O_{14}$ as **1**, on the basis of HR-ESI-MS data (*m/z* 639.2649 ($[M − H]^-$; calc. 639.2652)), indicating ten degrees of unsaturation. The ^{1}H- and ^{13}C-NMR data of **2** and **1** were almost the same except for the following differences. The NMR resonances for H–C(7), and C(14) and the propanoyl C=O of **2** were shifted by $\Delta\delta(H)$ +0.05 and $\Delta\delta(C)$ +0.4 and −1.1 ppm, respectively, as compared with those of **1** (*Table 1*). The resonances of H–C(14), and C(7) and one Ac C=O of **2** were shifted in turn by $\Delta\delta(H)$ −0.04, and $\Delta\delta(C)$ −0.4 and +1.1 ppm, respectively, as compared with those of **1**. This suggested that the AcO group at C(7) and the propanoyloxy group at C(14) were exchanged mutually in **2**. The assumption was verified unequivocally by the 2D-NMR experiments of **2**, in particular, by the HMBCs from H–C(7) to the propanoyl C=O ($\delta(C)$ 173.0), and from H–C(14) to the Ac C=O ($\delta(C)$ 170.9). Therefore, the structure of compound **2** was as depicted in *Fig. 1* determined and named pierisformotoxin B.

Pierisformotoxin C (**3**) was isolated as a white solid. Its molecular formula was determined as $C_{33}H_{46}O_{15}$ (eleven degrees of unsaturation) by means of ^{1}H-, ^{13}C-, and DEPT-NMR spectroscopy, and was verified by HR-ESI-MS data (*m/z* 681.2764 ($[M − H]^-$; calc. 681.2758)). The ^{1}H-NMR spectrum of **3** (*Table 2*) revealed the presence of five Ac Me groups ($\delta(H)$ 1.93, 2.00, 2.06, 2.10, and 2.13), four Me groups (*singlet*s at $\delta(H)$ 0.96, 1.11, 1.68, and 1.83), a propanoyl unit ($\delta(H)$ 1.13 (*t*), 2.33 (*q*)), and five O-bearing CH groups ($\delta(H)$ 5.25 (*s*), 5.63 (*d*, *J* = 8.1), 5.62–5.66 (*m*), 6.17 (*s*), and 6.26 (*d*, *J* = 8.0). The DEPT-NMR results indicated that there were 44 H-atoms bound to C-atoms and two exchangeable H-atoms. Apart from C-atom resonances of five *O*-Ac and one *O*-propanoyl moieties, the ^{13}C-NMR and DEPT spectra of **3** showed 20 C-atom signals, including those of four Me, two CH_2, eight CH groups (five O-bearing ones), and six quaternary C-atoms (three O-bearing ones and one keto C=O group; *Table 2*). This observation suggested that **3** was likely a grayanane-type diterpenoid that was substituted by five Ac and one propanoyl groups. This assumption was subsequently confirmed by conducting a set of 2D-NMR spectroscopic experiments (^{1}H,^{1}H-COSY, HSQC, HMBC, and ROESY spectra).

In the HMBC spectrum (*Fig. 4*), the H-atom signals for Me(18) and Me(19) were correlated with a keto C=O group ($\delta(C)$ 218.6 (C(3))), a quarternary C-atom ($\delta(C)$ 57.6 (C(4))), and an O-bearing quarternary C-atom ($\delta(C)$ 81.5 (C(5))). This result, in combination with the ^{1}H,^{1}H-COSY correlation observed for H–C(1) with $CH_2(2)$, as well as the HMBC of $CH_2(2)$ with C(3), indicated that the C=O group was located at C(3). The key HMBCs from Me(20) ($\delta(H)$ 1.68 (*s*)) to C(1), C(9), and C(10), from one exchangeable H-atom ($\delta(H)$ 4.04 (*d*, *J* = 1.6, HO–C(5))) to C(1), C(5), and C(6), and from another exchangeable H-atom ($\delta(H)$ 4.22 (*s*, HO–C(10))) to C(1), C(9), C(10), and C(20) showed the occurrence of two OH groups at C(5) and C(10). Four Ac groups were attached to C(6), C(7), C(11), and C(15), because H–C(6) ($\delta(H)$ 5.63 (*d*, *J* = 8.1)), H–C(7) (6.26 (*d*, *J* = 8.0)), H–C(11) (5.64 (*m*)), and H–C(15) (5.25 (*s*)) showed HMBC cross-peaks with the corresponding four AcO C=O C-atoms ($\delta(C)$

Table 2. *^{1}H- and ^{13}C-NMR Data for Compounds* **3** (at 400 (^{1}H) and 100 MHz (^{13}C), resp.) *and* **4** (at 500 (^{1}H) and 125 MHz (^{13}C), resp.). In (D$_5$)pyridine; δ in ppm, *J* in Hz. Atom numbering as indicated in *Fig. 1*.

Position	3		4	
	δ(H)	δ(C)	δ(H)	δ(C)
1	2.98 (*dd*, *J* = 6.4, 10.8)	45.3 (*d*)	3.37 (br. *s*)	51.9 (*d*)
2	2.48 (*dd*, *J* = 10.8, 14.4, H$_\alpha$), 2.29 (*d*, *J* = 14.0, H$_\beta$)	37.0 (*t*)	2.55 (*dd*, *J* = 15.0, 4.0, H$_\alpha$), 2.42 (*dd*, *J* = 15.0, 4.0, H$_\beta$)	35.5 (*t*)
3		218.6 (*s*)	3.89 (*d*, *J* = 4.5)	82.7 (*d*)
4		57.6 (*s*)		52.3 (*s*)
5		81.5 (*s*)		83.0 (*s*)
6	5.63 (*d*, *J* = 8.1)	73.8 (*d*)	5.91 (*d*, *J* = 9.0)	79.7 (*d*)
7	6.26 (*d*, *J* = 8.0)	66.8 (*d*)	4.91 (br. *s*)	77.5 (*d*)
8		53.1 (*s*)		56.5 (*s*)
9	1.94 (overlap)	60.4 (*d*)	2.46 – 2.50 (*m*)	53.4 (*d*)
10	–	75.0 (*s*)	–	78.0 (*s*)
11	5.62 – 5.66 (*m*)	68.0 (*t*)	2.10 (*dd*, *J* = 14.0, 7.0, H$_\alpha$), 1.62 – 1.66 (*m*, H$_\beta$)	22.7 (*t*)
12	2.13 – 2.18 (*m*)	32.0 (*t*)	2.51 – 2.55 (*m*, H$_\alpha$), 1.65 – 1.69 (*m*, H$_\beta$)	26.6 (*t*)
13	2.75 – 2.80 (*m*)	45.9 (*d*)	2.53 (overlap)	53.1 (*d*)
14	6.17 (*s*)	81.5 (*d*)	5.34 (*s*)	81.5 (*d*)
15	5.25 (*s*)	87.8 (*d*)	4.38 (*s*)	87.7 (*d*)
16		90.0 (*s*)		80.8 (*s*)
17	1.83 (*s*)	23.6 (*q*)	1.49 (*s*)	22.2 (*q*)
18	1.11 (*s*)	19.0 (*q*)	1.54 (*s*)	19.6 (*q*)
19	0.96 (*s*)	21.5 (*q*)	1.02 (*s*)	23.2 (*q*)
20	1.68 (*s*)	27.6 (*q*)	1.80 (*s*)	28.4 (*q*)
*H*O–C(5)	4.04 (*d*, *J* = 1.6)		5.09 (*s*)	
*H*O–C(10)	4.22 (*s*)			
*Ac*O–C(6)	2.10 (*s*)	20.6 (*q*), 169.6 (*s*)		
MeCH$_2$CO$_2$–C(6)			1.64 (*t*, *J* = 7.5), 2.50 (*q*, *J* = 7.5)	9.4 (*q*), 28.4 (*t*), 174.5 (*s*)
*Ac*O–C(7)	2.13 (*s*)	21.6 (*q*), 171.1 (*s*)		
*Ac*O–C(11)	2.06 (*s*)	22.0 (*q*), 169.7 (*s*)		
MeCH$_2$CO$_2$–C(14)	1.13 (*t*, *J* = 8.0), 2.33 (*q*, *J* = 8.0)	9.5 (*q*), 28.5 (*t*), 173.5 (*s*)		
*Ac*O–C(15)	2.00 (*s*)	21.0 (*q*), 171.0 (*s*)		
*Ac*O–C(16)	1.93 (*s*)	22.4 (*q*), 170.0 (*s*)		

169.6, 171.1, 169.7, and 171.0, resp.). HMBCs of the H-atom signal at δ(H) 6.17 (*s*, H–C(14)) with the C=O signal at δ(C) 173.5 (propanoyl) indicated that C(14) was propanoylated. Moreover, compared with the known compound **5** [23], the downfield shift of C(16) (δ(C) 90.0) indicated that HO–C(16) was also acetylated. Accordingly, the constitution of **3** could be established.
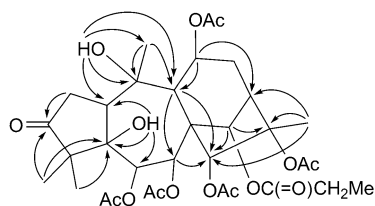
Fig. 4. *Key HMBCs of* **3**

The relative configurations of all stereogenic centers of **3** were identical to those of compound **5** as determined by the observed ROESY correlations and by comparison of NMR data of both compounds [23]. The strong ROESY correlations of HO–C(5) with H–C(7), Me(18), and Me(20) of H–C(7) with Me(20), and of H–C(15) with H–C(7), H–C(9), Me(17) and Me(20) indicated that HO–C(5), H–C(7), and H–C(15) all were β-oriented, and HO–C(10) had the α-orientation. The NOE correlations from H–C(1) and H–C(6) to Me(19), and from H–C(14) to H–C(6) suggested that H–C(1), H–C(6), and H–C(14) were α-oriented. Consequently, the structure of **3** was established as depicted in *Fig. 1* and named pierisformotoxin C.

Pierisformotoxin D (**4**), obtained as a white amorphous powder, had the molecular formula $C_{23}H_{38}O_9$ as deduced from HR-ESI-MS data (*m/z* 457.2434 ([$M - H$]$^-$; calc. 457.2437)). Its IR spectrum showed specific absorptions at 3424 cm^{-1}, which could be ascribed to OH group. The intense IR absorption band at 1738 cm^{-1} ($\nu_{C=O}$) revealed the presence of ester functionality, and the strong and broad band at 1180 cm^{-1} (ν_{C-O-C}^{as}) further confirmed the above conclusion. The ^{1}H- and ^{13}C-NMR data (*Table 2*) were very close to those of asebotoxin IV (**5**) [24]. Comparison of 1D-NMR data suggested that CH$_2$(15) in the latter compound was replaced by an O-bearing CH group, with an additional OH group at C(15) in **4**. This deduction was confirmed by HMBCs of H–C(15) with C(7), C(8), C(13), C(14), and Me(17), and of H–C(9), H–C(14), and Me(17) with C(15). The ROESY correlations between H–C(15), and H–C(9) and H$_\beta$–C(11) established HO–C(15) as being α-oriented. Other relative configurations of the stereogenic centers of **4** were assigned as the same as those of **5** on the basis of the similarity of all the ^{1}H and ^{13}C chemical shifts, and H-atom multiplicities for both compounds. Thus, the structure of compound **4** was assigned and designated as pierisformotoxin D.

The known compounds were determined as pierisoid A [8], pierisoid B [8], secorhodomollolide D [7], pierisformosin B [4], asebotoxin IV [24], asebotoxin V [24], asebotoxin VIII [25], grayanotoxin-I [26], grayanotoxin-III [27], 5,6-acetonylgraya-notoxin-I [28], grayanotoxin XXII [16], oleanolic acid [29], ursolic acid [30], β-sitosterol [31], bayogenin [32], arjunolic acid [33], (2*S*,3*R*)-*ent*-catechin [34], 2′,4-dihydroxy-4′-methoxy-6′-*O*-β-glucopyranoside dihydrochalcone [35], asebotin [36], bis-8,8′-catechinylmethane [37], epicatechin-(2$\beta \rightarrow O \rightarrow$7,4$\beta \rightarrow$8)-*ent*-epicatechin [38], proanthocyanidin A-1 [39], benzyl 2-hydroxy-4-*O*-[β-xylopyranosyl (1″ → 6′)-β-gluco-pyranosyl]benzoate [16], benzyl *O*-β-D-glucopyranoside [40], 2-hydroxybenzyl β-D-glucopyranoside [41], and 2,6-dimethoxy-4-hydroxyphenol 1-*O*-β-D-glucopyranoside [42] by comparison of their spectral data with literature values.

The cyclic adenosine monophosphate (cAMP) is an important second messenger regulating many biological processes. In humans, cAMP affects not only the higher

order of thinking, but also neurogenesis, memory, emotional disorder, and cognitive function. cAMP is formed from ATP, and its intracellular concentration is tightly regulated by two membrane-bound enzymes, adenylate cyclase and phosphodiesterase. Since *P. formosa* has been used to treat neuropsychiatric diseases in Chinese Traditional Medicine, the cAMP regulation activities of compounds **1**–**4** were evaluated by an *AlphaScreen*® assay, according to an established method as described in [43]. Compound **3** at the concentration of 10 or 2 μM, and compound **2** at 50 μM could significantly decrease the cAMP level in N1E-115 neuroblastoma cells ($p < 0.05$, *Table 3*).

Table 3. *The cAMP-Decreasing Activity of Compounds* **1**–**4**[a])

Compound	Concentration [μM]	*AlphaScreen*® counts/s (cps)	Standard error	*IR* [%]	*p* Value
No cell		44880	1001	42.1	
Only cell		35410	620	12.1	
DMSO		31600	402	–	
1	2	31500	800	− 0.3	0.932
	10	30860	1013	− 2.3	0.469
	50	30980	1343	− 1.9	0.589
2	2	30070	2140	− 4.8	0.765
	10	30740	559	− 2.7	0.399
	50	35980	457	13.9	< 0.001
3	2	34700	654	9.8	0.007
	10	34790	515	10.1	0.006
	50	32550	330	3.0	0.346
4	2	30620	1818	− 3.1	0.391
	10	31170	893	− 1.4	0.705
	50	31500	1149	− 0.5	0.899
Forskolin	10	12370	568	− 60.8	< 0.001
	250	9580	502	− 69.7	< 0.001
cAMP	1	2367	539	− 92.5	< 0.001
Adenosine	10	23050	582	− 27.0	< 0.001

[a]) The inhibitory ratio (*IR*) of the test compounds := [cps (test compound(− cps (DMSO control)]/cps (DMSO control) × 100.

Experimental Part

General. TLC: silica gel *GF254* (SiO$_2$; *Qingdao Marine Chemical Factory*, Qingdao, China); visualization by heating SiO$_2$ plates with 5% H$_2$SO$_4$ in EtOH. Column chromatography (CC): SiO$_2$ (200–300 mesh, *Qingdao Marine Chemical Factory*, Qingdao, China), *Lichroprep RP-18* (43–63 μm, *Merck*, D-

Darmstadt), *Sephadex LH-20* (*Amersham Biosciences AB*, S-Uppsala), and *MCI* (*MCI-gel CHP-20P*, 75–150 μm, *Mitsubishi Chemical Corporation*, Tokyo, Japan). Semi-prep. HPLC: *Agilent 1200* liquid chromatograph with a *ZORBAX SB-C₁₈* (5 μm, 9.4 × 250 mm, 3 ml/min, *Agilent*, USA) column. Optical rotations: *JASCO DIP-370* digital polarimeter (*JASCO Corporation*, Tokyo, Japan). IR Spectra: *Bio-Rad FTS-135* spectrophotometer with KBr pellets (*Bio-Rad Corporation*, USA); $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR spectra: *Bruker AM-400* or *DRX-500* instruments (*Bruker BioSpin Group*, Germany); δ in ppm rel. to Me₄Si as internal standard, *J* in Hz. FAB-MS: *VG Auto Spec-3000* spectrometer (Gly, *VG PRIMA*, Britain); in *m/z* (rel. %). HR-ESI-MS: *API Qstar Pulsar* instrument (*Applied Biosystem Corporation*, Canada); in *m/z*.

Plant Material. The flowers of *P. formosa* were collected in Jindian, Kunming, China, in May 2008. The sample was identified by Dr. *Yong-Peng Ma*, Kunming Institute of Botany, Chinese Academy of Sciences, and a voucher specimen (KMUST 2008050701) was deposited with the Laboratory of Phytochemistry, Biotechnology Research Center, Kunming University of Science and Technology.

Extraction and Isolation. Air-dried and powdered flowers of *P. formosa* (5.5 kg) was extracted with 70% acetone/H₂O (3 × 15 l, 1 d, each) at r.t. The concentrated syrup was suspended in H₂O and then subsequently extracted with AcOEt (3 × 2 l) and BuOH (3 × 2 l), resp. The AcOEt-soluble part (450 g) was decolorized on *MCI* gel with 90% MeOH/H₂O to obtain a yellow gum (427.5 g), which was then purified by CC (CHCl₃/acetone gradient system 1:0, 9:1, 8:2, 7:3, 6:4, and 1:1) to yield six fractions, *Frs. A–F. Fr. A* (100.5 g; CHCl₃/acetone 9:1) was submitted to repeated CC (SiO₂; CHCl₃/MeOH 20:1) to afford compounds **16** (58 g), **17** (10 g), and **18** (50 mg). *Fr. B* (15.8 g, CHCl₃/acetone 8:2) was subjected to CC (petroleum ether/i-PrOH 30:1, 20:1, and 10:1) to yield *Subfrs. B1–B3. Subfr. B2* was repeatedly chromatographed on SiO₂ (petroleum ether/AcOEt 5:1) and *Sephadex LH-20* (CHCl₃/MeOH 1:1) to afford **1** (4 mg), **2** (6 mg), **5** (15 mg), **6** (20 mg), **7** (50 mg), and **15** (7 mg). Compounds **3** (25 mg) and **14** (10 mg) were isolated from *Subfr. B3* by repeated CC (SiO₂; CHCl₃/MeOH 30:1, and then *RP-18*; MeOH/H₂O 45:55). *Fr. C* (6.5 g, CHCl₃/acetone 7:3) was subjected to CC (*Sephadex LH-20*; MeOH/H₂O 3:7, 6:4, and 9:1) to afford *Subfrs. C1–C3*. Compounds **8** (40 mg), **9** (35 mg), **10** (9 mg), **11** (11 mg), and **13** (75 mg) were obtained from *Subfr. C1* (450 mg) by CC (SiO₂; CHCl₃/MeOH 10:1). *Subfr. C2* (360 mg) was applied to semi-prep. HPLC (60% MeOH/H₂O) to obtain **12** (t_R 14.0 min; 15 mg). In addition, *Subfr. C3* was purified by semi-prep. HPLC (40% MeOH/H₂O) to yield **4** (t_R 56.17 min; 10 mg), **19** (t_R 53.2 min; 28 mg), **20** (t_R 53.5 min; 18 mg), and **21** (t_R 3.4 min; 25 mg). *Fr. D* (35.8 g; CHCl₃/acetone 6:4) was further separated to four fractions, *Frs. D1–D4*, by CC (*RP-18*; MeOH/H₂O 2:8, 3:7, 4:6, 5:5). From *Subfr. D2* (650 mg; MeOH/H₂O 3:7), compounds **24** (t_R 33.7 min; 125 mg), **25** (t_R 27.8 min; 23 mg), and **26** (t_R 24.2 min; 19 mg) were isolated by semi-prep. HPLC (23% MeOH/H₂O). *Subfr. D3* (250 mg; MeOH/H₂O 4:6) was subjected to semi-prep. HPLC (30% MeOH/H₂O) to furnish compounds **22** (t_R 26.4 min; 78 mg) and **23** (t_R 18.5 min; 105 mg).

The BuOH extract (425 g) was purified by CC (*Sephadex LH-20*; MeOH/H₂O 3:7, 6:4, 9:1) to give three fractions, *Frs. F1–F3. Fr. F1* (MeOH/H₂O 3:7, 15 g) was subjected to CC (*RP-18*; MeOH/H₂O 45:55), and semi-prep. HPLC (50% MeOH/H₂O) to give **27** (t_R 2.1 min; 6 mg). Semi-prep. HPLC (55% MeOH/H₂O) was applied to obtain **28** (t_R 5.08 min; 12 mg) and **29** (t_R 4.07 min; 10 mg) from *Fr. F2*. Compound **30** (20 mg) was isolated from *Fr. F3* by CC (*RP-18*; 58% MeOH/H₂O).

AlphaScreen® cAMP Assay. The effect of compounds **1–4** on cAMP levels in neuroblastma cells was tested using the *AlphaScreen®* cAMP assay kit (*Perkin-Elmer*) according to the procedure described in [39]. Briefly, when neuroblastoma N1E-115 cells were grown to *ca.* 80% confluence, they were harvested and suspended with stimulation buffer (HBSS, pH 7.4, containing 0.5 mᴍ IBMX, 5 mᴍ *HEPES*, and 0.1% BSA) at a density of 2000 cells/μl, and were further diluted by anti-cAMP acceptor beads soln. (0.2 units of anti-cAMP acceptor beads in 1 μl of stimulation buffer) into 1000 cells/μl. Then, the cell-beads mix was allocated into a 384-well white opaque microplate with 5 μl/well (*Perkin-Elmer*, No. 6007290). The test compounds (50, 10, or 2 μᴍ), or adenosine (10 μᴍ), or forskolin (10 μᴍ or 250 μᴍ), which is a generic activator of cAMP synthesis directly stimulating adenylate cyclase, were added to the well and incubated for 30 min at r.t. Three replicates were performed for each concentration. Then, streptavidin donor beads/biotinylated cAMP detection mix was added to the plate at 15 μl/well and incubated for 60 min in the dark. The mixture containing 5 μl of anti-cAMP acceptor beads, 5 μl of 5 μᴍ cAMP soln., and 15 μl of biotintylated-cAMP/streptavidin donor beads was used as a

positive control. After 2-h incubation in the dark, plates were read on a *2104 EnVision* ® Multilabel Plate Reader (*Perkin-Elmer*) at an excitation wavelength of 680 nm and an emission wavelength of 570 nm. The *AlphaScreen* ® counts per s (cps) decrease with increasing cAMP concentration (*e.g.*, positive control adenosine), and increase with decreasing cAMP concentration [39]. The cAMP level change due to the presence of test compounds was calculated. The inhibitory ratio (*IR*) of the test compounds was evaluated by the following equation: IR (%) = [cps (test compound) − cps (DMSO control)]/cps (DMSO control) × 100. The statistical tests were performed using one-way ANOVA analysis in software SPSS11.5 (SPSS, Chicago, IL).

Pierisformotoxin A (= rel-*(3aR,4S,4aS,7S,8R,9S,9aR,10R,11S,11aR,12S)-8,9,10,11-Tetrakis(acetyloxy)decahydro-4-hydroxy-11a-(1-hydroxy-1-methylethyl)-4,8-dimethyl-12-(1-oxopropoxy)-4H-7,9a-methanoheptaleno[3,2-b]furan-2(3H)-one*; **1**): White powder. R_f (CHCl₃/MeOH 20:1) 0.52. $[\alpha]_D^{18}$ = − 34.1 (*c* = 0.16, pyridine). IR (KBr): 3441, 1777, 1736, 1639, 1629, 1372, 1236. ¹H- and ¹³C-NMR: *Table 1*. FAB-MS (neg.): 639 ($[M − H]^-$), 597 ($[M − MeCO]^-$), 583 ($[M − MeCH_2CO]^-$), 541. HR-ESI-MS (neg.): 639.2661 ($[M − H]^-$, $C_{31}H_{43}O_{14}^-$; calc. 639.2652).

Pierisformotoxin B (= rel-*(3aR,4S,4aS,7S,8R,9S,9aR,10R,11S,11aR,12S)-8,9,11,12-Tetrakis(acetyloxy)decahydro-4-hydroxy-11a-(1-hydroxy-1-methylethyl)-4,8-dimethyl-10-(1-oxopropoxy)-4H-7,9a-methanoheptaleno[3,2-b]furan-2(3H)-one*; **2**): White powder. R_f (CHCl₃/MeOH 20:1) 0.50. $[\alpha]_D^{18}$ = − 37.7 (*c* = 0.16, pyridine). IR (KBr): 3441, 1737, 1639, 1629, 1370, 1060. ¹H- and ¹³C-NMR: *Table 1*. FAB-MS (neg.): 639 ($[M − H]^-$), 597 ($[M − MeCO]^-$), 583 ($[M − MeCH_2CO]^-$), 541. HR-ESI-MS (neg.): 639.2649 ($[M − H]^-$, $C_{31}H_{43}O_{14}^-$; calc. 639.2652).

Pierisformotoxin C (= rel-*(6β,7α,11β,14R)-6,7,11,15,16-Pentakis(acetyloxy)-5,10-dihydroxy-14-(1-oxopropoxy)grayanotoxan-3-one*; **3**): White powder. R_f (CHCl₃/MeOH 20:1) 0.63. $[\alpha]_D^{12}$ = − 5.4 (*c* = 0.3, acetone). IR (KBr): 3557, 3528, 3422, 2978, 1743, 1652, 1465, 1433, 1385, 1370, 1236, 1085, 1075, 1020. ¹H- and ¹³C-NMR: *Table 2*. FAB-MS (neg.): 681 ($[M − H]^-$), 639 ($[M − MeCO]^-$), 561, 501, 455, 385, 325. HR-ESI-MS (neg.): 681.2764 ($[M − H]^-$, $C_{33}H_{45}O_{15}^-$; calc. 681.2758).

Pierisformotoxin D (= rel-*(3β,6β,7α,14R)-3,5,7,10,14,15,16-Heptahydroxygrayanotoxan-6-yl Propanoate*; **4**): White powder. R_f (CHCl₃/MeOH 10:1) 0.45. $[\alpha]_D^{12}$ = − 27.0 (*c* = 0.2, acetone). IR (KBr): 3424, 2920, 1738, 1722, 1639, 1629, 1466, 1377, 1320, 1220, 1180, 1070, 1046. ¹H- and ¹³C-NMR: *Table 2*. FAB-MS (neg.): 457 ($[M − H]^-$), 401 ($[M − CH_2CH_2CO]^-$), 383 ($[M − CH_2CH_2CO − H_2O]^-$), 325. HR-ESI-MS (neg.): 457.2434 ($[M − H]^-$, $C_{23}H_{37}O_9^-$; calc. 457.2437).

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