Two New Triterpenoid Saponins Cytotoxic to Human Glioblastoma U251MG Cells from *Ardisia pusilla*

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Two new triterpenoid saponins, ardipusillosides IV and V (**1** and **2**, resp.), together with one known saponin, ardisiacrispin B (**3**), were isolated from the whole plants of *Ardisia pusilla* A. DC. Their structures were deduced by extensive spectral analysis and chemical evidences. Compound **1** contains a glycosylated glycerol residue which is a very rare structural feature among triterpenoid glycosides and has been so far found only in the genus *Ardisia*. All the saponins exhibited significant cytotoxicity against human glioblastoma U251MG cells, but did not affect the growth of primary cultured human astrocytes.

Introduction. – *Ardisia pusilla* A. DC (Myrsinaceae) is a widely occurring shrub in southern China. Its whole plants, also known as '*Jiu Jie Long*', have been used as an antidote in Chinese traditional medicine [1]. Other plants of this genus have also been used and are well-documented in traditional medicine in Southeast Asia [2]. Previous chemical studies have shown that triterpenoid saponins are the main components in this genus. To date, four triterpenoid saponins, ardipusillosides I, II, III, and an unnamed saponin, have been reported from *A. pusilla* [3]. Ardipusillosides I and II showed significant antitumor effects in both *Lewis* pulmonary carcinoma and hepatocarcinoma [4]. As part of our ongoing investigation on new antitumor glycosides from natural sources [5], we studied the bioactive triterpenoid saponins of this plant. In this article, we report the isolation and structure elucidation of two new triterpenoid saponins, ardipusillosides IV and V (**1** and **2**, resp.), along with one known saponin, ardisiacrispin B (**3**), as well as their potent cytotoxicity against human glioblastoma U251MG cells.

Results and Discussion. – The EtOH extract of *A. pusilla* was suspended in H_2O and partitioned successively with petroleum ether and BuOH. The BuOH extract was subjected to several chromatographic purification steps to afford **1**–**3**.

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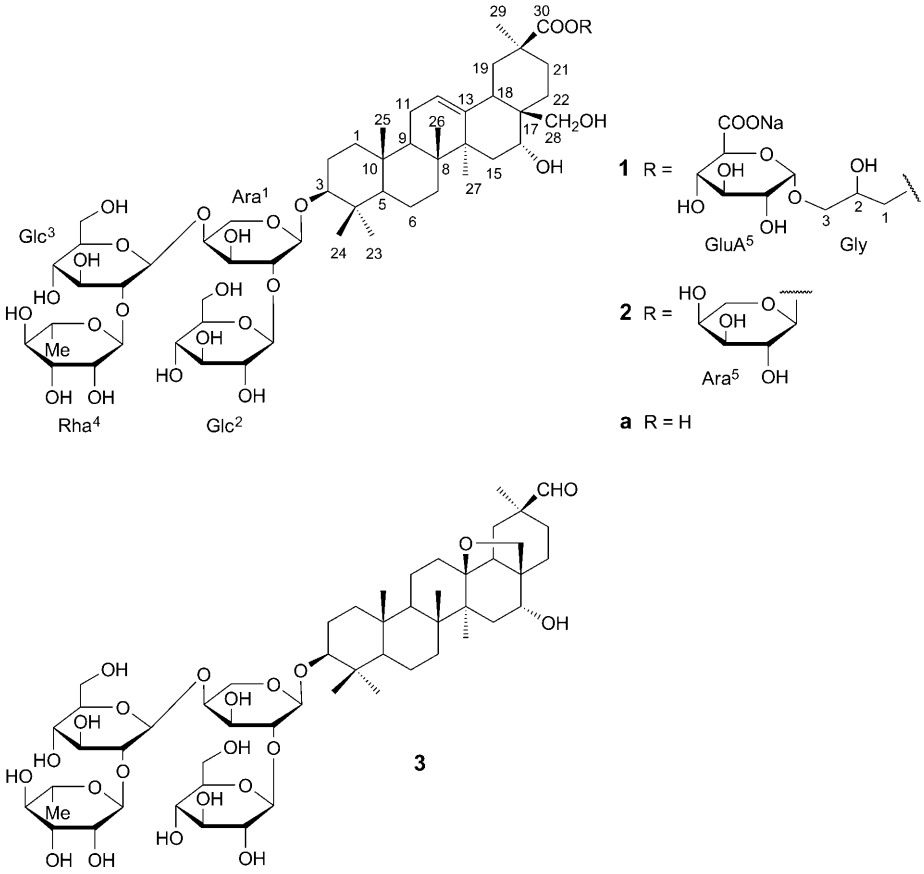

Ardipusilloside IV (**1**), a colorless amorphous powder, was positive to *Liebermann–Burchard* and *Molish* tests. The positive-ion mode HR-ESI-MS showed pseudomolecular-ion peak at *m/z* 1385.5955 ($[M+Na]^+$), which, together with the pseudomolecular-ion peak at *m/z* 1339 ($[M-Na]^-$) in the negative-ion mode ESI-MS and with the NMR data, led to the molecular formula $C_{62}H_{99}NaO_{31}$. The fragment-ion peak at *m/z* 1187 in the positive-ion mode ESI-MS corresponding to the loss of 198 mass units from 1385, was interpreted as due to the loss of a sodium uronate unit, suggesting that the uronic acid residue was in the form of a monosodium salt.

Saponin **1** displayed 62 C-atom signals in the ^{13}C-NMR spectrum, of which 30 could be assigned to the signals of the aglycone. It was evident that **1** was a triterpenoid saponin related to olean-12-ene skeleton based on the ^{1}H-NMR signals assigned to six tertiary Me groups at δ(H) 0.64 (*s*, Me(25)), 0.72 (*s*, Me(26)), 0.79 (*s*, Me(24)), 0.97 (*s*, Me(23)), 1.12 (*s*, Me(29)), and 1.55 (*s*, Me(27)), together with the ^{13}C-NMR signals for olefinic C-atoms at δ(C) 122.0 (C(12)) and 143.3 (C(13)). The presence of a C=O C-atom, C(30), was deduced from the ^{13}C-NMR resonance at δ(C) 177.7, and the long-range coupling between Me(29) and C(30) in the HMBC experiment. Further, OH groups at C(3) and C(16) could be attributed by the ^{13}C-NMR resonance at δ(C) 88.1

(C(3)) and 72.2 (C(16)). In the NOESY spectrum, the correlations between H–C(3) (δ 3.00) and Me(23) (δ 0.97), H–C(3), and H–C(5) (δ 0.47), and between H–C(16) (δ 4.53) and H-atoms of CH_2(28) (δ 3.29, 3.55) indicated the β-configuration of the OH group at C(3) and the α-configuration of the OH group at C(16). The assignments of the NMR signals associated with the aglycone moiety (*Table 1*) were derived from ^{1}H,^{1}H-COSY, TOCSY, HSQC, HMBC, and NOESY experiments, and the relative configurations at all stereogenic centers were established by analysis of the NOESY spectrum. By comparison of the NMR data with those reported [6], the aglycone of **1** was identified as (3β,16α)-3,16,28-trihydroxy-12-olean-30-oic acid (jacquinic acid). The glycosidation shifts for the signals due to C(3) ($\Delta\delta$(C) +10.0) and C(30) ($\Delta\delta$(C) −2.7) indicated that **1** is a bisdesmoside of jacquinic acid with glycosyl linkage at both the OH group at C(3) and the COOH group at C(30).

Alkaline hydrolysis of **1** afforded a prosapogenin **a** and a side-chain moiety (**1b**), which was originally linked to C(30) of the aglycone. Compound **a** was subjected to acidic hydrolysis to give L-arabinose (Ara), L-rhamnose (Rha), and D-glucose (Glc) in a ratio of 1:1:2 based on GC analysis of the corresponding trimethylsilyl derivatives using an L-*Chirasil-Val* column [7]. Hydrolysis of the side-chain moiety (**1b**) with HCl vapor on TLC plate revealed the presence of glucuronic acid (GluA) by comparison with an authentic sample. The above evidence suggested that **1** was a pentoside with a tetrasaccharide moiety linked to C(3) of the aglycone and a side chain including a sodium glucuronate residue attached to C(30) of the aglycone through an ester bond. The ^{1}H-NMR spectrum of **1** displayed five signals ascribable to the anomeric H-atoms with signals at δ(H) 4.77, 4.84, 5.11, 5.35, and 5.92, which were correlated in the HSQC experiment with the corresponding C-atoms with signals at δ(C) 103.0, 102.0, 103.2, 99.0, and 100.3, resp. The β-configurations for both glucose units were determined from their coupling constants of anomeric H-atoms (7.8 Hz). The H–C(1) non-splitting pattern of rhamnose unit and the three-bond HMBC correlations from H–C(1) (Rha^4) to C(3) (Rha^4) and C(5) (Rha^4) indicated that its anomeric H-atom was equatorial, thus possessing an α-configuration in the $^1C_4$ form [8]. The α-configuration for glucuronic acid moiety was clear from its small H–C(1) coupling constant (3.6 Hz). The α-configuration for the arabinose residue was evidenced by the correlations between H–C(1) (Ara^1) and H–C(3) (Ara^1), and between H–C(1) (Ara^1) and H–C(5) (Ara^1) in the NOESY experiment observed for the $^4C_1$ form, although its coupling constant of anomeric H-atom (4.8 Hz) was smaller than methyl-α-L-arabinopyranoside (8 Hz). This could be explained by the fast conformational equilibrium between its $^1C_4$ and $^4C_1$ conformers [9]. All H-atom signals due to sugar moieties were identified by careful analysis of the ^{1}H,^{1}H-COSY, TOCSY, and NOESY spectra, and the C-atom signals were assigned by HSQC and further confirmed by the HMBC spectrum. Data from the above experiments (*Table 1*) indicated that five sugar residues were in their pyranose forms. The absolute configuration of the sodium glucuronate residue was assumed to be D based on biogenetic considerations.

After mapping all of the signals for each sugar moiety, a 5-H spin system ascribable to a glycerol (Gly) residue was evident from the ^{1}H,^{1}H-COSY and TOCSY spectra in the region from δ(H) 3.8 to 4.5 that correlated, in the HSQC experiment, with C-atom signals at δ(C) 64.8 (CH_2(1) (Gly)), 67.9 (C(2) (Gly)), and 69.3 (CH_2(3) (Gly)). The glycerol fragment was confirmed by HMBC correlations as shown in the *Figure*.

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1**. At 600 and 150 MHz respectively, in C_5D_5N/D_2O 2 : 1; δ in ppm, J in Hz.

	δ(H)	δ(C)		δ(H)	δ(C)
CH_2(1)	0.62–0.66 (*m*, 1 H),	37.7	*Ara^{1}(1→C(3)):*		
	1.32–1.36 (*m*, 1 H)		H–C(1^{1})	4.77 (*d*, *J*=4.8)	103.0
CH_2(2)	1.75–1.80 (*m*, 1 H),	25.1	H–C(2^{1})	4.29–4.32 (*m*)	78.3
	1.82–1.86 (*m*, 1 H)		H–C(3^{1})	4.33–4.36 (*m*)	70.4
H–C(3)	3.00 (*dd*, *J*=4.4, 11.4)	88.1	H–C(4^{1})	4.37 (br. *s*)	73.0
C(4)	–	38.1	CH_2(5^{1})	3.90 (*d*, *J*=10.4, 1 H),	62.7
H–C(5)	0.47 (*d*, *J*=10.8)	54.6		4.27–4.31 (*m*, 1 H)	
CH_2(6)	1.27–1.33 (*m*)	17.7			
CH_2(7)	1.11–1.15 (*m*, 1 H),	31.9	*Glc^{2}(1→2):*		
	1.37–1.40 (*m*, 1 H)		H–C(1^{2})	5.11 (*d*, *J*=7.8)	103.2
C(8)	–	38.7	H–C(2^{2})	3.82–3.86 (*m*)	74.7
H–C(9)	1.41–1.46 (*m*)	45.9	H–C(3^{2})	4.09–4.12 (*m*)	75.8
C(10)	–	35.5	H–C(4^{2})	3.95–3.99 (*m*)	69.8
CH_2(11)	1.65–1.70 (*m*)	22.6	H–C(5^{2})	3.78–3.81 (*m*)	77.0
H–C(12)	5.46 (br. *t*)	122.0	CH_2(6^{2})	4.08–4.11 (*m*, 1 H),	61.0
C(13)	–	143.3		4.25 (br. *d*, *J*=11.4, 1 H)	
C(14)	–	40.4			
CH_2(15)	1.50 (*dd*, *J*=4.2, 14.4, 1 H),	33.2	*Glc^{3}(1→4):*		
	2.02 (*dd*, *J*=4.2, 14.4, 1 H)		H–C(1^{3})	4.84 (*d*, *J*=7.8)	102.0
H–C(16)	4.53 (br. *s*)	72.2	H–C(2^{3})	3.92–3.95 (*m*)	76.4
C(17)	–	38.9	H–C(3^{3})	4.16–4.19 (*m*)	77.2
H–C(18)	2.25 (*dd*, *J*=4.2, 14.4)	42.6	H–C(4^{3})	3.75 (*dd*, *J*=9.0, 9.2)	70.1
			H–C(5^{3})	3.65–3.69 (*m*)	76.6
CH_2(19)	2.01–2.06 (*m*, 1 H),	43.3	CH_2(6^{2})	3.96–4.00 (*m*, 1 H),	60.8
	2.49 (*dd*, *J*=13.2, 13.8, 1 H)			4.21 (br. *d*, *J*=11.4, 1 H)	
C(20)	–	43.6			
CH_2(21)	2.18–2.22 (*m*)	32.3	*Rha^{4}(1→2):*		
CH_2(22)	1.97–2.00 (*m*, 1 H),	30.8	H–C(1^{4})	5.92 (*s*)	100.3
	2.27–2.30 (*m*, 1 H)		H–C(2^{4})	4.48–4.52 (*m*)	71.5
Me(23)	0.97 (*s*)	27.0	H–C(3^{4})	4.40–4.43 (*m*)	70.6
Me(24)	0.79 (*s*)	15.5	H–C(4^{4})	4.04–4.08 (*m*)	72.6
Me(25)	0.64 (*s*)	14.5	H–C(5^{4})	4.63–4.67 (*m*)	68.7
Me(26)	0.72 (*s*)	15.7	Me(6^{4})	1.63 (*d*, *J*=6.6)	17.3
Me(27)	1.55 (*s*)	26.1			
CH_2(28)	3.29 (*d*, *J*=10.8, 1 H),	69.0	*GluA^{5} (1→CH$_2$(3)(Gly)):*		
	3.55 (*d*, *J*=10.8, 1 H)		H–C(1^{5})	5.35 (*d*, *J*=3.6)	99.0
Me(29)	1.12 (*s*)	27.6	H–C(2^{5})	3.98–4.02 (*m*)	71.5
C(30)	–	177.7	H–C(3^{5})	4.38–4.42 (*m*)	72.4
			H–C(4^{5})	4.34–4.37 (*m*)	71.5
Gly:			H–C(5^{5})	4.55 (*d*, *J*=10.2)	71.8
CH_2(1)	4.44 (br. *d*, *J*=11.4, 1 H),	64.8	C(6^{5})	–	175.7
	4.46 (br. *d*, *J*=11.4, 1 H)				
H–C(2)	4.30–4.33 (*m*)	67.9			
CH_2(3)	3.80–3.83 (*m*, 1 H),	69.3			
	4.14 (br. *d*, *J*=11.4, 1 H)				

Inspection of HMBC and NOESY spectra led to the determination of the sequence and binding sites of the oligosaccharide chain. In the HMBC spectrum, a cross-peak between C(3) of the aglycone and H–C(1) of arabinose indicated that Ara[1] was connected to C(3) of the aglycone. The linkage of the terminal glucose at C(2) of Ara[1] was indicated by the cross-peak H–C(1) (Glc[2])/C(2) (Ara[1]). Similarly, the linkages of the terminal rhamnose at C(2) of the inner glucose, in turn, linked to C(4) of Ara[1] were indicated by cross-peaks H–C(1) (Rha[4])/C(2) (Glc[3]), H–C(1) (Glc[3])/C(4) (Ara[1]). The conclusion was confirmed by NOESY experiment. It must be noted that, compared with the corresponding C-atoms of Glc[2], the glycosidation shifts for the signals due to C(2) (Glc[3]) ($\Delta\delta$(C) +1.7) and C(3) (Glc[3]) ($\Delta\delta$(C) +1.4) caused by the attachment of Rha[4] were too small and unusual to predict an interglycosidic site. It has been reported that this situation could be correlated with the distortion of the corresponding torsion angles [10]. The side-chain structure at C(30) of the aglycone was also elucidated by HMBC correlations between H-atoms of CH_2(1) (Gly) and C(30) of aglycone, and between H–C(1) of sodium glucuronate residue and CH_2(3) (Gly) (*Fig.*). The configuration of the glycerol remains to be determined. On the basis of the above evidences, the structure of ardipusilloside IV (**1**) was determined as (3β,16α)-16,28-dihydroxy-3-({α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}oxy)olean-12-en-30-oic acid 2-hydroxy-3-[(sodium α-D-glucopyranuronat)yloxy]propyl ester. Ardipusilloside IV contains a glycosylated glycerol residue, which is a very rare structural feature among triterpenoid glycosides. To date, only ardisicrenoside E and F, two saponins possessing the same structural feature previously isolated from *A. crenata*, have been reported. Compound **1** was the sodium salt of the demethylated derivative of ardisicrenoside E [10].

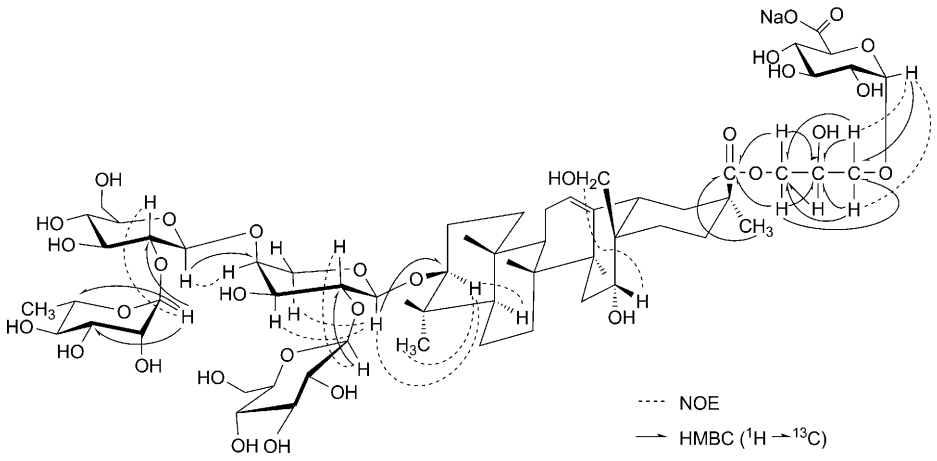

Figure. *Key NOESY and HMBC correlations for saponin* **1**

Ardipusilloside V (**2**) was obtained as a colorless amorphous powder with a molecular formula of $C_{58}H_{94}O_{27}$ determined by HR-ESI-MS. Fragment-ion peak at *m*/*z* 1113 ($[M+Na-132]^+$) in ESI-MS (positive-ion mode) indicated the presence of a terminal pentose residue in **2**. Alkaline hydrolysis of **2** afforded a prosapogenin and an L-arabinose identified by GC analysis of its corresponding trimethylsilyl derivative.

Further subjected to acidic hydrolysis, the prosapogenin yielded the same monosaccharides as for **a**, *i.e.*, L-arabinose, L-rhamnose, and D-glucose (1:1:2), identified by GC analysis. The ^{1}H-NMR, ^{13}C-NMR, and DEPT spectra of **2** (*Table 2*) revealed that **2** was also a bisdesmoside, with the same triterpene and the same tetrasaccharide moiety linked to C(3) of the aglycone as those of **1**, but differed in the side-chain fragment attached to C(30). Extensive NMR studies (^{1}H,^{1}H-COSY, TOCSY, HSQC, HMBC, and NOESY) indicated that the only difference between **1** and **2** was the replacement of the glycosyl glycerol moiety in **1** by an α-L-arabinose unit in **2**. A cross-peak of long-range coupling between a H-atom signal at δ(H) 6.19 (H–C(1) (Ara^5)) and a C=O C-atom signal at δ(C) 177.1 (C(30) of the aglycone) in HMBC spectrum substantiated that one of the arabinoses was located at C(30) through an ester bond. Consequently, the structure of **2** was established as (3β,16α)-16,28-dihydroxy-3-({α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}oxy)olean-12-en-30-oic acid α-L-arabinopyranosyl ester. To date, glucose is the only sugar residue bound to the aglycone by a glycosidic ester linkage among triterpenoid saponins from genus *Ardisia* reported. Saponin **2** is the first example of an arabinose residue located at the aglycone through an ester bond.

On the basis of its MS and NMR data compared with literature data, and by acid hydrolysis, followed by GC analysis of the corresponding trimethylsilylated monosaccharides, saponin **3** was identified as ardisiacrispin B, *i.e.*, (3β,13β,16α)-16-hydroxy-3-({α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}oxy)-13,28-epoxyoleanan-30-al, previously isolated from *A. crenata* [11].

The *in vitro* cytotoxicity of **1**–**3** and the prosapogenin **a**, *i.e.*, (3β,16α)-16,28-dihydroxy-3-({α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}oxy)olean-12-en-30-oic acid, against human glioblastoma U251MG cells and primary cultured human astrocytes was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide (MTT) colorimetric assay [3d][12]. The IC_{50} value of each saponin tested was determined on the basis of cell viability, after 48-h treatment. Nimustine hydrochloride (ACNU), a known anticancer agent commonly used in clinical treatment for malignant brain tumors, was used as the positive control. The results were listed in *Table 3*, and indicated that saponins **1**, **2**, and **3** displayed significant cytotoxicity against glioblastoma U251MG cells, while the prosapogenin **a** was inactive ($IC_{50} > 100$ μM).

Saponin **3** and **a** share the same tetrasaccharide chain, but differ in their oleanane skeleton. This led to significant difference in cytotoxicity against U251MG cells. Earlier studies on the cytotoxicity of **3** and **a** against other three human cancer cell lines have reached the same result. In addition, ardisiamamilloside F, an analogue of **3** with the similar structure except that the aldehyde group at C(30) in **3** was replaced by a carboxyl group, was also inactive [8]. Thus, it seems that the structure of the aglycone especially the aldehyde group at C(30) play an important role in terms of cytotoxic activity against tumor cells. However, the bisdesmosides **1** and **2** with the same aglycone and tetrasaccharide moiety at C(3) exhibited remarkable cytotoxicity. It seems that the presence of an additional sugar or glycosyl glycerol moiety at C(30) greatly increases the activity. Thus, our results indicated that the cytotoxic activity of such saponins was influenced by the structures of both the aglycones and the sugar parts, and very

Table 2. *^{1}H- and ^{13}C-NMR Data of* **2**. At 600/150 MHz resp., in C_5D_5N/D_2O 2 : 1; δ in ppm, J in Hz.

	δ(H)	δ(C)		δ(H)	δ(C)
CH_2(1)	0.66–0.70 (*m*, 1 H),	37.8	*Ara^1(1→C(3)):*		
	1.35–1.39 (*m*, 1 H)		H–C(1^1)	4.80 (*d*, *J*=4.8)	103.1
CH_2(2)	1.76–1.81 (*m*, 1 H),	25.2	H–C(2(1^1)	4.34–4.37 (*m*)	78.8
	1.84–1.88 (*m*, 1 H)		H–C(3(1^1)	4.30–4.33 (*m*)	70.7
H–C(3)	3.05 (*dd*, *J*=4.0, 11.4)	88.2	H–C(4(1^1)	4.40 (br. *s*)	73.4
C(4)	–	38.2	CH_2(5^1)	3.85–3.89 (*m*, 1 H),	62.7
H–C(5)	0.51 (*d*, *J*=10.8)	54.6		4.24–4.26 (*m*, 1 H)	
CH_2(6)	1.30–1.36 (*m*)	17.7			
CH_2(7)	1.13–1.17 (*m*, 1 H),	31.9	*Glc^2(1→2):*		
	1.40–1.44 (*m*, 1 H)		H–C(1(2^2)	5.15 (*d*, *J*=7.8)	103.7
C(8)	–	38.9	H–C(2(2^2)	3.86–3.90 (*m*)	74.9
H–C(9)	1.44–1.49 (*m*)	45.9	H–C(3(2^2)	4.17–4.20 (*m*)	76.1
C(10)	–	35.6	H–C(4(2^2)	3.98–4.02 (*m*)	70.1
CH_2(11)	1.69–1.74 (*m*)	22.6	H–C(5(2^2)	3.82–3.85 (*m*)	77.1
H–C(12)	5.52 (br. *t*)	122.1	CH_2(6$_2$)	4.13–4.17 (*m*, 1 H),	61.2
C(13)	–	143.4		4.28 (br. *d*, *J*=10.8, 1 H)	
C(14)	–	40.5			
CH_2(15)	1.53 (*dd*, *J*=4.2, 14.4, 1 H),	33.5	*Glc^3(1→4):*		
	2.05 (*dd*, *J*=4.2, 14.4, 1 H)		H–C(1^3)	4.88 (*d*, *J*=7.8)	102.0
H–C(16)	4.58 (br. *s*)	72.7	H–C(2^3)	3.97–4.00 (*m*)	76.5
C(17)	–	39.1	H–C(3^3)	4.14–4.17 (*m*)	77.5
H–C(18)	2.28 (*dd*, *J*=4.2, 14.4)	42.2	H–C(4^3)	3.79 (*dd*, *J*=9.0, 9.6)	70.4
CH_2(19)	2.05–2.10 (*m*, 1 H),	43.5	H–C(5^3)	3.68–3.72 (*m*)	76.9
	2.54 (*dd*, *J*=13.2, 14.4, 1 H)		CH_2(6^3)	4.01–4.05 (*m*, 1 H),	60.9
C(20)	–	43.6		4.22 (br. *d*, *J*=10.8, 1 H)	
CH_2(21)	2.22–2.26 (*m*)	32.4			
CH_2(22)	2.00–2.03 (*m*, 1 H),	30.5	*Rha^4(1→2):*		
	2.31–2.34 (*m*, 1 H)		H–C(1^4)	5.99 (*s*)	100.4
Me(23)	1.01 (*s*)	27.0	H–C(2^4)	4.51–4.55 (*m*)	71.4
Me(24)	0.83 (*s*)	15.5	H–C(3^4)	4.46 (*dd*, *J*=3.2, 9.6)	70.8
Me(25)	0.69 (*s*)	14.6	H–C(4^4)	4.06–4.10 (*m*)	72.8
Me(26)	0.76 (*s*)	15.8	H–C(5^4)	4.64–4.68 (*m*)	68.8
Me(27)	1.58 (*s*)	26.2	Me(6^4)	1.66 (*d*, *J*=6.0)	17.3
CH_2(28)	3.32 (*d*, *J*=10.8, 1 H),	68.9			
	3.57 (*d*, *J*=10.8, 1 H)		*Ara^5(1→C(30)):*		
Me(29)	1.14 (*s*)	27.5	H–C(1^5)	6.19 (*d*, *J*=7.2)	95.7
C(30)	–	177.1	H–C(2^5)	4.62 (br. *t*)	71.3
			H–C(3^5)	4.08–4.12 (*m*)	73.8
			H–C(4^5)	4.20 (br. *s*)	68.0
			CH_2(5^5)	3.88–3.92 (*m*, 1 H),	66.0
				4.30–4.34 (*m*, 1 H)	

sensitive to their precise functionalization. Therefore, more extensive studies are needed before a clear structure-activity relationship (SAR) can be reached.

It is worth to be mentioned that saponins **1**–**3** did not affect the growth of primary cultures of human astrocytes. In fact, exposure of the astrocytes to the highest concentration of **1**–**3** (100 μM) for 48 h did not result in any statistically significant

Table 3. *Cytotoxicity of Saponins* **1**, **2**, **3**, *and* **a** *against Human Glioblastoma U251MG Cells and Primary Cultured Astrocytes* in vitro

	IC_{50} [μM][a])				
	1	**2**	**3**	**a**	ACNU[b])
U251MG	1.55 ± 0.21	2.20 ± 0.35	3.33 ± 0.29	> 100	0.98 ± 0.06
Astrocytes	> 100	> 100	> 100	> 100	> 100

[a]) IC_{50} Values are means from three independent experiments (mean ± SD). [b]) Nimustine hydrochloride (ACNU) as positive control.

change in cell viability, with the % inhibition ranging from 4.3 to 8.5% ($p > 0.05$). Meanwhile, viability of astrocytes treated with 100 μM ACNU for 48 h decreased to 69.2% ($p < 0.05$). Glioblastoma multiforme is the most common and lethal primary brain malignancy and relatively resistant to chemotherapy. Therefore, the development of effective drugs to reverse its drug resistance and induce apoptosis is critical. We have reported that ardipusilloside III isolated also from *A. pusilla* could induce apoptosis of U251 cells, and both the BAD-mediated intrinsic apoptotic signaling pathway and the caspase-8-mediated extrinsic apoptotic signaling pathway were involved in the apoptosis [3d]. Clearly, the promising saponins **1**, **2**, and **3** also merit further study as potential anticancer agents.

Experimental Part

General. Column chromatography (CC): silica gel (200–300 mesh and 10–40 μM; *Qingdao*, China), *Sephadex LH-20* (*Pharmacia*), and *Lobar Lichroprep RP-18* (size B, 40–63 μM; *Merck*). TLC: precoated silica gel GF_{254} plates (10–40 μM; *Yantai*, China); detection by spraying with 20% aq. H_2SO_4, followed by heating. HPLC: *Dionex P680* liquid chromatograph equipped with a *UV170* UV/VIS detector monitored at 206 nm; *Elite Sino Chrom ODS-BP* column (250 × 10 mm i.d., 5 μM). M.p.: *XT5-XMT* apparatus; uncorrected. Optical rotations: *Perkin-Elmer 341* polarimeter. NMR: *Bruker AV-600*, at 600 (^{1}H) and 150 MHz (^{13}C); δ in ppm rel. to Me_4Si, *J* in Hz. ESI- and HR-ESI-MS: *Waters Q-TOF Micro YA019* mass spectrometer; in *m*/*z*. GC: *Finnigan Voyager* GC/MS apparatus with a L-*Chirasil-Val* column (25 m × 0.32 mm i.d.).

Plant Material. The whole plants of *A. pusilla* were collected in Jiajiang County, Sichuan Province, China, in September 2006, and identified by one of the authors, Prof. *Xiao-Juan Wang*. A voucher specimen (No. 06-SC03) was deposited with the Department of Pharmacy, School of Stomatology, Fourth Military Medical University, Xi'an, China.

Extraction and Isolation. Dried powder of the whole plants (3 kg) was refluxed with 95% EtOH (3 × 8 l, each for 2 h) and centrifuged. The combined extract was suspended in H_2O (1 l) and partitioned successively with petroleum ether and BuOH (each 3 × 1 l). The BuOH-soluble fraction (10 g) was subjected to CC (SiO_2; lower phases of $CHCl_3$/MeOH/H_2O 7:3:1 and 6.5:3.5:1): *Fr. 1–8*. *Fr. 3* and *7* mainly contained triterpenoid saponins. *Fr. 7* (0.3 g) was subjected to CC (*RP-18*; MeOH/H_2O 1:1): *Fr. 7.1–7.3*. *Fr. 7.1* (58 mg) was purified by HPLC (*Elite Sino Chrom ODS-BP*; 38% MeOH, 2.0 ml/min): **1** (12.3 mg, t_R 22.0 min). *Fr. 7.3* (80 mg) was purified by HPLC (75% MeOH, 2.0 ml/min): **2** (37.3 mg, t_R 13.5 min). *Fr. 3* (1.2 g) was submitted to CC (*Sephadex LH-20*; MeOH/H_2O 1:1) to remove the pigments and carbohydrates and finally purified by HPLC (90% MeOH, 2.0 ml/min): **3** (445 mg, t_R 9.5 min).

Ardipusilloside IV (*=(3β,16α)-16,28-Dihydroxy-3-({α-*L*-rhamnopyranosyl-(1→2)-β-*D*-glucopyranosyl-(1→4)-[β-*D*-glucopyranosyl-(1→2)]-α-*L*-arabinopyranosyl}oxy)olean-12-en-30-oic Acid 2-Hydroxy-3-[(sodium-α-*D*-glucopyranuronat)yloxy]propyl Ester*; **1**). Colorless amorphous powder. M.p.

235–236° (dec.). $[\alpha]_D^{22} = +25.2$ ($c = 0.15$, MeOH). ^{1}H- and ^{13}C-NMR: *Table 1*. Key HMBC and NOESY correlations: *Figure*. ESI-MS (pos.): 1385 ($[M+Na]^+$), 1187 ($[M+Na-GluANa]^+$), 1113 ($[1187-CH_2CHOHCH_2O]^+$), 1097 ($[1187-OCH_2CHOHCH_2O]^+$). ESI-MS (neg.): 1361 ($[M-H]^-$), 1339 ($[M-Na]^-$), 1163 ($[M-GluANa-H]^-$), 1089 ($[M-GluANa-CH_2CHOHCH_2OH]^-$), 1073 ($[1089-O]^-$). ESI-MS/MS (neg., 1339): 1193 ($[1339-Rha]^-$), 1177 ($[1339-Glc]^-$), 1089, 1073, 1031 ($[1177-Rha]^-$), 943 ($[1089-Rha]^-$), 927 ($[1089-Glc]^-$), 869 ($[1031-Glc]^-$), 781 ($[943-Glc]^-$), 619 ($[781-Glc]^-$), 601 ($[Ara+Rha+2\times Glc-H]^-$), 487 ($[aglycone-H]^-$). HR-ESI-MS: 1385.5955 ($[M+Na]^+$, $C_{62}H_{99}Na_2O_{31}^+$; calc. 1385.5965).

Ardipusilloside V (*=(3β,16α)-16,28-Dihydroxy-3-({α-*L*-rhamnopyranosyl-(1→2)-β-*D*-glucopyranosyl-(1→4)-[β-*D*-glucopyranosyl-(1→2)]-α-*L*-arabinopyranosyl}oxy)olean-12-en-30-oic Acid α-*L*-Arabinopyranosyl Ester*; **2**). Colorless amorphous powder. M.p. 232–233° (dec.). $[\alpha]_D^{22} = +12.8$ ($c = 0.13$, MeOH). ^{1}H- and ^{13}C-NMR: *Table 2*. ESI-MS (pos.): 1245 ($[M+Na]^+$), 1113 ($[M+Na-Ara]^+$). ESI-MS (neg.): 1221 ($[M-H]^-$), 1089 ($[M-H-Ara]^-$). ESI-MS/MS (neg., 1221): 1089 ($[1221-Ara]^-$), 1075 ($[1221-Rha]^-$), 1059 ($[1221-Glc]^-$), 943 ($[1089-Rha]^-$), 927 ($[1089-Glc]^-$), 781 ($[943-Glc]^-$), 619 ($[781-Glc]^-$), 487 ($[aglycone-H]^-$). HR-ESI-MS: 1245.5887 ($[M+Na]^+$, $C_{58}H_{94}NaO_{27}^+$; calc. 1245.5880).

Alkaline Treatment of **1** *and* **2**. Saponin **1** (5 mg) was dissolved in 1M NaOH soln. (2 ml) and heated at 100° for 1 h. The mixture was neutralized with a cation-exchange resin (*Dowex 50W-X2*, H^+) and evaporated *in vacuo*. The residue was subjected to CC (*RP-18*; H_2O and MeOH/H_2O 6:4): **1b** (<1 mg) and **a** (2.6 mg), resp. **1b** was applied to a TLC plate and hydrolyzed with HCl vapor at 80° for 20 min. The plate was subjected to co-TLC analysis ($CHCl_3$/MeOH/H_2O 15:6:2 (9 ml of the lower phase and 1 ml AcOH), detection: aniline phthalate) with authentic sugars. Glucuronic acid was identified (R_f 0.62). By the same method, saponin **2** (10 mg) furnished **a** (6.2 mg) and **2b**. Compound **2b** was dissolved in 1-(trimethylsilyl)-1*H*-imidazole and pyridine (0.1 ml). The soln. was stirred at 60° for 5 min and dried with a stream of N_2. The residue was partitioned between $CHCl_3$ and H_2O. The org. layer was analyzed by GC, and the peaks at 8.77 and 9.65 min enabled the identification of L-arabinose. t_R Values for authentic sugars after being treated simultaneously with 1-(trimethylsilyl)-1*H*-imidazole in pyridine were 8.68 and 9.60 min (D-arabinose), 8.76 and 9.64 min (L-arabinose), 9.39 and 10.28 min (D-rhamnose), 9.31 and 10.21 min (L-rhamnose), 14.56 min (D-glucose), and 14.50 min (L-glucose).

Acid Hydrolysis of **1** *and* **2** (*Prosapogenin* **a**). Each prosapogenin (2 mg) was heated in 2M CF_3COOH (1 ml) at 120° for 2 h. The mixture was evaporated to dryness, and the residue was partitioned between $CHCl_3$ and H_2O. The aq. phase was concentrated and the residue was treated with 1-(trimethylsilyl)-1*H*-imidazole in pyridine by the same procedure performed for **2b**. The sugar moieties of **1** and **2** were both determined to be L-arabinose, L-rhamnose, and D-glucose in a ratio of 1:1:2 by comparing the GC retention times of the corresponding trimethylsilylated hydrolysates with those of the authentic samples prepared in the same manner.

Bioassays. The cytotoxicity of **1**–**3** and **a** against human glioblastoma U251MG cells (originally obtained from Uppsala, Sweden) and primary cultured human astrocytes was evaluated by MTT (=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide) colorimetric assay described in [3d][12], with the anticancer agent nimustine hydrochloride (ACNU, *Sigma*) as positive control. Cultured primary astrocytes were obtained from a slightly impaired brain-tissue fragment of a volunteer with cerebral trauma who consented to the procedure as described in [3d]. Acquisition of the tissue was approved by the local medical research ethics committee at Xijing Hospital, Fourth Military Medical University. Target cells were grown to log phase in *DMEM* medium containing 10% fetal bovine serum and transferred to serum free medium in 96-well plates at a density of 4×10^3 cells/well. Cultures were preincubated for 24 h in a humidified 5% CO_2/95% O_2 atmosphere at 37°. Then, control or test soln. was put into each well, and the plates were incubated for an additional 48 h. At the end of exposure, MTT solved in PBS (=phosphate-buffered saline) was added to each well at a final concentration of 5 mg/ml, and then incubated for 4 h. The H_2O-insoluble dark blue formazan crystals formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density of each well was measured with a *Bio-Rad 680* microplate reader at 490 nm. The activities of saponins **1**, **2**, **3**, **a**, and ACNU were determined at 100, 10, 1, 0.1, and 0.01 μM (each concentration was tested in triplex wells), resp. Data were calculated as percentage of inhibition by the formula: % Inhibition=$(100-(OD_t/OD_s)\times100)$, OD_t and

OD_s being the mean optical densities of the test compounds and the solvent control, resp. The concentration inducing a 50% inhibition of cell growth (IC_{50}) was determined graphically for each experiment using the curve-fitting routine of the computer software Prism 4.0 (*GraphPad*) and the equation derived by *De Lean et al.* [13]. The IC_{50} value represented the mean of three independent experiments and was expressed as mean ± SD using *Student*'s *t* test.

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