Triterpenoids and Iridoid Glycosides from *Gentiana dahurica*

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One new and six known pentacyclic triterpenoids, **1** and **4**–**9**, resp., along with two new and three known iridoid glycosides, **2** and **3**, and **10**–**12**, resp., were isolated from the roots of *Gentiana dahurica* ('*Qin-Jiao*'). The new structures were elucidated by means of spectroscopic and chemical methods as 1*β*,2*α*,3*α*,24-tetrahydroxyursa-12,20(30)-dien-28-oic acid (**1**), 6′-*O*-acetylgentiopicroside (**2**), and 3′-acetylgentiopicroside (**3**). Isolated compounds were tested for their cytotoxicity against the MCF-7 human breast cancer cell line using the MTT assay. Among them, triterpenoids 2*α*,3*β*,24-trihydroxyurs-12-en-28-oic acid (**6**) and 3*β*,24-dihydroxyurs-12-en-28-oic acid (**9**) were found to have moderate cytotoxic effects with IC_{50} values of 20.9 and 21.7 μg/ml, respectively. Additionally, the chemotaxonomic significance of the identified secondary metabolites is briefly discussed.

Introduction. –'*Qin-Jiao*' is a traditional Chinese medicine (TCM) that has been commonly used for treatment of rheumatoid arthritis and tumefaction [1]. This TCM is composed of dried roots of several plant species of the genus *Gentiana* (Gentianaceae), especially *G. dahurica* Fisch. and *G. macrophylla* Pall. [1][2]. Chemical constituents of *G. macrophylla* have been extensively investigated [3][4], whereas only ursolic acid [5], a few iridoid glycosides [6], and some miscellaneous constituents [7][8] have been purified or detected from *G. dahurica*. During a reinvestigation on this plant as part of our continuing work towards the discovery of novel antitumor agents from natural products [9][10], a new triterpenoid, **1**, and two new iridoid glycosides, **2** and **3**, together with several known triterpenoids, **4**–**9**, and iridoid glycosides, **10**–**12** (*Fig. 1*), were obtained from the roots of *G. dahurica*. Here, we report the isolation and structure elucidation of these new compounds, and their cytotoxicity against MCF-7 human breast cancer cells.

Results and Discussion. – The EtOH extract from the roots of *G. dahurica* was successively subjected to column chromatography (CC) over silica gel and *Sephadex LH-20*, and semi-preparative HPLC to furnish seven triterpenoids and five iridoid glycosides. Comparison of their MS and NMR data as well as physical properties with those in the literature led to the identification of the known compounds as 1*β*,2*α*,3*α*,24-

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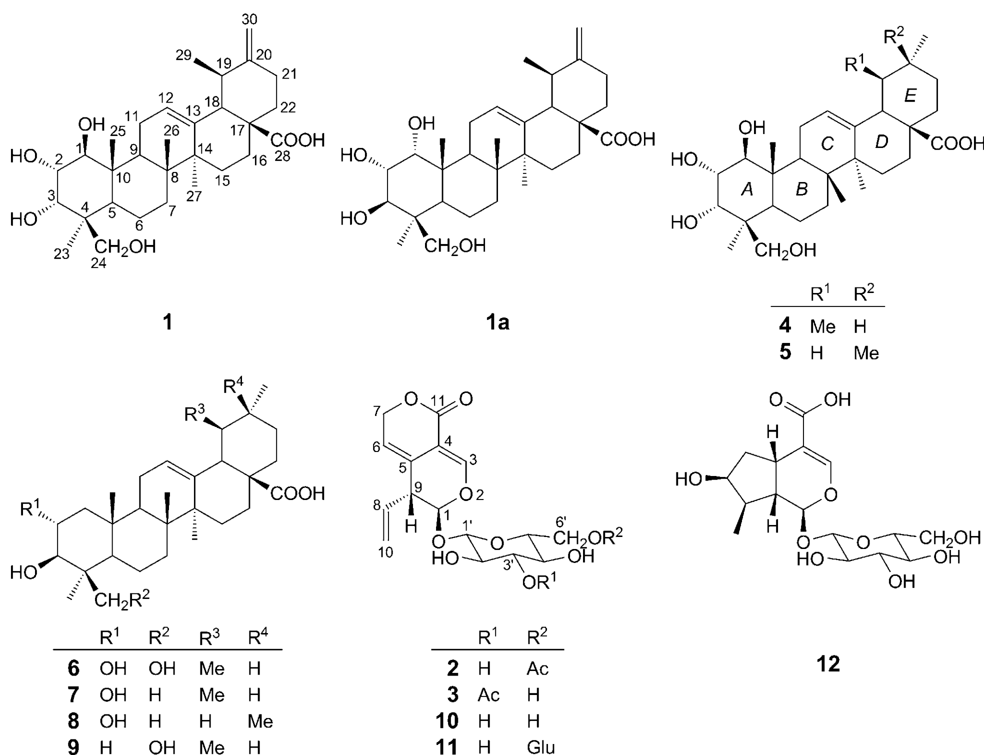

Fig. 1. *Chemical structures of compounds* **1**–**12**

tetrahydroxyurs-12-en-28-oic acid (**4**) [11][12], 1β,2α,3α,24-tetrahydroxyolean-12-en-28-oic acid (**5**) [12], 2α,3β,24-trihydroxyurs-12-en-28-oic acid (**6**) [13], 2α-hydroxyursolic acid (**7**) [14], maslinic acid (**8**) [14], 3β,24-dihydroxyurs-12-en-28-oic acid (**9**) [15], gentiopicroside (**10**) [16], 6′-*O*-β-D-glucopyranosyl gentiopicroside (**11**) [17], and loganic acid (**12**) [18]. Two-dimensional NMR spectra of compound **4** were also acquired to reconfirm the configuration of its OH groups. Although **10** and **12** were previously detected by LC/MS from roots of *G. dahurica* [6], all known compounds, **4**–**12**, were purified from this plant for the first time.

The molecular formula of compound **1** was determined to be $C_{30}H_{46}O_6$ based on a *pseudo*-molecular-ion peak at *m/z* 525.3186 ($[M + Na]^+$) in the positive-ion-mode HR-ESI mass spectrum. The ^{1}H- and ^{13}C-NMR spectra (*Table 1*) of **1** showed general features very similar to those of 1β,2α,3α,24-tetrahydroxyurs-12-en-28-oic acid (**4**) [11][12]. The most obvious difference between these two compounds was that a secondary Me group in ring *E* of **4** was replaced by an exocyclic CH_2 group in **1**, and hence signals of the two H-atoms were observed at δ(H) 4.79 and 4.74 (br. *s*) in the ^{1}H-NMR spectrum of **1**. In the COSY spectrum of **1**, a spin system was observed between H–C(12) at δ(H) 5.56 (br. *s*) and H–C(18) at δ(H) 2.75 (*d*, $J = 11.7$) *via* an allylic coupling, between H–C(18) and H–C(19) at δ(H) 2.43–2.41 (*m*), and between H–C(19) and Me(29) at δ(H) 1.09 (*d*, $J = 7.1$). Therefore, the exocyclic CH_2 group was

Table 1. *^{13}C-NMR Data* (recorded at 125 MHz) *of Compounds* **1**–**3**[a])

Position	**1** (DEPT)[b])	Position			**2** (DEPT)[c])	**2** (DEPT)[d])	**3** (DEPT)[e])
	δ(C)		δ(C)		δ(C)	δ(C)	δ(C)
1	80.7	16	24.6	1	96.9	97.3	98.6
2	71.3	17	48.0	3	148.7	149.4	150.7
3	74.3	18	55.3	4	103.4	103.8	104.9
4	44.5	19	37.4	5	124.8	125.6	127.0
5	49.0	20	153.7	6	116.3	115.9	117.2
6	18.6	21	32.5	7	69.2	69.6	70.9
7	33.9	22	39.4	8	134.0	133.0	135.0
8	40.4	23	23.5	9	44.4	45.2	46.6
9	48.8	24	64.9	10	117.9	118.7	118.6
10	43.4	25	13.2	11	162.7	164.0	166.2
11	27.4	26	17.5	1′	99.1	98.7	100.1
12	126.9	27	23.3	2′	72.7	72.9	72.8
13	137.6	28	179.0	3′	76.3	76.0	78.9
14	42.2	29	16.2	4′	69.7	69.8	69.7
15	28.4	30	104.5	5′	73.9	74.2	78.2
				6′	63.3	63.3	62.4
				AcO	170.3, 20.7	171.6, 20.9	172.6, 21.1

[a]) Assignments were achieved by a combination of 1D- and 2D-NMR techniques (COSY, HSQC, and HMBC). [b]) Recorded in C_5D_5N. [c]) Recorded in (D_6)DMSO. [d]) Recorded in $CDCl_3$. [e]) Recorded in CD_3OD.

located at C(20), and not at C(19). This was further confirmed by HMBCs observed between H–C(18) and C(19) (δ(C) 37.4), C(20) (δ(C) 153.7), and C(29) (δ(C) 16.2), respectively (*Fig. 2*).

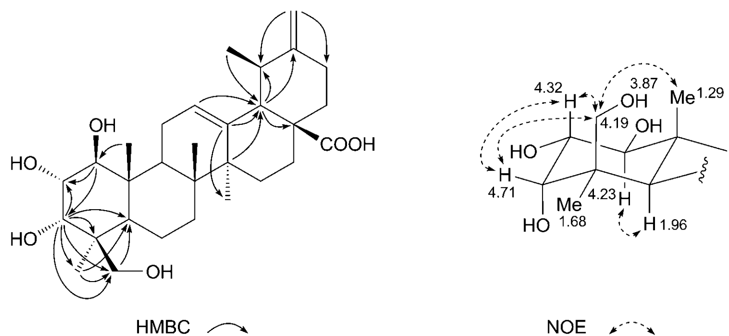

Fig. 2. *Selected HMBC and NOE correlations of compound* **1**

Similar to those of ring *A* in compound **4**, compound **1** also has three CH–O H-atoms resonating at δ(H) 4.23 (*d*, *J* = 9.8, H_α–C(1)), 4.32 (*dd*, *J* = 9.4, 2.5, H_β–C(2)), and 4.71 (br. *s*, H_β–C(3)) in the ^{1}H-NMR spectrum. In addition, a HO–CH_2 group at C(4) with signals at δ(H) 4.19 (H_a–C(24)) and 3.87 (H_b–C(24)) showed an *AB*

coupling ($J = 10.9$). The chemical shifts of the corresponding O-bearing C-atoms were subsequently deduced through HSQC experiment to be δ(C) 80.7 (C(1)), 71.3 (C(2)), 74.3 (C(3)), and 64.9 (C(24)). These assignments were confirmed by the 2D-NMR data: in the COSY spectrum of **1**, a spin system was detected between H–C(1) through H–C(3). In the HMBC spectrum, H–C(3) correlates with C(1), C(2), C(4) (δ(C) 44.5), C(5) (δ(C) 49.0), C(23) (δ(C) 23.5), and C(24) (*Fig. 2*). Location of the HO–CH_2 at C(24) was based on a clear NOE correlation observed between H_b–C(24) (δ(H) 3.87) and Me(25) (δ(H) 1.29 (*s*, 3 H)) in its NOESY spectrum (*Fig. 2*). This was in agreement with the experimental rule based on ^{13}C chemical shifts of the HO–CH_2 and the tertiary Me groups at C(4). In general, the chemical shift of the Me(23) group is at *ca.* δ(C) 23.5, if the OH group is at C(24) (δ(C) *ca.* 64.5), while the chemical shift of the Me(24) group is at *ca.* δ(C) 12.8, if the OH group is at C(23) (δ(C) *ca.* 67.9) [19].

The relative configuration of **1** was determined using coupling constants and the NOESY spectrum. The coupling constants found for H–C(1), H–C(2), and H–C(3) indicated that H–C(1) and H–C(2) have a *trans*-diaxial relationship, while H–C(3) adopts an equatorial orientation. In the NOESY spectrum of **1**, clear NOE correlations were observed between H–C(2) (δ(H) 4.32) and H_b–C(24) (δ(H) 3.87), between H–C(2) and H–C(3) (δ(H) 4.71), between H–C(3) and H_a–C(24) (δ(H) 4.19), and between H–C(1) (δ(H) 4.23) and H–C(5) (δ(H) 1.96) (*Fig. 2*). Thus, compound **1** was determined as 1β,2α,3α,24-tetrahydroxyursa-12,20(30)-dien-28-oic acid. The structure of **1** is similar to that of 1α,2α,3β,24-tetrahydroxyursa-12,20(30)-dien-28-oic acid (**1a**) (*Fig. 1*), a related triterpenoid isolated from *G. tibetica* KING [19]. However, compounds **1** and **1a** have opposite relative configurations at C(1) and C(3).

The molecular weight of compound **2** and its molecular formula $C_{18}H_{22}O_{10}$ were deduced from a positive-ion-mode HR-ESI-MS, which resulted in an $[M+H]^+$ ion peak at *m*/*z* 399.1285. The ^{1}H- and ^{13}C-NMR spectra data (*Table 1*) suggested that **2** is a derivative of gentiopicroside (**10**) [16]. The only difference is that **2** contains an AcO moiety (AcO: δ(H) 2.02 (*s*, 3 H); δ(C) 20.7, 170.3), which was supported by a strong absorption band at 1722 cm^{-1} in the IR spectrum. The acetylated position was deduced to be C(6′), because H-atom signals of CH_2(6′) in **2** were shifted downfield to δ(H) 4.26 (br. *d*, $J = 12.0$) and 4.08 (*dd*, $J = 11.5$, 6.0). This linkage position was supported by HMBCs between these two H-atoms and the ester CO C-atom. The β-glycosidic linkage was determined on the basis of the observed coupling constant (7.9 Hz) of the anomeric H-atom with the signal at δ(H) 4.54. The glycosidic position at C(1), and the relative configuration at C(1) and C(9) were reexamined by detailed 2D-NMR experiments, which were consistent with those of gentiopicroside (**10**). In addition, acid hydrolysis of **2** with 4N HCl gave only D-glucose as the sugar component, which was identified by direct comparison with an authentic sample using HPLC analysis and optical rotation detection. Therefore, compound **2** was determined as 6′-*O*-acetylgentiopicroside.

The molecular formula of compound **3** was found to be identical to that of **2**. The IR spectrum also exhibited an absorption band at 1726 cm^{-1} attributed to an ester CO group. The ^{1}H- and ^{13}C-NMR data were very similar to those of **2**. The β-glycosidic linkage was also determined according to the observed coupling constant (7.5 Hz) of the anomeric H-atom resonating at δ(H) 4.75 (H–C(1′)). In the COSY spectrum of **3**, the spin system of the sugar moiety was observed between H–C(1′) and H–C(2′) at

δ(H) 3.28 (*dd*, $J = 9.0, 8.0$), between H–C(2′) and H–C(3′) at δ(H) 4.93 (*dd*, $J = 9.5, 9.0$), between H–C(3′) and H–C(4′) at δ(H) 3.44 (*dd*, overlapped), between H–C(4′) and H–C(5′) at δ(H) 3.44–3.42 (*m*), as well as between H–C(5′) and H_a–C(6′) at δ(H) 3.89 (*dd*, $J = 11.0, 2.0$) and 3.68 (*dd*, $J = 11.5, 5.5$). The acetylated position was indicated by an HMBC between H–C(3′) and the ester CO C-atom (δ(C) 172.6). Acid hydrolysis of **3** with 4N HCl also yielded D-glucose. Therefore, **3** was characterized as 3′-acetylgentiopicroside. Both compounds **2** and **3** were detected in the crude EtOH extract by TLC and HPLC analyses, suggesting that they were not purification artifacts. Related naturally occurring acylated iridoid glycosides have also been recently isolated from *G. straminea* MAXIM. [20].

Earlier studies revealed that *Gentiana* plants are rich sources of triterpenoids [19][21][22] and iridoids [3][4][7][17][20][23–27]. In agreement with the previous findings, seven triterpenoids, **1** and **4**–**9**, and five iridoid glycosides, **2**, **3**, and **10**–**12**, were obtained from *G. dahurica*, indicating that these two classes of compounds may be considered as taxonomic markers for *Gentiana* genus. *G. dahurica* and *G. macrophylla* are two major representatives for the traditional Chinese medicine '*Qin-Jiao*' [1][2]. In the present study, the occurrence of the major secondary metabolite of gentiopicroside is in accordance with that of **10** in *G. macrophylla* [3]. Additionally, an endophytic fungus that can produce the same secoiridoid (*i.e.*, gentiopicrin) as the host plant was recently isolated from *G. macrophylla* [28]. If such a key iridoid-producing or triterpenoid-producing strain could be isolated from *G. dahurica*, one could envision using fermentation to economically produce the naturally occurring compounds (especially the cytotoxic triterpenoids) described here.

All isolated compounds, **1**–**12**, were tested *in vitro* against the MCF-7 human breast cancer cell line using the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay method, and none of the iridoid glycosides was found to show cytotoxic effects (IC_{50} values > 100 μg/ml; *Table 2*). Among the triterpenoids **1** and **4**–**9**, 2α,3β,24-trihydroxyurs-12-en-28-oic acid (**6**) and 3β,24-dihydroxyurs-12-en-28-oic acid (**9**) displayed moderate cytotoxic effects with IC_{50} values of 20.9 and 21.7 μg/ml, respectively. The inhibition of MCF-7 cell proliferation by 2α-hydroxyursolic acid (**7**) was formerly carried out by *Liu* and co-workers [29][30]. In their reports, compound **7** at doses of 20 μM and below did not exhibit cytotoxicity toward MCF-7 cells, but significantly inhibited the MCF-7 cell proliferation at doses of 20 μM and above, with an EC_{50} value of *ca* 37.1 μM [29]. In our study, this compound showed an IC_{50} value of 45.9 μg/ml. The IC_{50} value for maslinic acid (**8**) is 85.2 μg/ml, which is comparable to the reported data (*ca.* 34.3 μg/ml) obtained by *Wang et al.* in 2006 [31].

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Experimental Part

General. D-Glucose (CAS# 50-99-7, *Sigma-Aldrich Inc.*) was purchased from *Sigma-Aldrich Trading Co., Ltd*. (Shanghai). The solvents for chromatography were of anal. grade (*Shanghai Chemical Reagents Co. Ltd*, P. R. China), and those for HPLC were HPLC grade (*Jiangsu Hanbon Science & Technology Co. Ltd.*, P. R. China). TLC: Silica gel-precoated plates (GF_{254}, 0.25 mm, *Yantai Kang-Bi-Nuo Silysia*

Table 2. *Cytotoxicity of Compounds* **1**–**12** *against the MCF-7 Cell Line*

Compound[a])	IC_{50} [µg/ml][b])
1	> 100
2	> 100
3	> 100
4	66.0 ± 4.0
5	50.6 ± 2.9
6	20.9 ± 1.4
7	45.9 ± 2.1
8	85.2 ± 3.2
9	21.7 ± 1.4
10	> 100
11	> 100
12	> 100
ADM[c])	1.0 ± 1.9

[a]) The purity of the positive control and tested compounds ranged from 93.2–99.5%; as was determined by analytical HPLC with ELSD detection. [b]) IC_{50} Values (mean ± S.E.) refer to the 50% inhibition concentration, and were calculated from regression using six different concentrations with quadruplicate determinations. [c]) ADM (Adriamycin): positive control.

Chemical Ltd, P. R. China); spots were visualized using UV light (254/365 nm) and/or by spraying with 5% H_2SO_4/EtOH, followed by heating to 120°. Semi-prep. HPLC: *Beckman* system consisting of a *Beckman Coulter System Gold 508* autosampler, *Gold 126* gradient HPLC pumps with a *Beckman System Gold 166* single-wavelength UV detector (254 nm), a *Sedex 80* (*SEDERE*, France) evaporative light-scattering detector (ELSD). A *Beckman Coulter Ultrasphare ODS* column (dp 5 µm, 250 × 10 mm), a *YMC ODS* column (dp 5 µm, 250 × 10 mm), or a *Waters Sugar-Pak*™ *1* column (300 × 6.5 mm i.d.) was used. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh; *Qingdao Ji-Yi-Da Silysia Chemical Ltd.*, P. R. China) and *Sephadex LH-20* (*GE Healthcare Bio-Sciences AB*, Sweden). Optical rotations: *Perkin Elmer 341* polarimeter. UV Spectra: *Libra S35/35PC* UV/VIS spectrophotometer (*Biochrom*, France); λ (log ε) in nm. IR Spectra: *Nicolet NEXUS-670* FT-IR spectrophotometer; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker Avance DRX-500* spectrometer; chemical shifts in δ [ppm], referenced to the residual non-deuterated solvent signals; J in Hz. ESI-MS: *Bruker Esquire 3000plus* instrument; in m/z. HR-ESI-MS: *Bruker Daltonics micrOTOFQII* mass spectrometer; in m/z.

Plant Material. The dried roots of *G. dauhurica* were purchased from *Chongqing Long-Zhou TCM materials Co., Ltd.*, and were originally collected in 2004 from Gansu Province of P. R. China. The sample was identified by Prof. *Jian-Wei Chen* (College of Pharmacy, Nanjing University of TCM). A voucher specimen (NO. 080416) was deposited with the Herbarium of the Shanghai Key Laboratory of Brain Functional Genomics, East China Normal University.

Extraction and Isolation. The dried roots of *G. dahurica* (4.3 kg) were extracted four times with 95% EtOH (20 l) at r.t. to give brownish crude extracts (semi-dry, 850 g). Most of the extract (800 g) was suspended in H_2O (2 l), and then extracted with petroleum ether (PE; 3 × 3 l), AcOEt (3 × 4 l), and BuOH (3 × 4 l). The AcOEt extract (109 g) was chromatographed on a SiO_2 column eluted with CH_2Cl_2/MeOH gradient (15 : 1 to 1 : 1 to MeOH neat) to give eight fractions, *Frs. 1–8*. Compounds **7** (11.6 mg), **8** (2.3 mg), and **9** (4.5 mg) were isolated from *Fr. 2* by CC (SiO_2; PE/AcOEt 2 : 1, 1 : 1, AcOEt neat). *Fr. 3* was subjected to CC (SiO_2; CH_2Cl_2/MeOH gradient 15 : 1 to 1 : 1 to MeOH neat) to yield eight subfractions, *Frs. 3-A–3-H*. Compound **6** (23.0 mg) was isolated from *Fr. 3-D* (4.9 g) by CC (SiO_2; CH_2Cl_2/MeOH 15 : 1 isocratic gradient) and semi-prep. HPLC. The method was an isocratic elution with 20% (*v*/*v*) MeCN in H_2O over 2 min, followed by a linear gradient of MeCN from 20 to 95% over 20 min, then followed by an isocratic elution with 95% MeCN for 10 min (flow rate: 3 ml/min; *YMC ODS* column, 10 mm × 250 cm, **6**: t_R 22.6 min). *Fr. 3-G* (1.2 g) was subjected to CC (SiO_2; CH_2Cl_2/MeOH

20 : 1) to give **10** (132.1 mg), which was further purified by gel permeation chromatography on *Sephadex LH-20* in MeOH. *Fr. 4* was submitted to CC (SiO_2; AcOEt/EtOH 10 : 1 to 1 : 1) to give four subfractions, *Frs. 4-A – 4-D*. Compounds **1** (22.6 mg), **4** (8.5 mg), and **5** (26.0 mg) were obtained from *Fr. 4-C* (7.7 g), and were separated using semi-prep. HPLC eluting with 40% (*v*/*v*) MeCN in H_2O (Flow rate: 3 ml/min; *Ultrasphare ODS* column, 10 mm × 250 cm, **1**: t_R 20.7 min, **4**: t_R 30.7 min, **5**: t_R 28.7 min).

The BuOH fraction (440.0 g) was subjected to CC (SiO_2; AcOEt/EtOH 6 : 1 to 1 : 1 to EtOH neat) to give five fractions, *Frs. 1 – 5*. *Fr. 1* was successively subjected to CC (SiO_2; AcOEt/EtOH/AcOH/H_2O 20 : 1 : 1 : 0.5) to yield **12** (106.0 mg). *Fr. 2* was submitted to CC (SiO_2; CH_2Cl_2/MeOH/H_2O 8 : 0.5 : 0.05 to MeOH neat) to afford four subfractions, *Frs. 2-A – 2-D*. Compound **3** (28.0 mg) was purified from *Fr. 2-B* (1.2 g) using semi-prep. HPLC with 35% (*v*/*v*) MeOH in H_2O (flow rate: 3 ml/min; *Ultrasphare ODS* column, 10 mm × 250 cm, **3**: t_R 12.7 min). Compound **2** (30.1 mg) was obtained by CC (SiO_2; CH_2Cl_2/MeOH/H_2O 16 : 1 : 0.1 to 6 : 1 : 0.15) from *Fr. 2-C* (1.8 g). *Fr. 4* was subjected to CC (SiO_2; CH_2Cl_2/MeOH/H_2O 4 : 1 : 0.1) to give **11** (29.8 mg); this was further purified by semi-prep. HPLC with 21% (*v*/*v*) MeOH in H_2O (flow rate: 3 ml/min; *Ultrasphare ODS* column, 10 × 250 mm, **11**: t_R 23.0 min).

Acid Hydrolysis of **2** *and* **3**. A soln. of **2** (4.5 mg) in 4N HCl (1.0 ml) was heated (85°) for 12 h. The mixture was diluted with H_2O and extracted with AcOEt three times. The aq. layer was neutralized with 2M $NaHCO_3$ and then subjected to HPLC (column: *Waters Sugar-Pak™ 1* (300 × 6.5 mm); column temp.: 25°; detector: *Sedex 80 ELSD*; mobile phase: H_2O; flow rate: 0.2 ml/min). Similarly, acid hydrolysis of **3** (5.6 mg) was accomplished. The mono-sugar component in the aq. layer was confirmed by comparison with the authentic sample of D-glucose.

1β,2α,3α,24-Tetrahydroxyursa-12,20(30)-dien-28-oic Acid (**1**). Colorless powder (MeOH). $[\alpha]_D^{25}$ = +107 (*c* = 0.16, MeOH). IR (KBr): 3423 (br.), 2937, 1691, 1643, 1458, 1238, 1032. ^{1}H-NMR (500 MHz, C_5D_5N): 5.56 (br. *s*, H–C(12)); 4.79 (br. *s*, H_a–C(30)); 4.74 (br. *s*, H_b–C(30)); 4.71 (br. *s*, H_β–C(3)); 4.32 (*dd*, *J* = 9.4, 2.5, H_β–C(2)); 4.23 (*d*, *J* = 9.8, H_α–C(1)); 4.19 (*d*, *J* = 10.9, H_a–C(24)); 3.87 (*d*, *J* = 10.9, H_b–C(24)); 3.16 (br. *d*, *J* = 10.5, H_a–C(11)); 2.75 (*d*, *J* = 11.7, H_β–C(18)); 2.46 (*dd*, *J* = 10.5, 4.9, H_b–C(11)); 2.43 – 2.41 (*m*, H–C(19)); 2.40 – 2.39 (*m*, H_a–C(21)); 2.32 (*m*, H_a–C(15)); 2.26 – 2.24 (*m*, H_b–C(21)); 2.22 – 2.20 (*m*, H_a–C(16)); 2.19 (*dd*, overlapped, H–C(9)); 2.11 – 2.09 (*m*, H_a–C(22)); 2.08 – 2.06 (*m*, H_b–C(16)); 2.01 – 1.99 (*m*, H_b–C(22)); 1.97 – 1.95 (*m*, H–C(5)); 1.76 – 1.75 (*m*, H_a–C(6)); 1.68 (*s*, Me(23)); 1.65 – 1.63 (*m*, H_b–C(6)); 1.63 – 1.62 (*m*, H_a–C(7)); 1.42 – 1.41 (*m*, H_b–C(7)); 1.29 (*s*, Me(25)); 1.23 – 1.20 (*m*, H_b–C(15)); 1.16 (*s*, Me(27)); 1.10 (*s*, Me(26)); 1.09 (*d*, *J* = 7.1, Me(29)). ^{13}C-NMR: see *Table 1*. ESI-MS: 501 ($[M - H]^-$), 1003 ($[2M - H]^-$), 525 ($[M + Na]^+$), 1027 ($[2 M + Na]^+$). HR-ESI-MS: 525.3186 ($[M + Na]^+$, $C_{30}H_{46}NaO_6^+$; calc. 525.3192).

*6′-*O*-Acetylgentiopicroside* (= *(5*R*,6*S*)-5-Ethenyl-5,6-dihydro-1-oxo-1*H*,3*H*-pyrano[3,4-*c*]pyran-6-yl 6-*O*-Acetyl-β-D-glucopyranoside*; **2**). Colorless oil. $[\alpha]_D^{22}$ = – 17 (*c* = 0.15, MeOH). UV (MeOH): 222 (4.88), 269 (4.02). IR (KBr): 3394 (br.), 2925, 1722, 1639, 1381, 1249, 1075, 1039. ^{1}H-NMR (500 MHz, (D_6)DMSO): 7.40 (*s*, H–C(3)); 5.71 (*ddd*, *J* = 17.0, 10.0, 7.0, H–C(8)); 5.64 (br. *s*, H–C(6)); 5.43 (*d*, *J* = 2.5, H–C(1)); 5.21 – 5.19 (*m*, H–C(10)); 5.06 (br. *d*, *J* = 16.5, H_a–C(7)); 4.98 (br. *d*, *J* = 16.5, H_b–C(7)); 4.54 (*d*, *J* = 7.9, H–C(1′)); 4.26 (br. *d*, *J* = 12.0, H_a–C(6′)); 4.08 (*dd*, *J* = 11.5, 6.0, H_b–C(6′)); 3.42 – 3.40 (*m*, H–C(5′)); 3.32 (overlapped, H–C(9)); 3.17 (*dd*, *J* = 9.0, 8.0, H–C(3′)); 3.10 (*dd*, *J* = 9.0, 9.0, H–C(4′)); 2.96 (*dd*, *J* = 8.5, 8.0, H–C(2′)); 2.02 (*s*, AcO). ^{13}C-NMR: see *Table 1*. ESI-MS: 397 ($[M - H]^-$), 399 ($[M + H]^+$). HR-ESI-MS: 399.1285 ($[M + H]^+$, $C_{18}H_{23}O_{10}^+$; calc. 399.1291).

*3′-*O*-Acetylgentiopicroside* (= *(5*R*,6*S*)-5-Ethenyl-5,6-dihydro-1-oxo-1*H*,3*H*-pyrano[3,4-*c*]pyran-6-yl 3-*O*-Acetyl-β-D-glucopyranoside*; **3**). Colorless oil. $[\alpha]_D^{22}$ = – 13 (*c* = 0.06, MeOH). UV (MeOH): 222 (4.38), 268 (3.69). IR (KBr): 3419 (br.), 2925, 1726, 1613, 1381, 1253, 1077, 1039. ^{1}H-NMR (500 MHz, CD_3OD): 7.45 (*s*, H–C(3)); 5.75 (*ddd*, *J* = 17.0, 10.0, 7.0, H–C(8)); 5.66 (*d*, *J* = 3.0, H–C(1)); 5.62 (br. *s*, H–C(6)); 5.24 (*d*, *J* = 17.0, H_a–C(10)); 5.21 (*d*, *J* = 10.0, H_b–C(10)); 5.06 (br. *d*, *J* = 17.5, H_a–C(7)); 4.99 (br. *d*, *J* = 17.5, H_b–C(7)); 4.93 (*dd*, *J* = 9.5, 9.0, H–C(3′)); 4.75 (*d*, *J* = 8.0, H–C(1′)); 3.89 (*dd*, *J* = 11.0, 2.0, H_a–C(6′)); 3.68 (*dd*, *J* = 11.5, 5.5, H_b–C(6′)); 3.44 (*dd*, overlapped, H–C(4′)); 3.44 – 3.42 (*m*, H–C(5′)); 3.31 (overlapped, H–C(9)); 3.28 (*dd*, *J* = 9.0, 8.0, H–C(2′)); 2.09 (*s*, AcO). ^{13}C-NMR: see *Table 1*. ESI-MS: 399 ($[M + H]^+$), 421 ($[M + Na]^+$). HR-ESI-MS: 421.1111 ($[M + Na]^+$, $C_{18}H_{22}NaO_{10}^+$; calc. 421.1111).

1β,2α,3α,24-Tetrahydroxyurs-12-en-28-oic Acid (**4**). Colorless powder (MeOH). $[\alpha]_D^{20}$ = +39 (*c* = 0.40, MeOH). ^{1}H-NMR (500 MHz, C_5D_5N): 5.53 (br. *s*, H–C(12)); 4.66 (br. *s*, H_β–C(3)); 4.27 (*dd*,

$J = 9.5$, 2.3, $H_\beta-C(2)$); 4.17 (*d*, $J = 9.2$, $H_\alpha-C(1)$); 4.15 (*d*, $J = 11.1$, $H_a-C(24)$); 3.82 (*d*, $J = 11.1$, $H_b-C(24)$); 3.15 (br. *dd*, $J = 10.6$, 3.3, $H_a-C(11)$); 2.57 (*d*, $J = 11.1$, $H_\beta-C(18)$); 2.43 (br. *dd*, $J = 10.6$, 7.9, $H_b-C(11)$); 2.28–2.26 (*m*, $H_a-C(15)$); 2.17 (br. *d*, $J = 10.2$, 5.5, H–C(9)); 2.06–2.04 (*m*, $H_a-C(16)$); 1.96–1.94 (*m*, $H_b-C(16)$); 1.93–1.91 (*m*, H–C(5)); 1.89–1.87 (*m*, H–C(22)); 1.72–1.69 (*m*, $H_a-C(6)$); 1.63 (*s*, Me(23)); 1.62–1.60 (*m*, $H_a-C(7)$); 1.60–1.58 (*m*, $H_b-C(6)$); 1.40–1.30 (*m*, H–C(19), $H_b-C(7)$, $H_a-C(21)$, $H_b-C(21)$); 1.25 (*s*, Me(25)); 1.21–1.19 (*m*, $H_b-C(15)$); 1.13 (*s*, Me(27)); 1.08 (*s*, Me(26)); 0.92 (*d*, $J = 6.1$, Me(30)); 0.87 (*d*, $J = 6.1$, Me(29)). ^{13}C-NMR data (C_5D_5N/CD_3OD) are consistent with those in [10][11]. ESI-MS: 503 ($[M-H]^-$), 1007 ($[2M-H]^-$), 527 ($[M+Na]^+$), 1031 ($[2M+Na]^+$). HR-ESI-MS: 527.3348 ($[M+Na]^+$, $C_{30}H_{48}NaO_6^+$; calc. 527.3349).

Cytotoxicity Assay. The MCF-7 human breast cancer cell line was purchased from *American Type Culture Collection* (*ATCC*). The cell line was maintained in *Dulbecco*'s Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 units/ml streptomycin (*GIBCOBRL Gaithersburg*, MD, USA), in a 37° incubator under an atmosphere of 5% CO_2.

Growth inhibition of compounds on the cell line was assessed by a standard MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide)-based colorimetric assay [10][32]. Briefly, the test tumor cells in exponential growth were plated at a final concentration of 2.5×10^3 cells/well in 96-well tissue culture dishes for 24 h. The cells were then treated with compounds at varying concentrations. To exclude phototoxicity [33], the operation process was kept away from bright light, and the cells were incubated in a dark incubator. After 48 h, the cells were incubated in fresh cell culture medium and washed carefully [34]. Then, 100 μl of 0.5 mg/ml MTT was added. After an additional 4 h incubation, the supernatant was discarded, and 150 μl of DMSO were added. After 0.5 h, the optical density (*OD*) was measured at 570 nm using an enzyme-immunoassay instrument. Adriamycin (CAS# 25316-40-9) was used as the positive control. The IC_{50} values were calculated from the curves generated by plotting the percentage of the viable cells *vs.* the test concentration on a logarithmic scale using SigmaPlot 10.0 software.

REFERENCES

[1] Jiangsu New Medical College, 'A Dictionary of the Traditional Chinese Medicine', Shanghai Science and Technology Press, Shanghai, 1986, p. 1764.

[2] H. Y. Hsu, Y. P. Chen, S. J. Shen, C. S. Hsu, C. C. Chen, H. C. Chang, 'Oriental Materia Medica: A Concise Guide. Keats Publishing', New Canaan, Connecticut, 1986, p. 334.

[3] Q. L. Chen, Z. Y. Shi, G. Z. Tu, W. J. Sun, *China J. Chin. Mater. Med.* **2005**, *30*, 1519 (*Chem. Abstr.* **2007**, *147*, 318314).

[4] R. X. Tan, J.-L. Wolfender, L. X. Zhang, W. G. Ma, N. Fuzzati, A. Marston, K. Hostettmann, *Phytochemistry* **1996**, *42*, 1305.

[5] M. S. Y. Khan, A. A. Anees, K. Javed, *Asian J. Chem.* **1997**, *9*, 27.

[6] J. Sun, W. H. Xu, Y. H. Wang, Y. J. Han, G. C. Chen, *Chin. J. Anal. Lab.* **2008**, *27*, 51 (*Chem. Abstr.* **2009**, *151*, 273125).

[7] J. Yang, J. Ma, D. X. Zhou, L. He, *Chin. Tradit. Herb. Drugs* **2006**, *37*, 187.

[8] J. Sun, F. Li, W. Xu, G. Zhou, J. You, G. Chen, *Chromatographia* **2009**, *70*, 1627.

[9] S.-B. Wu, Y.-P. Ji, J.-J. Zhu, Y. Zhao, G. Xia, Y.-H. Hu, J.-F. Hu, *Steroids* **2009**, *74*, 761.

[10] S.-B. Wu, F. Pang, Y. Wen, H.-F. Zhang. Z. Zhao, J.-F. Hu, *Planta Med.* **2010**, *76*, 82.

[11] H. A. Jung, H. Y. Chung, S. S. Kang, S. K. Hyun, H. S. Kang, J. S. Choi, *Arch. Pharmacal Res.* **2005**, *28*, 1127.

[12] H. El Lahlou, N. Hirai, M. Tsuda, H. Ohigashi, *Phytochemistry* **1999**, *52*, 623.

[13] Y. H. Xiao, A. L. Zhang, G. L. Zhang, *Nat. Prod. Res. Dev.* **2007**, *19*, 978.

[14] S. Taniguchi, Y. Imayoshi, E. Kobayashi, Y. Takamatsu, H. Ito, T. Hatano, H. Sakagami, H. Tokuda, H. Nishino, D. Sugita, S. Shimura, T. Yoshida, *Phytochemistry* **2002**, *59*, 315.

[15] Y. Shi, H. L. Wang, B. R. Ma, *Chin. Tradit. Herb. Drugs* **1993**, *24*, 386.

[16] C. A. Boros, F. R. Stermitz, *J. Nat. Prod.* **1991**, *54*, 1173.

[17] R. Kakuda, T. Iijima, Y. Yaoita, K. Machida, M. Kikuchi, *J. Nat. Prod.* **2001**, *64*, 1574.
[18] C. A. Boros, F. R. Stermitz, *J. Nat. Prod.* **1990**, *53*, 1055.
[19] Y.-J. Zhang, C.-R. Yang, *Phytochemistry* **1994**, *36*, 997.
[20] M. Xu, M. Zhang, Y.-J. Zhang, C.-R. Yang, *Helv. Chim. Acta* **2009**, *92*, 321.
[21] Q.-X. Wu, X. Liu, Y.-P. Shi, *Chem. Biodiversity* **2007**, *4*, 175.
[22] Y. Toriumi, R. Kakuda, M. Kikuchi, Y. Yaoita, M. Kikuchi, *Chem. Pharm. Bull*. **2003**, *51*, 89.
[23] J.-A. Kim, N. S. Son, J. K. Son, Y. D. Jahng, H. W. Chang, T. S. Jang, M. K. Ma, S.-H. Lee, *Arch. Pharmacal Res.* **2009**, *32*, 863.
[24] M. Kikuchi, R. Kakuda, M. Kikuchi, Y. Yaoita, *J. Nat. Prod.* **2005**, *68,* 751.
[25] D. Krstić, T. Janković, I. Aljančić, K. Šavikin-Fodulović, N. Menković, S. Milosavljević, *Biochem. Syst. Ecol.* **2004**, *32*, 937.
[26] J. M. Li, F. A. Li, X. Y. Li, H. Zhang, Q. J. Wei, Z. X. Wang, L. Q. Mi, C. N. Xiao, *Nat. Prod. Res. Dev.* **2004**, *16*, 225.
[27] M. Wu, P. Wu, M. Liu, H. Xie, Y. Jiang, X. Wei, *Phytochemistry* **2009**, *70*, 746.
[28] H. Yin, Q. Zhao, F.-M. Sun, T. An, *Phytomedicine* **2009**, *16*, 793.
[29] H. Yoon, R. H. Liu, *J. Agric. Food Chem.* **2008**, *56*, 8412.
[30] X. He, R. H. Liu, *J. Agric. Food Chem.* **2007**, *55*, 4366.
[31] D. Wang, M. Xia, Z. Cui, *Chem. Pharm. Bull.* **2006**, *54*, 775.
[32] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55.
[33] M. Colombain, V. Goll, F. Muyard, C. Girard, F. Bévalot, L. Richert, *Planta Med.* **2001**, *67*, 644.
[34] R. Bruggisser, K. von Daeniken, G. Jundt, W. Schaffner, H. Tullberg-Reinert, *Planta Med.* **2002**, *68*, 445.

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