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Five new cucurbitane triterpenoids with cytotoxic activity from *Hemsleya jinfushanensis*

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ABSTRACT

Five new cucurbitane triterpenoids (hemslepenside B–E (**1**–**3** and **5**)), one new cucurbitane (16,25-*O*-diacetyl-cucurbitane F (**4**)), and six analogues (**6**–**11**) were isolated from the roots of *Hemsleya jinfushanensis*. The structures of **1**–**5** were elucidated using infrared absorption spectroscopy (IR), high resolution electrospray ionization mass spectrometry (HR-ESI-MS), and nuclear magnetic resonance spectroscopy (NMR). These five new compounds exhibited cytotoxic effects against lung adenocarcinoma (H460), colon cancer (SW620) and human prostate cancer cells (DU145), and compound **4** showed significant cytotoxic activity, with IC_{50} values of 0.046, 0.18 and 0.87 μg/mL. This finding suggests that cucurbitane triterpenoids isolated from *H. jinfushanensis* should be studied further as potential anti-cancer drugs.

1. Introduction

Hemsleya pengxianensis var. *jinfushanensis*, which is a traditional Chinese medicinal plant, is widely distributed in southeast China. This plant was traditionally used for the treatment of bronchitis, bacillary dysentery and tuberculosis (NPC, 1977). One of the main types of chemical constituents of this plant, cucurbitane-type compounds, exhibit a wide range of bioactivities, such as cytotoxic activity (Xu et al., 2014; Wu et al., 2002) and anti-HIV-1 activity (Tian et al., 2008). Our studies focus on isolating and investigating the bioactivity of cucurbitane-type compounds and led to the isolation of five new cucurbitane triterpenoids (hemslepenside B–E (**1**–**3,** and **5**) and 16,25-*O*-diacetyl-cucurbitane F (**4**)), together with six known analogues (scandenogenin A (**6**) (Kasai et al., 1988), jinfushanoside G (**7**) (Chen et al., 2012), jinfushanoside B (**8**) (Chen et al., 2005), delavanosides D (**9**) (Chen et al., 2007), 23,24-dihydro-cucurbitane F-16,25-diacetate (**10**) (Qiu and Gao, 2003), and 25-*O*-acetyl-23,24-dihydro-cucurbitane F-2-*O*-β-D-glucopyranoside (**11**) (Jiang, 1981)) (Fig. 1). The 11 identified cucurbitane triterpenoids were evaluated for cytotoxic activity against the human tumor cell lines H460, SW620 and DU145.

2. Results and discussion

Hemslepenside B (**1**) (HRESIMS *m/z*: 636.4474 $[M+NH_4]^+$, calcd for $C_{36}H_{56}O_8NH_4$ 636.4475) was obtained as a white amorphous powder and had a molecular formula of $C_{36}H_{58}O_8$. The IR spectrum (KBr) of **1** exhibited stretching frequencies for OH (3423 cm^{-1}), CHO (1684 cm^{-1}), and CH=CH (1637 cm^{-1}). The ^{13}C NMR (Table 2) and distortionless enhancement by polarization transfer (DEPT) spectra showed resonances for seven methyls, nine methylenes, fourteen methines and six quaternary carbons, including two olefinic carbons at δ_C 144.5 and 156.6 and one carbonyl carbon at δ_C 196.2. The ^1H NMR (Table 1) spectrum showed the presence of seven methyl signals at δ_H 0.78 (3H, s), 0.82 (3H, s), 0.97 (3H, d, J = 6.6 Hz), 1.00 (3H, s), 1.10 (3H × 2, s), and 1.65 (3H, s). Further features were observed at δ_H 5.40 (1H, d, J = 5.0 Hz, an olefinic proton), 5.12 (1H, t, J = 7.1 Hz, an olefinic proton) and δ_H 9.37 (1H, s, an aldehyde proton), which suggested that **1** possesses a tetracyclic-cucurbitane skeleton. The NMR data obtained for **1** were similar to those obtained for jinfushanoside G (**7**) (Chen et al., 2012), except for the presence of an aldehyde proton [δ_H 9.37 (1H, s)] at C-26 in **1** instead of a hydroxymethyl in **7**. This finding was confirmed using the heteronuclear multiple bond correlation (HMBC) (Fig. 2) from δ_H 9.37 (H-26) to δ_C 139.2 (C-25) and 9.7 (C-27). Furthermore, HMBC correlations between δ_H 4.12 (1H, d, J = 7.8 Hz, Glc-H-1) and δ_C 87.36 (C-3), as well as δ_H 3.27 (1H, H-3, brs) and δ_C 106.27 (Glc-C-1), suggested that an additional β-orientation of hexose was linked at C-3, and enzymatic

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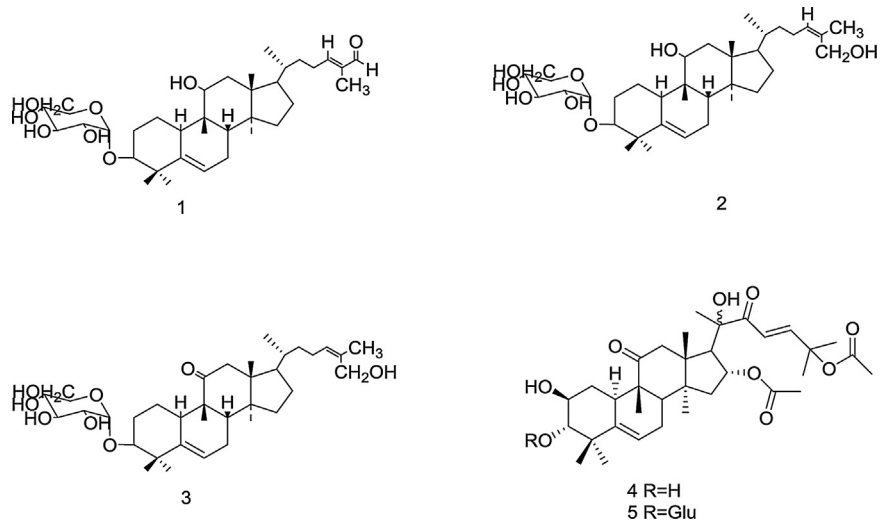

Fig. 1. Structures of compounds **1**–**5**.

Table 1
^{1}H (400 MHz) data for compounds **1**–**5** in d_6-DMSO.

Position	**1**	**2**	**3**	**4**	**5**
1	1.01(1H, m)	1.27(1H, m)	1.31(1H, m)	0.83(1H, m)	0.83(1H, m)
	2.21(1H, m)	2.22(1H,m)	1.84(1H, m)	1.57(1H, m)	1.56(1H, m)
2	1.68(1H, m)	1.71(1H, m)	1.46(1H, m)	4.45(3H, s)	3.27(1H, m)
	1.85(1H, m)	1.85(1H, m)	1.85(1H, m)		
3	3.27(1H, brs)	3.26(1H, brs)	3.27(1H, brs)	4.58(3H, s)	2.83(1H, d, 9.2)
4					
5					
6	5.34(1H, brd, 5.4)	5.34(1H, brd, 5.6)	5.47(1H, d, 5.0)	5.59(1H, m)	5.64(1H, m)
7	1.67(1H, m)	1.72(1H, m)	1.87(1H, m)	1.87(1H, m)	1.84(1H, m)
	2.35(1H, m)	2.32(1H, m)	2.09(1H, m)	2.30(1H, m)	2.27(1H, m)
8	1.53(1H, m)	1.47(1H, m)	1.84(1H, m)	1.85(1H, m)	1.82(1H, m)
9					
10	2.35(1H, m)	2.34(1H, m)	1.85(1H, m)	2.35(1H, m)	2.36(1H, m)
11	3.65(1H, m)	3.09(1H, m)			
12	1.67(1H, m)	1.67(1H, m)	2.19(1H, d, 14.1)	2.38(1H, m)	2.32(1H, m)
	2.5(1H, m)	2.51(1H, m)	3.09(1H, d, 14.4)	3.34(1H, m)	3.36(1H, m)
13					
14					
15	1.00(1H, m)	1.02(1H, m)	1.23(1H, m)	1.21(1H, m)	1.22(1H, m)
	1.21(1H, m)	1.10(1H, m)	1.34(1H, m)	1.79(1H, m)	1.84(1H, m)
16	0.99(1H, m)	0.96(1H, m)	1.81(1H, m)	5.27(1H, t, 12.0)	5.25(1H, t, 12.0)
	1.27(1H, m)	1.23(1H, m)	2.00(1H, m)		
17	1.55(1H, s)	1.48(1H, m)	1.61(1H, m)	2.61(1H, m)	2.62(1H, d, 7.6)
18	0.82(3H, s)	0.81(3H, s)	0.64(3H, s)	0.82(3H, s)	0.83(3H, s)
19	1.00(3H, s)	1.01(3H, s)	0.95(3H, s)	0.94(3H, s)	0.94(3H, s)
20	1.56(1H, m)	1.38(1H, m)	2.32(1H, m)		
21	0.94(3H, d, 8.0)	0.89(3H, d, 6.2)	0.84(3H, d, 6.2)	1.27(3H, s)	1.27(3H, s)
22	1.54(1H, m)	1.00(1H, m)	0.99(1H, m)		
	2.37(1H, m)	1.35(1H, m)	1.30(1H, m)		
23	1.58(1H, m)	1.85(1H, m)	1.81(1H, m)	6.79(1H, d, 16.0)	6.74(1H, d, 16.0)
	2.24(1H, m)	2.02(1H, m)	2.32(1H, m)		
24	6.65(1H, t, 7.2)	5.11(1H, t, 14)	5.12(1H, t, 7.1)	6.95(1H, d, 16.0)	6.94(1H, d, 16.0)
25					
26	9.37(1H, s)	1.66(3H, s)	1.66(3H, s)	1.48(3H, s)	1.48(3H, s)
27	1.65(3H, s)	3.89(2H, d, 5.1)	3.89(2H, d, 5.2)	1.49(3H, s)	1.49(3H, s)
28	0.78(3H, s)	0.77(3H, s)	0.97(3H, s)	1.13(3H, s)	1.13(3H, s)
29	0.97(3H, s)	1.11(3H, s)	0.99(3H, s)	1.07(3H, s)	1.18(3H, s)
30	1.10(3H, s)	0.97(3H, s)	1.14(3H, s)	0.85(3H, s)	0.88(3H, s)
16-OOCCH$_3$					
				1.78(3H, s)	1.78(3H, s)
25-OOCCH$_3$					
				1.95(3H, s)	1.86(3H, s)
3-glc					
1′	4.12(1H, d, 7.8)	4.14(1H, d, 7.8)	4.12(1H, d, 7.8)		4.32(1H, d, 7.8)
2′	2.92(1H, m)	2.91(1H, m)	3.01(1H, m)		3.07(1H, m)
3′	3.14(1H, m)	3.07(1H, m)	3.15(1H, m)		3.16(1H, m)
4′	2.97(1H, m)	3.02(1H, m)	3.11(1H, m)		3.01(1H, m)
5′	3.02(1H, m)	3.04(1H, m)	3.06(1H, m)		3.13(1H, m)
6′	3.61(1H, m)	3.61(1H, m)	3.66(1H, m)		3.63(1H, m)
	3.4(1H, m)	3.41(1H, m)	3.48(1H, m)		3.36(1H, m)

Table 2
^{13}C (150 MHz) data for compounds **1**–**5** in d_6-DMSO.

Position	1	2	3	4	5
1	26.53	26.55	22.02	34.46	33.82
2	29.09	29.1	28.14	70.25	70.88
3	87.36	87.38	86.76	80.74	93.16
4	42.32	42.33	42.14	42.64	42.54
5	144.5	144.51	141.68	142.6	142.09
6	118.07	118.08	118.31	118.39	119.25
7	24.73	24.74	24.42	24.21	24.31
8	43.54	43.56	44.13	42.8	42.68
9	39.89	39.9	49.02	48.13	48.17
10	36.41	37.19	35.92	33.79	33.49
11	77.48	77.48	214.5	213.31	213.39
12	41.01	40.93	49.32	49.39	49.49
13	47.51	47.46	49.32	48.51	48.52
14	49.83	49.82	49.66	50.69	50.73
15	34.75	34.76	34.88	43.82	43.84
16	28.35	28.4	28.13	74.14	74.19
17	50.58	50.63	49.95	55.42	55.49
18	17.35	17.36	17.48	20.56	20.63
19	26.43	26.43	20.62	20.67	20.63
20	35.17	36.08	35.5	79.16	79.21
21	19.2	19.34	18.87	25.17	25.22
22	36.18	36.41	37.06	203.98	204.07
23	26.3	24.64	24.54	121.53	121.55
24	156.63	127.14	127.14	151.09	151.18
25	139.22	136.04	136.17	80.22	80.28
26	196.21	22.13	22.19	27.21	27.25
27	9.75	62.11	60.37	27.29	27.33
28	19.57	19.58	19.16	19.19	19.17
29	28.06	26.43	28.88	25.78	25.61
30	26.43	28.07	26.11	22.64	23.7
16-OOCCH$_3$				170.63	170.71
				22.57	22.61
25-OOCCH$_3$				170.24	170.32
				21.7	21.75
3-glc					
1′	106.27	106.27	106.19		106.4
2′	74.75	74.75	74.81		75.6
3′	78.11	78.11	78.14		77.91
4′	71.07	71.06	71.12		70.57
5′	77.48	77.51	77.57		77.83
6′	62.12	60.31	62.16		62.01

hydrolysis of **1** afforded D-(+)-glucose as the sugar moiety. Thus, compound **1** was determined to be 3β-hydroxycucurbita-5,24(*E*)-diene-11-hydroxy-26-al-3-*O*-β-D-glucopyranoside, which is also named hemslepenside B.

Hemslepenside C (**2**) was obtained as a white amorphous powder. The molecular formula of this compound was determined to be $C_{36}H_{60}O_8$ based on positive ion HRESIMS *m/z*: $[M+NH_4]^+$ 638.4630, calcd for 638.4632. IR absorptions at 3383, 2930 and 1629 cm^{-1} indicated the presence of hydroxyl, methyl and ethenyl groups. The NMR data (Tables 1 and 2) obtained for **2** were very similar to those obtained for jinfushanoside G (**7**) (Chen et al., 2012), except for the obvious variations at the end of the side chains; $\delta_{C\text{-}26}$ was shifted upfield from 68.1 in **7** to 22.1 in **2**, while $\delta_{C\text{-}27}$ was shifted downfield from 14.0 in **7** to 62.1 in **2**, which led to the deduction of the interchanging of groups of CH_2OH and CH_3 at C-26 and C-27 in compounds **2** and **7**, respectively. Combined with the fact that these two compounds have the same molecular formula, we deduced that these compounds were a pair of cis and trans isomers of $\Delta^{24(25)}$. To confirm this deduction, **2** and **7** were subjected to nuclear overhauser enhancement spectroscopy (NOESY) experiments. The NOESY (Fig. 2) correlation was observed between δ_H 5.11 (1H, H-24) and δ_H 1.66 (3H, CH_3-26) in **2**, and there was no correlation between δ_H 5.11 (1H, H-24) and δ_H 3.89 (2H, CH_2OH-27) in **2**. In contrast, the NOESY correlation was observed between δ_H 5.71 (1H, H-24) and δ_H 4.30 (2H, CH_2OH-26) in **7**, and there was no correlation between δ_H 5.71 (1H, H-24) and δ_H 1.83 (3H, CH_3-27) in **7**. The results of the NOESY experiments confirmed that **2** possesses the cis configuration and **7** possesses the trans configuration. Enzymatic hydrolysis of **2** afforded D-(+)-glucose as the composition sugar. The signal at δ_H 4.14 (1H, d, J = 7.8 Hz) suggested the β-orientation for the anomeric proton. Thus, compound **2** was determined to be 3β-hydroxycucurbita-5,24(*E*)-diene-11,27-hydroxy-26-methyl-3-*O*-β-D-glucopyranoside, which is also named hemslepenside C.

Table 3
IC_{50} values (μg/mL) of compounds **1**–**11** against three different human cancer cell lines.

Compound	IC_{50}[a] (μg/mL)		
	H460	SW620	DU145
1	2.12 ± 0.26	0.82 ± 0.24	2.14 ± 0.35
2	6.9 ± 0.13	2.22 ± 0.23	6.3 ± 0.36
3	2.41 ± 0.08	1.21 ± 0.38	3.83 ± 0.53
4	0.046 ± 0.28	0.18 ± 0.10	0.87 ± 0.12
5	9.18 ± 0.14	100.69 ± 0.34	79.25 ± 0.24
6	1.38 ± 0.68	3.23 ± 0.27	2.24 ± 0.94
7	2.22 ± 0.16	347.53 ± 0.59	179.5 ± 0.63
8	1.51 ± 0.11	2.44 ± 0.39	4.06 ± 0.45
9	5.53 ± 0.35	2.2 ± 0.10	5.48 ± 0.35
10	3.98 ± 0.46	4.36 ± 0.19	15.8 ± 0.10
11	3.96 ± 0.28	5.68 ± 0.20	81.48 ± 0.45
Cisplatin[b]	0.12 ± 0.10	0.28 ± 0.71	0.34 ± 0.43
DMSO (0.8%)	0	0	0

[a] Means ± S.D. from three independent experiments (n = 3).
[b] Positive control.

Hemslepenside D (**3**) was determined to be a white amorphous powder with the molecular formula of $C_{36}H_{58}O_8$ based on positive ion HRESIMS [*m/z*: 636.4476 $[M+NH_4]^+$, calcd for 636.4475. The molecular weight of **3** was equal to that of delavanosides D (**9**) (Chen et al., 2007), and the main difference in the NMR data (Tables 1 and 2) between **3** and **9** exists in the end of the side chains, with $\delta_{C\text{-}26}$ shifted upfield from 14 in **9** to 59.87 in **3** and $\delta_{C\text{-}27}$ shifted downfield from 68.0 in **9** to 21.51 in **3**. Based on these findings, we deduced that **3** and **9** were a pair of *cis* and *trans* isomers of $\Delta^{24(25)}$. The NOESY experiments for **3** and **9** further confirmed this deduction. δ_H 5.12 (1H, H-24) and δ_H 1.66 (3H, CH_3-26) in **3** showed a significant correlation, while no correlation was observed between δ_H 5.12 (1H, H-24) and δ_H 3.89 (2H, CH_2OH-27) in **3**. In contrast, the NOESY (Fig. 2) correlation was observed between δ_H 5.71 (1H, H-24) and δ_H 4.31 (2H, CH_2OH-26) in **9**, and there was no correlation between δ_H 5.71 (1H, H-24) and δ_H 1.82 (3H, CH_3-27) in **9**. These results demonstrated that **3** possesses the cis configuration and **9** possesses the trans configuration. HMBC correlations between δ_H 4.12 (Glc-H-1) and δ_C 86.76 (C-3) suggested that an additional hexose was linked at C-3, with the signal at δ_H 4.12 (1H, d, J = 7.8 Hz), suggesting the β-orientation for the anomeric proton, and enzymatic hydrolysis of **3** afforded D-(+)-glucose as the composition sugar. Thus, compound **3** was determined to be 3β-hydroxycucurbita-5,24(*E*)-diene-11-one-26-methy-27-hydroxyl-3-*O*-β-D-glucopyranoside, which is also named hemslepenside D.

16,25-*O*-Diacetyl-cucurbitane F (**4**) was isolated as a white amorphous powder and had a molecular formula of $C_{34}H_{50}O_9$ (HRESIMS *m/z*: 620.3796 $[M+NH_4]^+$, calcd for $C_{34}H_{50}O_9$ NH_4 620.3799). The IR spectrum exhibited resonances for OH (3447 cm^{-1}), C=O (1738 and 1692 cm^{-1}). The ^1H NMR (Table 1) and ^{13}C NMR (Table 2) spectra and DEPT experiments exhibited ten methyl groups (δ_C: 18.97, 20.34, 20.45, 21.48, 22.35, 22.42, 23.99, 25.56, and 26.99), four methylene groups, nine methine groups, and eleven quaternary carbons, which were assigned to a triterpene skeleton. A comparison of the ^{13}C and ^1H NMR spectra of this compound with those of 16,25-*O*-diacetyl-cucurbitane F-2-*O*-β-D-glucopyranoside (Xu et al., 2014) revealed that they were

similar, except for the presence of a D-glucopyranoside. The signal of C-2 was shifted upfield from 83.3 to δ_C 70.04 (t), which further confirmed that no glucopyranosyl moiety was linked at C-2 in **4**. Thus, compound **4** was determined to be 16,25-*O*-diacetyl-cucurbitane F.

Hemslepenside E (**5**) was obtained as a white amorphous powder with the molecular formula of $C_{40}H_{60}O_{14}$, in agreement with the positive ion HRESIMS (positive) *m/z*: 782.4329 $[M+NH_4]^+$, calcd for 782.4327. A comparison of the NMR data (Tables 1 and 2) obtained for **5** and those obtained for **4** indicated that the two compounds were very similar, except for the presence of one additional sugar unit in **5**. It was apparent that the signal of C-3 in **5** was shifted downfield by 12.6 in comparison to **4**, which indicated that D-glucose was linked to C-3. The above deduction was confirmed by the HMBC (Fig. 2) correlation between δ_H 4.32 (1H, Glc-H-1) and δ_C 93.14 (C-3). Partial enzymatic hydrolysis of **5** also yielded **4**, together with D-(+)-glucose, and the sugar was determined based on a comparison with a trade sample. Thus, the structure of **5** was determined to be 2β,3α,20-trihydroxycucurbita-5,23(*E*)-diene-11,22-dione-16,25-diacetyl-3-*O*-β-D-glucopyranoside, which is also named hemslepenside E.

The in vitro experiment revealed that the isolated compounds (**1**–**11**) exhibited obvious cytotoxic activity against H460, SW620 and DU145. As shown in Table 3, most of the compounds displayed cytotoxic activity against the cancer cell lines, and compound **4** exhibited the strongest cytotoxicity (IC_{50} = 0.046, 0.18, and 0.87 μg/ml). Compound **4** was even more active than cisplatin (IC_{50} = 0.12, 0.28, and 0.84 μg/ml) against the H460 and SW620 cell lines, while **5**, which contains the same aglycone as **4** except for one inactive glucopyranoside, was inactive. This finding suggested that glycosidation may reduce the cytotoxic activity. Based on the cytotoxicity results, the relationship between elementary structure and activity could be elucidated as follows. By comparing the structure and cytotoxic activities of **5** with those of **4**, we confirmed that the glycosidation at C-3 may reduce cytotoxic activity. A comparison of the structure and cytotoxic activities of two pairs of isomer compounds (**2** and **7**; **3** and **9**) confirmed that the cis configuration at 24 exhibited stronger activity than its isomer with the trans configuration. By comparing the structure and cytotoxic activities of **1** with those of **7**, we deduced that the aldehyde function at C-26 would enhance cytotoxicity. The IC_{50} values of compound **1** were 2.12, 0.82, and 2.14 μg/ml, and the cytotoxicity of **1** may be related to the aldehyde function at C-26, which is a unique structural feature of this compound in comparison to its analogues. Compound **2**, which possesses the cis configuration, exhibited stronger cytotoxicity against the SW620 and DU145 cell

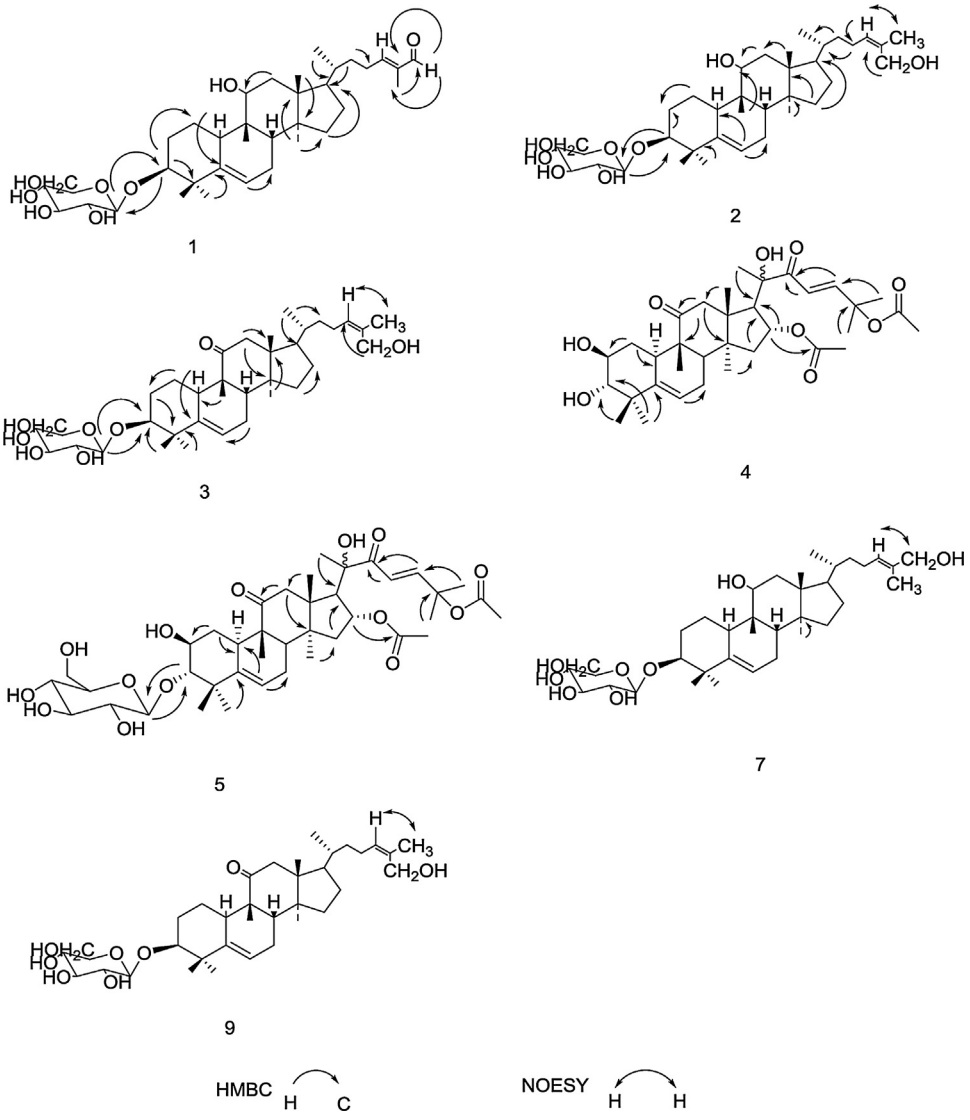

Fig. 2. Key HMBC and NOESY correlations of compounds **1**–**5**,**7**, and **9**.

lines than **7,** which has the trans configuration. In addition, in another pair of isomers, compound **3** (*cis*) showed stronger activity than **9** (*trans*) against all of the tested cell lines, which suggested that the *cis* configuration may increase activity. Compounds **6**, **8** and **10** showed moderate cytotoxic potency, and **5**, **7**, and **11** were inactive in some cell lines (IC_{50} > 20 μg/ml).

3. Experimental

3.1. General

IR spectra were measured using a Thermo Nicolet NEXUS-670 FTIR spectrometer. Optical rotations were run on a JASCO P-2000 polarimeter. HR-ESI-MS was determined using Thermo Scientific Accela PDA and LTQ-Orbitrap XL mass spectrometers (Thermo Fisher Scientific, Germany). NMR spectra were performed on a Bruker Avance III HD NMR spectrometer (Bruker BioSpin). Preparative HPLC was run on a LC-6AD Shimadzu Liquid chromatograph equipped with an SPD-20A UV/VIS Detector and an ODS column (YMC-Pack ODS-A column, 250 mm × 20 mm, 5 μm). Column chromatography (CC) was performed using silica gel (300–400 mesh, Qingdao Marine Chemistry Ltd., China), an ODS C18 (50 μm, YMC Co., Ltd., Kyoto, Japan).

3.2. Plant material

Rhizomes of *H. jinfushanensis* were collected from Chongqing in the People's Republic of China in September 2012 and identified by Dr. Si-rong Yi from the Chongqing Institute of Pharmaceutical Plants. A voucher specimen (voucher number: HA201209B) was deposited in the Institute of Materia Medica at the Shandong Academy of Medical Science in China.

3.3. Extraction and isolation

Air-dried rhizomes (3.0 kg) of *H. jinfushanensis* were cut into slices, minced, extracted exhaustively with 95% EtOH under reflux (3 × 10 L) and filtered. The extract was concentrated to dryness in a vacuum using a rotatory evaporator, concentrated to an aqueous suspension and further extracted successively with EtOAc, $CHCl_3$ and *n*-BuOH. The EtOAc fraction (200 g) was absorbed on 300 g of silica gel (200–300 mesh) and chromatographed over a silica gel (10 × 120 cm, 2 kg) column using a gradient system of $CHCl_2$ (8 L), $CHCl_2$–MeOH (99:1/8 L, 98:2/10 L, 95:5/12 L, 93:7/10 L, 90:10/12 L, 85:15/8 L, 80:20/6 L, and 100:0/5 L) to give five fractions (Fr.A–Fr.E). Fr.A (25 g) was separated by a silica gel (300–400 mesh) and eluted with a gradient system of $CHCl_2$–MeOH (99:1, 98:2, 95:5, 93:7, 90:10, 85:15, 80:20, and 100:0, each 800 mL), resulting in six fractions (Fr.A1–Fr.A6). Fr.A-2 (6.8 g) was absorbed on silica gel (10 g) and eluted with $CHCl_2$–MeOH (99:1, 98:2, 95:5, 93:7, 90:10, 85:15, 80:20, and 100:0, each 500 mL) to afford five subfractions (Fr.A-2-1 to Fr.A-2-5). Subfraction A-2-4 (900 mg) was purified via pre-HPLC (MeOH:H_2O = 65:35, 7 mL/min, 210 nm) to give compounds **4** (30 mg, t_R = 35.7 min), **6** (21 mg, t_R = 40.2 min) and **10** (15 mg, t_R = 44.9 min). Fr.B (15.3 g) was absorbed on 20 g of silica gel and chromatographed with $CHCl_2$–MeOH (99:1, 98:2, 95:5, 93:7, 90:10, 85:15, 80:20, and 100:0, each 300 mL) to give Fr. B1–B4. Fr. B-2 was further fractionated on an RP-18 column and eluted with MeOH-H_2O (MeOH:H_2O = 75:25, 7 mL/min, 210 nm) to afford compounds **1** (28.0 mg, t_R = 51.1 min) and **5** (18.0 mg, t_R = 32.7 min). Fraction C (31.7 g) was fractionated using silica gel column chromatography (CC) with a gradient elution of $CHCl_2$, $CHCl_2$–MeOH (99:1, 98:2, 95:5, 93:7, 90:10, 85:15, 80:20, and 100:0, each 1.2 L) to yield 6 fractions (Fr.C1–Fr.C6). Fr.C-3 was further purified via ODS CC and pre-HPLC (MeOH:H_2O = 70:30, 7 mL/min, 210 nm) to give compounds **3** (32.5 mg, t_R = 29.2 min) and **9** (43.0 mg, t_R = 36.3 min). Fr.C-4 was further subjected to pre-HPLC to give compound **11** (48.0 mg, 65:35, t_R = 24.4 min). Fr.D (45 g) was eluted with $CHCl_2$, $CHCl_2$–MeOH (99:1, 98:2, 95:5, 93:7, 90:10, 85:15, 80:20, and 100:0, each 1.5 L), resulting in 11 subfractions (Fr.D1–Fr. D11). Compounds **2** (15.0 mg, t_R = 10.9 min), **7** (46.0 mg, t_R = 21.6 min) and **8** (62.0 mg, t_R = 28.9 min) were obtained from Fr.D-2 using ODS CC and pre-HPLC (MeOH:H_2O = 75:25, 7 mL/min, 210 nm).

3.4. Structural elucidation of compounds ***1–5***

3.4.1. Hemslepenside B (***1***)

White amorphous powder; $[\alpha]_D^{25}$: +28.15 (c = 0.1, MeOH); IR (KBr) ν_{max} 3423, 2947, 2705, 1684, 1637, 1458, 1382, 1251, 1167, 1074 and 1033 cm^{-1}; ^1H NMR data see Table 1; ^{13}C NMR data see Table 2; and ESI-HRMS m/z 636.4474 ($[M+NH_4]^+$) (calcd for $C_{36}H_{58}O_8NH_4$, 636.4475).

3.4.2. Hemslepenside C (***2***)

White amorphous powder; $[\alpha]_D^{25}$: +44.37 (c = 0.1, MeOH); IR (KBr) ν_{max} 3383, 2930, 1629, 1466, 1381, 1168, 1075, 1010 and 943 cm^{-1}; ^1H data see Table 1; ^{13}C NMR data see Table 2; and ESI-HRMS m/z 638.4630 ($[M+NH_4]^+$) (calcd for $C_{36}H_{60}O_8$ NH_4, 638.4632).

3.4.3. Hemslepenside D (***3***)

White amorphous powder; $[\alpha]_D^{25}$: +44.53 (c = 0.1, MeOH); IR (KBr) ν_{max} 3406, 2963, 2882, 1691, 1462, 1384, 1167, 1074 and 1015 cm^{-1}; ^1H data see Table 1; ^{13}C NMR data see Table 2; and ESI-HRMS m/z 636.4476 ($[M+NH_4]^+$) (calcd for $C_{36}H_{58}O_8$ NH_4, 636.4475).

3.4.4. 16,25-O-Diacetyl-cucurbitane F (***4***)

White amorphous powder; $[\alpha]_D^{25}$: +11.38 (c = 0.1, MeOH); IR (KBr) ν_{max} 3450, 2976, 2930, 1738, 1692, 1631, 1431, 1371, 1248, 1058 and 1024 cm^{-1}; ^1H data see Table 1; ^{13}C NMR data see Table 2; and ESI-HRMS m/z 620.3796 ($[M+NH_4]^+$) (calcd for $C_{34}H_{49}O_9$ NH_4, 620.3799).

3.4.5. Hemslepenside E (***5***)

White amorphous powder; $[\alpha]_D^{25}$: +47.15 (c = 0.1, MeOH); IR (KBr) ν_{max} 3447, 2975, 2879, 1738, 1692, 1629, 1432, 1371, 1252, 1024 cm^{-1}; ^1H data see Table 1; ^{13}C NMR data see Table 2; and ESI-HRMS m/z 782.4329 ($[M+NH_4]^+$) (calcd for $C_{40}H_{60}O_{14}$ NH_4, 782.4327).

3.5. Determination of the glucose configuration in compounds ***1–3*** *and* ***5***

3.5.1. Enzymatic hydrolysis

Compounds **1**–**3** and **5** (4 mg) were hydrolyzed with cellulase (Worthington, USA, each 20 mg) in H_2O (each 4 mL), and the mixtures were incubated for 120 h at 40 °C. The reaction mixture was extracted with EtOAc, and the aqueous layers were evaporated to dryness to obtain the sugar fractions. The sugar residue and authentic samples of D-(+)-glucose and L-(−)-glucose were dissolved in H_2O (each 1 mL) and mixed with EtOH (each 1 mL) that contained (*S*)-(−)-α-methylbenzylamine (7 μl) and $NaBH_3CN$ (8 mg), respectively. The mixture was incubated for 4 h at 40 °C. Glacial acetic acid (0.2 mL) was then added and concentrated to dryness. Acetic anhydride (0.3 mL) and pyridine (0.3 mL) were added to the residue and acetylated at room temperature for 24 h. After the pyridine was removed, the aqueous solution of the reaction mixture was subjected to a Cleanert C18-N column (Agela), and H_2O, 20% CH_3CN and 50% CH_3CN (15, 15 and 10 mL) were used as the eluents.

*3.5.2. HPLC analysis of compounds **1**–**3** and **5***

The 50% CH_3CN fraction was analyzed via HPLC under the following conditions: Agilent SB-C18 column (4.6 × 250 mm, 5 μm); mobile phase, 40% CH_3CN; flow rate, 0.8 mL/min; and DAD detection, 230 nm. The absolute configuration of the monosaccharide was determined by comparing the retention time t_R (min) of the derivatives (1-[(*S*)-*N*-acetyl-a-methylbenzylamino]-1-deoxyglucitol acetate derivatives) with those of authentic samples: 20.31 min (derivative of D-glucose) and 19.35 min (derivative of L-glucose). All of the sugar moieties were determined to be D-(+)-glucose based on the finding that the retention time of the sugars in compounds **1**, **2**, **3** and **5** were 20.63, 20.67, 20.62, 20.65 min, which was determined to correspond to D-(+)-glucose.

3.6. Cytotoxicity assay

The H460, SW620 and DU145 cell lines were obtained from the Institute of Materia Medica in the Chinese Academy of Medical Sciences. The cells were cultured in RPMI-1640 medium (Gibco, USA) containing 15% heat-inactivated fetal bovine serum (Tianhang, Zhejiang, China), 50 U/mL penicillin and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Tumor cells in the exponential growth phase were seeded in 96-well micro plates at a density of 1×10^4 cells/well for 24 h. After incubation, each test sample (cisplatin and 11 cucurbitane triterpenoids) was added to the medium at final concentrations of 100, 10, 1, 0.1, and 0.01 μg/mL, and cisplatin served as a positive control. After 48 h of incubation, 10 μL of an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) solution (5 mg/ml in PBS) were added to each well, and the samples were re-incubated for an additional 4 h (37 °C). The MTT dye was removed, and DMSO (200 μL/well) was added. Gentle stirring in a gyratory shaker enhanced dissolution, and the optical density (OD) was measured using a multifunctional-counter instrument (PerkinElmer Victor 1420, USA) at 570 nm. The inhibition (%) was calculated as $(OD_0–OD_S)/OD_0 \times 100\%$, where OD_0 and OD_S are the values of the cells without and with the test agent. The results (IC_{50}) were defined as the concentration of samples that inhibited 50% of control growth after the incubation period. All 11 compounds were evaluated, and the obtained data are presented as the mean of three independent experiments. In addition, each compound was tested in three parallel wells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.10.019.

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