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Purine-containing cucurbitane triterpenoids from *Cucurbita pepo cv dayangua*

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Abstract

Phytochemical investigation of the fruits of *Cucurbita pepo cv dayangua* led to the isolation of cucurbitaglycosides A (1) and B (2). This is the first report of cucurbitane triterpenoids with a purine unit. Their structures were elucidated mainly based on interpretation of HRESIMS results, as well as 1D and 2D NMR spectra. Cucurbitaglycosides A and B showed cytotoxic activity against the human epithelial carcinoma cell line HeLa with IC50 of 17.2 and 28.4 μg/mL, respectively.

Keywords: *Cucurbita pepo cv dayangua*; Cucurbitaceae; Cucurbitane triterpenoids; Adenine; Adenosine; Cytotoxic activity

1. Introduction

Cucurbita pepo cv dayangua, which is distributed throughout the Duo Lun county of the Inner Mongolia autonomous region (Yang et al., 2000), has been used as a traditional folk medicine to treat cold and alleviate ache (Yang et al., 2000). Previous pharmacological tests have shown that it possesses antiviral, anti-inflammatory, and analgesic effects (Wang et al., 2001; Ding et al., 2002). In our search for new antitumor agents, we found that the ethanolic extract of fruits of this plant had a dose-dependent inhibitory effect on the growth of HeLa cells. Phytochemical investigation of this ethanolic extract led to isolation of several cucurbitane and hexanorcucurbitane glycosides and other types of triterpenoids from the fruits of *Cucurbita pepo cv dayangua* (Wang et al., 2005; Ge et al., 2006). Herein, is reported the isolation, structural elucidation, and biological activities of **1** and **2**.

Cucurbitacins possess the biogenetically unusual 10α-cucurbit-5-ene-[19(10 → 19β)-*abeo*-10α-lanostane] skeleton (Pryzek, 1979), which has mainly been reported in the Cucurbitaceae but is also known to occur in other plant families (Lavie and Glotter, 1971; Guha and Sen, 1975; Bauer and Wagner, 1983). Cucurbitacins are well-known for their cytotoxic behavior and broad range of bioactivities, such as antitumor, antiinflammatory, antimicrobial, antihelminthic, and cardiovascular properties (Agil et al., 1999; Peters et al., 1999, 2003; Oh et al., 2002; Blaskovich et al., 2003; Jayaprakasam et al., 2003). Cucurbitaglycosides A (1) and B (2) have unusual side-chains with attached adenosine/adenine and represent the first examples of this type of cucurbitacin.

The ethanolic extract of air-dried fruits was subjected to column chromatography on macroporous absorption resin (AB-8), silica gel and Sephadex LH-20 to give cucurbitaglycosides A (6.3 mg) and B (7.5 mg) as colorless powders.

2. Results and discussion

Compound**1** was obtained as a white amorphous powder, m.p. 148–150 °C (MeOH). $[\alpha]_D^{20}$ − 43.8 (*c* 0.05, MeOH). The absorption bands in its IR spectrum

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suggested the presence of hydroxyl and/or amino (3383 cm^{-1}), and conjugated carbonyl (1685 and 1666 cm^{-1}) functional groups. The positive ESIMS exhibited a $[M+H]^+$ at *m/z* 944.4. The molecular formula $C_{46}H_{65}N_5O_{16}$ was established by HRESIMS at *m/z* 944.4514 $[M+H]^+$ (calcd. 944.4505 for $C_{46}H_{66}N_5O_{16}$). The ^{13}C NMR spectra (APT, DEPT) indicated the presence of 46 carbon signals (Table 1), including eight methyl, six methylene, 18 methine and 14 quaternary carbons. The presence of two trisubstituted double bonds was suggested by signals at δ_c 137.0, 146.9 (C), 120.9 and 120.7 (CH). Resonances for α,β unsaturated ketonic carbonyls and two ketonic carbonyls (Fig. 1) were observed at δ_c 197.0 and δ_c 214.0, 213.9, respectively.

The structure of **1** was deduced based on analyses of the ^1H–^1H COSY, HMQC and HMBC spectra. In the ^1H–^1H COSY spectrum, a methine proton at δ_H 3.66 (H-10) correlated with an olefinic proton at δ_H 6.44 (H-1). In the HMBC spectrum, the methine proton at δ_H 3.66 (H-10) correlated with olefinic carbons at δ_c 146.9 (C-2) and δ_c 120.7 (C-1). The proton at δ_H 6.44 (H-1) correlated with an olefinic carbon at δ_c 146.9 (C-2) and a carbonyl carbon at δ_c 197.0 (C-3). The above correlations demonstrated that **1** contains partial structure A (Fig. 2). Methyl group protons at δ_H 1.14, 1.28 (H_3-28, 29) showed correlations (HMBC) with a quaternary carbon at δ_c 49.7 (C-4) and an olefinic carbon at δ_c 137.0 (C-5), which suggested the presence of partial structure B (Fig. 2). Meanwhile, two protons δ_H 6.44, 3.66 (H-1, 10) showed correlations to C-5 and the geminal methyl protons at δ_H 1.14, 1.28 (H_3-28,29) were correlated with the carbonyl carbon at δ_c 197.0 (C-3). This information allowed us to elucidate the structure of ring A, and also indicated the presence of an double bond at $\Delta^{5,6}$. Similarly, the partial structures of rings B, C and D of the aglycone moiety were established (Fig. 2).

In the HMBC spectrum, the methyl protons at δ_H 1.55 and 1.50 (H_3-26, H_3-27) correlated with the same oxygenated quaternary carbon at δ_c 72.9 (C-25) and the methine carbon at δ_c 55.9 (C-24), which allowed the assignment of C-24, 25, 26, 27. The methyl proton at δ_H 1.56 (H-21) showed long-range correlations to the oxygenated quaternary carbon at δ_c 80.2 (C-20), the carbonyl carbon at δ_c 214.0 (C-22) and the methine carbon at δ_c 58.8 (C-17), while methylene protons at δ_H 3.77 and 4.05 (H_2-23) correlated with the carbonyl carbon at δ_c 214.0 (C-22) and the methine carbon at δ_c 55.9 (C-24). Thus the structure of the side-chain was deduced as shown in Fig. 2.

The presence of the β-D-glucopyranosyl moiety was supported by ^{13}C NMR spectroscopic data and was further confirmed by acid hydrolysis of **1** in 2 N HCl at 80 °C for 6 h, which resulted in release of glucose as identified by PC and TLC comparisons of the hydrolyzate with an authentic sugar sample. The configuration of the glucopyranosyl was assigned to be β-D-based on the coupling constant of the anomeric proton (Sang et al., 1999), and the preponderance of the β-D-glucopyranosyl unit in the natural products. An HMBC correlation was observed between an anomeric proton signal at δ_H 4.89 (H-1′) and an olefinic carbon at δ_C 146.9 (C-2), which indicated that the glucopyranosyl moiety was connected at C-2 of the triterpene nucleus.

The presence of five sp^2 carbon signals (δ_c 156.2, 152.9, 149.2, 140.5, 123.5) in the downfield region of the ^{13}C spectrum, combined with the molecular formula $C_{46}H_{66}O_{16}N_5$ derived from HRESIMS, indicated the presence of an

Table 1
NMR spectroscopic data of compound **1** in C_5D_5N

No.	^{13}C/ppm[a]	^1H/ppm[b] mult. (J/Hz)	HMBC $^1H \rightarrow {}^{13}C$[c]
1	120.7	6.44 (1H, *s*)	C5, 2, 3
2	146.9		
3	197.0		
4	49.7		
5	137.0		
6	120.9	5.64 (1H, *m*)	
7	23.9	2.12 (1H, *m*), 1.97 (1H, *m*)	C9
8	41.8	1.91 (1H, *m*)	C30, 7, 10, 14, 6
9	48.7		
10	35.6	3.66 (1H, *s*)	C19, 9, 1, 5, 2
11	213.9		
12	49.5	3.21 (1H, *d*, 14.7)	C18, 13, 14, 11
		2.81 (1H, *d*, 14.7)	
13	50.9		
14	48.6		
15	46.5	1.88 (1H, *m*), 1.64 (1H, *m*)	C30, 16
16	72.4	5.03 (1H, *m*)	
17	58.9	2.86 (1H, *d*, 7.0)	C18, 13, 16
18	20.3	1.17 (3H, *s*)	C12, 13, 17
19	20.2	0.99 (3H, *s*)	C10, 8, 9, 11
20	80.3		
21	28.1	1.56 (3H, *s*)	C17, 20, 22
22	214.0		
23	39.3	4.05 (1H, *m*), 3.77 (1H, *m*)	C22, 24
24	55.9	5.55 (1H, *t*, 9.5)	C1″, 22
25	72.9		
26	25.2	1.55 (3H, *s*)	C27, 25, 24
27	27.6	1.50 (3H, *s*)	C24, 25
28	20.8	1.41 (3H, *s*)	C29, 4, 5, 3
29	27.6	1.28 (3H, *s*)	C28, 4, 5, 3
30	18.3	1.44 (3H, *s*)	C8, 15, 14
Gl1′	100.6	5.50 (1H, *d*, 7.7)	C2
2′	74.5	4.24 (1H, *t*, 8.1)	
3′	78.7	4.07 (1H, *m*)	
4′	70.7	4.50 (1H, *t*, 9.1)	
5′	78.5	4.31 (1H, *m*)	
6′	62.0	4.72 (1H, *m*), 4.58 (1H, *m*)	
1″	156.2		NH 7.87 (1H, 9.1)
3″	152.9	8.56 (1H, *s*)	C5″, 1″
5″	149.2		
7″	140.5	8.66 (1H, *s*)	C9″, 5″
9″	123.2		
1‴	90.9	6.66 (1H, *d*, 5.3)	
2‴	75.4	5.46 (1H, br *s*)	
3‴	70.4	4.97 (1H, *m*)	
4‴	87.9	4.73 (1H, *m*)	
5‴	63.1	4.29 (1H, *m*)	
		4.12 (1H, *m*)	

[a] Recorded at 150 MHz.
[b] Recorded at 600 MHz.
[c] Determined by HMBC experiments.

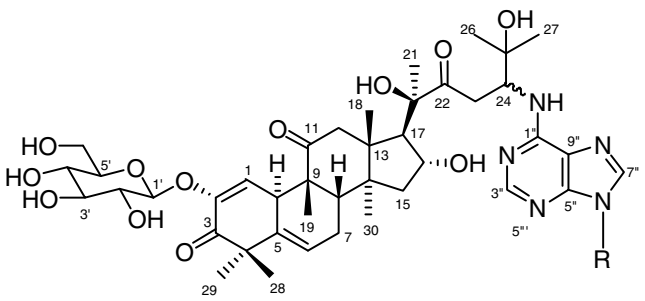

Fig. 1. Compounds **1** and **2**.

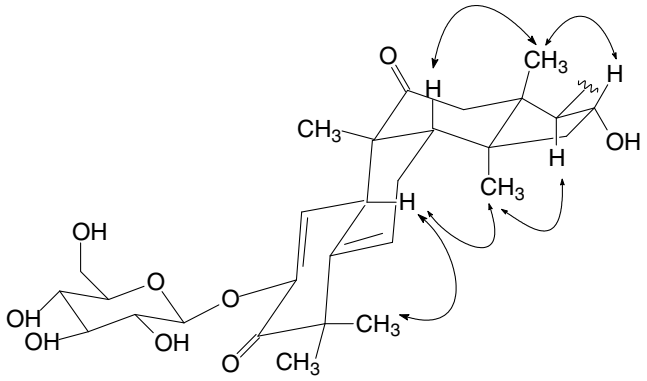

Fig. 3. Key NOEs correlations of compound **1**.

unsaturated heterocycle. In the HMBC spectrum, the olefinic proton at δ_H 8.56 (1H, *s*, H-3″) correlated with carbons at δ_c 149.2 (C-5″) and 156.2 (C-1″), and another olefinic proton at δ 8.66 (1H, *s*, H-7″) showed long-range correlations to the carbons at δ_c 123.2 (C-9″) and 149.2 (C-5″). This information was consistent with the presence of a purine ring system (Rong and Zhu, 2002). The carbon signals at δ_c 90.9, 75.4, 70.4, 87.9 and 63.1 were assigned to a ribonucleoside unit based on the ^{13}C chemical shift (Jones et al., 1970). The long-range correlation (HMBC) from the anomeric proton at δ_H 6.66 (H-1‴) to the olefinic carbons of the purine moiety at δ_c 149.2 (C-5″) and 140.5 (C-7″) indicated that the ribose unit was connected to N-6″ of the purine moiety. The presence of adenine and ribose moieties was further confirmed by acidic hydrolysis of 1 in 2 N HCl at 100 °C for 6 h, which resulted in release of adenine and ribose as identified by TLC comparisons of the hydrolyzate with authentic samples (Zhao et al., 1987; Wu et al., 1996; Wang, 1959). An HMBC correlation from the proton at δ_c 5.55 (H-24) to the olefinic carbon at δ 156.2 (C-1″), in conjunction with chemical shifts of C-24, that the amino group at C-1″ of the purine moiety was connected to C-24 of the triterpene nucleus.

Analysis of the NOESY spectrum of one (Fig. 3) showed that it has the expected relative stereochemistry for a cucurbitacin (Molholland et al., 1997; Pryzek, 1979; Chen et al., 2005). Thus cucurbitaglycoside A is 6N-(2-β-D-glucopyranosyloxy-16*R*,20*R*,25-trihydroxycucurbita-1,5-diene-3,11,22-trione-24-yl)-adenosine (**1**).

Compound **2** was obtained as a white amorphous powder, m.p. 142–143 °C (MeOH), $[\alpha]_D^{20}$ – 53.8 (*c* 0.06, MeOH). The IR spectrum also showed the presence of hydroxyl and/or amino (3383 cm^{-1}), and conjugated carbonyl (1685 and 1666 cm^{-1}) functional groups similar to those of **1**. HRESIMS *m/z* at 812.9437 $[M+H]^+$established that its molecular formula was $C_{41}H_{58}O_{12}N_5$. Its structure was elucidated based on comparison of the NMR spectroscopic data with those of **1**. The ^1H and ^{13}C NMR spectral

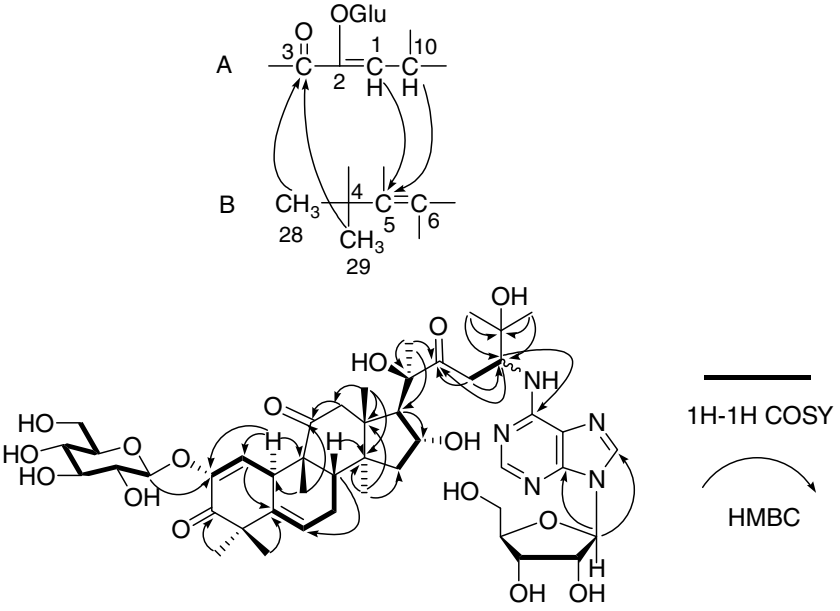

Fig. 2. Partial structures A and B and key ^1H–^1H COSY (thick lines), HMBC (arrows), correlations of compound **1**.

data of **2** (Table 1) were comparable to those of compound **1**, except that NMR signals due to the ribose unit were absent. The presence of the adenine moiety of **2** was further confirmed using the same methods as for the structure elucidation of **1**. Therefore compound **2**, cucurbitaglycoside B, is 6N-(2-β-D-glucopyranosyloxy-16*R*,20*R*,25-trihydroxycucurbita-1,5-diene-3,11,22-trione-24-yl)-adenine (**2**), which was further substantiated by HMQC, HMBC and NOESY experiments, was designated as Cucurbitaglycoside B. The detailed NMR spectroscopic data are presented in Table 2.

In in vitro bioassays, cucurbitaglycosides A (1) and B (2) showed weak cytotoxic activity against HeLa cells with IC50 values of 17.2 and 28.4 μg/mL, respectively.

While more than 200 cucurbitane triterpenoids with various of biological activities have been isolated from plants of different families, this is the first report of cucurbitane triterpenoids with a purine unit. Since it is the first isolation of cucurbitane triterpenoids with a purine unit, the possibility that **1** and **2** are artefacts needs to be excluded. The EtOH extracts of *Cucurbita pepo cv dayangua* were freshly prepared with cold and boiling EtOH, respectively. cucurbitane triterpenoids **1** and **2** could be detected by HPLC in both extracts. Therefore, we concluded that **1** and **2** are not artefacts of the extraction process.

Table 2
NMR spectroscopic data of compound **2** in C_5D_5N

No.	^{13}C/ppm[a]	^{1}H/ppm[b] mult. (J/Hz)	HMBC $^{1}H \rightarrow {}^{13}C$[c]
1	121.0	6.46 (1H, *s*)	C5, 2, 3
2	146.9		
3	197.4		
4	49.3		
5	137.1		
6	120.2	5.64 (1H, *m*)	
7	23.9	2.12 (1H, *m*), 1.97 (1H, *m*)	C 1
8	41.9	1.88 (1H, *m*)	C30, 7, 10, 1, 6
9	49.2		
10	35.7	3.64 (1H, *s*)	C19, 4, 1, 5, 2
11	213.9		
12	49.5	3.06 (1H, *d*, 14.3) 2.66 (1H, *d*, 14.3)	C18, 13, 11
13	50.8		
14	48.7		
15	46.3	1.84 (1H, *m*), 1.68 (1H, *m*)	C30, 13, 16
16	70.5	4.93 (1H, *m*)	C14, 20
17	58.7	2.90 (1H, *d*, 7.1)	C18, 13, 20
18	20.4	1.07 (3H, *s*)	C14, 13, 17
19	20.2	0.98 (3H, *s*)	C10, 8, 11
20	80.5		
21	25.1	1.47 (3H, *s*)	C17, 20, 22
22	213.4		NH 4.35
23	38.1		
24	61.0	5.60 (1H, *m*)	NH 4.35
25	72.4		
26	28.3	1.54 (3H, *s*)	27, 24, 25
27	27.8	1.23 (3H, *s*)	26, 24, 25
28	20.9	1.41 (3H, *s*)	29, 5, 4, 3
29	27.8	1.34 (3H, *s*)	28, 4, 5, 3
30	18.3	1.45 (3H, *s*)	8, 15, 14
Glc1′	100.7	5.53 (1H, *d*, 7.7)	2
2′	74.7	4.25 (1H, *t*, 8.1)	1′
3′	78.8	4.07 (1H, *m*)	
4′	70.8	4.46 (1H, *t*, 9.1)	6′, 3′
5′	78.4	4.33 (1H, *m*)	4′, 2′
6′	62.1	4.69 (1H, *m*), 4.56 (1H, *m*)	
3″	153.1	8.66 (1H, *s*)	5″, 1″
5″	149.5		
7″	142.2	8.55 (1H, *s*)	9″, 5″
9″	120.4		

[a] Recorded at 150 MHz.
[b] Recorded at 600 MHz.
[c] Determined by HMBC experiments.

3. Experimental

3.1. General experimental methods

Melting points were measured on a Yanaco MP-S3 micromelting point apparatus and are uncorrected. The NMR spectra were obtained on a Bruker ARX-600 (600 MHz for ^{1}H and 150 MHz for ^{13}C) spectrometer in C_5D_5N solution with tetramethylsilane (TMS) as an internal standard. HRESIMS data were measured with a Bruker AOEXIII 7.0 TESLA FTMS. Separation and purification were performed by column chromatography on silica gel (Merck 3374, 70–230 mesh,) and Sephadex LH-20 (Pharmacia)CC. Optical density was measured by a microplate autoreader (Nanjing, China).

3.2. Plant material

The fruits of *Cucurbita pepo cv dayangua* were collected from the agricultural center of Jilin University, Jilin Province, China, in August 2002. The specimen was botanically identified by An-min Lu (Institute of Medicinal Plant Development of the Chinese Academy of Medical Science, China). A voucher specimen has been deposited in the Herbarium of the Institute of Medicinal Plant Development of the Chinese Academy of Medical Science, China.

3.3. Extraction and isolation of plant constituents

The air-dried fruits (15 kg) were extracted with 95% aqueous EtOH at room temperature. The concentrated extract was applied to a column of macroporous absorption resin (AB-8) and eluted with EtOH–H_2O (7:3, v/v), and the eluate was then evaporated to dryness under vacuum to give a residue (200 g). The residue was fractionated via silica gel CC eluting with a gradient increasing MeOH (0–50%) in CHCl3 to give 15 fractions. Fractions 8 and 11 (2 and 1.5 g, respectively) were further purified using Sephadex LH-20CC eluting with $CHCl_3$–MeOH (1:1) to give cucurbitaglycosides A (6.3 mg) and B (7.5 mg) as white powders.

Cucurbitaglycoside A(**1**), White amorphous powder, m.p. 148–150 °C (MeOH), UV (MeOH) λ_{max} (log ε) 203(4.45), 266(4.41) nm, for ^{1}H and ^{13}C NMR (C_5D_5N) spectroscopic data, see Table 1, HRESIMS *m/z* 944.4514 $[M+H]^+$ (calcd 944.4505 for $C_{46}H_{66}N_5O_{16}$).

Cucurbitaglycoside B(**2**), White amorphous powder, m.p. 142–143 °C (MeOH), UV (MeOH) λ_{max} (log ε) 205(4.49), 269(4.47) nm, ^{1}H and ^{13}C NMR (C_5D_5N) see Table 2, HRESIMS *m/z* 812.9437 $[M+H]^+$ (calcd for $C_{41}H_{58}N_5O_{12}$: 812.9429).

3.4. Cytotoxic activity assay

Human HeLa cells were seeded into each well of a 96-well microplate in 100 μL of Eagle's minimum essential medium (Gibco Co. Ltd.) containing 10% fetal bovine serum (Gibco Co. Ltd.), 1% glutamine (Sigma) and gentamycin (100 μg/ml) (Shanghai 4th Pharm Ltd.). The plates were then cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Drugs were added to the culture at various concentrations 1 day after subculture. At 4 days, 20 μL of MTT solution (5 mg/mL) (Sigma) was added to each well. After a further 4 h of incubation, DMSO 100 μL was added to the wells and the formazan crystals in each were dissolved by vibration. Optical density was measured by a microplate autoreader (Nanjing, China) at 590 nm and 700 nm. Data were obtained from triplicate wells. Adriamycin (HCl salt) (Zhejiang Haizheng Pharm Ltd.) was used as a control treatment.

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