

Cucurbitane-Type Triterpenes with Anti-proliferative Effects on U937 Cells from an Egyptian Natural Medicine, *Bryonia cretica*: Structures of New Triterpene Glycosides, Bryoniaosides A and B

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The 90% aqueous ethanol extract of an Egyptian natural medicine, the roots of *Bryonia cretica* L., was found to exhibit a strong inhibitory effect on the proliferation of human leukemia U937 cells. By bioassay-guided fractionation, we isolated two new cucurbitane-type triterpene glycosides, bryoniaosides A and B, were isolated from the roots of *Bryonia cretica* L. together with 16 known cucurbitane-type triterpenes and glycosides. The chemical structures of bryoniaosides A and B were determined on the basis of chemical and spectroscopic evidence. Effects of principal cucurbitane-type triterpenes (cucurbitacins B, D, E, and J, 23,24-dihydrocucurbitacins B and E, and hexanorcucurbitacin D) on proliferation of the cells were examined. Cucurbitacins B and E showed the greater cytotoxic effects with IC₅₀ values of 9.2 and 16 nM after 72 h, and their IC₅₀ values were equivalent to that of camptothecin. An α,β -conjugated ketone moiety at the 22–24-positions and an acetoxy group at the 25-position are essential for the strong activity.

Key words *Bryonia cretica*; bryoniaoside; cucurbitacin; U937 cell; structural requirement; anti-proliferative effect

Bryonia cretica L., is a Cucurbitaceae plant growing widely in the western Mediterranean coastal region and in Egypt.¹⁾ This plant has been used in folk medicine as a drastic purgative, emetic, bitter tonic and anti-diabetic agent.²⁾ Previous chemical study of *B. cretica* revealed the presence of cucurbitacins B (3), E (5), I, and L.³⁾ In the course of our studies on bioactive constituents from Egyptian natural medicines,^{4–15)} the 90% aqueous ethanolic (90% EtOH) extract from the roots of *B. cretica* showed strong inhibition of the proliferation of human leukemia U937 cells (IC₅₀ value at 72 h = 0.035 μ g/ml).

Here we describe the chemical elucidation of two new cucurbitane-type triterpene glycosides, bryoniaosides A (1) and B (2), and anti-proliferative effects of several cucurbitane-type triterpenes (3–9) on U937 cells together with several

structural requirements for the activity.

Results and Discussion

The 90% EtOH extract of the roots of *B. cretica* (11.5% from the dried roots) was partitioned into an ethyl acetate (EtOAc)–H₂O (1 : 1, v/v) mixture to furnish the EtOAc-soluble fraction and an aqueous layer. The aqueous layer was extracted with *n*-butanol (*n*-BuOH) to give *n*-BuOH and H₂O-soluble fractions. As shown in Table 1, the EtOAc-soluble fraction exhibited marked activity (IC₅₀ value at 72 h = 0.050 μ g/ml), but the *n*-BuOH- and H₂O-soluble fraction lacked the activity (IC₅₀ values at 72 h > 1 μ g/ml).

The EtOAc-soluble fraction was subjected to normal-phase and reversed-phase silica gel column chromatography and repeated HPLC to give two new cucurbitane-type triter-

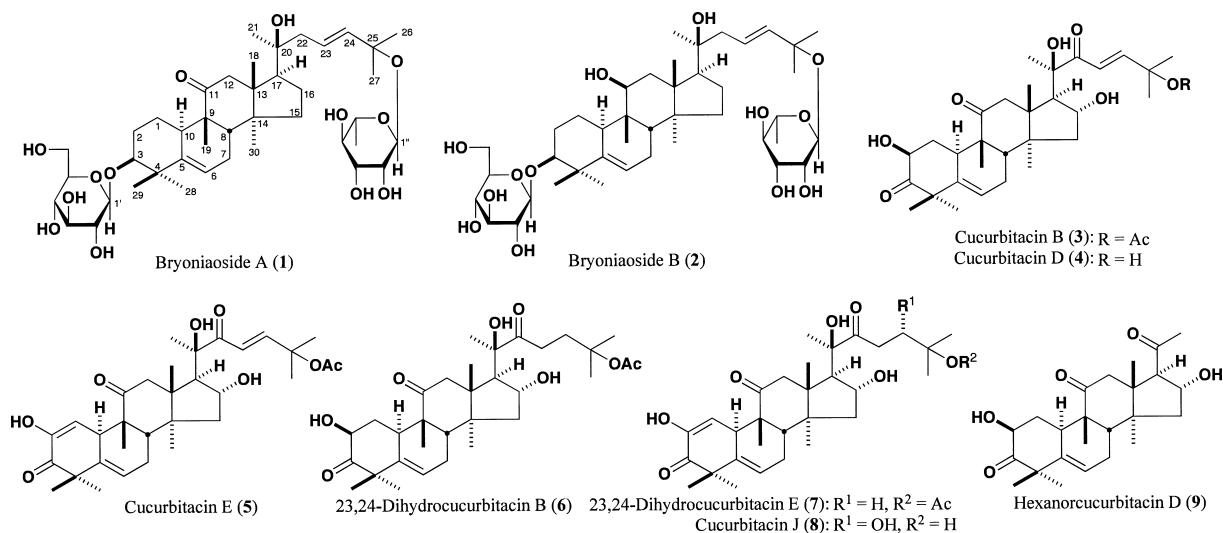


Chart 1. Chemical Structures of Cucurbitane-Type Triterpenes (1–9) from *B. cretica*

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Table 1. Inhibitory Effects of the 90% EtOH Extract and Its Fractions from *B. cretica* on Proliferation of U937 Cells

		Conc. ($\mu\text{g/ml}$)						IC ₅₀ ($\mu\text{g/ml}$)
		0	0.01	0.03	0.1	0.3	1.0	
Inhibition (%)								
90% EtOH ext.	24 h	0.0 $\pm$ 2.8	-6.4 $\pm$ 4.6	-9.7 $\pm$ 5.5	37.2 $\pm$ 2.4**	62.4 $\pm$ 1.6**	73.0 $\pm$ 1.1**	0.17
	48 h	0.0 $\pm$ 1.4	-5.4 $\pm$ 2.5	—	48.3 $\pm$ 5.9**	82.7 $\pm$ 1.0**	87.9 $\pm$ 0.9**	ca. 0.10
	72 h	0.0 $\pm$ 1.4	3.2 $\pm$ 2.1	46.7 $\pm$ 4.3**	77.8 $\pm$ 0.6**	92.2 $\pm$ 0.4**	94.7 $\pm$ 0.4**	0.035
EtOAc-soluble fr.	24 h	0.0 $\pm$ 3.8	15.3 $\pm$ 4.3**	-2.5 $\pm$ 2.7	19.3 $\pm$ 3.0**	46.0 $\pm$ 2.6**	63.1 $\pm$ 0.5**	0.34
	48 h	0.0 $\pm$ 6.4	12.8 $\pm$ 13.9	12.2 $\pm$ 6.8	64.7 $\pm$ 3.5**	87.0 $\pm$ 0.6**	90.5 $\pm$ 0.4**	0.075
	72 h	0.0 $\pm$ 2.7	-0.5 $\pm$ 2.1	26.1 $\pm$ 1.4**	77.1 $\pm$ 0.7**	92.3 $\pm$ 0.3**	93.7 $\pm$ 0.3**	0.050
BuOH-soluble fr.	72 h	0.0 $\pm$ 1.2	-3.1 $\pm$ 1.9	2.2 $\pm$ 3.8	-3.2 $\pm$ 1.4	-4.0 $\pm$ 1.6	11.4 $\pm$ 2.3**	—
H ₂ O-soluble fr.	72 h	0.0 $\pm$ 0.8	7.6 $\pm$ 4.1	9.3 $\pm$ 0.5*	9.0 $\pm$ 0.9	7.9 $\pm$ 3.1	0.4 $\pm$ 2.0	—

Each value represents the mean $\pm$ S.E.M. ($n=4$). Significantly different from the control * $p<0.05$, ** $p<0.01$.

pene glycosides, bryoniaosides A (**1**, 0.0091%) and B (**2**, 0.0028%), together with 16 known cucurbitane-type triterpenes, cucurbitacins B^{16–18} (**3**, 0.020%), D^{16,17,19,20} (**4**, 0.015%), E^{16,17} (**5**, 0.011%), G²⁰ (0.0051%), H²⁰ (0.015%), and J^{21,22} (**8**, 0.017%), 23,24-dihydrocucurbitacins B^{16,23} (**6**, 0.0035%), D¹⁹ (0.011%), and E^{16,19} (**7**, 0.0043%), 3-epi-cucurbitacins B²⁴ (0.0024%) and D²⁵ (0.0037%), hexanor-cucurbitacin D²⁰ (**9**, 0.0024%), and 2-*O*- β -D-glucopyranosyl-cucurbitacins B^{18,26,27} (0.030%), D²⁷ (0.0057%), I^{28–30} (0.0039%), and J²² (0.0035%).

Structures of Bryoniaosides A (1) and B (2) Bryoniaoside A (**1**) was obtained as a white powder and exhibited a positive optical rotation ($[\alpha]_D^{24} +34.4^\circ$ in MeOH). The IR spectrum of **1** showed absorption bands at 1693 and 1640 cm^{-1} ascribable to carbonyl and olefin functions, and broad bands at 3540 and 1028 cm^{-1} , suggestive of a glycoside structure. In the positive-ion fast atom bombardment (FAB)-MS of **1**, a quasimolecular ion peak was observed at m/z 803 ($M+\text{Na}$)⁺, and a high-resolution positive-ion FAB-MS analysis revealed the molecular formula of **1** to be C₄₂H₆₈O₁₃. The acid hydrolysis of **1** with 1.0 M hydrochloric acid (HCl) liberated L-rhamnose and D-glucose, which were identified by HPLC analysis using an optical rotation detector. The proton and carbon signals in the ¹H- and ¹³C-NMR (Table 2, CD₃OD) spectra of **1**, which were assigned based on various NMR experiments,³¹⁾ showed signals assignable to eight methyls [δ 0.90, 1.02, 1.12, 1.25, 1.25, 1.25, 1.26, 1.26 (3H each, all s, H₃-18, 29, 30, 26, 27, 28, 19, 21)], a methine [δ 3.41 (1H, m, H-3)] and two quaternary carbons [δ 71.2 (C-25), 75.8 (C-20)] bearing an oxygen function, a trisubstituted olefin [δ 5.67 (1H, br d, $J=ca.$ 6 Hz, H-6)], and an *trans*-olefin pair [δ 5.59 (1H, m, H-23), 5.60 (1H, d, $J=15.9$ Hz, H-24)], together with a β -glucopyranosyl moiety and a α -rhamnopyranosyl moiety [δ 1.18 (3H, d, $J=6.1$ Hz, Rha-H₃-6), 4.37 (1H, d, $J=7.2$ Hz, Glc-H-1), 5.52 (1H, d, $J=1.5$ Hz, Rha-H-1)]. The carbon skeleton and the positions of functional groups were revealed by the heteronuclear multiple-bond correlations (HMBC) experiment, which showed long-range correlations between the following protons and carbons (Glc-H-1 and C-3; Rha-H-1 and C-25) (Fig. 1). Next, the stereostructure of the aglycone part of **1** was characterized by nuclear Overhauser enhancement spectroscopy (NOESY) experiment, which showed NOE correlations between the following proton pairs (H-8 and H₃-18, 19; H-10 and H₃-28, H₃-30; H-17 and H₃-30). The stereostructure of the 20-position in **1** was deduced by comparison of ¹³C-NMR

Table 2. ¹³C-NMR Data (125 MHz, CD₃OD) of Bryoniaosides A (**1**) and B (**2**)

Position	1	2
1	22.5	22.6
2	29.5	30.3
3	86.8	87.4
4	42.7	40.7
5	140.5	143.6
6	120.6	120.5
7	24.8	25.2
8	44.6	43.9
9	50.9	48.1
10	36.9	37.4
11	218.1	79.3
12	49.9	41.2
13	51.1	49.0
14	50.1	50.7
15	35.0	34.8
16	22.8	27.6
17	51.4	52.4
18	19.4	18.8
19	26.4	26.4
20	75.8	76.3
21	25.7	26.7
22	48.5	48.5
23	123.5	123.8
24	142.5	142.1
25	71.2	71.2
26	29.9 ^{a)}	29.9 ^{a)}
27	30.1 ^{a)}	30.1 ^{a)}
28	26.7	26.1
29	28.8	28.1
30	20.6	20.0
3-O-Glc		
1	105.2	105.1
2	77.0	77.2
3	77.6	77.7
4	72.2	72.1
5	80.1	80.3
6	62.7	62.8
25-O-Rha		
1	100.9	101.1
2	72.0	72.1
3	72.2	72.3
4	73.9	73.9
5	69.9	70.0
6	18.7	18.7

a) May be interchangeable within the same column.

data around the 20-position of **1** with those of related triterpenes.^{32–35)} On the basis of this evidence, the structure of bryoniaoside A (**1**) was determined to be as shown.

Bryoniaoside B (**2**) was also obtained as a white powder with negative optical rotation ($[\alpha]_D^{24} -2.6^\circ$ in MeOH). The molecular formula, $C_{42}H_{70}O_{13}$, of **2** was determined from positive-ion FAB-MS [m/z 805 ($M+Na$) $^+$] and high-resolution FAB-MS measurements. The acid hydrolysis of **2** with 1.0 M HCl liberated L-rhamnose and D-glucose, which were identified by HPLC analysis using an optical rotation detector. The proton and carbon signals in the 1H - and ^{13}C -NMR spectra (Table 2, CD_3OD) of **2** were superimposable on those of **1**, except for the signals due to the 11-hydroxyl group {eight methyls [δ 1.03, 1.05, 1.12, 1.18, 1.25, 1.25, 1.26, 1.26 (3H each, all s, H₃-29, 18, 30, 28, 26, 27, 19, 21)], two

methines [δ 3.41 (1H, m, H-3), 3.82 (1H, m, H-11)] and two quaternary carbons [δ_C 71.2 (C-25), 76.3 (C-20)] bearing an oxygen function, a trisubstituted olefin [δ 5.56 (1H, brd, $J=ca.$ 6 Hz, H-6)], and a *trans*-olefin pair [δ 5.60 (1H, d, $J=16.1$ Hz, H-24), 5.61 (1H, m, H-23)], together with a β -glucopyranosyl moiety and a α -rhamnopyranosyl moiety [δ 1.18 (3H, d, $J=6.1$ Hz, Rha-H₃-6), 4.36 (1H, d, $J=7.5$ Hz, Glc-H-1), 5.50 (1H, d, $J=1.5$ Hz, Rha-H-1)]. The planar structure of **2** was confirmed by 1H - 1H correlation spectroscopy (COSY) and HMBC experiments (Fig. 1). Finally, the stereostructure of **2** was characterized by NOESY experiment, which showed the NOE correlations between the fol-

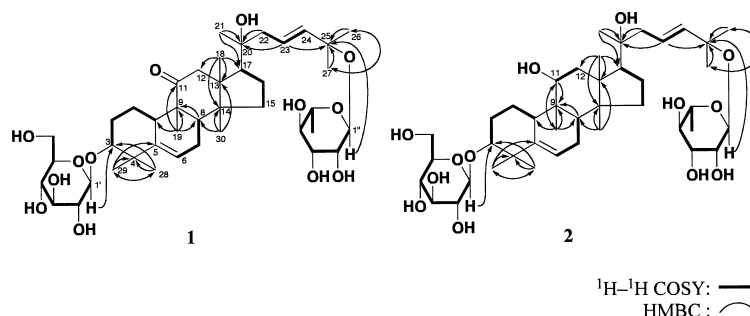


Fig. 1. 1H - 1H COSY and HMBC Correlations **1** and **2**

Table 3. Inhibitory Effects of Cucurbitacins B (**3**), D (**4**), E (**5**), and J (**8**), 23,24-Dihydrocucurbitacins B (**6**) and E (**7**), and Hexanorcucurbitacin D (**9**) on Proliferation of U937 Cells

	Incubation time	Conc. (nM)						IC ₅₀ (nM)
		0	1	3	10	30	100	
Cucurbitacin B (3)	24 h	0.0±1.9	9.3±2.4	-16.5±2.5	9.0±2.2*	56.0±0.5**	73.8±0.4**	25
	48 h	0.0±3.4	-8.4±1.1	-2.6±2.4	31.1±2.0**	70.5±1.1**	86.4±0.5**	15
	72 h	0.0±1.0	24.4±25.1	6.1±1.8	54.2±0.7**	80.0±0.4**	94.1±0.2**	9.2
Cucurbitacin E (5)	24 h	0.0±8.7	-3.1±3.8	-4.2±3.1	-3.8±2.9	50.2±1.0**	68.4±0.1**	29
	48 h	0.0±1.0	2.6±0.9	-0.4±1.0	19.3±2.8**	70.5±1.3**	89.8±1.1**	22
	72 h	0.0±0.7	15.6±14.1	20.7±17.5	27.8±1.0	84.0±5.9**	97.7±2.9**	16
Camptothecin	24 h	0.0±3.3	-12.6±2.6	-0.6±4.7	6.5±3.2	14.6±4.2*	44.0±2.9**	160 ^a
	48 h	0.0±2.6	-3.0±3.3	3.3±3.3	55.7±2.8**	91.1±0.3**	94.2±0.2**	9.1
	72 h	0.0±2.4	1.4±1.3	0.8±2.0	65.7±1.6**	96.7±0.1**	97.3±0.1**	8.6
	Incubation time	Conc. (μM)						IC ₅₀ (μM)
		0	0.01	0.03	0.1	0.3	1	
Cucurbitacin D (4)	24 h	0.0±1.7	-5.0±0.5	-2.5±2.7	-6.8±4.3	12.1±4.4*	54.2±0.6**	0.89
	48 h	0.0±4.9	-4.5±5.3	-4.6±6.7	-12.6±10.3	29.4±8.0**	72.3±4.1**	0.53
	72 h	0.0±3.0	4.2±1.9	5.0±1.0	6.2±0.1	48.1±3.6**	88.7±0.5**	0.33
23,24-Dihydrocucurbitacin B (6)	24 h	0.0±7.1	—	—	—	-4.0±10.5	51.3±1.7**	ca. 1.0
	48 h	0.0±3.6	-3.1±2.1	-12.1±0.6	-2.8±2.5	34.3±6.2**	77.5±0.9**	0.42
	72 h	0.0±3.7	8.8±3.1	12.5±2.2**	23.9±1.7**	62.1±1.8**	84.8±0.5**	0.22
	Incubation time	Conc. (μM)						IC ₅₀ (μM)
		0	1	3	10	30	100	
23,24-Dihydrocucurbitacin E (7)	24 h	0.0±3.7	-7.5±1.9	11.8±4.1*	62.8±1.0**	83.7±0.9**	92.2±0.7**	5.8
	48 h	0.0±1.5	-6.5±1.6	26.0±3.7**	83.6±0.6**	96.5±0.7**	97.3±0.1**	4.5
	72 h	0.0±0.7	-3.3±1.8	44.0±1.6**	88.4±0.3**	97.3±0.3**	99.1±0.1**	3.3
Cucurbitacin J (8)	24 h	0.0±3.7	-18.8±3.3	8.7±2.9	70.9±1.0**	73.9±1.2**	91.7±0.8**	7.9
	48 h	0.0±2.7	-2.9±4.0	49.1±3.9**	86.0±1.2**	90.6±1.1	95.6±0.9**	4.4
	72 h	0.0±3.7	10.0±1.3*	62.3±3.5**	92.4±0.1**	95.5±0.1**	99.4±0.1**	2.4
Hexanorcucurbitacin D (9)	24 h	0.0±6.0	—	7.5±4.8	55.4±2.3**	68.7±4.0**	86.9±1.5**	9.4
	48 h	0.0±2.8	-3.1±2.6	45.2±2.3**	87.8±0.5**	88.4±0.9**	97.6±0.4**	4.6
	72 h	0.0±1.9	12.0±4.9*	60.8±3.7**	91.9±0.3**	94.6±0.5**	98.9±0.1**	2.4

Each value represents the mean±S.E.M. (n=4). Significantly different from the control * $p<0.05$, ** $p<0.01$. a) Inhibition (%) at 300 nm was 57.7±1.1 ($p<0.01$).

lowing proton pairs (H-8 and H₃-18, 19; H-10 and H-11, H₃-28, H₃-30; H-11 and H₃-30; H-17 and H₃-30). Consequently, the structure of bryoniaoside B (**2**) was characterized to be as shown.

Effects of Several Cucurbitane-Type Triterpenes on Proliferation of U937 Cells Among the constituents isolated, effects of several principal cucurbitane-type triterpenes (**3**–**9**) on the proliferation of U937 cells were examined. As shown in Table 3, all compounds tested showed concentration-dependent and time-dependent inhibition of the cell proliferation. The compounds **3** and **5** had the greater effects with IC₅₀ values at 72 h of 9.2 and 16 nM, respectively, while the 23,24-dihydro derivatives (**6**, **7**) and the 25-deacetyl derivative (**4**) were markedly less active with IC₅₀ values at 72 h of 0.22, 3.3, and 0.33 μ M, respectively (Table 3).

Previously, cucurbitacins B (**3**), E (**5**), and I and related compounds were reported to show cytotoxic effects in several cell lines including A549, MDA-MB-468, HepG2, and KB, but not in U937 cells, and their apoptosis-inducing activity mediated by the inhibition of Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling at a high concentration (1–10 μ M).^{36–41} Bartalis and Halaweish reported that the glycosides of cucurbitacins E (**5**) and I were less toxic than the aglycones.³⁶ In addition, Chen *et al.* recently reported the importance of an α,β -unsaturated ketone in the side chains and the 25-acetoxy group in a preliminary analysis of structure–activity relationships.³⁸ Consistent with the results of these studies,^{36,38} cucurbitacin E 2-*O*- β -D-glucopyranoside from the fruit of *Citrullus colocynthis*¹⁵ was not effective less than 30 μ M (inhibition: $-7.7 \pm 1.3\%$ at 30 μ M after 72 h). Our results also demonstrate that an α,β -conjugated ketone moiety at the 22–24 positions and an acetoxy group at the 25 position are essential for the greater activity [**3** (9.2 nM) > **6** (0.22 μ M); **5** (16 nM) > **7** (3.3 μ M); **3** (9.2 nM) > **4** (0.33 μ M)]. In addition, the structure of the A ring is also important [**3** (9.2 nM) > **5** (16 nM); **6** (0.22 μ M) > **7** (3.3 μ M)].

In the present study, cucurbitacins B (**3**) and E (**5**) from *B. cretica* showed strong cytotoxic effects in U937 cells at low concentrations (IC₅₀=9.2 and 16 nM), and their IC₅₀ values were equivalent to that of camptothecin. The mechanism of action of cucurbitane-type triterpenes, including JAK/STAT3 signaling, should be examined further using U937 cells.

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (*l*=5 cm); IR spectra, Shimadzu FTIR-8100 spectrometer; FAB-MS and high-resolution FAB-MS, JEOL JMS-SX 102A mass spectrometer; ¹H-NMR spectra, JNM-LA500 (500 MHz); ¹³C-NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index detector; HPLC column, YMC-Pack ODS-A (250×4.6 mm i.d.) and (250×20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following materials were used for chromatography: ordinary-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh); TLC, pre-coated TLC plates with Silica gel 60F₂₅₄ (Merck, normal-phase) and Silica gel RP-18 F_{254S} (Merck, reversed-phase); HPTLC, pre-coated TLC plates with Silica gel 60F₂₅₄ (Merck, normal-phase), and Silica gel RP-18 WF_{254S} (Merck, reversed-phase). Detection was achieved by spraying with 1% Ce(SO₄)₂–10% aqueous H₂SO₄ followed by heating.

Plant Material The roots of *Bryonia cretica* were identified by one of the authors, Professor Osama B. Abdel-Halim (College of Pharmacy, Taibah

University, Saudi Arabia). A voucher specimen (No. Y-03) of this natural medicine is on file in our laboratory.

Extraction and Isolation The dried roots of *B. cretica* (1.0 kg) was finely cut and extracted three times with 90% aqueous ethanol under reflux for 3 h. Evaporation of the solvent under reduced pressure gave the aqueous ethanolic extract (115.0 g, 11.5% from dried roots). The aqueous ethanolic extract (100.0 g) was partitioned in an EtOAc–H₂O (1:1, v/v) mixture. The aqueous layer was extracted with *n*-BuOH and removal of the solvent *in vacuo* from the EtOAc-, *n*-BuOH-, and H₂O-soluble portions yielded 35.0 g (4.0%), 33.0 g (3.8%), and 32.0 g (3.7%) of the residue, respectively. The EtOAc-soluble fraction (30.0 g) was subjected to ordinary-phase silica gel column chromatography [900 g, *n*-hexane–EtOAc (10:1–5:1–1:1–1:10, v/v)–CHCl₃–MeOH–H₂O (10:3:1–7:3:1, v/v/v, lower layer)–MeOH] to afford 10 fractions [Fr. 1 (6.8 g), Fr. 2 (0.6 g), Fr. 3 (1.5 g), Fr. 4 (3.1 g), Fr. 5 (4.4 g), Fr. 6 (2.2 g), Fr. 7 (4.1 g), Fr. 8 (2.8 g), Fr. 9 (2.3 g), Fr. 10 (2.1 g)]. Fraction 2 (0.6 g) was separated by reversed-phase silica gel column chromatography [18 g, MeOH–H₂O (40:60–50:50–70:30, v/v)–MeOH] and finally HPLC [MeOH–H₂O (55:45, v/v) or CH₃CN–H₂O (40:60, v/v)] to give cucurbitacin E (**5**, 85 mg, 0.011%) and 23,24-dihydrocucurbitacins B (**6**, 26 mg, 0.0035%) and E (**7**, 32 mg, 0.0043%). Fraction 3 (1.5 g) was purified by HPLC [MeOH–H₂O (55:45, v/v)] to give cucurbitacin B (**3**, 150 mg, 0.020%) and 3-epi-cucurbitacin B (18 mg, 0.0024%). Fraction 4 (3.1 g) was separated by reversed-phase silica gel column chromatography [95 g, MeOH–H₂O (40:60–50:50–70:30, v/v)–MeOH] and finally HPLC [MeOH–H₂O (50:50, v/v) or MeOH–H₂O (55:45, v/v)] to give cucurbitacin D (**4**, 110 mg, 0.015%) and 23,24-dihydrocucurbitacin D (80 mg, 0.011%). Fraction 5 (4.4 g) was separated by reversed-phase silica gel column chromatography [95 g, MeOH–H₂O (40:60–50:50–80:20, v/v)–MeOH] and finally HPLC [MeOH–H₂O (50:50, v/v) or MeOH–H₂O (55:45, v/v)] to give cucurbitacins H (112 mg, 0.015%) and J (**8**, 130 mg, 0.017%) and hexanorcucurbitacin D (**9**, 18 mg, 0.0024%). Fraction 6 (2.2 g) was separated by reversed-phase silica gel column chromatography [70 g, MeOH–H₂O (40:60–50:50–80:20, v/v)–MeOH] and finally HPLC [CH₃CN–H₂O (30:70, v/v)] to give 3-epi-cucurbitacin D (28 mg, 0.0037%). Fraction 7 (4.1 g) was separated by reversed-phase silica gel column chromatography [85 g, MeOH–H₂O (40:60–50:50–80:20, v/v)–MeOH] and finally HPLC [CH₃CN–H₂O (30:70, v/v)] to give cucurbitacin G (38 mg, 0.0051%) and 2-*O*- β -D-glucopyranosylcucurbitacin B (220 mg, 0.030%). Fraction 8 (2.8 g) was separated by reversed-phase silica gel column chromatography [70 g, MeOH–H₂O (30:70–50:50–80:20, v/v)–MeOH] and finally HPLC [CH₃CN–H₂O (30:70 or 40:60, v/v)] to give 2-*O*- β -D-glucopyranosylcucurbitacins D (43 mg, 0.0057%) and I (29 mg, 0.0039%). Fraction 9 (2.3 g) was separated by reversed-phase silica gel column chromatography [70 g, MeOH–H₂O (30:70–50:50–70:30, v/v)–MeOH] and finally HPLC [CH₃CN–H₂O (30:70, v/v)] to give 2-*O*- β -D-glucopyranosylcucurbitacin J (26 mg, 0.0035%). Fraction 10 (2.1 g) was separated by reversed-phase silica gel column chromatography [70 g, MeOH–H₂O (30:70–50:50–80:20, v/v)–MeOH] and finally HPLC [MeOH–H₂O (55:45 or 65:35, v/v)] to give bryoniaosides A (**1**, 68 mg, 0.0091%) and B (**2**, 21 mg, 0.0028%).

Bryoniaoside A (**1**): A white powder, [α]_D²⁴ +34.4° (*c*=2.50, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₂H₆₈O₁₃Na (M+Na)⁺: 803.4558. Found: 803.4554. IR (KBr): 3540, 1693, 1640, 1028 cm^{−1}. ¹H-NMR (500 MHz, CD₃OD) δ : 0.90, 1.02, 1.12, 1.25, 1.25, 1.25, 1.26, 1.26 (3H each, all s, H₃-18, 29, 30, 26, 27, 28, 19, 21), 1.18 (3H, d, *J*=6.1 Hz, Rha-H₃-6), 1.92 (1H, br d, *J*=ca. 8 Hz, H-8), 3.41 (1H, m, H-3), [3.62 (1H, dd, *J*=5.2, 11.7 Hz), 3.80 (1H, dd, *J*=2.1, 11.7 Hz), Glc-H₂-6], 4.37 (1H, d, *J*=7.2 Hz, Glc-H-1), 5.52 (1H, d, *J*=1.5 Hz, Rha-H-1), 5.59 (1H, m, H-23), 5.60 (1H, d, *J*=15.9 Hz, H-24), 5.67 (1H, br d, *J*=ca. 6 Hz, H-6). ¹³C-NMR (125 MHz, CD₃OD) δ _C: given in Table 1. Positive-ion FAB-MS *m/z*: 803 (M+Na)⁺.

Bryoniaoside B (**2**): A white powder, [α]_D²⁴ −2.6° (*c*=1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₂H₆₈O₁₃Na (M+Na)⁺: 805.4714. Found: 805.4706. IR (KBr): 3540, 1465, 1220, 1028 cm^{−1}. ¹H-NMR (500 MHz, CD₃OD) δ : 1.03, 1.05, 1.12, 1.18, 1.25, 1.25, 1.26, 1.26 (3H each, all s, H₃-29, 18, 30, 28, 26, 27, 19, 21), 1.18 (3H, d, *J*=6.1 Hz, Rha-H₃-6), 1.66 (1H, br d, *J*=ca. 8 Hz, H-8), 3.41 (1H, m, H-3), [3.63 (1H, dd, *J*=5.5, 12.0 Hz), 3.80 (1H, dd, *J*=2.0, 12.0 Hz), Glc-H₂-6], 3.82 (1H, m, H-11), 4.36 (1H, d, *J*=7.5 Hz, Glc-H-1), 5.50 (1H, d, *J*=1.5 Hz, Rha-H-1), 5.56 (1H, br d, *J*=ca. 6 Hz, H-6), 5.60 (1H, d, *J*=16.1 Hz, H-24), 5.61 (1H, m, H-23). ¹³C-NMR (125 MHz, CD₃OD) δ _C: given in Table 1. Positive-ion FAB-MS *m/z*: 805 (M+Na)⁺.

Acid Hydrolysis of 1 and 2 A solution of **1** or **2** (each 2 mg) in 1 M HCl (1 ml) was heated under reflux for 3 h. After cooling, the reaction mixture

was neutralized with Amberlite IRA-400 (OH⁻ form) and the resin was filtered. On removal of the solvent from the filtrate under reduced pressure, the residue was partitioned in an EtOAc–H₂O (1:1, v/v) mixture giving an EtOAc-soluble fraction and an aqueous phase. The solvent was removed *in vacuo* from the aqueous phase, which was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC NH₂-60-5, 4.6 mm i.d.×250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; mobile phase, CH₃CN–H₂O (85:15, v/v); flow rate, 0.8 ml/min]. The identification of L-rhamnose (i) and D-glucose (ii) from **1** and **2** present in the aqueous layer was carried out by comparing their retention time and optical rotation with those of authentic samples [*t*_R: (i) 7.8 min (negative optical rotation) and (ii) 13.9 min (positive optical rotation)], respectively.

Cell Culture Human leukemia U937 cells (Cell No. JCRB9021) were obtained from Health Science Research Resources Bank (Osaka, Japan). They were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin.

Cytotoxicity Cytotoxicity was assayed as described previously.⁴²⁾ Briefly, after a 20, 44, or 68-h incubation of U937 cells (5×10³ cells/100 µl/well) with test compounds in RPMI1640 medium supplemented with 10% FBS in 96-well microplates, 10 µl of WST-8 solution (Cell Counting Kit-8TM) was added to each well. After a further 4 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad) at 450 nm (reference: 655 nm). Camptothecin was used as a reference compound. Inhibition (%) was calculated with the following formula and the IC₅₀ value was determined graphically.

$$\text{inhibition (\%)} = (A - B) / A \times 100$$

A and *B* indicate the optical density of vehicle and test compound-treated groups (*n*=4).

Statistical Analysis For the statistical analysis, a one-way analysis of variance followed by Dunnett's test was used (Tables 1, 3). Probability (*p*) values less than 0.05 were considered significant.

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References and Notes

- 1) Tackholm V., "Student Flora of Egypt," 2nd ed., Cairo University, Cairo, 1974, p. 399.
- 2) Uphof J. C., "Dictionary of Economic Plants," ed. by Engelmann H. R., Hafner Publishing Co., New York, 1959, p. 61.
- 3) Halim A. F., Mansour E. S., El-Fattah H. A., *Mansoura J. Pharm. Sci.*, **6**, 22–36 (1989).
- 4) Murakami T., Kishi A., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **48**, 994–1000 (2000).
- 5) Yoshikawa M., Murakami T., Kishi A., Kageura T., Matsuda H., *Chem. Pharm. Bull.*, **49**, 863–870 (2001).
- 6) Murakami T., Kishi A., Yoshikawa M., *Chem. Pharm. Bull.*, **49**, 974–978 (2001).
- 7) Morikawa T., Xu F., Matsuda H., Yoshikawa M., *Heterocycles*, **57**, 1983–1988 (2002).
- 8) Yoshikawa M., Xu F., Morikawa T., Ninomiya K., Matsuda H., *Bioorg. Med. Chem. Lett.*, **13**, 1045–1049 (2003).
- 9) Yoshikawa M., Morikawa T., Xu F., Ando S., Matsuda H., *Heterocycles*, **60**, 1787–1792 (2003).
- 10) Xu F., Morikawa T., Matsuda H., Ninomiya K., Yoshikawa M., *J. Nat. Prod.*, **67**, 569–576 (2004).
- 11) Abdel-Halim O. B., Morikawa T., Ando S., Matsuda H., Yoshikawa M., *J. Nat. Prod.*, **67**, 1119–1124 (2004).
- 12) Morikawa T., Xu F., Kashima Y., Matsuda H., Ninomiya K., Yoshikawa M., *Org. Lett.*, **6**, 869–872 (2004).
- 13) Morikawa T., Xu F., Ninomiya K., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **52**, 494–497 (2004).
- 14) Morikawa T., Abdel-Halim O. B., Matsuda H., Ando S., Muraoka O., Yoshikawa M., *Tetrahedron*, **62**, 6435–6442 (2006).
- 15) Yoshikawa M., Morikawa T., Kobayashi H., Matsuhira K., Nakamura S., Matsuda H., *Chem. Pharm. Bull.*, **55**, 428–434 (2007).
- 16) Yamada Y., Hagiwara K., Iguchi K., Takahashi Y., *Chem. Lett.*, **1978**, 319–322 (1978).
- 17) Che C.-T., Fang X., Phoebe C. H. Jr., Kinghorn A. D., Farnsworth N. R., *J. Nat. Prod.*, **48**, 429–434 (1985).
- 18) Ahmad M. U., Huo M. E., Sutradhar R. K., *Phytochemistry*, **36**, 421–423 (1994).
- 19) Velde V. V., Lavie D., *Tetrahedron*, **39**, 317–321 (1983).
- 20) Fujita S., Kasai R., Ohtani K., Yamasaki K., Chiu M.-H., Nie R.-L., Tanaka O., *Phytochemistry*, **38**, 465–472 (1995).
- 21) Gamalath C. B., Gunatilaka A. A. L., Alvi K. A., Atta-ur-Rahman, Balasubramanian S., *Phytochemistry*, **27**, 3225–3229 (1988).
- 22) Kanchanapoom T., Kasai R., Yamasaki K., *Phytochemistry*, **59**, 215–228 (2002).
- 23) Bauer R., Berganza L. H., Seligmann O., Wagner H., *Phytochemistry*, **24**, 1587–1591 (1985).
- 24) Kitajima J., Mukai A., Masuda Y., Tanaka Y., *Yakugaku Zasshi*, **109**, 265–270 (1989).
- 25) Sarker S. D., Whiting P., Sik V., Dinan L., *Phytochemistry*, **50**, 1123–1128 (1999).
- 26) Laurie W. A., McHale D., Sheridan J. B., *Phytochemistry*, **24**, 2659–2661 (1985).
- 27) Yamada Y., Hagiwara K., Iguchi K., Suzuki S., Hsu H.-Y., *Chem. Pharm. Bull.*, **26**, 3107–3112 (1978).
- 28) Hatam N. A. R., Whiting D. A., Yousif N. J., *Phytochemistry*, **28**, 1268–1271 (1989).
- 29) Sachdev-Gupta K., Radke C. D., Renwick J. A. A., *Phytochemistry*, **33**, 1385–1388 (1993).
- 30) Mai L. P., Guénard D., Franck M., Tri M. V., Gaspard C., Sévenet T., *Nat. Prod. Lett.*, **16**, 15–19 (2002).
- 31) The ¹H- and ¹³C-NMR spectra of **1** and **2** were assigned with the aid of distortionless enhancement by polarization transfer (DEPT), homocorrelation spectroscopy (¹H–¹H COSY), heteronuclear multiple-quantum coherence (HMQC), and HMBC experiments.
- 32) Teng R., Li H., Chen J., Wang D., He Y., Yang C., *Magn. Reson. Chem.*, **40**, 483–488 (2002).
- 33) Chen J.-T., Li H.-Z., Wang D., Zhang Y.-J., Yang C.-R., *Helv. Chim. Acta*, **89**, 1142–1148 (2006).
- 34) Yoshikawa M., Sugimoto S., Nakamura S., Matsuda H., *Chem. Pharm. Bull.*, **55**, 571–576 (2007).
- 35) Nakamura S., Sugimoto S., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **55**, 1342–1348 (2007).
- 36) Bartalis J., Halaweish F. T., *J. Chromatogr. B*, **818**, 159–166 (2005).
- 37) Meng D., Qiang S., Lou L., Zhao W., *Planta Med.*, **74**, 1741–1744 (2008).
- 38) Chen C., Qiang S., Luo L., Zhao W., *J. Nat. Prod.*, **72**, 824–829 (2009).
- 39) Blaskovich M. A., Sun J., Cantor A., Turkson J., Jove R., Sebti S. M., *Cancer Res.*, **63**, 1270–1279 (2003).
- 40) Sun J., Blaskovich M. A., Jove R., Livingston S. K., Coppola D., Sebti S. M., *Oncogene*, **24**, 3236–3245 (2005).
- 41) Shi X., Franko B., Franz C., Amin H. M., Lai R., *Br. J. Haematol.*, **135**, 26–32 (2006).
- 42) Matsuda H., Yoshida K., Miyagawa K., Asao Y., Takayama S., Nakashima S., Xu F., Yoshikawa M., *Bioorg. Med. Chem.*, **15**, 1539–1546 (2007).