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 IC_{50} values of 9.2 and 16 nM after 72 h, and their IC_{50} 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 (IC_{50} value at 72 h=0.035 μg/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)–H_2O (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 H_2O-soluble fractions. As shown in Table 1, the EtOAc-soluble fraction exhibited marked activity (IC_{50} value at 72 h= 0.050 μg/ml), but the *n*-BuOH- and H_2O-soluble fraction lacked the activity (IC_{50} values at 72 h>1 μg/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-

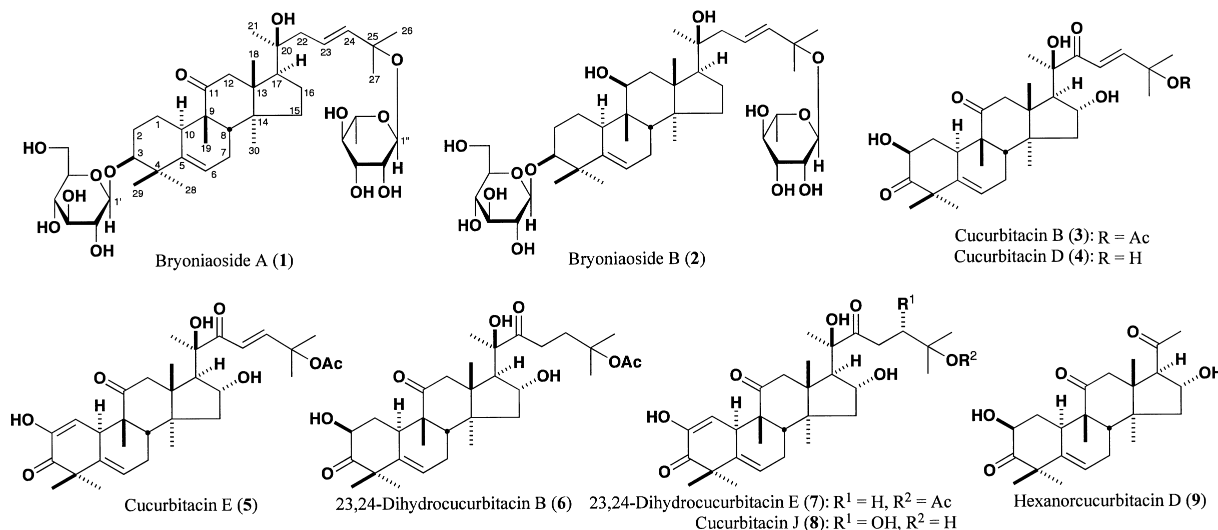

Chart 1. Chemical Structures of Cucurbitane-Type Triterpenes (**1**—**9**) from *B. cretica*

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

	Incubation time	Conc. (μg/ml)						IC_{50} (μg/ml)
		0	0.01	0.03	0.1	0.3	1.0	
Inhibition (%)								
90% EtOH ext.	24 h	0.0±2.8	−6.4±4.6	−9.7±5.5	37.2±2.4**	62.4±1.6**	73.0±1.1**	0.17
	48 h	0.0±1.4	−5.4±2.5	—	48.3±5.9**	82.7±1.0**	87.9±0.9**	*ca.* 0.10
	72 h	0.0±1.4	3.2±2.1	46.7±4.3**	77.8±0.6**	92.2±0.4**	94.7±0.4**	0.035
EtOAc-soluble fr.	24 h	0.0±3.8	15.3±4.3**	−2.5±2.7	19.3±3.0**	46.0±2.6**	63.1±0.5**	0.34
	48 h	0.0±6.4	12.8±13.9	12.2±6.8	64.7±3.5**	87.0±0.6**	90.5±0.4**	0.075
	72 h	0.0±2.7	−0.5±2.1	26.1±1.4**	77.1±0.7**	92.3±0.3**	93.7±0.3**	0.050
BuOH-soluble fr.	72 h	0.0±1.2	−3.1±1.9	2.2±3.8	−3.2±1.4	−4.0±1.6	11.4±2.3**	—
H_2O-soluble fr.	72 h	0.0±0.8	7.6±4.1	9.3±0.5*	9.0±0.9	7.9±3.1	0.4±2.0	—

Each value represents the mean±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%), G[20] (0.0051%), H[20] (0.015%), and J[21,22] (**8**, 0.017%), 23,24-dihydrocucurbitacins B[16,23] (**6**, 0.0035%), D[19] (0.011%), and E[16,19] (**7**, 0.0043%), 3-epi-cucurbitacins B[24] (0.0024%) and D[25] (0.0037%), hexanor-cucurbitacin D[20] (**9**, 0.0024%), and 2-*O*-β-D-glucopyranosyl-cucurbitacins B[18,26,27] (0.030%), D[27] (0.0057%), I[28—30] (0.0039%), and J[22] (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° 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 $(M+Na)^+$, and a high-resolution positive-ion FAB-MS analysis revealed the molecular formula of **1** to be $C_{42}H_{68}O_{13}$. 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 ^{1}H- and ^{13}C-NMR (Table 2, CD_3OD) 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, H_3-18, 29, 30, 26, 27, 28, 19, 21)], a methine [δ 3.41 (1H, m, H-3)] and two quaternary carbons [δ_C 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-H_3-6), 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 H_3-18, 19; H-10 and H_3-28, H_3-30; H-17 and H_3-30). The stereostructure of the 20-position in **1** was deduced by comparison of ^{13}C-NMR 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.

Table 2. ^{13}C-NMR Data (125 MHz, CD_3OD) 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.

Bryoniaoside B (**2**) was also obtained as a white powder with negative optical rotation ($[\alpha]_D^{24}$ −2.6° 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 ^{1}H- 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, H_3-29, 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, br d, *J*=*ca.* 6 Hz, H-6)], and an *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-H_3-6), 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 ^{1}H–^{1}H 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-

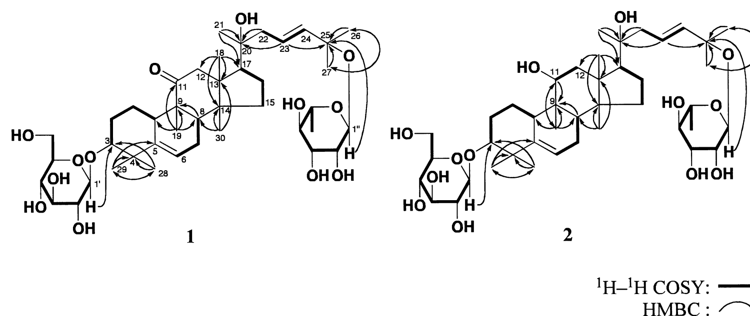

Fig. 1. ^{1}H–^{1}H COSY and HMBC Correlations **1** and **2**

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 H_3-18, 19; H-10 and H-11, H_3-28, H_3-30; H-11 and H_3-30; H-17 and H_3-30). 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 IC_{50} 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 IC_{50} values at 72 h of 0.22, 3.3, and 0.33 μM, 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 μM).[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 μM (inhibition: $-7.7\pm1.3\%$ at 30 μM 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 μM); **5** (16 nM)>**7** (3.3 μM); **3** (9.2 nM)>**4** (0.33 μM)]. In addition, the structure of the A ring is also important [**3** (9.2 nM)>**5** (16 nM); **6** (0.22 μM)>**7** (3.3 μM)].

In the present study, cucurbitacins B (**3**) and E (**5**) from *B. cretica* showed strong cytotoxic effects in U937 cells at low concentrations (IC_{50}=9.2 and 16 nM), and their IC_{50} 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; ^{1}H-NMR spectra, JNM-LA500 (500 MHz); ^{13}C-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, precoated TLC plates with Silica gel $60F_{254}$ (Merck, normal-phase) and Silica gel RP-18 F_{254S} (Merck, reversed-phase); HPTLC, pre-coated TLC plates with Silica gel $60F_{254}$ (Merck, normal-phase), and Silica gel RP-18 WF_{254S} (Merck, reversed-phase). Detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 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–H_2O (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 H_2O-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)–$CHCl_3$–MeOH–H_2O (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–H_2O (40 : 60—50 : 50—70 : 30, v/v)–MeOH] and finally HPLC [MeOH–H_2O (55 : 45, v/v) or CH_3CN–H_2O (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–H_2O (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–H_2O (40 : 60—50 : 50—70 : 30, v/v)–MeOH] and finally HPLC [MeOH–H_2O (50 : 50, v/v) or MeOH–H_2O (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–H_2O (40 : 60—50 : 50—80 : 20, v/v)–MeOH] and finally HPLC [MeOH–H_2O (50 : 50, v/v) or MeOH–H_2O (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–H_2O (40 : 60—50 : 50—80 : 20, v/v)–MeOH] and finally HPLC [CH_3CN–H_2O (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–H_2O (40 : 60—50 : 50—80 : 20, v/v)–MeOH] and finally HPLC [CH_3CN–H_2O (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–H_2O (30 : 70—50 : 50—80 : 20, v/v)–MeOH] and finally HPLC [CH_3CN–H_2O (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–H_2O (30 : 70—50 : 50—70 : 30, v/v)–MeOH] and finally HPLC [CH_3CN–H_2O (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–H_2O (30 : 70—50 : 50—80 : 20, v/v)–MeOH] and finally HPLC [MeOH–H_2O (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, $[\alpha]_D^{24}$ +34.4° (c=2.50, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{42}H_{68}O_{13}Na$ $(M+Na)^+$: 803.4558. Found: 803.4554. IR (KBr): 3540, 1693, 1640, 1028 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.90, 1.02, 1.12, 1.25, 1.25, 1.25, 1.26, 1.26 (3H each, all s, H_3-18, 29, 30, 26, 27, 28, 19, 21), 1.18 (3H, d, J=6.1 Hz, Rha-H_3-6), 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-H_2-6], 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). ^{13}C-NMR (125 MHz, CD_3OD) δ_C : given in Table 1. Positive-ion FAB-MS *m/z*: 803 $(M+Na)^+$.

Bryoniaoside B (**2**): A white powder, $[\alpha]_D^{24}$ −2.6° (c=1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{42}H_{70}O_{13}Na$ $(M+Na)^+$: 805.4714. Found: 805.4706. IR (KBr): 3540, 1465, 1220, 1028 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.03, 1.05, 1.12, 1.18, 1.25, 1.25, 1.26, 1.26 (3H each, all s, H_3-29, 18, 30, 28, 26, 27, 19, 21), 1.18 (3H, d, J=6.1 Hz, Rha-H_3-6), 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-H_2-6], 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). ^{13}C-NMR (125 MHz, CD_3OD) 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–H_2O (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 NH_2-60-5, 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, CH_3CN–H_2O (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 (5×10^3 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-8™) 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 IC_{50} 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) Gamlath C. B., Gunatilaka A. A. L., Alvi K. A., Atta-ur-Rahman, Balasubramaniam 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 ^{1}H- and ^{13}C-NMR spectra of **1** and **2** were assigned with the aid of distortionless enhancement by polarization transfer (DEPT), homocorrelation spectroscopy (^{1}H–^{1}H 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).