Anti-Inflammatory and Anti-Tumor-Promoting Effects of Cucurbitane Glycosides from the Roots of *Bryonia dioica*

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Seven new triterpene glycosides, bryoniosides A−G (**1**−**7**), have been isolated along with two known triterpene glycosides, cabenoside D (**8**) and bryoamaride (**9**), from a methanol extract of the roots of *Bryonia dioica*. The structures of **1**−**7** were determined on the basis of spectroscopic and chemical methods. Six compounds, **2**, **3**, **5**, and **7**−**9**, and 11 compounds, **1**−**9**, bryodulcosigenin (**10**), and bryosigenin (**11**), respectively, were evaluated for their inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (1 μg/ear) in mice and on Epstein−Barr virus early antigen (EBV-EA) activation induced by TPA. All compounds tested showed marked anti-inflammatory effects, with 50% inhibitory doses (ID_{50}) of 0.2−0.6 mg per ear. In addition, all of the compounds tested except for compound **5** showed potent inhibitory effects on EBV-EA induction (100% inhibition at 1×10^3 mol ratio/TPA).

Bryonia dioica Jacq. (white bryony), a cucurbitaceous plant, is a climbing perennial herb with tuberous roots which occurs in temperate Europe, North Africa, and western Asia.[1] The roots of this plant, Bryonia Radix, are used as purgative, emmenagogue, and a treatment for gout.[2] We have recently reported the isolation and characterization of eight sterols[3] and four triterpenoids[4] from the roots, and a triterpenoid[5] and four sterols[6] from the aerial parts of this plant. We now report from an ethyl acetate-soluble fraction of the methanol extract of the roots the isolation and structure elucidation of seven new cucurbitane-type triterpene glycosides, bryoniosides A−G (**1**−**7**), along with two known glycosides, cabenoside D [(24*R*)-3β,24,25-trihydroxycucurbit-5-en-11-one (bryodulcosigenin)-3-*O*-β-D-glucopyranoside; **8**][7] and bryoamaride [(20*S*)-2,16α,20,25-tetrahydroxycucurbita-1,5-diene-3,11,22-trione-2-*O*-β-D- glucopyranoside; **9**].[8,9] Inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice of six of these compounds, **2**, **3**, **5**, and **7**−**9**, and on Epstein−Barr virus early antigen (EBV-EA) activation induced by TPA of 11 compounds, **1**−**9** and two aglycons, bryodulcosigenin [(24*R*)-3β,24,25-trihydroxycucurbit-5-en-11-one; **10**] and bryosigenin [3β,25-dihydroxycucurbit-5-ene-11,24-dione; **11**], were evaluated as preliminary screen for their potential cancer chemopreventive activities.

Besides the nine compounds **1**−**9** isolated in the present study, the roots of *B. dioica* have so far been reported to contain 14 other cucurbitane-type triterpenes: 25-*O*-acetyl bryoamaride,[8] bryodiosides A, B, and C,[9] bryodulcoside,[10] bryonoside,[9,11] bryoside,[11] cucurbitacin I,[8] cucurbitacin I 2-*O*-β-D-glucopyranoside,[8] cucurbitacin K,[12] cucurbitacin L,[8,12] 10α-cucurbitadienol,[3] elaterinide,[8] and tetrahydrocucurbitacin I.[12]

Results and Discussion

The EtOAc-soluble fraction showed the most potent inhibitory effects [I.R. (inhibitory ratio) = 90% at 1 mg/ear] among the four fractions of the MeOH extract of *B. dioica* roots on TPA-induced inflammation in mice in a preliminary screen. Column chromatography followed by preparative reverse-phase HPLC afforded seven new cucurbitane-type triterpene glycosides named bryoniosides A−G (**1**−**7**), in addition to two known compounds, cabenoside D (**8**)[7] and bryoamaride (**9**),[8,9] which were identified by ^{13}C and ^{1}H NMR spectral data comparison with literature results.

Bryonioside A (**1**) was assigned a molecular formula of $C_{36}H_{60}O_9$, as determined from its ^{13}C DEPT NMR data and the $[M + Na]^+$ ion at *m/z* 659.4135 in the high-resolution (HR) FABMS (positive-ion mode). The ^{1}H NMR spectrum (Table 1) of **1** exhibited signals due to seven tertiary methyl groups [δ_H 0.75, 1.05, 1.16, 1.28, 1.44, and 1.56 (6H)], one secondary methyl group (δ_H 0.94, d, J = 6.4 Hz), one methylene group (δ_H 2.55 and 2.99, each 1H, d, J = 14.2 Hz) adjacent to a carbonyl group, two hydroxy methine groups (δ_H 3.73, br s, and 4.03, dd, J = 3.7 and 4.2 Hz), and an olefin (δ_H 5.71, d, J = 5.7 Hz). It also showed a doublet signal at δ_H 5.25 (J = 7.8 Hz) ascribable to an anomeric proton. These results, combined with the observed ^{13}C NMR data (Table 2), suggested **1** to be a glucoside of **10**.[9] The ^{13}C NMR spectral comparison of **1** with **2**, for which the structural assignment is described subsequently, showed a glycosylation shift[13,14] for the C-24 signal (+7.9 ppm from δ_C 72.7 to 80.6), implying that a β-glucopyranosyl group is linked to the OH-24 in **1**. A long-range $^3J_{C-H}$ correlation between the anomeric ^{1}H NMR signal (H-G1, δ_H 5.25) and the ^{13}C NMR signal of C-25 (δ_C 80.6) observed in the HMBC spectrum of **1** was consistent with a glucosidic linkage. Accordingly, bryonioside A (**1**) was formulated as (24*R*)-3β,24,25-trihydroxycucurbit-5-en-11-one-25-*O*-β-D-glucopyranoside.

Bryonioside B (**2**), $C_{42}H_{70}O_{13}$ (HRFABMS *m/z* 805.4714 $[M + Na]^+$), upon acid hydrolysis, furnished **8** and **10**, and two sugars, D-glucose and L-rhamnose, demonstrating that **2** possesses the basic structure of **8** with one α-rhamnosyl unit. In the ^{13}C NMR spectrum, glucosyl C-G2 signal of **2** appeared at lower field by +5.0 ppm compared with that of **8** because of a glycosylation shift,[13,14] indicating an

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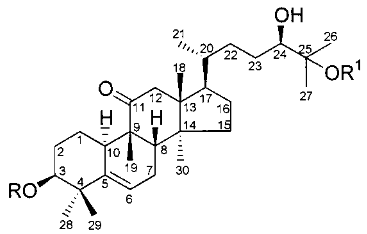
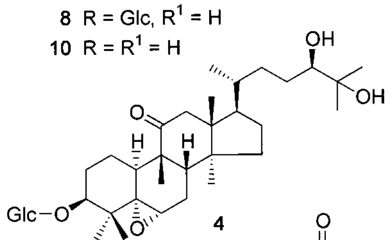


1 R = H, R^1 = Glc
2 R = Rha-(1→2)-Glc, R^1 = H
3 R = Rha(4-Ac)-(1→2)-Glc, R^1 = H
8 R = Glc, R^1 = H
10 R = R^1 = H



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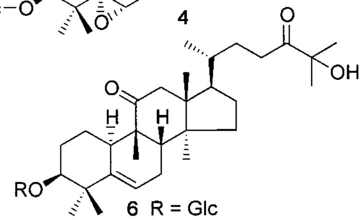
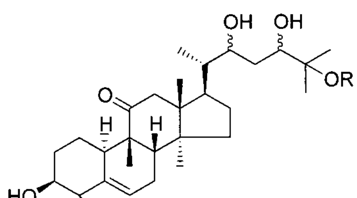
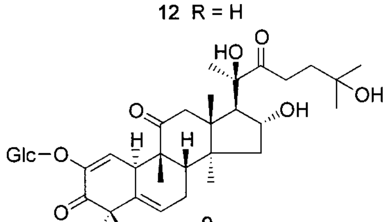


6 R = Glc
7 R = Rha-(1→2)-Glc
11 R = H



5 R = Glc
12 R = H



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α-rhamnopyranosyl group to be located at C-G2 of glucose. The HMBC spectrum of **2** showed a long-range $^3J_{C-H}$ correlation between the ^{1}H NMR signal of H-2 (δ_H 4.25) of the glucosyl moiety and the anomeric ^{13}C NMR signal (δ_C 101.0) of the rhamnosyl moiety, indicating that C-R1 of rhamnose is linked to C-2G of glucose. Hence, bryonioside B (**2**) was assigned as (24*R*)-3*β*,24,25-trihydroxycucurbit-5-en-11-one-3-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside.

Bryonioside C (**3**) exhibited a [M + Na]$^+$ ion 42 mass units higher than bryonioside B (**2**) in the HRFABMS at *m*/*z* 847.4819, consistent with the presence of an additional acetyl group [δ_H 2.27, 3H (s); δ_C 170.8 (O*C*OMe) and 21.4 (OCO*Me*)]. The ^{1}H and ^{13}C NMR spectra of **3** were almost superimposable on those of **2** except for certain signals of their α-rhamnosyl moieties. A ^{1}H and ^{13}C NMR spectral comparison of **3** with **2** revealed an acylation shift[14] at the C-R4 position [+1.63 ppm (H-R4), +2.0 ppm (C-R4), −2.8 ppm (C-R3), and −2.5 ppm (C-R5)] for the rhamnosyl moiety of **3**. Therefore, the hydroxyl group at the C-4 position of the rhamnosyl moiety was acetylated in this compound. Accordingly, bryonioside C (**3**) was formulated as (24*R*)-3*β*,24,25-trihydroxycucurbit-5-en-11-one-3-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*β*-D-glucopyranoside.

Bryonioside D (**4**) was assigned the molecular formula $C_{36}H_{60}O_{10}$ (HRFABMS *m*/*z* 675.4086 [M + Na]$^+$), corresponding to one oxygen atom (16 mass units) more than **8**. The ^{1}H and ^{13}C NMR spectra of **4** were superimposable with those of **8** except for the presence of two carbons bearing an oxygen atom [δ_C 51.5 (d) and 64.8 (s); δ_H 3.16 (1H, br d, J = 5.3 Hz)] in **4** instead of an olefin group at C-5 [δ_C 118.4 (C-6) and 141.2 (C-5)] of **8**, suggesting the presence of an epoxy ring at C-5. The location of the epoxy group at C-5 was confirmed unambiguously from the HMBC spectrum of **4**, in which the quaternary carbon at δ_C 64.8 showed significant cross-peaks, due to $^3J_{C-H}$ correlations, with the ^{1}H NMR signals at δ_H 1.19 (H-28), 1.25 (H-29), and 2.42 (H-10), and the tertiary carbon at δ_C 118.4 with the ^{1}H NMR signal at δ_H 1.77 (H-8). The epoxy group was determined as being α-oriented by the NOESY spectrum of **4**, with significant NOE correlations being observed between the ^{1}H NMR signals at δ_H 1.25 [H-29 (4*β*-Me)]/3.16 (H-6)/1.77 (H-8*β*). Bryonioside D (**4**) was formulated, therefore, as (24*R*)-5α,6α-epoxy-3*β*,24,25-trihydroxycucurbit-5-en-11-one-3-*O*-*β*-D-glucopyranoside (5α,6α-epoxycabenoside D).

The ^{1}H and ^{13}C NMR spectral data of bryonioside E (**5**), $C_{36}H_{60}O_{10}$ (HRFABMS *m*/*z* 675.4084 [M + Na]$^+$), a compound possessing one oxygen atom (16 mass units) more than **1**, were almost superimposable on those of **1** with the exception of some signals of the side-chain, and this suggested that **5** is a hydroxylated analogue of **1**. Diagnostic cross-peaks due to $^3J_{C-H}$ correlations were observed in the HMBC spectrum of **5** for the oxygenated carbon at δ_C 69.6 (d) with the ^{1}H NMR signals at δ_H 1.22 (H-21) and 4.73 (H-24), and the ^{1}H NMR signal correlations of δ_H 1.22 (H-21)/2.06 (H-20)/4.63/1.85, 1.97 (H-23)/4.73 (H-24) observed in the ^{1}H−^{1}H COSY spectrum of **5** located unambiguously the hydroxyl group at C-22 (δ_C 69.6; δ_H 4.63, br d, J = 10.1 Hz). Thus, bryonioside E (**5**) is a (22*ξ*,24*ξ*)-3*β*,22,24,25-tetrahydroxycucurbit-5-en-11-one-25-*O*-*β*-D-glucopyranoside in which the stereochemistry at C-22 and C-24 remained undetermined. Acid hydrolysis of **5** yielded a sapogenin **12** and D-glucose. 2D NMR experiments (run in $CDCl_3$) and FABMS analysis of **12** were consistent with the structure (22*ξ*,24*ξ*)-3*β*,22,24,25-tetrahydroxycucurbit-5-en-11-one. The ^{1}H and ^{13}C NMR spectral data for **12** are shown in Tables 1 and 2, respectively.

Bryonioside F (**6**), $C_{36}H_{58}O_9$ (HRFABMS *m*/*z* 657.3980 [M + Na]$^+$), having two hydrogen atoms (2 mass units) less than **8**, showed almost superimposable ^{1}H and ^{13}C NMR signals with those of **8** except for some of the side-chain signals. A carbonyl ^{13}C NMR signal appeared at δ_C 216.4 for **6** and was assigned to C-24 based on the HMBC spectrum, where a significant cross-peak ($^3J_{C-H}$) was observed for this signal with the ^{1}H NMR signal at δ_H 1.58 (H-26 and H-27). Hence, bryonioside F (**6**) was characterized as 3*β*,25-dihydroxycucurbit-5-ene-11,24-dione-3-*O*-*β*-D-glucopyranoside (bryosigenin[15] 3-*O*-*β*-D-glucopyranoside).

Bryonioside G (**7**) exhibited a [M + Na]$^+$ at *m*/*z* 803.4560 in the HRFABMS corresponding with a molecular formula of $C_{42}H_{68}O_{13}$. In the ^{1}H and ^{13}C NMR spectra of **7**, signals due to the aglycon moiety were in good agreement with those of **6**, while signals due to the sugar moieties were superimposable with those of **2**. Acid hydrolysis of **7** furnished **11**,[15] as an aglycon, and two sugars, D-glucose and L-rhamnose. From the foregoing, the structure 3*β*,25-dihydroxycucurbit-5-ene-11,24-dione-3-*O*-α-L-rhamnopyra-

Table 1. ^{1}H NMR Spectral Data (δ values; 500 MHz; pyridine-d_5) of Compounds **1**−**7** and **12**[a]

proton(s)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**12**[b]
1	1.64, 2.07	1.57, 1.89	1.55, 1.83	1.60, 2.23	1.65, 2.09	1.52, 1.92	1.56, 1.90	1.39, 1.50
2	1.86, 1.96	1.84, 2.30	1.83, 2.40	1.88, 2.42	1.87, 1.99	1.80, 2.39	1.83, 2.31	1.67, 1.80
3	3.73 (br s)	3.63 (br s)	3.62 (br s)	3.71 (br s)	3.73 (br s)	3.65 (br s)	3.62 (br s)	3.48 (br s)
6	5.71 (d, 5.7)	5.95 (br d, 5.8)	5.76 (br d, 5.9)	3.16 (br d, 5.3)	5.58 (br d, 5.4)	5.53 (br d, 6.0)	5.97 (br d, 5.6)	5.67 (br d, 5.8)
7	1.87, 2.34	1.87, 2.73	1.95, 2.46	1.73, 2.15	1.81, 2.30	1.76, 2.17	1.89, 2.74	1.92, 2.40
8	1.89	1.90	1.94	1.77	1.86	1.80	1.89	1.93
10	2.55	2.46	2.49	2.42	2.56	2.46	2.46	2.30
12α	2.99 (d, 14.2)	2.94 (d, 14.3)	2.93 (d, 14.2)	2.93 (d, 15.0)	3.04 (d, 14.0)	2.92 (d, 14.4)	2.92 (d, 14.6)	2.95 (d, 14.3)
12β	2.55 (d, 14.2)	2.50 (d, 14.3)	2.52 (d, 14.2)	2.48 (d, 15.0)	2.61 (d, 14.0)	2.46 (d, 14.4)	2.48 (d, 14.6)	2.43 (d, 14.3)
15	1.18, 1.33	1.15, 1.28	1.24, 1.33	1.11, 1.30	1.15, 1.31	1.17, 1.27	1.17, 1.30	1.40, 1.27
16	1.37, 2.03	1.76, 1.83	1.84 (2H)	1.76, 1.85	1.52, 2.32	1.35, 1.96	1.32, 1.96	1.47, 1.94
17	1.75	1.72	1.73	1.70	2.02	1.68	1.70	1.72
18	0.75 (s)	0.74 (s)	0.79 (s)	0.66 (s)	0.78 (s)	0.70 (s)	0.70 (s)	0.77 (s)
19	1.28 (s)	1.40 (s)	1.32 (s)	1.37 (s)	1.27 (s)	1.16 (s)	1.40 (s)	1.13 (s)
20	1.45	1.49	1.50	1.50	2.06	1.40	1.39	1.73
21	0.94 (d, 6.4)	0.93 (d, 6.4)	0.94 (d, 6.4)	0.92 (d, 6.4)	1.22 (d, 6.1)	0.86 (d, 6.4)	0.85 (d, 6.1)	0.91 (d, 6.1)
22	1.20, 1.95	1.60, 1.78	1.72, 1.83	1.67, 1.79	4.63 (br d, 10.1)	1.40, 1.96	1.41, 1.96	4.03 (br d, 10.3)
23	1.55, 1.71	1.33, 2.03	1.36, 2.04	1.33, 2.01	1.85, 1.97	2.92, 2.98	2.97 (2H)	1.32, 1.43
24	4.03 (dd, 3.7, 4.2)	3.47 (d, 7.9)	3.78 (d, 7.6)	3.77 (d, 9.8)	4.73 (br d, 10.1)			3.66 (dd, 1.8, 9.9)
26	1.56 (s)	1.53 (s)	1.55 (s)	1.56 (s)	1.61 (s)	1.58 (s)	1.57 (s)	1.18 (s)
27	1.56 (s)	1.53 (s)	1.56 (s)	1.54 (s)	1.65 (s)	1.58 (s)	1.58 (s)	1.25 (s)
28	1.44 (s)	1.08 (s)	1.13 (s)	1.19 (s)	1.43 (s)	1.12 (s)	1.08 (s)	1.02 (s)
29	1.16 (s)	1.51 (s)	1.54 (s)	1.25 (s)	1.16 (s)	1.56 (s)	1.55 (s)	1.17 (s)
30	1.05 (s)	0.99 (s)	1.01 (s)	1.03 (s)	1.03 (s)	0.96 (s)	0.97 (s)	1.02 (s)
G1		4.88 (d, 7.3)	4.88 (dt, 7.5, 3.5)	4.88 (d, 7.8)		4.88 (d, 7.8)	4.91 (d, 7.5)	
G2		4.25	4.25	3.99		3.96	4.27	
G3		4.27	4.26	4.23		4.19	4.30	
G4		4.13	4.15	4.22		4.22	4.16	
G5		3.84	3.86	3.95		3.96	3.86	
G6		4.32, 4.46	4.36, 4.49	4.41, 4.55		4.40, 4.55	4.34, 4.49	
R1		6.67 (br s)	6.74 (br s)				6.74 (d, 7.6)	
R2		4.62	4.77				4.63	
R3		4.75	4.69				4.78	
R4		4.28	5.91 (t, 10.0)				4.30	
R5		4.62	4.72				4.64	
R6		1.70 (d, 6.1)	1.50 (d, 6.1)				1.72 (d, 6.1)	
R4-OAc			2.27 (s)					
G′1	5.25 (d, 7.8)				5.22 (d, 8.0)			
G′2	4.06				4.03			
G′3	4.25				4.17			
G′4	4.25				4.18			
G′5	3.98				3.85			
G′6	4.37, 4.53				4.32, 4.49			

[a] *J* values (Hz) determined are shown in parentheses. [b]Determined in $CDCl_3$.

nosyl-(1→2)-β-D-glucopyranoside [bryosigenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside] was deduced for **7**.

The assigned structures for the seven novel cucurbitane-type triterpene glycosides **1**−**7** were supported from extensive NMR experiments, including DEPT, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY. Although the absolute configurations of sugar moieties of the seven triterpene glycosides **1**−**7** were not determined in this study, we have chosen the configurations of D and L for the glucose and rhamnose moieties, respectively, in keeping with those mostly encountered among plant glycosides.[16]

The inhibitory effects on TPA-induced inflammation in mice were examined for the MeOH extract of the *B. dioica* roots, and the *n*-hexane, EtOAc, *n*-butanol, and H_2O fractions obtained from the MeOH extract. Of these, the EtOAc-soluble fraction was shown to possess a marked inhibitory effect (I.R. = 90% at 1 mg/ear). The inhibitory effects in the same biological test system were further examined for the six triterpene glycosides, **2**, **3**, **5**, and **7**−**9**, isolated from the EtOAc-soluble fraction and compared with those of reference compounds, quercetin (3,5,7,3′,4′-pentahydroxyflavone), a known inhibitor of TPA-induced inflammation in mice, as well as a commercially available anti-inflammatory drug, indomethacin. The inhibitory effects of the *B. dioica* triterpenoids (ID_{50} = 0.2−0.7 mg/ear) evaluated were stronger than that of quercetin (1.6 mg/ear), and data for **2**, **3**, **5**, and **7** were comparable in potency to the effects of indomethacin (0.3 mg/ear). The inhibitory effects against TPA-induced inflammation have been demonstrated to closely parallel those of the inhibition of tumor promotion on two-stage carcinogenesis promoted by TPA following initiation with 7,12-dimethylbenz[*a*]anthracene (DMBA), a well-known initiator, in a mouse skin model,[17,18] and thus, these anti-inflammatory triterpenes from *B. dioica* roots may be expected to possess high anti-tumor-promoting effects in the same animal model.

The inhibitory effects on EBV-EA activation induced by TPA were further examined as a preliminary evaluation of the potential anti-tumor-promoting activities for the 11 cucurbitane-type triterpenes, **1**−**11**, and the results are shown in Table 3 along with comparable data for β-carotene, a vitamin A precursor that has been studied intensively in cancer chemoprevention using animal models.[19] All of the compounds exhibited potent inhibitory effects (88−100% inhibition of induction at 1×10^3 mol ratio/TPA, about 63−76% inhibition at 5×10^2 mol/TPA, and about 23−31% at 1×10^2 mol ratio/TPA) on EBV-EA induction by TPA with preservation of the high viability (70%) of the Raji cells which were equivalent to or more potent than β-carotene. Among these, two aglycons, **10** and **11**, showed the most potent inhibitory effects on EBV-EA activation (11% and 7% inhibition even at 1×10 mol ratio/TPA, respectively). This may suggest that deglycosylation enhances the inhibitory effects on EBV-EA activation for these cucurbitane-type triterpenes.

Table 2. ^{13}C NMR Data (δ values, 125 MHz, pyridine-d_5) for Compounds **1**−**7** and **12**

carbon	1	2	3	4	5	6	7	12[a]
aglycon								
1	21.3	22.4	22.3	20.5	21.3	22.1	22.4	20.6
2	29.8	28.9	28.8	29.7	29.9	28.5	28.8	28.5
3	75.6	86.1	86.5	86.4	75.6	87.2	86.1	76.1
4	41.9	42.1	42.0	41.0	41.9	42.0	42.1	41.6
5	141.5	140.0	140.9	64.8	141.5	141.2	140.0	139.6
6	119.0	120.0	118.6	51.5	119.0	118.5	120.0	120.6
7	24.2	24.3	24.6	23.1	24.2	24.1	24.3	24.0
8	44.1	44.2	44.0	42.7	44.1	43.9	44.1	43.8
9	49.2	49.0	48.8	48.6	49.2	49.0	49.0	48.8
10	36.0	35.9	35.9	33.7	36.0	35.9	35.9	35.4
11	213.9	214.0	213.6	213.8	213.9	213.6	213.9	214.3
12	48.8	48.8	48.7	48.7	48.8	48.7	48.7	48.3
13	49.7	49.2	49.0	49.1	49.9	49.1	49.1	49.6
14	49.7	49.6	49.5	49.1	48.8	49.6	49.5	48.4
15	34.4	34.6	34.4	34.5	34.7	34.5	34.5	34.5
16	28.2	28.7	28.8	28.7	27.4	27.9	28.0	26.9
17	50.0	49.9	49.9	50.2	46.8	49.7	49.6	46.4
18	17.0	17.0	16.9	16.7	17.0	16.9	16.9	16.8
19	20.2	20.5	20.3	19.4	20.2	20.3	20.5	20.0
20	36.3	36.0	36.0	36.0	43.1	35.8	35.8	42.3
21	18.6	18.6	18.6	18.6	13.0	18.4	18.4	12.3
22	34.6	34.0	33.9	34.0	69.6	30.4	30.3	70.0
23	28.9	28.1	28.1	27.7	33.1	33.3	33.2	31.3
24	75.8	79.1	79.0	79.1	72.1	216.4	216.0	74.9
25	80.6	72.7	72.7	72.2	80.6	76.8	76.8	73.1
26	22.6	25.5	25.3	26.0	23.0	27.3	27.3	23.9
27	23.0	25.9	26.0	26.2	23.0	27.3	27.3	26.8
28	26.3	28.3	28.4	20.8	26.3	28.3	28.2	27.3
29	28.0	26.2	26.1	25.4	28.0	25.9	25.5	25.4
30	18.2	18.4	18.3	19.8	18.4	18.2	18.3	18.4
C_3-Glc (G)								
G1		105.0	105.2	106.8		107.4	105.0	
G2		80.4	80.3	75.6		75.5	80.4	
G3		76.4	76.5	78.6		78.8	76.3	
G4		72.1	71.8	71.7		71.8	72.0	
G5		78.1	78.2	78.5		78.3	78.2	
G6		62.8	62.6	62.9		63.0	62.7	
Rha (R)								
R1		101.0	100.9				101.0	
R2		72.4	72.5				72.3	
R3		72.6	69.8				72.6	
R4		74.2	76.2				74.1	
R5		69.6	67.1				69.6	
R6		19.3	19.0				19.4	
R4-OCO*Me*		21.4						
R4-OCOMe		170.8						
C_{25}-Glc (G′)								
G′1	97.6				97.7			
G′2	75.6				75.6			
G′3	79.0				78.9			
G′4	71.8				71.7			
G′5	78.5				78.5			
G′6	62.8				62.8			

[a]Determined in $CDCl_3$.

From the foregoing, it can be concluded that the EtOAc-soluble fraction of the *B. dioica* root extract and several of its cucurbitane-type triterpene constituents have potential importance from the point of view of cancer chemoprevention.

Experimental Section

General Experimental Procedures. Crystallizations were performed in MeOH. Melting points were measured on a Yanagimoto micro mp apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-370 polarimeter in MeOH at 25 °C. IR spectra were recorded on a JASCO IR-300 IR spectrometer in KBr disks. NMR spectra were recorded with a JEOL LA-500 spectrometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in pyridine-d_5 or in $CDCl_3$, and chemical shifts were expressed in δ (ppm) referring to tetramethylsilane (TMS). FABMS and HRFABMS (positive-ion mode) were analyzed by a JEOL JMS-BU20 spectrometer using glycerol as matrix. Silica gel (Silica gel 60, Merck) and octadecyl silica (Chromatorex-ODS, 100−200 mesh; Fuji Silysia Chemical, Ltd., Aichi, Japan) were used for open column chromatography. GLC was performed using a DB-17 fused-silica capillary column (30 m × 0.3 mm i.d., column temperature 150 °C). Reversed-phase HPLC was carried out on an octadecyl silica gel column (PEGASIL ODS II column, 25 cm × 10 mm i.d.; Senshu Scientific Co., Ltd., Tokyo, Japan) at 25 °C with MeOH−H_2O (4:1; HPLC I) as mobile phase at 3 mL/min.

Table 3. Inhibitory Effects of Compounds **1**−**11** on TPA-Induced Inflammation in Mice and on the Induction of Epstein−Barr Virus Early Antigen

compound	inhibition of inflammation: ID_{50}[a] (mg/ear)	inhibition of inflammation: I.R.[b] (%)	percentage of EBV-EA induction[c], concentration (mol raito/TPA): 1000	500	100	10
1			3.4 (60)	30.4	75.1	100
2	0.2	94	0 (60)	29.9	72.7	97.4
3	0.3	83	0 (60)	30.2	73.5	100
4			0 (60)	28.6	70.0	95.3
5	0.2	94	11.6 (60)	37.0	77.2	100
6			0 (60)	29.2	71.4	97.6
7	0.2	90	0 (60)	30.6	74.2	100
8	0.6	66	0 (60)	26.1	69.3	94.1
9	0.7	51	0 (60)(60)	28.7	71.7	96.0
10			0 (70)	23.6	69.3	88.9
11			0 (70)	28.9	73.5	92.7
quercetin[d]	1.6	40				
indomethacin[d]	0.3	96				
β-carotene[d]			8.6 (70)	34.2	82.1	100

[a] ID_{50}: 50% inhibitory dose. [b]I.R.: inhibition ratio at 1.0 mg/ear. [c]Values represent relative percentages to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells. [d]Reference compound.

Plant Material and Chemicals. The roots of *Bryonia dioica* Jacq. (Cucurbitaceae) were collected in July, 1995.[5] A voucher specimen has been deposited in the College of Science and Technology, Nihon University. Compounds were purchased as follows: 12-*O*-tetradecanoylphorbol-13-acetate (TPA) from ChemSyn Laboratories (Lenexa, KS), quercetin and indomethacin from Sigma Chemical Co. (St. Louis, MO), the EBV cell culture reagent, *n*-butyric acid, D-glucose, and L-rhamnose from Nacalai Tesque, Inc. (Kyoto, Japan), and TMS-HT (hexamethyldisilazane and trimethylchlorosilane in anhydrous C_5H_5N) from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan).

Animals. Female ICR mice were obtained from Japan SLC (Shizuoka, Japan). The animals were housed in an air-conditioned specific pathogen-free room (22−23 °C lit from 08:00 to 20:00). Food and water were available ad libitum.

Assay of TPA-Induced Inflammation Ear Edema. TPA (1 μg) dissolved in acetone (20 μL) was applied to the right ear only of ICR mice by means of a micropipet. A volume of 10 μL was delivered to both the inner and outer surfaces of the ear. The samples or the vehicle, MeOH−$CHCl_3$−H_2O (1:2:1, 20 μL), as a control, were applied topically about 30 min before TPA treatment. For ear thickness determination, a pocket thickness gauge with a range of 0−9 mm, graduated at 0.01 mm intervals and modified so that the contact surface area was increased to reduce the tension, was applied to the tip of the ear. The ear thickness was measured before treatment (a) and 6 h after TPA treatment (b = TPA alone; b' = TPA plus sample). The following values were then calculated:

Edema A as induced by TPA alone ($b - a$)

Edema B as induced by TPA plus sample ($b' - a$)

Inhibitory Ratio (I.R.) (%) = [(Edema A − Edema B)/Edema A] × 100

Each value was the mean of individual determinations from five mice. The 50% inhibitory dose (ID_{50}) values were determined by the method of probit-graphic interpolation for four dose levels.

Statistical Analysis. Statistical analysis was carried out by Student's *t*-test.

In Vitro Assay for Epstein−Barr Virus Early Antigen Activation Effect. The inhibition of Epstein−Barr virus early antigen (EBV-EA) activation was assayed using Raji cells (virus nonproducer), the EBV genome-carrying human lymphoblastoid cells, which were cultivated in 10% fetal bovine serum-Roswell Park Memorial Institute (FBS RPMI) 1640 medium solution. The indicator cells (Raji) (1×10^6/mL) were incubated at 37 °C for 48 h in 1 mL of the medium containing *n*-butyric acid (4 mM, inducer) and 32 pmol of TPA [20 ng/mL in dimethyl sulfoxide (DMSO) and a known amount of test compound in DMSO]. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-EA-positive sera from nasopharyngeal carcinoma patients and were detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the experiments were repeated twice. The average EA induction was compared with that of positive control experiments with *n*-butyric acid plus TPA in which EA induction was ordinarily around 30%.

Extraction and Isolation. Air-dried and ground roots of *B. dioica* (593 g) were extracted with MeOH in a Soxhlet extractor. The resultant dried extract (216 g), which showed an I.R. (inhibitory ratio) = 27% at 1 mg/ear on the assay of TPA-induced inflammation ear edema in mice, was partitioned between *n*-hexane−MeOH−H_2O (95:95:10), which gave *n*-hexane (8 g; I.R. = 67% at 1 mg/ear) and MeOH−H_2O fractions. The latter fraction, after evaporation of the solvent, was partitioned between EtOAc−H_2O (1:1), yielding EtOAc (32 g; I.R. = 90% at 1 mg/ear) and H_2O fractions. The H_2O fraction was extracted with *n*-BuOH, which yielded *n*-BuOH (100 g; I.R. = 12% at 1 mg/ear) and residual H_2O fractions (82 g; I.R. = 13% at 1 mg/ear). Chromatography on Si gel of a portion (15 g) of the EtOAc fraction yielded fraction I (2.1 g) from eluant of *n*-hexanes−EtOAc (1:1, v/v), fraction II (8.6 g) from EtOAc eluant, and fraction III (5.1 g) from MeOH eluant. Fraction II upon chromatography on ODS column with MeOH−H_2O (1:1 → 1:0, v/v) as eluants followed by preparative HPLC eventually yielded nine compounds: **1** (11 mg; t_R 21.0 min in HPLC), **2** (222 mg; t_R 10.0 min), **3** (93 mg; t_R 14.1 min), **4** (30 mg; t_R 8.2 min), **5** (94 mg; t_R 5.4 min), **6** (15 mg; t_R 27.4 min), **7** (80 mg; t_R 15.6 min), **8** (58 mg; t_R 14.6 min), and **9** (867 mg; t_R 2.3 min).

Bryonioside A (1): needles, mp 155−156 °C; $[\alpha]^{25}_D$ +105.3° (MeOH; *c* 0.30); IR (KBr) ν_{max} 3421 (OH), 1689 (C=O), 825 (>C=CH−) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m/z* 659.4135 [M + Na]$^+$ (calcd for $C_{36}H_{60}O_9$·Na, 659.4132).

Bryonioside B (**2**): needles, mp 149−151 °C; $[\alpha]^{25}_D$ +22.3° (MeOH; *c* 1.05); IR (KBr) ν_{max} 3428 (OH), 1686 (C=O), 812 (>C=CH−) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m/z* 805.4714 [M + Na]$^+$ (calcd for $C_{42}H_{70}O_{13}$·Na, 805.4710).

Bryonioside C (3): needles, mp 172−176 °C; $[\alpha]^{25}_D$ +64.2° (MeOH; *c* 0.24); IR (KBr) ν_{max} 3421 (OH), 1731 (OAc), 1689 (C=O), 1242 (OAc) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m/z* 847.4819 [M + Na]$^+$ (calcd for $C_{44}H_{72}O_{14}$·Na, 847.4815).

Bryonioside D (**5α,6α-epoxycabenoside D**; **4**): needles, mp 124−156 °C; $[\alpha]^{25}_D$ +75.0° (MeOH; *c* 0.24); IR (KBr) ν_{max} 3417 (OH), 1689 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m/z* 675.4086 [M + Na]$^+$ (calcd for $C_{36}H_{60}O_{10}$·Na, 675.4081).

Bryonioside E (**5**): needles, mp 162−164 °C; $[\alpha]^{25}_D$ +54.5° (MeOH; *c* 0.33); IR (KBr) ν_{max} 3419 (OH), 1687 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m/z* 675.4084 [M + Na]$^+$ (calcd for $C_{36}H_{60}O_{10}$·Na, 675.4081).

Bryonioside F (6): needles, mp 192−193 °C; $[\alpha]^{25}_D$ +62.4° (MeOH; *c* 0.31); IR (KBr) ν_{max} 3424 (OH), 1699 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m/z* 657.3980 [M + Na]$^+$ (calcd for $C_{36}H_{58}O_9$·Na, 657.3975).

Bryonioside G (7): needles, mp 162−164 °C; $[\alpha]^{25}_D$ +48.8° (MeOH; *c* 0.32); IR (KBr) ν_{max} 3423 (OH), 1693 (C=O) cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m/z* 803.4560 [M + Na]$^+$ (calcd for $C_{42}H_{68}O_{13}$·Na, 803.4554).

Acid Hydrolysis of Compounds 2, 5, and 6. Compound **2** (50 mg) was refluxed in 1 M H_2SO_4−MeOH (4 mL) for 2 h on a water bath. The reaction mixture was diluted with ice water and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. Preparative-HPLC of the residue gave two hydrolysis products, cabenoside D (**8**)[7] (8 mg) and bryodulcosigenin (**10**)[9,15] (12 mg; t_R 45.9 min in HPLC). The H_2O layer was neutralized with Na_2CO_3 in H_2O and concentrated under reduced pressure. The residue was dissolved in H_2O, passed through an ion-exchange resin (Amberlite MB-3) column, concentrated (dried overnight), and then treated with TMS-HT (hexamethyldisilazane and trimethylchlorosilane in anhydrous C_5H_5N). The TMSi derivatives of the monosaccharides were identified as glucose and rhamnose by co-GLC with standard monosaccharides. By the same method, compounds **5** and **6** afforded (22ξ,24ξ)-22-hydroxybryodulcosigenin (**12**; t_R 7.8 min) and bryosigenin[15] (**11**; t_R 75.7 min), respectively, as aglycons, and glucose as a monosaccharide.

(22ξ,24ξ)-22-Hydroxybryodulcosigenin (**12**): ^1H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 513 [M + Na]$^+$.

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