Three New Quassinoid Derivatives and Related Compounds as Antitumor Promoters from *Brucea javanica*

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Three new quassinoid derivatives [desmethyl-brusatol (**1**), desmethyl- bruceantinoside A (**2**), and butyl ester (**3**) of bruceoside D (**6**)] were isolated from the extract of *Brucea javanica*. Their structures were elucidated by spectral evidence and chemical transformation to or from known compounds. The three new quassinoid derivatives and related compounds have shown inhibitory effects against TPA-induced Epstein–Barr virus early antigen (EBV-EA) activation.

Quassinoids, which are bitter principles of Simaroubaceae, have been extensively invesitigated from a structural viewpoint, their useful biological activities, and interesting structure-activity relationships.[1] Following our earlier isolation of bruceosides A, B, and C, as well as brusatol (**4**) and cleomiscosin A, from *Brucea javanica* (L.) Merr. (Simaroubaceae) ("Ya-Tan-Tzu")[2—4] and the isolation of sixteen quassinoid glucosides by Takahashi et al.,[5—12] we reported on the isolation of three new quassinoid glucosides [bruceosides D (**6**), E (**7**), and F (**8**)[13]] from the same species. The quassinoid bruceoside-C demonstrated potent cytotoxicity against human epidermoid carcinoma of the nasopharynx (KB) (ED_{50} < 0.1 $\mu g\,ml^{-1}$) and CNS carcinoma (TE-671) (ED_{50} = 0.29 $\mu g\,ml^{-1}$) as well as murine lymphocytic leukemia (P-388) (ED_{50} = 5.11 $\mu g\,ml^{-1}$). Bruceosides D (**6**), E (**7**), and F (**8**) were also evaluated for cytotoxicity in the NCI's in vitro human cell line panel, which included fifty-eight cell lines representing nine cancer types. These quassinoids showed selective cytotoxicity in leukemia, non-small cell lung cancer, prostate cancer, and breast cancer cell lines. The log GI_{50} values (log concentration which reduced cell growth to 50%) ranged from −4 to −5.72. Fifteen known quassinoids (bruceins D and E, bruceosides A, B, and C, bruceantinoside A, yadanziolide C, yadanziosides A, B, C, E, F, G, L, and M), which we isolated from the same species, showed inhibitory effect against TPA-induced Epstein–Barr virus early antigen (EBV-EA) activation.[14]

We are very interested in the bioactive constituents of Ya-Tan-Tzu, and carried out a detailed investigation on the components of the *n*-BuOH extract, and recently succeeded to isolate a new quassinoid, desmethyl-brusatol (**1**), and two new quassinoid glucosides, desmethyl-bruceantinoside A (**2**) and butyl ester (**3**) of bruceoside D (**6**). Their structures were elucidated by spectral evidence and chemical transformation to or from the known compounds: compound **1** to brusatol (**4**), compound **2** to bruceantinoside A (**5**), and compound **3** from bruceoside D (**6**). This paper deals with their isolation, their structure elucidation, and inhibitory effect against TPA-induced EBV–EA activation of the three new quassinoid derivatives (**1**—**3**) and related compounds (**4** and **6**—**8**)[3,13] which were isolated form the same species (Fig. 1).

Results and Discussion

Compound **1** was obtained as a colorless amorphous solid by column chromatography (CC) using Sephadex LH-20 and repeated preparative TLC and HPLC. Its IR spectrum showed the presence of hydroxy (3410 cm^{-1}), δ-lactone and ester (1735 cm^{-1}), and α,β-unsaturated carbonyl (1680 and 1640 cm^{-1}) groups. The UV spectrum of compound **1** exhibited an absorption maximum at 278 nm due to a conjugated enone system. Its molecular formula was established to be $C_{25}H_{30}O_{11}$ from its HRSIMS spectrum (*m/z* 506.1784). The ^{1}H NMR spectrum (Table 1) of compound **1** disclosed the presence of 3-methyl-2-butenoyl (senecioyl) [δ = 2.16 (3′-Me), 1.52 (3′-Me), and 5.92 (H-2′)] and two methyl [δ = 1.90 (4-Me) and 1.62 (10-Me)] groups. The ^{13}C NMR spectrum (Table 2) of compound **1** also indicated the presence of a senecioyl [δ = 166.0 (C-1′), 116.5 (C-2′), 157.5 (C-3′), 20.3 (C-4′), and 26.9 (C-5′)] group. The ^{1}H and ^{13}C NMR signals of compound **1** were similar to those

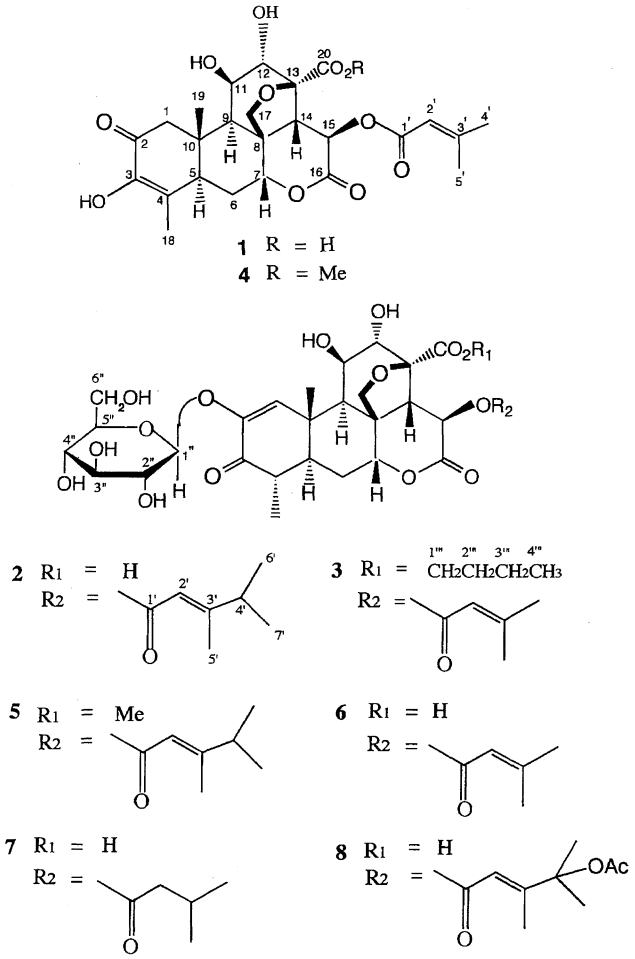

Fig. 1. Structures of compounds **1**—**8**.

of brusatol (**4**), except for the chemical shift values at C-13. Brusatol (**4**) contains a COOMe group at C-13, which shows ^{1}H and ^{13}C NMR signals at δ = 3.76 and 52.4, respectively. However, these signals were not present in the spectra of compound **1**. These results suggested that the C-13 moiety of compound **1** was a free COOH and hence compound **1** was assumed to be desmethyl-brusatol. The structure of compound **1** was confirmed from the fact that brusatol (**4**) was obtained by methylation of compound **1** with CH_2N_2.

Compound **2** was obtained as a colorless amorphous solid by CC using Sephadex LH-20 and repeated preparative TLC and HPLC. Its IR spectrum showed the presence of hydroxy (3400 cm^{-1}), δ-lactone and ester (1740 cm^{-1}), and α,β-unsaturated carbonyl (1675 and 1650 cm^{-1}) groups. The UV spectrum of compound **2** exhibited an absorption maximum at 254 nm due to a conjugated enone system. Its molecular formula was established as $C_{33}H_{44}O_{16}$ from its HRSIMS spectrum (*m/z* 696.2608). The EIMS spectrum showed a fragment ion peak $[M-C_6H_{10}O_5-H]^+$ at *m/z* 533, which suggested a glycoside structure.

The ^{1}H NMR spectrum (Table 1) of compound **2** showed that it bears a *trans*-3,4-dimethyl-2- pentenoyl [δ = 2.14 (3′-Me), 0.69 (4′-Me×2), 2.4 (H-4′), and 5.86 (H-2′)] and two methyl [δ = 1.19 (4-Me) and 1.66 (10-Me)] groups. The ^{13}C NMR spectrum (Table 2) also indicated the presence of a *trans*-3,4-dimethyl-2-pentenoyl [δ = 174.1 (C-1′), 113.8 (C-2′), 168.6 (C-3′), 38.0 (C-4′), 16.6 (C-5′), 20.5 (C-6′), and 20.5 (C-7′)] group and a β-D-glucose [δ = 101.9 (C-1″), 74.8 (C-2″), 78.6 (C-3″), 71.3 (C-4″), 79.0 (C-5″), and 62.3 (C-6″)] moiety. Both the ^{1}H and ^{13}C NMR spectra of compound **2** were similar to those of bruceantinoside A (**5**) except for the chemical shift values at C-13. Bruceantinoside A (**5**) contains a COOMe group at C-13, which shows ^{1}H and ^{13}C NMR signals at δ = 3.78 and 52.4, respectively. However, these signals were not present in the spectra of compound **2**. From these results, compound **2** was assumed to be desmethyl-bruceantinoside A. The structure of compound **2** was confirmed from the fact that bruceantinoside A (**5**) was obtained by methylation of compound **2** with CH_2N_2.

Compound **3** was obtained as a colorless amorphous solid by CC using Sephadex LH-20 and repeated preparative TLC and HPLC. Its IR and UV spectra were similar to those of compounds **1** and **2**. Its molecular formula was established as $C_{33}H_{48}O_{16}$ from its HRSIMS spectrum (*m/z* 724.2916). The EIMS spectrum suggested a glycoside structure like that of compound **2**. The ^{1}H NMR spectrum (Table 1) of compound **3** showed that it bears a senecioyl [δ = 2.16 (3′-Me), 1.62 (3′-Me), and 5.91 (H-2′)], two methyl [δ = 1.17 (4-Me) and 1.62 (10-Me)], and a butyl ester [δ = 4.30 (H-1‴), 1.59 (H-2‴), 1.29 (H-3‴), and 0.77 (3‴-Me)] groups. The ^{13}C NMR spectrum (Table 2) of compound **3** indicated the presence of a senecioyl [δ = 169.0 (C-1′), 116.2 (C-2′), 158.2 (C-3′), 20.1 (C-4′), and 27.1 (C-5′)], a butyl ester [δ = 170.9 (C-20), 65.5 (C-1‴), 30.8 (C-2‴), 19.3 (C-3‴), and 13.8 (C-4‴)], and a β-D-glucose [δ = 102.0 (C-1″), 74.7 (C-2″), 78.6 (C-3″), 71.3 (C-4″), 79.0 (C-5″), and 62.4 (C-6″)] moieties. Both the ^{1}H and ^{13}C NMR spectra of compound **3** were similar to those of bruceoside D (**6**), except for the chemical shift values at C-13. Bruceoside D (**6**) contains a free COOH group at C-13, whereas compound **3** contains a butyl ester group, which shows ^{1}H and ^{13}C NMR signals at δ = 4.3, 1.59, 1.29, and 0.77 and 65.5, 30.8, 19.3, and 13.8, respectively. From these results, compound **3** was assumed to be a butyl ester of bruceoside D (**6**). The structure of compound **3** was confirmed from the fact that compound **3** was obtained by butylation of bruceoside D (**6**).

Compounds **4**,[3] **6**,[13] **7**,[13] and **8**[13] were isolated from the same plant. Compounds **1**, **2**, **6**, **7**, and **8** might be obtained by the hydrolysis of brusatol (**4**), bruceantinoside A (**5**), bruceoside A, yadanzioside A, and yadanzioside G, respectively, in the process of the first EtOH extraction of the plant, and compound **3** might be obtained in the process of *n*-BuOH extraction.

The three new quassinoid derivatives (**1**, **2**, and **3**) and related compounds (**4**, **6**, **7**, and **8**) were assayed for their inhibitory effect against TPA-induced EBV–EA activation and their IC_{50} values are shown in Table 3. All the quassinoids used in this investigation (IC_{50} = 6.8—143) were more active than the reference compounds,[17] curcumin (IC_{50} = 289), β-carotene (IC_{50} = 340), and glycyrrhizin (IC_{50} = 531). The structure-activity relationships concerning the moiety of C-

Table 1. ^{1}H NMR Spectra[a] of Compounds **1**—**6**

Proton	Compound					
	1[b]	**2**[b]	**3**[c]	**4**[b]	**5**[b]	**6**[c]
H-1		7.32 br s	7.29 s	—	7.29 s	7.31 s
H-1α	2.44 d (15)	—		2.50 d (16)	—	
H-1β	3.25 d (15)	—		3.28 d (16)	—	
H-4	—	2.41 m	2.40 m	—	2.40 m	2.42 d q
H-5	3.0 d (10)	d)	d)	2.9 d (4)	d)	d)
H-6α	2.24 br s	2.04 br d (14.8)	2.02 d (15)	2.29 m	2.04 br d (14.8)	2.04 br d
H-6β	1.59 br s	1.71 br s	1.65 m	1.60 s	1.67 s	1.68 br d
H-7	4.89 br s	5.0 br s	4.97 br s	4.92 br s	4.86 br s	4.99 br s
H-9	2.59 br s	2.57 d (4)	2.53 d (4.5)	2.59 d (4.4)	2.54 d (4.4)	2.55 d (5.0)
H-11	4.79 s	5.25 d (4.4)	5.20 d (3)	4.78 d (4.8)	5.17 d (4.4)	5.23 d (5.0)
H-12	5.10 br s	5.20 br s	5.15 m	5.08 s	5.0 s	5.20 br d
H-14	4.21 d (7.2)	d)	d)	3.91 d (7.2)	d)	d)
H-15	d)	d)	d)	d)	d)	d)
H-17α	3.70 br s	3.95 br s	3.91 m	3.70 s	3.90 s	3.95 m
H-17β	5.10 br s	5.13 d (6.4)	5.08 d (7.5)	d)	5.08 d (7.6)	5.12 d (7.5)
4-Me	1.90 s	1.19 d (6.8)	1.17 d (7.0)	1.95 d (1.2)	1.18 d (6.4)	1.18 d (6.5)
10-Me	1.62 s	1.66 s	1.62 s	1.67 s	1.63 s	1.64 s
20-OMe	—	—	—	3.76 s	3.78 s	—
H-2′	5.92 s	5.86 s	5.91 m	5.85 s	5.86 s	5.79 m
H-4′	—	2.4 m	—	—	2.48 m	—
3′-Me	2.16 s	2.14 s	2.16 s	2.15 s	2.16 s	2.09 s
	1.52 s	—	1.68 s	1.63 s	—	1.45 s
4′-Me	—	0.69 d (6)	—	—	0.85 d (6.8)	—
	—	0.69 d (6)	—	—	0.85 d (6.8)	—
H-1″	—	5.40 d (6.8)	5.36 d (7.5)	—	5.35 d (7.6)	5.39 d (7.0)
H-2″	—	4.29 m	4.28 m	—	4.32 m	4.28 m
H-3″	—	4.26 m	4.27 m	—	4.26 m	4.27 m
H-4″	—	4.25 m	4.26 m	—	4.22 m	4.26 m
H-5″	—	3.95 m	3.93 m	—	3.91 m	3.94 m
H-6″	—	4.23 m	4.23 m	—	4.24 m	4.23 m
		4.49 d (11.2)	4.49 d (10.5)		4.48 d (8.4)	4.49 dd (12.3)
H-1‴	—	—	4.30 m	—	—	—
H-2‴	—	—	1.59 m	—	—	—
H-3‴	—	—	1.29 m	—	—	—
3‴-Me	—	—	0.77 t (7.3)	—	—	—

a) Values are in ppm (C_5D_5N). The coupling constant (J values) in parentheses are Hz. b) 400 MHz. c) 500 MHz. d) Not assignable.

13 are as follows. Desmethyl-brusatol (**1**) (IC_{50} = 7.7) and desmethyl-bruceantinoside A (**2**) (IC_{50} = 143) were respectively less active than brusatol (**4**) (IC_{50} = 6.8) and bruceantinoside A (**5**) (IC_{50} = 50).[14] Similarly, the butyl ester (**3**) (IC_{50} = 90) of bruceoside D (**6**) was more active than bruceoside D (**6**) (IC_{50} = 123) whose moiety at C-13 is COOH. Thus, esterification of the COOH group at C-13 is more effective than to be a free COOH group. But, the difference of the IC_{50} values between **1** and **4** is very small, whereas the difference between **3** and **6** is slightly larger. This fact suggests that higher lipophilicity at C-13 is more effective for an enhancement of activity.

Experimental

The melting points were determined on an MRK air-bath-type melting-point apparatus and are uncorrected. Specific rotations were obtained on a JASCO DIP-370 digital polarimeter (length = 0.5 dm). IR spectra were recorded on a JASCO IR-810 spectrophotometer and UV spectra were obtained on a Hitachi 320-S or Shimadzu UV 3101 PC spectrophotometers. ^{1}H and ^{13}C NMR spectra were determined on JEOL JNM-A 400 or Varian VXR-500 instruments in C_5D_5N using TMS as an internal standard. Mass spectra were recorded on a Hitachi M-80 instrument. HRSIMS spectra were obtained in glycerol or a mixture of glycerol and thioglycerol. SiO_2 gel (Merck, type 60, 70—230 mesh) was used for CC. Precoated SiO_2 gel plates (Merck, $60F_{254}$, 0.25 mm thickness) were used for analytical TLC. Plates of 1 mm and 2 mm thickness were used for prep. TLC. $CHCl_3$–MeOH–H_2O (50 : 14 : 3, v/v, lower phase) was used as the TLC solvent. Components were detected on TLC plates using a UV lamp (254 and 365 nm) and the indicator bromocresol Green [BCG, 0.3% solution of MeOH–H_2O (4 : 1, v/v)]. Analytical HPLC was performed on a Tosoh liquid chromatograph equipped with a UV detector at 254 nm and a reverse-phase column (TSK-gel ODS-80Ts) using a mixed solvent of MeOH–H_2O–AcOH or MeOH–H_2O. Medium-pressure liquid chromatography (MPLC) was carried out with Micro Pump (Kusano Kagakukikai Co.) equipped with a reverse-phase column (Lobar Lichroprep. RP-18). Preparative HPLC and recycling HPLC were performed on Tosoh or Gilson liquid chromatographs, equipped with a reverse-phase column (Lichrosorb RP-18 or Dynamax-60A) at 254 nm. The same solvent as for analytical HPLC was employed

Table 2. ^{13}C NMR Spectra[a] of Compounds **1**—**6**

Carbon	Compound											
	1[b]		**2**[b]		**3**[c]		**4**[b]		**5**[b]		**6**[c]	
C-1	50.2	(CH_2)	129.4	(CH)	129.2	(CH)	50.2	(CH_2)	129.4	(CH)	129.3	(CH)
C-2	193.0	(C=O)	148.9	(C)	148.9	(C)	193.0	(C=O)	148.9	(C)	148.4	(C)
C-3	146.0	(C)	194.6	(C=O)	194.5	(C=O)	146.0	(C)	194.6	(C=O)	194.6	(C=O)
C-4	128.2	(C)	41.4	(CH)	41.4	(CH)	128.3	(C)	41.4	(CH)	41.4	(CH)
C-5	42.5	(CH)	43.9	(CH)	43.8	(CH)	42.5	(CH)	43.9	(CH)	43.8	(CH)
C-6	29.6	(CH_2)	30.2	(CH_2)	30.1	(CH_2)	29.6	(CH_2)	30.0	(CH_2)	30.1	(CH_2)
C-7	83.9	(CH)	83.7	(CH)	83.4	(CH)	83.6	(CH)	83.4	(CH)	83.7	(CH)
C-8	41.4	(C)	46.7	(C)	46.8	(C)	41.4	(C)	46.7	(C)	46.7	(C)
C-9	42.4	(CH)	40.6	(CH)	40.6	(CH)	42.1	(CH)	40.5	(CH)	40.6	(CH)
C-10	46.4	(C)	39.7	(C)	39.7	(C)	46.2	(C)	39.7	(C)	39.7	(C)
C-11	73.7	(CH)	73.2	(CH)	73.7	(CH)	73.2	(CH)	73.2	(CH)	73.2	(CH)
C-12	76.1	(CH)	76.8	(CH)	76.1	(CH)	75.9	(CH)	76.0	(CH)	76.7	(CH)
C-13	83.0	(C)	82.6	(C)	82.8	(C)	82.8	(C)	82.9	(C)	82.5	(C)
C-14	49.8	(CH)	51.8	(CH)	50.0	(CH)	51.6	(CH)	51.7	(CH)	50.2	(CH)
C-15	67.8	(CH)	68.3	(CH)	68.0	(CH)	68.3	(CH)	68.2	(CH)	68.3	(CH)
C-16	168.9	(C=O)	168.4	(C=O)	168.4	(C=O)	168.3	(C=O)	168.2	(C=O)	168.2	(C=O)
C-17	71.5	(CH_2)	73.8	(CH_2)	73.7	(CH_2)	73.8	(CH_2)	73.6	(CH_2)	73.8	(CH_2)
C-18	13.4	(Me)	12.6	(Me)	12.6	(Me)	13.4	(Me)	12.6	(Me)	12.6	(Me)
C-19	15.9	(Me)	18.1	(Me)	18.0	(Me)	15.8	(Me)	18.0	(Me)	18.0	(Me)
C-20	174.0	(C=O)	173.4	(C=O)	170.9	(C=O)	171.3	(C=O)	171.2	(C=O)	173.5	(C=O)
20-OMe	—		—		—		52.4	(Me)	52.4	(Me)	—	
C-1′	166.0	(C=O)	174.1	(C=O)	169.0	(C=O)	165.4	(C=O)	165.0	(C=O)	174.0	(C=O)
C-2′	116.5	(CH)	113.8	(CH)	116.2	(CH)	116.0	(CH)	113.6	(CH)	116.3	(CH)
C-3′	157.5	(C)	168.6	(C)	158.2	(C)	158.4	(C)	165.0	(C)	157.7	(C)
C-4′	20.3	(Me)	38.0	(CH)	20.2	(CH)	20.2	(Me)	38.1	(CH)	26.9	(CH)
C-5′	26.9	(Me)	16.6	(Me)	27.1	(Me)	27.0	(Me)	16.7	(Me)	20.1	(Me)
C-6′	—		20.5	(Me)	—		—		20.7	(Me)	—	
C-7′	—		20.5	(Me)	—		—		20.7	(Me)	—	
C-1″	—		101.9	(CH)	102.0	(CH)	—		102.0	(CH)	101.9	(CH)
C-2″	—		74.8	(CH)	74.7	(CH)	—		74.7	(CH)	74.7	(CH)
C-3″	—		78.6	(CH)	78.6	(CH)	—		79.0	(CH)	78.5	(CH)
C-4″	—		71.3	(CH)	71.3	(CH)	—		71.2	(CH)	71.2	(CH)
C-5″	—		79.0	(CH)	79.0	(CH)	—		78.6	(CH)	78.9	(CH)
C-6″	—		62.3	(CH_2)	62.4	(CH_2)	—		62.4	(CH_2)	62.3	(CH_2)
C-1‴	—		—		65.5	(CH_2)	—		—		—	
C-2‴	—		—		30.8	(CH_2)	—		—		—	
C-3‴	—		—		19.3	(CH_2)	—		—		—	
C-4‴	—		—		13.8	(Me)	—		—		—	

a) Values are in ppm (C_5D_5N). b) 100.4 MHz. c) 125.7 MHz.

Table 3. Inhibitory Effects of Quassinoids (**1**—**4** and **6**—**8**) against TPA-Induced EBV–EA Activation

Compound	%EBV–EA positive cells					IC_{50}
	Compound concentration (mol ratio/32 pmol TPA)					
	1000	500	100	10	0[a]	
Brusatol (**4**)	0 (80)[b]	0	2	11	40	6.8
Desmethyl-brusatol (**1**)	0 (80)[b]	0	3	14	40	7.7
Butyl ester (**3**) of Bruceoside D	0 (80)[b]	2	19	29	40	90
Bruceoside E (**7**)	0 (80)[b]	3	19	30	40	91
Bruceoside D (**6**)	0 (80)[b]	5	21	32	40	123
Desmethyl-bruceantinoside A (**2**)	0 (80)[b]	5	22	33	40	143
Bruceoside F (**8**)	0 (80)[b]	5	22	33	40	143

a) Control. b) Values in the parentheses are viability percentage of Raji cells.

in all cases.

Materials. Plant material is described earlier.[4] The fruit of *B. javanica* was procured and identified by C. H. Huang. A voucher specimen is available for inspection at the herbarium of the School of Pharmacy, Kaohsiung Medical College, Kaohsiung, Taiwan.

Raji cells, TPA, and compounds **1**—**4** and **6**—**8** whose structures

are shown in Fig. 1 were used for the in vitro EBV–EA activation test.

Extraction and Isolation. The fruit (45.5 kg) of *B. javanica* was ground and extracted with hot EtOH. The EtOH solution was concentrated to give a syrup, which was partitioned between H_2O and hexane. The hexane solution was evaporated to afford a hexane extract (6.86 kg) as a brown viscous oil. The aqueous solution was extracted successively with $CHCl_3$ and then *n*-BuOH. The $CHCl_3$ and *n*-BuOH solutions were concentrated to dryness to give a $CHCl_3$ extract (992 g) as a brown gum and a *n*-BuOH extract (1.45 kg) as a brown resin.

A part (445 g) of the crude *n*-BuOH extract was subjected to SiO_2 gel CC using EtOAc–Et_2O (1 : 1, v/v) to give four fractions, then $CHCl_3$–MeOH–H_2O (50 : 14 : 3, v/v, lower phase) to yield eleven fractions, and finally MeOH to afford two fractions.

From the analytical TLC and IR spectra of each fraction, quassinoid glycosides were assumed to be in the eleven fractions and the major quassinoids were isolated.[4] An HPLC chromatogram of the 9th fraction showed the presence of a very minor amount of a new quassinoid, which was compound **3**. The first MeOH fraction contained acidic components as indicated by analytical TLC using BCG.

The first MeOH fraction (108 g) was dissolved in MeOH and filtered. The filtrate was evaporated to afford a brown resinous substance (89 g), which contained three major components [bruceoside D (**6**), E (**7**), and F (**8**)][13] along with very minor amounts of new components as shown by analytical HPLC (MeOH–H_2O–AcOH, 40 : 60 : 1, v/v). The resinous substance (89 g) was subjected to CC using Sephadex LH-20 (MeOH) to remove polymeric compounds. Then three fractions Fr. 1 (68.4 g), 2 (14.1 g), and 3 (1.0 g) were obtained. Compounds **1** and **2** were isolated from these fractions as follows.

Isolation of Compound 1. The Fr. 1 (68.4 g) was subjected to CC using Sephadex LH-20 (MeOH) and four subfractions Fr. 1-1 (28.9 g), 1-2 (7.3 g), 1-3 (11.3 g), and 1-4 (17.2 g) were obtained. The Fr. 1-1 (28.9 g) was further separated in the same manner to afford Fr. 1-1-1 (13.5 g) and 1-1-2 (10.9 g). Fr. 1-1-2 (10.9 g) was then subjected to MPLC (MeOH–H_2O–AcOH, 40 : 60 : 1, v/v) to give Fr. 1-1-2-1 (4.24 g), 1-1-2-2 (0.568 g), 1-1-2-3 (0.07 g), and 1-1-2-4 (3.43 g). The Fr. 1-1-2-3 (70 mg) was then subjected to prep. TLC to afford Fr. 1-1-2-3-1 (24 mg), 1-1-2-3-2 (13 mg), 1-1-2-3-3 (25 mg), and 1-1-2-3-4 (4 mg). Finally Fr. 1-1-2-3-3 (25 mg) was subjected to repeated prep. HPLC (MeOH–H_2O–AcOH, 30 : 70 : 1, v/v) to obtain a pure compound **1** (2.5 mg). Then the Fr. 1-2 (7.3 g) was also subjected in the same manner to CC using Sephadex LH-20, MPLC, prep. TLC, and prep. HPLC to obtain a pure compound **1** (1.6 mg). Totally 4.1 mg of compound **1** was obtained.

Desmethyl-brusatol (1). Colorless amorphous solid; mp 221—224 °C (decomp); $[\alpha]_D^{26}$ +66.8° (*c* 0.08, MeOH); UV (MeOH) λ_{max} (ε) 278 (6687) nm; IR (KBr) ν_{max} 3410 (OH), 1735 (δ-lactone and ester C=O), 1680 and 1640 cm^{-1} (α,β-unsaturated C=O); HRSIMS *m/z* M^+ 506.1784 ($C_{25}H_{30}O_{11}$, error −0.2). ^1H and ^{13}C NMR data, see Tables 1 and 2.

Isolation of Compound 2. The Fr. 1-4 (17.2 g) was subjected to CC using Sephadex LH-20 (MeOH) to afford Fr. 1-4-1 (2.4 g), 1-4-2 (12.1 g), and 1-4-3 (1.9 g). Then the Fr. 1-4-1 (2.4 g) was subjected to prep. TLC to give Fr. 1-4-1-1 (297 mg), 1-4-1-2 (195 mg), 1-4-1-3 (141 mg), 1-4-1-4 (614 mg), and 1-4-1-5 (920 mg). Finally, Fr. 1-4-1-3 (141 mg) was subjected to repeated prep. HPLC (MeOH–H_2O–AcOH, 40 : 60 : 1, v/v) to obtain pure compound **2** (1.1 mg). Then, the Fr. 2 (14.1 g) was also subjected to CC using Sephadex LH-20, MPLC, and repeated HPLC to obtain a pure compound **2** (2.7 mg). In total, 3.8 mg of compound **2** was obtained.

Desmethyl-bruceantinoside A (2). Colorless amorphous solid; mp 170—173 °C (decomp); $[\alpha]_D^{27}$ 0° (*c* 0.062, MeOH); UV (MeOH) λ_{max} (ε) 254 (5035) nm; IR (KBr) ν_{max} 3400 (OH), 1740 (δ-lactone and ester C=O), 1675 and 1650 cm^{-1} (α,β-unsaturated C=O); HRSIMS *m/z* M^+ 696.2608 ($C_{33}H_{44}O_{16}$, error −1.8). ^1H and ^{13}C NMR data, see Tables 1 and 2.

Isolation of Compound 3. A part (22 g) of Fr. 9 (67 g) obtained by CC (Si gel) using $CHCl_3$–MeOH–H_2O (50 : 14 : 3, v/v, lower phase) was subjected to CC (Sephadex LH-20) using MeOH to afford three subfractions, Fr. 9-1 (1.5 g), 9-2 (11.2 g), and 9-3 (7.9 g). The Fr. 9-2 (11.2 g) was then subjected to prep. TLC to give eight fractions, Fr. 9-2-1 (0.84 g), 9-2-2 (1.69 g), 9-2-3 (1.87 g), 9-2-4 (3.52 g), 9-2-5 (0.73 g), 9-2-6 (0.98 g), 9-2-7 (0.38 g), and 9-2-8 (0.69 g). Finally, a part (381 mg) of the Fr. 9-2-5 (0.73 g) was subjected to repeated prep. HPLC (MeOH–H_2O, 55 : 45, v/v) to obtain a pure compound **3** (10.3 mg).

Butyl Ester of Desmethyl-bruceoside A (3). Colorless amorphous solid; mp 172—175 °C (decomp); $[\alpha]_D^{26}$ +0.78° (*c* 0.258, MeOH); UV(MeOH) λ_{max} (ε) 250 (8333) nm; IR (KBr) ν_{max} 3400 (OH), 1735 (δ-lactone and ester C=O), 1680 and 1650 cm^{-1} (α,β-unsaturated C=O); HRSIMS *m/z* M^+ 724.2916 ($C_{35}H_{48}O_{16}$, error −2.3). ^1H and ^{13}C NMR data, see Tables 1 and 2.

Methylation of Compound 1. An Et_2O (10 ml) solution of CH_2N_2 prepared from *N*-methyl-*N*-nitrosourea (500 mg, 4.8 mmol) was added to MeOH (1 ml) solution of compound **1** (2.5 mg, 0.0049 mmol) at 0 °C. After the reaction mixture was stirred at 0 °C for 2.5 h, the solvent was evaporated to give a crude product. Purification of the crude product by prep. HPLC (MeOH–H_2O, 50 : 50, v/v) afforded a pure compound **4** (1.7 mg, 68% yield).

Brusatol (4). Colorless amorphous solid; mp 274—276 °C; IR (KBr) ν_{max} 3400 (OH), 1745 (δ-lactone and ester C=O), and 1685 and 1650 cm^{-1} (α,β-unsaturated C=O). ^1H and ^{13}C NMR data, see Tables 1 and 2. The physical and spectral data of compound **4** are identical with those of the known compound brusatol (**4**).[3,16]

Methylation of Compound 2. Methylation of compound **2** (2.0 mg, 0.0029 mmol) was carried out in the same manner as described above, and afforded compound **5** (1.3 mg, 65% yield).

Bruceantinoside A (5). Colorless amorphous solid; mp 150 °C (decomp); IR (KBr) ν_{max} 3410 (OH), 1725 (δ-lactone and ester C=O), and 1678 and 1635 cm^{-1} (α,β-unsaturated C=O). ^1H and ^{13}C NMR data, see Tables 1 and 2. The physical and spectral data of compound **5** are identical with those of the known compound bruceantinoside A (**5**).[15]

Butyl Ester (3) from Bruceoside D (6). Bruceoside D (**6**) (50 mg, 0.074 mmol) was dissolved in *n*-BuOH (10 ml) and the solution was refluxed for 18 h. Then, the solvent was evaporated to give a resinous substance (56 mg). The resinous substance was subjected to prep. TLC and then prep. HPLC (MeOH–H_2O, 50 : 50, v/v) to afford compound **3** (0.7 mg, 1.4% yield) whose IR and ^1H NMR spectra coincided with those of the isolated compound **3**.

EBV–EA Activation Test. The inhibition of EBV–EA activation was assayed using a literature method.[18] The cells were incubated at 37 °C for 48 h in a medium containing butyric acid (4 nmol), TPA (32 pmol), and various amounts of test compounds. Smears were made from the cell suspension and the EBV–EA inducing cells were stained by means of an indirect immunofluorescence technique.[19]

In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were

performed for each data point. The EBV–EA inhibitory activity of the test compound was compared with that of the control experiment with butyric acid plus TPA. In the control experiments, the EBV–EA activities were ordinarily around 40%, and these values were taken as a positive control. The viability of cells was assayed by the trypan-blue staining method. For the determination of cytotoxicity, the cell viability was required to be more than 60%.[20]

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