Regular Article

Ebenamariosides A–D: Triterpene Glucosides and Megastigmanes from the Leaves of *Diospyros maritima*

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The 1-BuOH-soluble fraction of the methanol (MeOH) extract of *Diospyros maritima* was separated by chromatographic techniques to give three new oleanane-type and one new ursane-type triterpene glucoside, named ebenamariosides A–D (1–4); two megastigmanes were also isolated. The structures of triterpene glucosides was elucidated with extensive investigation by one and two dimensional NMR spectroscopy and the structures were confirmed by partial enzymatic hydrolyses to give the corresponding mono-glucosides and aglycones. The structures of the megastigmanes, including their absolute stereochemistries, were elucidated by spectroscopic evidence and by the modified Mosher's method. Two megastigmanes were chemically correlated and their absolute structures were unambiguously determined. The cytotoxicity of the triterpene glucosides and their degradation products were assayed. They did not show any significant activity.

Key words *Diospyros maritima*; Ebenaceae; ebanamarioside; triterpene glucoside; megastigmane

Introduction

Diospyros maritima Blume (Ebenaceae) is a tall evergreen tree with a height of about 10 m, distributed in Okinawa, Taiwan, Malaysia, Micronesia and Australia.[1] In summer, it bears green sap fruits of 2 to 3 cm in diameter, which then turn to a dark orange colour in autumn. It is known that the fruits contain a toxic naphthoquinone derivative, plumbagin, and their constituents have been extensively investigated by Higa *et al.*[2–4] Recently, from the leaves and branches of a related Thai medicinal plant, *D. mollis*, the isolation of naphthoquinone glycosides was reported.[5] In our continuing work on Okinawan resource plants, the constituents of the leaves of *D. maritima* were investigated to give eight *ent*-kaurane-type diterpenoid glycosides, called diosmariosides A–H.[6] Further extensive work resulted in the isolation of four new triterpene saponins, named ebenamariosides A–D (**1–4**) and two megastigmanes (**5**, **6**), along with two known flavonol glycosides, kaempferol 3-*O*-*β*-D-(2″,6″-di-*O*-*α*-L-rhamonopyranosyl)glucopyranoside (**7**)[7] and 3-*O*-*β*-D-(2″,6″-di-*α*-L-rhamonopyranosyl)galactopyranoside (**8**)[8] (Fig. 1). The cytotoxicity of the triterpenoids was assayed using the human lung adenocarcinoma cell line A549 and the parasitic protozoan *Leishmania major*.

Results and Discussion

The leaves of *D. maritima* extracted with MeOH and the MeOH extracts were separated by solvent partition according to the polarity of the constituents. The relatively polar 1-BuOH-soluble fraction was separated by various kinds of chromatography to afford four new triterpene saponins (**1–4**) and two new megastigmanes (**5**, **6**), together with two known flavonol glycosides (**7**, **8**). The structures of new triterpene derivatives were elucidated using one- and two-dimensional

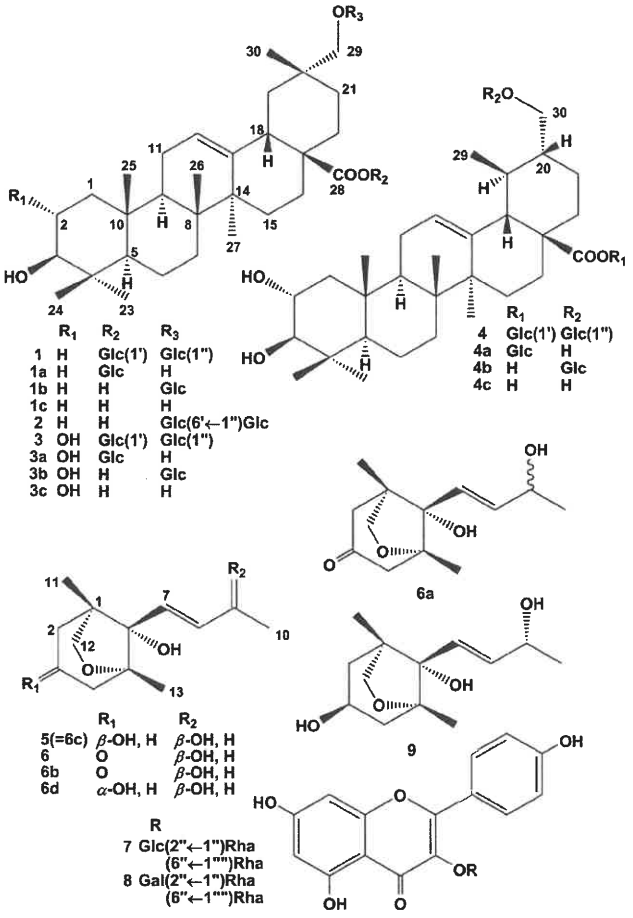

Fig. 1. Compounds Isolated and Related Ones

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spectroscopies. The structures of megastigmanes were elucidated by NMR and circular dichroism (CD) spectroscopies, and as well as by the modified Mosher method. The biological activity of four new triterpene saponins and their derivatives was assayed against the human lung adenocarcinoma cell line A549 and the parasitic protozoan *Leishmania major*.

Ebenamarioside A (**1**), $[\alpha]_D^{25}$ +15.1, was isolated as a colorless amorphous powder and its molecular formula was determined to be $C_{42}H_{68}O_{14}$ by observation of a quasi-molecular ion peak ($[M+Na]^+$) in the high-resolution (HR) electrospray-ionisation (ESI) mass spectrometry. The IR spectrum showed strong absorption bands assignable to hydroxy (3439 cm^{-1}) and ester carbonyl (1745 cm^{-1}) functional groups. In the ^{1}H-NMR spectrum, signals assignable to six singlet methyls, oxymethylene protons (δ_H 3.40 and 3.91), oxymethine proton (δ_H 3.45), olefinic proton (δ_H 5.43) and two anomeric protons [δ_H 6.35 (d, J = 8.3 Hz) and 4.85 (d, J = 7.8 Hz)] were observed (Table 1). Since HPLC analysis of the hydrolysate of **1** revealed the presence of D-glucose as a sole sugar component, two D-glucose molecules were expected to be present in **1**. In the ^{13}C-NMR spectrum, other than 12 signals assignable to those of glucopyranose units, 30 signals observed comprised of six methyls, eleven methylenes including one oxymethylene, four methines with an oxygenated one, six quaternary carbons, one trisubstituted double bond and a carboxyl functional group. From the above evidence and the degrees of unsaturation (Δ = 7), ebenamarioside A (**1**) was assumed to be an oleanolic acid derivative with a primary hydroxy group. In the heteronuclear multiple bond connectivity (HMBC) spectrum, geminal oxymethylene protons showed correlation with a methyl carbon (C-30, δ_C 19.7), methylene carbons, C-19 (δ_C 41.1) and 21 (δ_C 29.2) as well as a quaternary carbon at δ_C 35.5 (Fig. 2). The significant correlation in the phase sensitive-nulear Overhauser effect spectroscopy (PS-NOESY) spectrum between H-18 (δ_H 3.27) and H_3-30 (δ_H 1.10) on C-30 enabled us to place the oxymethylene carbon at the C-29 position (Fig. 2). Similarly, the oxymethine proton was placed at the 3-position from diagnostic HMBC between H-3 and C-4, C-23 and C-24 (Fig. 2). From the axial (10.2 Hz) and equatorial (4.6 Hz) coupling constants of H-3, the hydroxy group at the 3-position was in a β equatorial orientation. The positions of the sugar linkages were established to be on the carboxyl group at the C-28 and the hydroxy group at C-29 from HMBC correlations H-1′ (δ_H 6.35) on δ_C 95.8 and C-28 (δ_C 176.4), and H-1″ (δ_H 4.85) on δ_C 105.5 and C-29 (δ_C 81.4), respectively. The mode of linkage was assigned to be β from the coupling constants of the anomeric protons. Therefore, the structure of ebenamarioside A (**1**) was elucidated to be 3β,29-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester 29-*O*-β-D-glucopyranoside, as shown in Fig. 1. Partial enzymatic hydrolysis of **1** using crude β-glucosidase liberated two monoglucosidic compounds (**1a** and **1b**) and an aglycone (**1c**). The structure of compound **1a** was elucidated to be mesembryanthenoidigenic acid 28-*O*-β-D-glucopyranosyl ester, isolated from *Salicornia europaea*,[9] whereas that of **1b** mesembryanthenoidigenic acid 29-*O*-β-D-glucopyranoide, whose isolation have not yet been reported. The aglycone (**1c**) was spectroscopically identified with mesembryanthenoidigenic acid, isolated from a South American cactus, *Rhipsalis mesembryanthemoides*.[10,11]

Ebenamarioside B (**2**), $[\alpha]_D^{27}$ −5.6, was isolated as a colorless amorphous powder and its elemental composition was the same as that of **1**. NMR spectra were similar to those of **1**. Two anomeric protons and carbons (δ_H 4.79 on δ_C 105.5 and δ_H 5.16 on δ_C 105.9) were also observed in the ^{1}H-, ^{13}C- and heteronuclear single quantum correlation NMR spectra and D-glucose was a sole sugar unit. The distinct difference between **1** and **2** in the NMR signal was the carboxy carbons (C-28), such as δ_C 176.4 in **1** was shifted down to δ_C 180.2 in **2** and the anomeric carbon signal from an ester linkage (δ_C 95.8) appeared in **1** was not observed in **2**. While, in the HMBC spectrum, one of the anomeric proton at δ_H 5.16 was correlated with C-6′ (δ_C 70.2) and then the other anomeric proton at δ_H 4.79 with C-29 (δ_C 81.4). Thus, the structure of **2** was 3β,20-dihydroxyolean-12-en-28-oic acid 29-*O*-β-D-(6′-*O*-β-D-glucopyranosyl)glucopyranoside, namely mesembryanthenoidigenic acid 29-*O*-β-gentiobioside, as shown in Fig. 1.

Ebenamarioside C (**3**), $[\alpha]_D^{27}$ +14.0, was isolated as a colorless amorphous powder and its elemental composition wad determined to be $C_{42}H_{68}O_{15}$, with one more oxygen atom than those of **1** and **2**. NMR spectroscopic data for the C, D and E-rings were essentially the same as those of **1** and **2**. In the ^{1}H-NMR spectrum, two oxymethine protons (δ_H 3.39 on δ_C 83.8 and δ_H 4.10 on δ_C 68.6) as well as oxymethylene protons (δ_H 3.39 and 3.89) were observed (Table 1). The position of the oxymethine protons were placed at the vicinal positions from the ^{1}H–^{1}H correlation spectroscopy correlation (COSY) (Fig. 3) and from the evidence of HMBC correlations, namely, H_3-23 (δ_H 1.25) and 24 (δ_H 1.08) and C-3 (δ_C 83.8) (Fig. 3). From the PS-NOESY correlations between H-2 (δ_H 4.10) and H_3-25 (axial) (δ_H 1.02), H-3 (δ_H 3.39) and H_3-23 (axial), and H_3-23 and H-5 (axial) (δ_H 1.00) (Fig. 3), the hydroxy groups at the 2- and 3-positions were placed in equatorial positions, which were further confirmed by the large coupling constant of the vicinal protons (J = 9.2 Hz). Two oxymethines were found to be coupled each other from the ^{1}H–^{1}H-COSY spectroscopic evidence. The positions of the oxymethylene protons and sugar linkages were assigned by the similar manner used for ebenamarioside A (**1**). Therefore, the structure of **3** was elucidated to be 2α,3β,29-trihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester 29-*O*-β-D-glucopyranoside, as shown in Fig. 1. On enzymatic hydrolysis of **3** using crude naringinase, 28-*O*-β-D-glucopyranosyl ester (**3a**) was obtained. Glucoside **3a** is a known compound, isolated from the stem bark of *Terminalia superba* (=**3a**′)[12]; however, its ^{13}C-NMR data for MeOH-d_4 were slightly different from those of **3** to confirm the structure (Table 2). ^{13}C-NMR data of **3a** for pyridine-d_5 and dimethyl sulfoxide (DMSO)-d_6 were also slightly different from those of **3a**′ (Table 2). Furthermore, the optical rotation value reported for **3a**′ was $[\alpha]_D$ −18.1 (*c* 0.1, MeOH) which showed an opposite sign to that of **3a**. In our report, the structure of **3a** was carefully elucidated with a highly detailed survey of the one- and two-dimensional NMR spectra. On the other hand, enzymatic hydrolysis of **3** using crude β-glucosidase gave 2α,3β,29-trihydroxyolean-12-en-28-oic acid 29-*O*-β-D-glucopyranoside (**3b**) and an aglycone (**3c**). Glucoside **3b** has not been isolated as a natural product and the aglycone (**3c**) was a known one, isolated from the pericarps of *Akebia trifoliata*.[13]

Ebenamarioside D (**4**), $[\alpha]_D^{26}$ −5.6, was isolated as a colorless amorphous powder and its elemental composition was determined to be $C_{42}H_{68}O_{15}$, which was the same as that of

Table 1. ^{1}H-NMR Spectroscopic Data for Ebenamariosides A–D (**1–4**) (600 MHz, Pyridine-d_5)

H	1	2	3	4
1	0.99 ddd 12.7, 12.7, 4.1	1.04 m	1.28 m	1.25 m
	1.55 m	1.57 m	2.24 dd 11.9, 4.2	2.24 dd 12.3, 4.0
2	1.85 2H m	1.85 m	4.10 ddd 11.9, 9.2, 4.2	4.10 ddd 12.3, 9.3, 4.0
3	3.45 dd 10.2, 4.6	3.47 dd 10.2, 5.7	3.39 d 9.2	3.38 d 9.3
5	0.85 brd 11.8	0.86 brd 12.0	1.00 m	0.99 m
6	1.37 m	1.37 m	1.40 m	1.38 m
	1.55 m	1.57 m	1.54 m	1.52 m
7	1.37 m	1.32 m	1.38 m	1.38 m
	1.49 ddd 12.8, 12.8, 3.4	1.50 m	1.49 m	1.54 m
9	1.64 dd 11.1, 6.7	1.66 dd 8.9, 8.9	1.74 dd 10.2, 7.3	1.70 m
11	1.95 2H m	1.95 m	1.95 m	2.02 2H m
		2.17 m	2.09 brdd 13.4, 10.2	
12	5.43 dd 3.5 3.5	5.48 brs	5.39 brs	5.42 brs
15	1.15 m	1.19 m	1.15 m	1.12 m
	2.37 ddd 13.7, 13.7, 3.6	2.17 m	2.35 ddd 13.4, 13.4. 3.5	2.43 ddd 13.5, 13.5, 4.7
16	1.95 m	1.98 2H m	1.98 2H m	1.93 m
	2.10 ddd 13.7, 13.7, 3.6			2.03 m
18	3.27 dd 13.8, 4.2	3.36 dd 13.7, 3.8	3.25 dd 13.6, 3.6	2.54 d 11.3
19	1.43 dd 13.6, 4.2	1.50 m	1.41 m	1.70 m
	2.01 dd 13.8, 13.6	2.09 m	2.00 m	—
20	—	—	—	1.26 m
21	1.35 m	1.43 m	1.33 m	1.56 m
	1.67 ddd 13.7, 13.7, 4.1	1.79 m	1.65 ddd 13.9, 13.9, 3.6	1.93 m
22	1.82 m	1.85 m	1.79 brd 13.4	1.73 m
	1.87 m	2.06 m	1.87 ddd 13.9, 13.9, 3.6	1.96 m
23	1.23 3H s	1.25 3H s	1.25 3H s	1.25 3H s
24	1.04 3H s	1.04 3H s	1.08 3H s	1.07 3H s
25	0.93 3H s	0.90 3H s	1.02 3H s	1.02 3H s
26	1.14 3H, s	1.03 3H s	1.12 3H s	1.15 3H s
27	1.21 3H s	1.27 3H s	1.19 3H s	1.12 3H s
29	3.40 d 9.2	3.41 d 9.2	3.39 d 9.0	0.93 3H d 6.3
	3.91 d 9.2	4.04 d 9.2	3.89 d 9.0	
30	1.10 3H s	1.22 3H s	1.09 3H s	3.84 dd 9.4, 3.1
				4.00 m
1′	6.35 d 8.3	4.79 d 7.7	6.32 d 8.2	6.25 d 8.2
2′	4.22 dd 8.8, 8.3	4.02 dd 8.6, 7.7	4.20 dd 8.8, 8.2	4.19 dd 8.6, 8.2
3′	4.38 dd 9.0, 8.8	4.21 dd 8.9, 8.6	4.28 dd 8.9, 8.8	4.27 m
4′	4.25 dd 9.2, 9.0	4.16 dd 9.2, 8.9	4.36 dd 9.1, 8.9	4.34 dd 9.3, 9.1
5′	3.99 ddd 9.2, 4.6, 2.3	4.21 m	4.03 m	4.00 m
6′	4.43 dd 12.0, 4.6	4.37 dd 11.4, 5.8	4.42 dd 11.0, 5.4	4.38 dd 12.0, 4.3
	4.48 dd 12.0, 2.3	4.88 brd 11.4	4.46 brd 11.0	4.44 dd 12.0, 2.1
1″	4.85 d 7.8	5.16 d 7.9	4.83 d 7.7	4.85 d 7.7
2″	4.07 dd 8.2, 7.8	4.07 m	4.05 m	4.04 dd 8.2. 7.7
3″	4.30 dd 8.6, 8.2	4.25 m	4.24 m	4.27 m
4″	4.42 dd 8.9, 8.6	4.26 m	4.24 m	4.23 dd 9.1, 8.9
5″	4.05 ddd 8.9, 5.4, 2.3	3.95 m	3.98 m	3.99 m
6″	4.43 dd 11.8, 5.4	4.39 dd 11.9, 5.2	4.41 dd 11.4, 5.5	4.41 dd 12.1, 5.7
	4.59 dd 11.8, 2.3	4.53 brd 11.9	4.58 brd 11.4	4.58 dd 12.1, 2.2

3. The ^{1}H-NMR spectrum showed resonances for five singlet methyls and one doublet methyl, two oxygenated methines, two anomeric protons and one olefinic proton along with two oxymethylene protons coupled in a geminal system, but also coupled with one more proton [δ_H 3.84 (dd, J = 9.4, 3.1 Hz) and 4.00 (m)]. Although six quaternary carbons were observed in the ^{13}C-NMR spectra of aforementioned oleanane-type aglycones, only five quaternary ones were present in the molecule, and two more methine (C-19 and -20) and one less methylene carbons were observed in **4**, when the functionality was compared with that of **3**. ^{13}C-NMR chemical shifts of the A and B rings were essentially the same as those of **3** and the presence of a double bond between C-12 and C-13 was also similar to aforementioned triterpene aglycones. The proton spin-spin coupling sequences from the doublet methyl signal (δ_H 0.93) to oxymethylene protons *via* two methine signals (δ_H 1.70 and 1.26) were observed in the ^{1}H–^{1}H COSY spectrum (Fig. 4). The HMBC correlations between the doublet methyl proton and C-18 (δ_C 53.3), C-19 (δ_C 34.4) and C-20 (δ_C 44.6), and the oxymethylene protons and C-19,

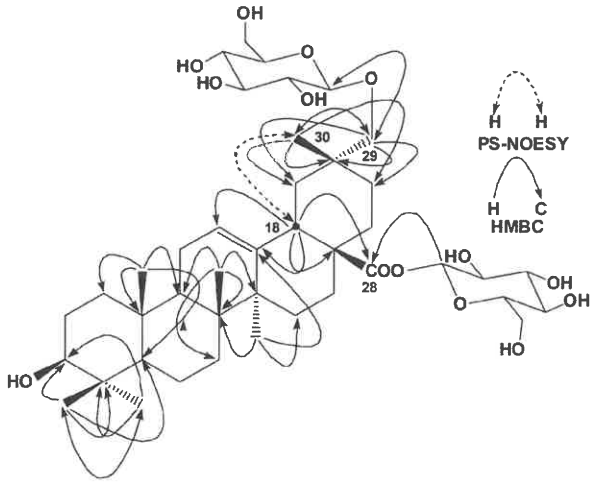

Fig. 2. Two Dimensional NMR Correlations of Ebenamarioside A (**1**)

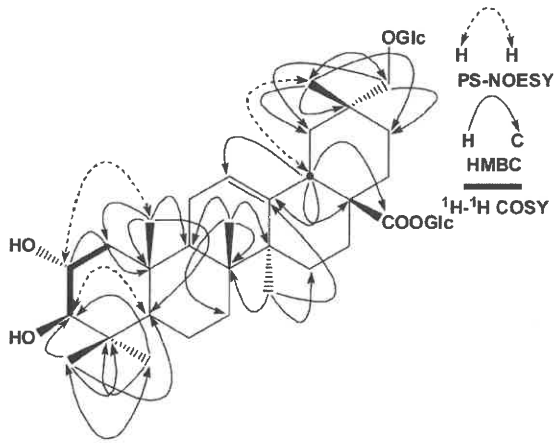

Fig. 3. Two Dimensional NMR Correlations of Ebenamarioside C (**3**)

C-20 and C-21 (δ_C 25.6) established the scaffold of the ring E to possess the ursane-type carbon frame work (Fig. 4). This was also supported by the significant PS-NOESY correlations H-18 (δ_H 2.54) and H_3-29 (δ_H 0.93), and H-18 and H-20 (δ_H 1.26). The sugar linkages were assigned by the similar manner used for ebenamariosides A and C (**1**, **3**). Therefore, the structure of ebenamarioside D (**4**) was elucidated to be 2α,3β,30-trihydroxyursan-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester 30-*O*-β-D-glucopyranoside, as shown in Fig. 1. Enzymatic hydrolysis using crude β-glucosidase gave a mixture of monoglucosidic compounds (**4a** and **4b**) and 2α,3β,30-trihydroxyurs-12-en-28-oic acid as an aglycone (**4c**), which is known as a microbial transformation product from corosolic acid by *Streptomyces asparaginoviolaceus*.[14] The mixture of monoglucosidic compounds was separated by silica gel CC and HPLC to give 2α,3β,30-trihydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**4a**) and 2α,3β,30-trihydroxyurs-12-en-28-oic acid 30-*O*-β-D-glucopyranoside (**4b**). These two monoglucosidic compounds were first described in this experiment.

Compound **5**, $[\alpha]_D^{26}$ +10.2, was isolated as a colorless powder and its elemental composition was determined to be $C_{13}H_{22}O_4$. In the ^{1}H-NMR spectrum, two singlet (δ_H 0.88 and 1.12) and one doublet (δ_H 1.28, J = 6.4 Hz) methyls, two olefinic protons [δ_H 6.03 (dd, J = 15.5, 4.5 Hz) and 6.06 (d, J = 15.5 Hz)] coupled in a trans geometry, two sets of methylene protons (δ_H 1.64 and 1.80, and 1.75 and 2.00), two oxygenated methylene protons (δ_H 3.68 and 3.77) and two oxygenated methine (δ_H 4.09 and 4.35) protons were found (Table 3). The ^{13}C-NMR spectrum displayed 13 signals including three methyls, three methylenes, two methines with oxygen atoms, two olefinic carbons, two oxygenated tertiary and one quaternary carbon (Table 3). The number of carbons and degrees of unsaturation suggested that compound **5** was a megastigmane with a bicyclic scaffold. Two ^{1}H–^{1}H COSY correlations [–C(2)H_2-C(3)HOH-C(4)H_2– and –C(7)H = C(8)H-C(9)HOH-C(10)H_3] and HMBC correlations between H_2-11 (δ_H 3.68 and 3.77) and C-5 (δ_C 87.5) and other diagnostic correlations shown in Fig. 5a suggested **5** was 5,11-eopxy-3,6,9-trihydroxymegastigman-7-ene. The relative stereochemistry was established by the PS-NOESY spectrum. Correlations between H-7 (δ_H 6.06) and H-2ax (δ_H 1.64), H-4ax (δ_H 1.75), H_3-12 (δ_H 0.88) and H_3-13 (δ_H 1.12) suggested these substituents were in the same face and those between H-11b (δ_H 3.68) and H-2eq (δ_H 1.80), H-3 (δ_H 4.09) and H-4eq (δ_H 2.00) these were in the same face and the opposite face to the side chain (Fig. 5b). A related compound (**9**) which showed superimposable NMR spectra with those of **5** was isolated from *Asclepias fruticosa* and the absolute structure of **9** was determined by the modified Mosher's method[15] to have 1*R*,3*S*,5*R*,6*S*,9*R* configurations.[16] Compound **5** was also subjected to the modified Mosher method and, as a result, **5** was found to have 1*R*,3*S*,5*R*,6*S*,9*S* configurations (Fig. 6). Therefore, **5** has the opposite configuration at the 9-position to that of **9** and thus it was found to be a new compound in nature.

Compound **6**, $[\alpha]_D^{25}$ −0.74, was isolated as a colorless syrup and its elemental composition was determined to be $C_{13}H_{20}O_4$ which was two hydrogen fewer than that of **5**. ^{13}C-NMR spectrum also displayed 13 signals including three methyls, three methylenes, one oxygenated methine, two oxygenated tertiary carbons, one quaternary carbon, one ketone, instead of oxygenated methine and one disubstituted double bond, whose NMR chemical shifts for $CDCl_3$ were almost superimposable to those of drummondol [**6a**, $[\alpha]_D^{23}$ −21.0 (MeOH)] isolated from *Sesbania drummondii* by Powell and Smith, Jr.,[17] whose geometry at the 9-position and the absolute configuration of bicyclo[3,2,1]octane region remains to be determined. Meanwhile, Çaliş *et al.* isolated drummondol 9-*O*-β-D-glucopyranoside from *Capparis spinosa* and the absolute configuration of the aglycone (**6b**) of the 9-position was determined to be 9*S* by the modified Mosher method.[16,18] That of the ring region was discussed using the Cotton effects in the CD spectrum of **6b**, compared with those of (+)-(*S*)-abscisic acid metabolites. Compound **6** showed similar Cotton effects [$\Delta\varepsilon$ (nm): +0.64 (241), −0.30 (296)] to those of **6b**[18] and hence the absolute stereochemistries of **6** and **6b** were expected to be the same at the 6-position. $NaBH_4$ reduction of **6** gave two products (**6c** and **6d**). Hydride was introduced from the less hindered 3*si* face to form the major compound **6d** and the minor compound **6c** was obtained by the reduction of **6** from the 3*re* face. The ^{1}H-NMR signal of the H-3 proton of **6d** was appeared as a doublet of a doublet, J = 5.7, 5.7 Hz, indicating that the α-hydroxy group formed at the 3-position was in the pseudo axial orientation due to steric hindrance toward the epoxide ring.[19,20] Meanwhile, the NMR spectroscopic data of the minor one (**6c**) were identical with those of **5** and similarly

Table 2. ^{13}C-NMR Spectroscopic Data for Ebenamariosides A–D (**1–4**) and Their Derivatives (150 MHz, Pyridine-d_5)

C	1	1b	2	3	3a	3a[a)]	3a[b)]	3a[c)]	3b	4	4a	4b
1	39.0	39.0	39.0	47.8	47.9	48.2	47.0	46.8	47.8	48.1	48.1	48.0
2	28.1	28.1	28.1	68.6	68.6	69.5	66.8	67.0	68.6	68.6	68.6	68.6
3	78.1	78.1	78.5	83.8	83.8	84.5	81.9	82.1	83.8	83.8	83.8	83.8
4	39.4	39.4	39.4	40.0	40.0	40.4	39.1	38.9	39.9	39.8	39.8	39.9
5	55.8	55.8	55.8	55.9	55.9	56.7	55.1	54.7	55.9	55.9	55.9	55.9
6	18.8	18.8	18.8	18.8	18.9	19.6	18.2	17.9	18.9	18.8	18.9	18.8
7	33.2	33.3	33.3	33.1	33.1	32.4	32.2	32.1	33.2	33.5	33.5	33.5
8	40.0	39.8	39.8	39.8	39.9	40.5	39.0	38.8	39.8	40.2	40.3	40.0
9	48.1	48.1	48.1	48.1	48.2	49.1	47.4	47.0	48.1	48.1	48.1	48.1
10	37.4	37.4	37.4	38.5	38.6	39.3	36.9	37.5	38.5	38.4	38.5	38.5
11	23.8	23.8	23.8	23.5	23.5	24.0	23.5	22.4	23.8	23.8	23.9	23.7
12	123.0	122.7	122.6	122.9	122.8	123.7	122.7	121.4	122.5	126.4	126.2	125.9
13	144.1	144.8	144.8	144.0	144.4	145.0	143.8	143.5	144.8	138.1	138.5	139.0
14	42.1	42.2	42.2	42.1	42.2	42.9	41.5	41.2	42.2	42.5	42.6	42.5
15	28.3	28.4	28.3	28.2	28.3	28.9	28.0	27.1	28.3	28.6	28.7	28.6
16	23.5	23.8	23.8	23.9	24.0	24.7	23.2	22.9	23.9	24.7	24.7	25.0
17	47.4	47.1	47.1	47.2	47.5	48.3	46.4	46.2	47.0	48.2	48.4	47.9
18	41.0	41.2	41.2	40.9	41.2	41.9	42.2	39.9	41.2	53.3	53.4	53.6
19	41.1	41.3	41.2	41.0	41.0	41.4	41.7	39.9	41.3	34.4	33.7	34.6
20	35.5	35.8	35.7	35.5	36.4	36.9	35.3	35.3	35.7	44.6	47.2	45.0
21	29.2	29.4	39.3	29.2	28.9	29.3	31.8	27.8	29.4	25.6	25.5	25.9
22	31.8	32.5	32.5	31.7	32.1	33.9	33.7	30.9	32.5	36.5	36.8	37.2
23	28.8	28.8	28.8	29.3	29.3	29.3	28.8	28.7	29.3	29.4	29.4	29.4
24	16.6	16.6	16.6	17.6	17.7	17.8	17.3	17.0	17.7	17.7	17.8	17.7
25	15.7	15.6	15.6	16.9	17.0	17.2	16.0	16.3	16.9	17.1	17.1	17.0
26	17.6	17.5	17.5	17.5	17.6	17.7	16.9	16.6	17.5	17.7	17.7	17.5
27	26.1	26.2	26.2	26.0	26.1	26.3	25.7	25.5	26.2	23.7	23.8	23.9
28	176.4	180.2	180.2	176.4	176.5	178.0	175.9	175.1	180.3	176.2	176.3	179.9
29	81.4	81.6	81.4	81.3	73.7	74.3	74.7	72.1	81.6	17.1	17.1	17.3
30	19.7	19.8	19.8	19.7	19.7	19.6	19.9	19.0	19.8	73.2	65.0	73.5
1′	95.8		105.5	95.8	95.8	95.8	95.8	94.0		95.7	95.8	
2′	74.2		75.2	74.1	74.2	74.0	74.1	72.3		74.0	74.1	
3′	79.0		78.5	78.9	79.0	78.7	79.1	77.6		78.8	79.0	
4′	71.1		71.7	71.1	71.2	71.1	71.3	69.4		71.2	71.2	
5′	79.4		77.3	79.3	79.4	78.3	78.6	76.6		79.1	79.3	
6′	62.2		70.2	62.2	62.2	62.4	62.2	60.6		62.3	62.3	
1″	105.5	105.5	105.9	105.4					105.5	104.8		105.1
2″	75.3	75.4	75.2	75.2					75.4	75.2		75.3
3″	78.7	78.7	78.6	78.6					78.7	78.6		78.7
4″	71.7	71.8	71.7	71.7					71.8	71.7		71.8
5″	78.6	78.6	78.1	78.5					78.6	78.5		78.6
6″	62.9	62.9	62.7	62.9					62.9	62.9		63.0

a) Data for CD_3OD. *b*) Data were taken from ref. 12 (CD_3OD). *c*) Data for DMSO-d_6.

6c was subjected to the modified Mosher's method to give (*R*)- and (*S*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) esters of **6c**, which were the identical compounds with **5a** and **5b** from **5**. Therefore, the absolute configurations of drummondol (**6**) isolated in this experiment was confirmed to be 1*R*,5*R*,6*S*,9*S*, which was the same as the aglycone of (9*S*)-drummondol 9-*O*-β-D-glucopyranoside from *C. spinosa* (Fig. 1) and **6** is expected to be a new compound as a non-glucosidic form; however, a direct correlation with the original drummondol (**6a**) was precluded.

The cytotoxic activity of isolated ebenamariosides (**1–4**), their derivatives (**1a**, **1b**, **1c**, **3a**, **3b**, **3c**, **4a** **4b** and **4c**), and compounds **5** and **6** was assayed using the human lung adenocarcinoma cell line A549. Compounds **1c** and **4c** showed slight activity with IC_{50} values of $174 \pm 16\,\mu$M and $107 \pm 8\,\mu$M, respectively, where that of the positive control etoposide was $23.3 \pm 4.3\,\mu$M, while other compounds did not show any activity at $100\,\mu$g/mL. Unfortunately, none of compounds were active toward *L. major* at $100\,\mu$g/mL.

Closing Remarks From the leaves of *Diospyros maritima*, four triterpene saponins, named ebenamariosides A–D (**1–4**) and two megastigmanes (**5**, **6**) were isolated. The structures of ebenamariosides were carefully elucidated by interpretation of one- and two-dimensional NMR spectroscopies and enzymatic hydrolysis of **1**, **3** and **4** using crude β-glucosidase and naringinase gave corresponding monoglucosides and aglycones. The structures of megastigmanes were confirmed by the modified Mosher's method and the Cotton effect in the CD spectrum. Assays of inhibitory activities for triterpene derivatives toward human lung adenocarcinoma cell line, A549 and

Leishmania major did not show any significant activity.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO P-2200 digital polarimeter. IR spectra were measured on JASCO FT/IR-6100 spectrophotometers. ^{1}H- and ^{13}C-NMR spectra were taken on a Bruker Avance III at 600 MHz and 150 MHz, respectively, with tetramethylsilane as an internal standard. CD spectra were obtained with a JASCO J-720 spectropolarimeter. Positive and negative-ion HR-ESI-MS were performed with a Thermo Fisher Scientific LTQ Orbitrap XL. Silica gel column chromatography (CC) was performed on silica gel 60 (70–230 mesh) (E. Merck, Darmstadt, Germany) and reversed-phase octadecylsilanized (ODS) open CC on Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) ($\Phi = 50$ mm, $L = 25$ cm). HPLC was performed on an ODS column [Inertsil ODS-3 (GL Science Inc., Tokyo, Japan; $\Phi = 10$ mm, $L = 25$ cm, 4.0 mL/min), Cosmosil

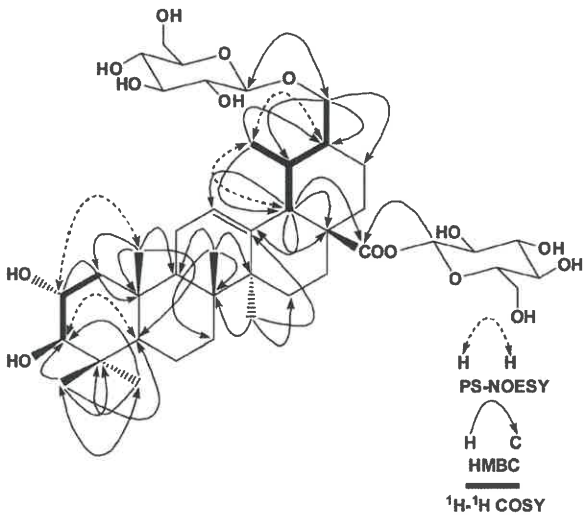

Fig. 4. Two Dimensional NMR Correlations of Ebenamarioside D (**4**)

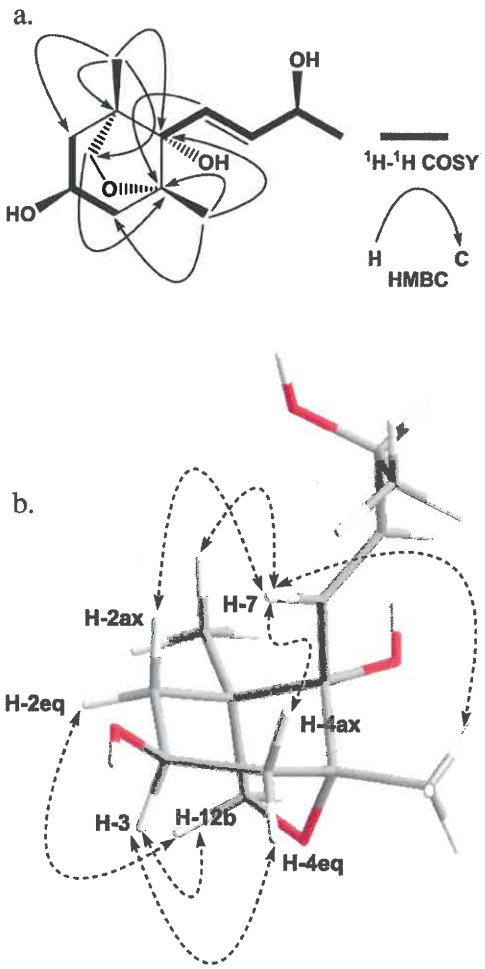

Fig. 5. a) ^{1}H–^{1}H COSY and HMBC Correlations of **5**; b) PS-NOESY Correlations of **5**

a) Significant long range ^{1}H–^{1}H correlations due to formation of W-figure were observed between H-2ax and H-12a and H-2 eq and H-4 eq.

Table 3. NMR Spectroscopic Data for Megastimane Derivatives (**5**, **6**) (C: 150 MHz, H: 600 MHz, CD_3OD)

	5		6	
	C	H	C	H
1	48.8	—	49.5	—
2	44.4	1.64 (ddd, 13.6, 10.5, 2.1)	53.2	2.35 (dd, 18.1, 2.6)
		1.80 (ddd, 13.6, 7.2, 1.4)		2.65 (dd, 18.1, 2.9)
3	66.0	4.09 (dddd, 10.5, 10.5, 7.2, 7.2)	211.4	—
4	45.8	1.75 (dd, 13.6, 10.5)	53.9	2.43 (dd, 18.1, 2.6)
		2.00 (ddd, 13.6, 7.2, 1.4)		2.78 (d, 18.1)
5	87.5	—	87.5	—
6	82.6	—	82.4	—
7	127.0	6.06 (d, 15.5)	125.7	6.02 (dd, 15.4, 1.5)
8	139.6	6.03 (d, 15.5, 4.5)	140.7	6.17 (dd, 15.4, 5.5)
9	69.2	4.35 (qd, 6.4, 4.5)	68.9	4.38 (dqd, 6.4, 5.5, 1.5)
10	24.0	1.28 (d, 6.4)	24.0	1.28 (3H, d, 6.4)
11	16.3	0.88 (3H, s)	19.2	1.18 (3H, s)
12	77.1	3.68 (d, 7.4)	78.4	3.65 (d, 7.5)
		3.77 (dd, 7.4, 2.1)		3.91 (dd, 7.5, 2.9)
13	19.5	1.12 (3H, s)	19.2	1.18 (3H, s)

Multiplicities and coupling constants in Hz are in the parentheses.

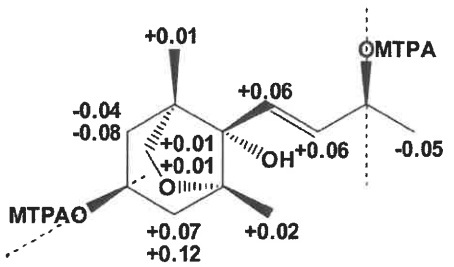

Fig. 6. Results of the Modified Mosher's Method of Compound **5**

Figures are δ_{5b}–δ_{5a} in ppm.

πNAP (Nacalai Tesque; Φ = 10 mm, L = 25 cm, 4.0 mL/min) and Cosmosil PBr (Nacalai Tesque; Φ = 10 mm, L = 25 cm, 4.0 mL/min)], and the eluate was monitored with photodiode arrtay (200–400 nm) and refractive index monitors. Crude β-glucosidase (Sumizyme BGA) was a generous gift from Shin Nihon Chemical Co., Ltd. (Anjo, Aichi, Japan) (Lot No. 0930708-03). Crude naringinase was from Amano Enzyme Inc. (Nagoya, Aichi, Japan) as a gift (Lot No. NAG1252306). MTPAs were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Plant Material Leaves of *D. maritima* were collected in Taketomi-cho, Yaeyama-gun, Okinawa, Japan, in November, 2003 and a voucher specimen was deposited in the Herbarium of Pharmaceutical Sciences, Graduate School of Biomedical and Health Sciences, Hiroshima University (03-DM-Okinawa-1105). The plant was identified by one of the authors (M.A.).

Extraction and Isolation Air-dried leaves of *D. maritima* (7.80 kg) were extracted with MeOH (45 L) three times. The MeOH extract was concentrated to 6 L and then washed with *n*-hexane (6 L, 245 g). The methanolic layer was concentrated to a viscous gum. The gummy mass was suspended in H_2O (6 L), and then partitioned with ethyl acetate (EtOAc) (6 L) and 1-butanol (1-BuOH) (6 L), successively, to give 397 g and 216 g of EtOAc and 1-BuOH-soluble fractions. The remaining water-layer was concentrated to give a H_2O-soluble fraction (245 g). The 1-BuOH-soluble fraction was subjected to a Diaion HP-20 CC (Φ = 80 mm, L = 50 cm), and eluted with H_2O–MeOH (4 : 1, 5 L), (3 : 2, 5 L), (2 : 3, 5 L), and (1 : 4, 5 L), and MeOH (5 L), 1 L-fractions being collected.

The residue (17.5 g) in fractions 4–7 of a Diaion HP-20 CC was subjected to silica gel CC (Φ = 40 mm, L = 55 cm), and eluted with $CHCl_3$ (3 L), $CHCl_3$–MeOH (99 : 1, 3 L), (49 : 1, 3 L), (97 : 3, 3 L), (19 : 1, 3 L), (37 : 3, 3 L), (9 : 1, 3 L), (7 : 1, 3 L), (17 : 3, 3 L), (33 : 7, 3 L), (4 : 1, 3 L), (3 : 1, 3 L), and (7 : 3, 3 L), 500 mL-fractions being collected. Compounds **6** (266 mg) and **5** (437 mg) were obtained in fractions 18–19 and 23–26, respectively.

The residue (29.2 g) in fractions 11–14 of a Diaion HP-20 CC was subjected to silica gel CC (Φ = 50 mm, L = 54.5 cm), and eluted with $CHCl_3$ (3 L), $CHCl_3$–MeOH (99 : 1, 3 L), (49 : 1, 3 L), (97 : 3, 3 L), (19 : 1, 3 L), (37 : 3, 3 L), (9 : 1, 3 L), (7 : 1, 3 L), (17 : 3, 3 L), (33 : 7, 3 L), (4 : 1, 3 L), (3 : 1, 3 L), and (7 : 3, 3 L), 500 mL-fractions being collected. The residue (3.00 g out of 6.74 g) in fractions 57–65 of silica gel CC was separated by ODS CC (Φ = 50 mm, L = 25 cm), and eluted with a linear gradient solvent system from MeOH–H_2O (1 : 9, 2 L) to MeOH–H_2O (9 : 1, 2 L), 10 g-fractions being collected. The residue (160 mg) in fractions 223–234 was purified by HPLC (Inertsil ODS-3, H_2O–MeOH, 1 : 1) to give 36.4 mg of **3** from the peak at 10.2 min. The residue (88.1 mg) in fractions 240–249 was purified by HPLC (Cosmosil πNAP, H_2O–MeOH, 2 : 3) to give 32.8 mg of **4** from the peak at 10.2 min. The residue (2.81 g) in fractions 66–71 of silica gel CC was separated by ODS CC (Φ = 50 mm, L = 25 cm), and eluted with a linear gradient solvent system from MeOH–H_2O (1 : 9, 2 L) to MeOH–H_2O (9 : 1, 2 L), 10 g-fractions being collected. The residue (136 mg) in fractions 106–129 was purified by HPLC (Cosmosil PBr, H_2O–MeOH, 3 : 2) to give 15.0 mg of **7** and 19.9 mg of **8** from the peaks at 25.0 min and 26.4 min, respectively.

The residue (64.5 g) in fractions 5–19 of a Diaion HP-20 CC was subjected to silica gel CC (Φ = 80 mm, L = 40 cm), and eluted with $CHCl_3$ (6 L), $CHCl_3$–MeOH (99 : 1, 6 L), (49 : 1, 6 L), (97 : 3, 6 L), (19 : 1, 6 L), (37 : 3, 6 L), (9 : 1, 6 L), (7 : 1, 6 L), (17 : 3, 6 L), (33 : 7, 6 L), (4 : 1, 6 L), (3 : 1, 6 L), and (7 : 3, 6 L), 1 L-fractions being collected. The residue (3.00 g out of 12.3 g) in fractions 53–62 was separated by ODS CC (Φ = 50 mm, L = 25 cm), and eluted with a linear gradient solvent system from MeOH–H_2O (1 : 9, 2 L) to MeOH–H_2O (9 : 1, 2 L), 10 g-fractions being collected. The residue (63.0 mg) in fractions 243–246 was purified by HPLC (Inertsil ODS-3, H_2O–MeOH, 3 : 7) to give 10.8 mg of **1** from the peak at 5.9 min. The residue (196 mg) in fractions 247–263 was purified by HPLC (Inertsil ODS-3, H_2O–MeOH–CH_3COOH, 7 : 13 : 0.1) to give 3.8 mg of **2** from the peak at 18.4 min.

Ebenamarioside A (1) Colorless amorphous powder, $[\alpha]_D^{25}$ +15.1 (c = 0.72, MeOH); IR ν_{max} (film) cm^{-1}: 3439, 2928, 2871, 1745, 1636, 1458, 1072; ^{1}H-NMR (600 MHz, pyridine-d_5): Table 1; ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2; HR-ESI-MS (positive-ion mode): m/z: 819.4499 $[M+Na]^+$ (Calcd for $C_{42}H_{68}O_{14}Na$: 819.4501).

Ebenamarioside B (2) Colorless amorphous powder, $[\alpha]_D^{27}$ −5.6 (c = 0.43, MeOH); IR ν_{max} (film) cm^{-1}: 3393, 2936, 2872, 1686, 1043; ^{1}H-NMR (600 MHz, pyridine-d_5): Table 1; ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2; HR-ESI-MS (negative-ion mode): m/z: 795.4529 $[M-H]^-$ (Calcd for $C_{42}H_{67}O_{14}$: 795.4525).

Ebenamarioside C (3) Colorless amorphous powder, $[\alpha]_D^{27}$ +14.0 (c = 0.10, pyridine); IR ν_{max} (film) cm^{-1}: 3353, 2927, 2876, 1705, 1045; ^{1}H-NMR (600 MHz, pyridine-d_5): Table 1; ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2; HR-ESI-MS (positive-ion mode): m/z: 835.4447 $[M+Na]^+$ (Calcd for $C_{42}H_{68}O_{15}Na$: 835.4450).

Ebenamarioside D (4) Colorless amorphous powder, $[\alpha]_D^{26}$ −5.6 (c = 0.86, MeOH); IR ν_{max} (film) cm^{-1}: 3400, 2932, 2877, 1740, 1071; ^{1}H-NMR (600 MHz, pyridine-d_5): Table 1; ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2; HR-ESI-MS (positive-ion mode): m/z: 835.4449 $[M+Na]^+$ (Calcd $C_{42}H_{68}O_{15}Na$: 835.4450).

Compound **5**

Colorless amorphous powder, $[\alpha]_D^{26}$ +10.2 (c = 0.33, MeOH); IR ν_{max} (film) cm^{-1}: 3379, 2930, 2876, 1450, 1375, 1135, 1043; ^{1}H-NMR (600 MHz, CD_3OD): Table 3; ^{13}C-NMR (150 MHz, CD_3OD): Table 3; HR-ESI-MS (positive-ion mode): m/z: 265.1411 $[M+Na]^+$ (Calcd $C_{13}H_{22}O_4Na$: 265.1410).

Compound **6**

Colorless syrup, $[\alpha]_D^{26}$ approx. 0.00 (c = 0.81, MeOH); IR ν_{max} (film) cm^{-1}: 3414, 2932, 2877, 1715, 1455, 1242, 1042; ^{1}H-NMR (600 MHz, CD_3OD): Table 3; ($CDCl_3$) δ: 6.22 (1H,

dd, $J=15.4$, 5.4 Hz, H-8), 5.91 (1H, dd, $J=15.4$, 1.2 Hz, H-7), 4.45 (1H, qd, $J=6.4$, 5.4 Hz, H-9), 3.91 (1H, dd, $J=8.3$, 3.0 Hz, H-12a), 3.75 (1H, d, $J=8.3$ Hz, H-12b), 2.65 (1H, d, $J=18.3$ Hz, H-4a), 2.60 (1H, dd, $J=18.3$, 2.0 Hz, H-4b), 2.55 (1H, dd, $J=18.1$, 3.0 Hz, H-2a), 2.42 (1H, dd, $J=18.1$, 2.0 Hz, H-2b), 1.33 (3H, d, $J=6.4$ Hz, H_3-10), 1.21 (3H, s, H_3-13); 0.99 (3H, s, H_3-11); ^{13}C-NMR (150 MHz, CD_3OD): Table 3; ($CDCl_3$) δ: 208.6 (C-3), 139.8 (C-8), 123.7 (C-7), 85.5 (C-5), 81.6 (C-6), 77.2 (C-12), 68.1 (C-9), 52.6 (C-4), 52.5 (C-2), 47.7 (C-1), 24.0 (C-10), 18.7 (C-13), 15.6 (C-11); CD ($c=6.46\times10^{-5}$ M, MeOH) $\Delta\varepsilon$ (nm): +0.64 (241), −0.30 (296); HR-ESI-MS (positive-ion mode): *m/z*: 263.1254 $[M+Na]^+$ (Calcd $C_{13}H_{20}O_4Na$: 263.1254).

Sugar Analysis About 500 μg each of **1–4** was hydrolyzed with 1 M HCl (0.1 mL) at 90°C for 2 h. The reaction mixtures were partitioned with an equal amount of EtOAc (0.1 mL), and the water layers were analyzed by HPLC with a chiral detector (JASCO OR-4090) on an amino column [InertSustain NH_2, 4.6 × 250 mm (GL Science Inc.), CH_3CN–H_2O (4 : 1), flow rate: 1 mL/min]. All the hydrolyzates gave a peak for D-glucose at 10.9 min with positive optical rotation signs. The peaks were identified by co-chromatography with an authentic sample.

Enzymatic Hydrolysis of 1, 3 and 4 to 1a, 1b and 1c, 3a, 3b and 3c, and 4a, 4b and 4c, Respectively Ebenamarioside A (**1**) (9.8 mg) in 1 mL of H_2O hydrolyzed with 15 mg of crude glucosidase at 37°C for 72 h. The reaction mixture was subjected to silica gel CC ($\Phi=2$ cm, $L=15$ cm) with increasing amounts of MeOH in $CHCl_3$ [$CHCl_3$–MeOH (9 : 1, 50 mL), (9 : 1, 100 mL to 7 : 3, 100 mL, linear gradient), (7 : 3, 50 mL) and (1 : 1, 100 mL)], 10-mL fractions being collected. An aglycone (**1c**) (2.8 mg) was obtained in fractions 5–6 and the monosaccharide mixture (**1a** and **1b**) (5.5 mg) in fractions 9–12. The mixture fraction was purified by HPLC (ODS-3, H_2O–MeOH, 4 : 1) to give 1.9 mg of **1a** and 3.0 mg of **1b** from the peaks at 4.8 min and 8.7 min, respectively. Mesembryanthenoidigenic acid 28-*O*-β-D-glucopyranosyl ester (**1a**): Amorphous powder, $[\alpha]_D^{25}$ +31.4 ($c=0.09$, MeOH), HR-ESI-MS (positive-ion mode) *m/z*: 657.3975 $[M+Na]^+$ (Calcd for $C_{36}H_{58}O_9Na$: 657.3973)[9]; mesembryanthenoidigenic acid 30-*O*-β-D-glucopyranoside (**1b**): Amorphous powder, $[\alpha]_D^{25}$ +14.5 ($c=0.15$, MeOH), IR ν_{max} (film) cm^{-1}: 3370, 2929, 2867, 1686, 1636, 1457, 1077; ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 5.47 (1H, dd, $J=3.3$, 3.3 Hz, H-12), 4.61 (1H, dd, $J=11.7$, 2.1 Hz, H-6″a), 4.44 (1H, dd, $J=11.7$, 5.3 Hz, H-6″b), 4.87 (1H, d, $J=7.7$ Hz, H-1″), 4.28 (1H, dd, $J=8.7$, 8.4 Hz, H-4″), 4.27 (1H, dd, $J=8.4$, 8.3 Hz, H-3″), 4.10 (1H, dd, $J=8.3$, 7.7 Hz, H-2″), 4.00 (1H, m, H-5″), 3.95 (1H, d, $J=9.3$ Hz, H-29a), 3.46 (1H, dd, $J=10.2$, 5.6 Hz, H-3), 3.44 (1H, d, $J=9.3$ Hz, H-29b), 3.37 (1h, dd, $J=13.6$, 3.9 Hz, H-18), 2.19 (1H, ddd, $J=13.2$, 13.0, 3.6 Hz, H-15a), 2.12 (1H, ddd, $J=13.0$, 13.0, 3.0 Hz, H-16a), 2.08 (1H, ddd, $J=14.0$, 14.0, 4.0 Hz, H-22a), 2.04 (1H, dd, $J=13.9$, 13.8 Hz, H-19a), 1.93 (3H, m, H_2-11 and H-16b), 1.84 (3H, m, H_2-2 and H-22b), 1.75 (1H, ddd, $J=13.6$, 13.3, 3.6 Hz, H-21a), 1.66 (1H, dd, $J=11.0$, 6.7 Hz, H-9), 1.57 (1H, m. H-6a), 1.56 (1H, m, H-1a), 1.51 (1H, ddd, $J=12.6$, 12.6, 3.5 Hz, H-7a), 1.45 (1H, dd, $J=13.6$, 4.2 Hz, H-19b), 1.43 (1H, m, H-21b), 1.38 (1H, m, H-6b), 1.33 (1H, m, H-7b), 1.26 (3H, s, H_3-27), 1.25 (3H, s, H_3-23), 1.21 (3H, s, H_3-30), 1.18 (1H, m, H-15b), 1.04 (3H, s, H_3-24), 1.03 (3H, s, H_3-26), 1.01 (1H, m, H-1b), 0.91 (3H, s, H_3-25), 0.87 (1H, brd, $J=10.4$ Hz, H-5); ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2, HR-ESI-MS (positive-ion mode) *m/z*: 633.4000 $[M-H]^-$ (Calcd for $C_{36}H_{57}O_9$: 633.3997); mesembryanthenoidigenic acid (**1c**): $[\alpha]_D^{25}$ +24.9 ($c=0.14$, MeOH), HR-ESI-MS (positive-ion mode) *m/z*: 471.3473 $[M-H]^-$ (Calcd for $C_{30}H_{47}O_4$: 471.3469).[10,11]

Ebenamarioside C (**3**) (9.5 mg) in 1 mL of H_2O was hydrolyzed with crude naringinase (15 mg) at 37°C for 72 h. The reaction mixture was subjected silica gel CC ($\Phi=2$ cm, $L=15$ cm) with increasing amounts of MeOH in $CHCl_3$ [$CHCl_3$-MeOH (9 : 1, 50 mL), (9 : 1, 100 mL to 7 : 3, 100 mL, linear gradient), (7 : 3, 50 mL) and (1 : 1, 100 mL)], 10-mL fractions being collected. 29-*O*-β-D-glucopyranosyl estre (**3a**) was obtained in fractions 11–13. Similarly ebenamarioside C (**3**) (15 mg) in 1 mL of H_2O was hydrolyzed with crude β-glucosidase and silica gel CC with the same condition as above gave 5.2 mg of an aglycone (**3c**) and 5.6 mg of 29-*O*-β-D-glucopyranoside (**3b**) in fractions 5–8 and 11–13, respectively. 2α,3β,29-Trihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**3a**): Amorphous powder, $[\alpha]_D^{24}$ +18.7 ($c=0.08$, MeOH), ^{13}C-NMR (150 MHz, pyridine-d_5, MeOH-d_4 and DMSO-d_6): Table 1, HR-ESI-MS (positive-ion mode) *m/z*: 673.3922 $[M+Na]^+$ (Calcd for $C_{36}H_{58}O_{10}Na$: 673.3922). 2α,3β,29-Trihydroxyolean-12-en-28-oic acid 29-*O*-β-D-glucopyranoside (**3b**): Amorphous powder; $[\alpha]_D^{24}$ +9.6 ($c=0.28$, MeOH); IR ν_{max} (film) cm^{-1}: 3379, 2934, 2872, 1687, 1459, 1050; ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 5.43 (1H, dd, $J=3.3$, 3.3 Hz, H-12), 4.87 (1H, d, $J=7.7$ Hz, H-1″), 4.60 (1H, dd, $J=11.8$, 2.2 Hz, H-6″a), 4.44 (1H, dd, $J=11.8$, 5.3 Hz, H-6″b), 4.28 (2H, m, H-3″ and 4″), 4.10 (2H, m, H-2 and 2″), 4.00 (1H, m, H-5″), 3.94 (1H, d, $J=9.1$ Hz, H-29a), 3.43 (1H, d, $J=9.1$ Hz, H-29b), 3.40 (1H, d, $J=9.6$ Hz, H-3), 3.35 (1H, dd, $J=13.3$, 3.1 Hz, H-18), 2.25 (1H, dd, $J=12.4$, 4.2 Hz, H-1a), 2.18 (1H, ddd, $J=13.3$, 13.0, 3.1 Hz, H-15a), 2.10 (1H, m, H-11a), 2.05 (1H, m, H-22a), 2.01 (1H, m, H-19a), 1.98 (2H, m, H_2-16), 1.95 (1H, m, H-11b), 1.84 (1H, brd, $J=13.6$ Hz, H-22b), 1.76 (1H, m, H-9), 1.75 (1H, m, H-21a), 1.56 (1H, m, H-6a), 1.51 (1H, m, H-7a), 1.43 (2H, m, H-19b and 21b), 1.38 (1H, m, H-6b), 1.31 (1H, m, H-7b), 1.28 (1H, m, H-1b), 1.27 (3H, s, H_3-23), 1.23 (3H, s, H_3-27), 1.21 (3H, s, H_3-30), 1.20 (1H, m, H-15b), 1.08 (3H, s, H_3-24), 1.02 (3H, s, H_3-26), 1.01 (1H, m, H-5), 0.99 (3H, s, H_3-25, ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2; HR-ESI-MS (positive-ion mode) *m/z*: 649.3951 $[M-H]^-$ (Calcd for $C_{36}H_{57}O_{10}$: 649.3946). 2α,3β,29-Trihydroxyolean-12-en-28-oic acid (**3c**): Amorphous powder; $[\alpha]_D^{24}$ +34.6 ($c=0.26$, MeOH); HR-ESI-MS (positive-ion mode) *m/z*: 487.3422 $[M-H]^-$ (Calcd for $C_{30}H_{47}O_5$: 487.3418).

Ebenamarioside C (**4**) (16.6 mg) in 1 mL of H_2O was hydrolyzed with crude β-glucosidase 37°C for 72 h. The reaction mixture was separated by silica gel CC with the same condition as above to give 4.1 mg of an aglycone (**4c**) and 9.4 mg of a mixture of two monoglucosidic compounds (**4a** and **4b**) in fractions 5–8 and 10–15, respectively. The mixture was purified by HPLC (ODS-3, H_2O–MeOH, 4 : 1) to afford 6.3 mg of 28-*O*-β-D-glucopyranosyl ester (**4a**) and 1.9 mg of 30-*O*-β-D-glucopyranoside (**4b**) from the peaks at 3.9 min and 9.2 min, respectively. 2α,3β,30-Trihydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**4a**): Amorphous powder; $[\alpha]_D^{24}$ +17.9 (*c* 0.31, MeOH); IR ν_{max} (film) cm^{-1}: 3373, 2925, 2877, 1732, 1456, 1073; ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 6.31 (1H, d, $J=8.1$ Hz, H-1′), 5.48 (1H, dd, $J=3.4$, 3.4 Hz, H-12), 4.47 (1H, dd, $J=11.8$, 2.4 Hz, H-6′a), 4.41 (1H, dd, $J=11.8$, 4.4 Hz, H-6′b), 4.39 (1H, dd, $J=9.5$, 8.9 Hz, H-4′),

4.31 (1H, dd, J=8.9, 8.7 Hz, H-3′), 4.23 (1H, dd, J=8.7, 8.1 Hz, H-2′), 4.11 (1H, ddd, J=11.1, 9.6, 4.3 Hz, H-2), 4.04 (1H, ddd, J=9.5, 4.4, 2.4 Hz, H-5′), 3.93 (1H, dd, J=10.7, 2.9 Hz, H-30a), 3.88 (1H, dd, J=10.7, 5.6 Hz, H-30b), 3.39 (1H, d, J=9.4 Hz, H-3), 2.65 (1H, d, J=11.5 Hz, H-18), 2.49 (1H, ddd, J=13.8, 13.7, 4.4 Hz, H-15a), 2.26 (1H, dd, J=12.5, 4.4 Hz, H-1a), 2.21 (1H, ddd, J=13.5, 13.4, 4.2 Hz, H-16a), 2.07 (1H, m, H-22a), 2.06 (1H, m, H-16b), 2.05 (2H, m, H_2-11), 2.01 (1H, m, H-19), 1.86 (2H, m, H_2-21), 1.84 (1H, m, H-22b), 1.74 (1H, dd, J=10.0, 7.4 Hz, H-9), 1.54 (1H, m, H-7a), 1.52 (1H, m, H-6a), 1.40 (1H, m, H-7b), 1.38 (1H, m, H-6b), 1.27 (1H, m, H-1b), 1.26 (3H, s, H_3-23), 1.20 (3H, s, H_3-27), 1.19 (3H, s, H_3-26), 1.18 (1H, m, H-15b), 1.16 (1H, m, H-20), 1.11 (3H, d, J=6.4 Hz, H_3-29), 1.09 (3H, s, H_3-24), 1.04 (3H, s, H_3-25), 1.01 (1H, d, J=12.1 Hz, H-5); ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2; HR-ESI-MS (positive-ion mode) m/z: 673.3925 $[M+Na]^+$ (Calcd for $C_{36}H_{58}O_{10}Na$: 673.3922).

2α,3β,30-Trihydroxyurs-12-en-28-oic acid 30-O-β-D-glucopyranoside (**4b**): Amorphous powder; $[\alpha]_D^{25}$ +5.5 (c=0.10, MeOH); IR ν_{max} (film) cm^{-1}: 3370, 2930, 2871, 1686, 1457, 1050; ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 5.45 (1H, dd, J=3.1, 3.1 Hz, H-12), 4.90 (1H, d, J=7.8 Hz, H-1″), 4.62 (1H, dd, J=11.7, 2.2 Hz, H-6″a), 4.45 (1H, dd, J=11.7, 5.3 Hz, H-6″b), 4.30 (1H, dd, J=8.8, 8.9 Hz, H-3″), 4.27 (1H, dd, J=8.9, 8.9 Hz, H-4″), 4.10 (1H, m, H-2), 4.09 (1H, m, H-2″), 4.07 (1H, m, H-30a), 4.03 (1H, m, H-5″), 3.89 (1H, dd, J=9.5, 3.5 Hz, H-30b), 3.40 (1H, d, J=9.4 Hz, H-3), 2.65 (1H, d, J=11.3 Hz, H-18), 2.32 (1H, ddd, J=13.8, 13.8, 4.4 Hz, H-15a), 2.24 (1H, dd, J=12.5, 4.4 Hz, H-1a), 2.07 (1H, m, H-16a), 2.04 (1H, m, H-21a), 2.03 (1H, m, H-22a), 2.02 (2H, m, H_2-11), 1.96 (1H, m, H-16b), 1.94 (1H, m, H-22b), 1.75 (2H, m, H-9 and 19), 1.66 (1H, m, H-21b), 1.55 (1H, m, H-6a), 1.54 (1H, m, H-7a), 1.39 (1H, m, H-20), 1.38 (1H, m, H-6b), 1.37 (1H, m, H-7b), 1.28 (3H, s, H_3-23), 1.28 (1H, m, H-1b), 1.17 (3H, s, H_3-27), 1.16 (1H, m, H-15b), 1.08 (3H, s, H_3-24), 1.04 (3H, s, H_3-26), 1.03 (1H, m, H-5), 1.01 (3H, d, J=6.4 Hz, H_3-29), 0.98 (3H, s, H_3-25); ^{13}C-NMR (150 MHz, pyridine-d_5): Table 2; HR-ESI-MS (positive-ion mode) m/z: 649.3948 $[M-Na]^-$ (Calcd for $C_{30}H_{57}O_{10}$: 649.3946).

2α,3β,30-Trihydroxyurs-12-en-28-oic acid (**4c**): Amorphous powder; $[\alpha]_D^{23}$ +17.7 (c=0.13, EtOH); HR-ESI-MS (positive-ion mode) m/z: 487.3423 $[M-H]^-$ (Calcd for $C_{30}H_{47}O_5$: 487.3418).

Preparation of (*R*)- and (*S*)-MTPA Esters (5a and 5b) from 5 A solution of **5** (1.0 mg) in 0.5 mL of dry CH_2Cl_2 were reacted with (*R*)-MTPA (21.5 mg) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (13.4 mg) and *N*,*N*-dimethyl-4-aminopyridine (4-DMAP) (16.1 mg). The mixture was then occasionally stirred at 37°C for 24 h. After the addition of $CHCl_3$ (1.5 mL), the reaction mixture was successively washed with H_2O (1 mL), 1 M HCl (1 mL), $NaHCO_3$-saturated H_2O (1 mL), and brine (1 mL). The organic layer was dried with Na_2SO_4 and evaporated under reduced pressure. The residue was purified by preparative TLC [silica gel (0.25 mm thickness), being applied for 8 cm width, with development with $CHCl_3$–MeOH (19 : 1) for 9 cm and then eluting with $CHCl_3$–MeOH (1 : 1)] to furnish an ester **5a** (0.5 mg) from the band at R_f=0.67. Through the same procedure, **5b** (0.6 mg, R_f=0.59) were prepared from **5** (1.0 mg) using (*S*)-MTPA (25.4 mg), EDC (15.8 mg), and 4-DMAP (15.5 mg), respectively.

(*R*)-MTPA ester of **5** (**5a**): amorphous powder; ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 7.35–7.55 (5H, m, aromatic protons), 6.05 (1H, d, J=15.2 Hz, H-7), 6.02 (1H, dd, J=15.2, 4.9 Hz, H-8), 5.66 (1H, qd, J=6.5, 4.9 Hz, H-9), 5.44 (1H, dddd, J=10.5, 10.5, 7.2, 7.2 Hz, H-3), 3.87 (1H, d, J=8.2 Hz, H-12a), 3.77 (1H, dd, J=8.2, 2.0 Hz, H-12b), 3.53 (3H, s, –OMe), 3.51 (3H, s, –OMe), 2.18 (1H, ddd, J=13.6, 7.2, 1.5 Hz, H-4), 2.03 (1H, ddd, J=13.6, 7.2, 1.5 Hz, H-2), 1.73 (1H, dd, J=13.6, 10.5 Hz, H-4), 1.70 (1H, ddd, J=13.6, 10.5, 2.0 Hz, H-2), 1.45 (3H, d, J=6.5 Hz, H_3-10), 1.08 (3H, s, H_3-13), 0.90 (3H, s, H_3-11); HR-ESI-MS (positive-ion mode) m/z: 697.2206 $[M+Na]^+$ (Calcd for $C_{33}H_{36}O_8F_6Na$: 697.2207).

(*S*)-MTPA ester of **5** (**5b**): amorphous powder; ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 7.35–7.55 (10H, m, aromatic protons), 6.11 (1H, d, J=15.5 Hz, H-7), 6.08 (1H, dd, J=15.5, 5.5 Hz, H-8), 5.63 (1H, qd, J=6.5, 5.5 Hz, H-9), 5.44 (1H, dddd, J=10.7, 10.7, 7.1, 7.1 Hz, H-3), 3.88 (1H, d, J=8.3 Hz, H-12a), 3.78 (1H, dd, J=8.3, 2.1 Hz, H-11b), 3.52 (3H, s, –OMe), 3.49 (3H, s, –OMe), 2.25 (1H, ddd, J=13.6, 7.1, 1.5 Hz, H-4), 1.99 (1H, ddd, J=13.6, 7.1, 1.5 Hz, H-2), 1.85 (1H, ddd, J=13.6, 10.7 Hz, H-4), 1.62 (1H, ddd, J=13.6, 10.7, 2.1 Hz, H-2), 1.40 (3H, d, J=6.5 Hz, H_3-10), 1.10 (3H, s, H_3-13), 0.91 (3H, s, H_3-11); HR-ESI-MS (positive-ion mode) m/z: 697.2206 $[M+Na]^+$ (Calcd for $C_{33}H_{36}O_8F_6Na$: 697.2207).

$NaBH_4$ Reduction of 6 To a solution of **6** (11.0 mg) in MeOH (1 mL) was added 8.2 mg of $NaBH_4$ and the reaction mixture was stirred for 5 min at 25°C. Excess $NaBH_4$ was quenched by the addition of 1 mL of acetone and then the reaction mixture was evaporated to dryness. The resultant residue was purified by HPLC [Inertsil ODS-3, 6×250 mm, H_2O–MeOH (1 : 4), flow rate: 1.6 mL/min] to give 3.1 mg of **6c** (=**5**) and 5.8 mg of **6d** from the peaks at 10.9 min and 13.3 min. respectively.

Compound **6c**: amorphous powder; $[\alpha]_D^{24}$ +7.1 (c=0.31, MeOH); HR-ESI-MS (positive-ion mode) m/z: 265.1409 $[M+Na]^+$ (Calcd for $C_{13}H_{22}O_4Na$: 265.1410).

Compound **6d**: amorphous powder; $[\alpha]_D^{24}$ −5.5 (c=0.29, MeOH); IR ν_{max} (film) cm^{-1}: 3402, 2929, 2889, 1604, 1453, 1381, 1101, 1053; ^{1}H-NMR (600 MHz, CD_3OD) δ: 6.02 (1H, dd, J=15.5, 5.6 Hz, H-8), 5.82 (1H, dd, J=15.5, 1.0 Hz, H-7), 4.33 (1H, qd, J=6.4, 5.6 Hz, H-9), 4.12 (1H, d, J=6.9 Hz, H-12a), 4.02 (1H, dd, J=5.7, 5.7 Hz, H-3), 3.76 (1H, dd, J=6.9, 2.3 Hz, H-12b), 2.13 (1H, dd, J=15.5, 5.7 Hz, H-4a), 2.02 (1H, ddd, J=15.3, 5.7, 2.2 Hz, H-2a), 1.84 (1H, dd, J=15.5, 2.2 Hz, H-4b), 1.73 (1H, dd, J=15.3, 2.3 Hz, H-2b), 1.25 (3H, d, J=6.4 Hz, H_3-10), 1.13 (3H, s, H_3-13), 0.86 (3H, s, H_3-11); ^{13}C-NMR (150 MHz, CD_3OD) δ: 139.4 (C-8), 126.8 (C-7), 87.2 (C-5), 82.5 (C-6), 76.3 (C-12), 69.1 (C-9), 66.0 (C-3), 48.0 (C-1), 45.1 (C-4), 44.9 (C-2), 24.1 (C-10), 19.7 (C-13), 16.3 (C-11); HR-ESI-MS (positive-ion mode) m/z: 265.1408 $[M+Na]^+$ (Calcd for $C_{13}H_{22}O_4Na$: 265.1410).

Cytotoxic Activity toward Human Lung Adenocarcinoma, A549 Cells Cytotoxic activity toward lung adenocarcinoma cells was determined by colorimetric cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Lung adenocarcinoma cell line A549 was purchased from the JCRB Cell Bank, Japan. A549 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated FCS, and kanamycin (100 μg/mL) and amphotericin B (5.6 μg/mL). In a 96-well plate, 1 μL aliquots of sample solutions and the cancer cells

(5×10^3 cells/well) in 100 μL medium were added to each well, and then the plate incubated at 37°C under a 5% CO_2 atmosphere for 72 h. A solution (100 μL) of MTT (0.5 mg/mL) was then added to each well and the incubation was continued for a further 1 h. The absorbance of each well was measured at 540 nm using a Molecular Devices Versamax tunable microplate reader. DMSO was used as a negative control and doxorubicin as a positive control. The cytotoxic activity was calculated as:

$$\% \text{ inhibition} = [1 - (A_{test} - A_{blank}) / (A_{control} - A_{blank})] \times 100$$

where $A_{control}$ is the absorbance of the control DMSO well, A_{test} the absorbance of the test wells, and A_{blank} the absorbance of the cell-free wells.

Anti-*Leishmania* Activity The anti-*Leishmania major* activity toward promastigotes was determined by the colorimetric cell viability MTT assay. The promastigotes at the logarithmic growth phase were cultured in M199 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin. In a 96-well plate, 1 μL aliquot of sample solutions and *L. major* cells (1×10^5 cells/well) in 100 μL medium were added to each well, and then the plate was incubated at 27°C under an ambient atmosphere for 72 h. A solution of MTT (100 μL) was then added to each well and the incubation was continued overnight. The formazan product of MTT reduction was then dissolved in DMSO and then the absorbance was measured using a Molecular Devices Versamax tunable microplate reader. DMSO was used as a negative control and amphotericin B as a positive control. The experiment was performed in triplicate. The anti-*Leishmania major* activity was quantified as the percentage of the control absorbance of reduced dye at 540 nm. The inhibitory activity was calculated as:

$$\% \text{ inhibition} = [1 - (A_{test} - A_{blank}) / (A_{control} - A_{blank})] \times 100$$

where $A_{control}$ is the absorbance of the control (DMSO) well, A_{test} the absorbance of the test wells, and A_{blank} the absorbance of the cell-free wells.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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