New *ent*-Kaurene-Type Diterpenoids Possessing Cytotoxicity from the New Zealand Liverwort *Jungermannia* Species

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Two new *ent*-kaurene-type and a new rearranged *ent*-kaurene-type diterpenoids possessing cytotoxicity against a human leukemia cell line have been isolated from the New Zealand liverwort *Jungermannia* species, together with previously known *ent*-kaurene-type diterpenoids. Their structures were established based on extensive NMR techniques.

Key words *Jungermannia* species; liverwort; *ent*-kaurene; diterpenoid; cytotoxicity; HL-60 cell

Most liverworts contain lipophilic mono-, sesqui-, and diterpenoids and phenolic compounds in the oil bodies.[1—3] These compounds often show interesting biological activities such as cytotoxic, antifungal, 5-lipoxygenase inhibitory, muscle relaxing, and antimicrobial activity.[1—5] They are also valuable as chemosystematic and genetic markers.[1—5] We have already reported the isolation and structural identification of a number of terpenoids and aromatic compounds with novel skeletons.[1—5]

As a part of the search for novel skeletal compounds and their biologically active compounds in liverworts, we focused on the genus *Jungermannia* L. belonging to the Jungermanniaceae (Jungermanniales) since they contain various terpenoids.[6] *Jungermannia* species are not only morphologically but also chemically interesting because they are polymorphic and their chemical constituents depend on the collection site.[6] There are more numerous endemic liverwort species in New Zealand in comparison with those found in Japan. Therefore we have been interested in their chemical constituents. The fractionation of the ether extract of unidentified *Jungermannia* species resulted in the isolation of two new *ent*-kaurene-type (**1**, **2**) and a new rearranged *ent*-kaurene-type diterpenoid named jungermannenone A (**3**), along with previously known *ent*-kaurenes, *ent*-11α-hydroxy-16-kauren-15-one (**4**)[7] and (16*R*)-*ent*-11α-hydroxykauran-15-one (**5**).[7] Here we report the structure determination of the new compounds and their cytotoxic activity.

The molecular formula of compound **1** was found to be $C_{20}H_{28}O_2$ (obs. *m*/*z* 300.2090 $[M]^+$) by high-resolution electron-impact mass spectrometry (HR-EI-MS), indicating seven degrees of unsaturation. The IR spectrum displayed a hydroxy (3487 cm^{-1}) and a carbonyl group (1721 cm^{-1}). The acetylation of **1** afforded a monoacetate **6**, which had an acetyl group (δ 2.05 s) in the ^{1}H-NMR spectrum. The ^{1}H- and ^{13}C-NMR (Tables 1, 2) spectra of **1** showed the presence of an *exo*-methylene (δ 115.3 t, 151.9 s), a trisubstituted olefin (δ 120.3 d, 149.0 s), a carbonyl carbon (δ 203.9 s), and a methine (δ 78.5)-bearing oxygen atom as well as three tertiary methyls, six methylenes, two methines, and three quaternary carbons. The above spectral evidence and the similarity of ^{1}H-NMR spectra with compounds **4** and **5** led us to assume that compound **1** is a kaurane-type diterpenoid.

The ^{1}H–^{1}H correlated spectroscopy (^{1}H–^{1}H COSY) and the heteronuclear multiple-quantum coherence (HMQC) of **1** showed three partial structures i) –C(9)=CH(11)–CH_2(12)–CH(13)–CH_2(14)–, ii) –CH(OH)(1)–CH_2(2)–CH_2(3)–, and iii) –CH(5)–CH_2(6)–CH_2(7)–, respectively. Moreover, the heteronuclear multiple-bond correlation (HMBC) experiment clarified the connectivity of each partial structure, as shown in Fig. 1. Accordingly, the structure of **1** was determined to be 1-hydroxy-9(11),16-kauradien-15-one. The phase-sensitive nuclear Overhauser enhancement and exchange spectroscopy (NOESY) of **1**, as shown in Fig. 1, clarified that the configuration of the hydroxy group at C-1 is α. The circular dichroism (CD) spectrum of **1** indicated first positive (352 nm) and second negative (268 nm) Cotton effects which were seen in *ent*-9(11),16-kauradiene-6,15-dione (**7**)[8] de-

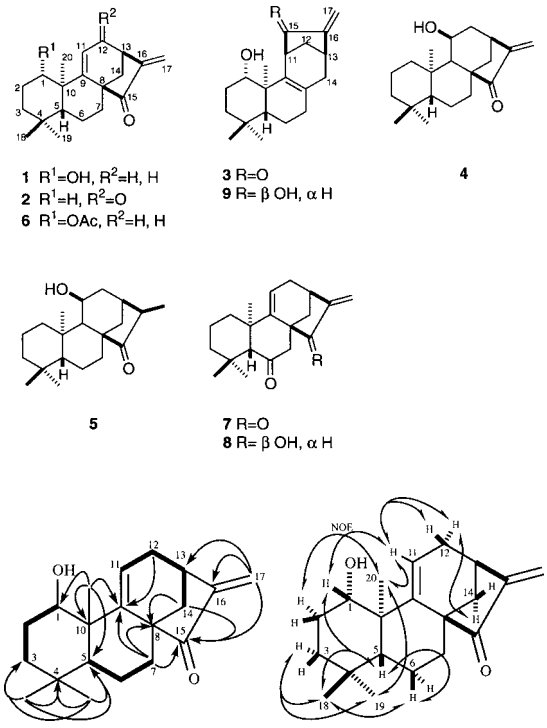

Fig. 1. ^{1}H–^{1}H COSY (Bold Lines) and HMBC (Arrows), and NOE Correlations (Half Arrows) of **1**

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Table 1. ^{1}H-NMR Data of **1**—**3** (600 MHz, $CDCl_3$)

H	1	2	3
1	3.60 br t like	1.91 br d like (12.9), α 1.18 ddd (12.9, 12.9, 4.1), β	3.47 ddd (11.5, 6.0, 4.4)
2	1.24—1.29 m, α 1.58 m, β	1.50 d quint. (14.0, 3.8), α 1.59 m, β	1.79 dddd (14.0, 14.0, 7.4, 4.1), α 1.57—1.62 m, β
3	1.34 dt (13.5, 3.6),[a] α 1.24—1.29 m, β	1.40 d sex. (13.2, 1.6), α 1.11 ddd (13.2, 13.2, 3.6), β	1.42 ddd (13.5, 4.1, 3.0), α 1.25 ddd (13.5, 13.5, 4.1), β
5	1.50 dd (12.1, 6.0)	1.44 dd (12.4, 6.9)	0.96 dd (12.1, 1.6)
6	1.55 m, α 2.24 dddd (18.7, 10.2, 9.1, 6.3), β	1.63 m, α 2.29 m, β	1.37 m, α 1.57—1.62 m, β
7	1.73—1.81 2H, m	1.79 ddd (14.3, 10.7, 8.2), α 1.94 br t like (14.3), β	1.87—1.91 2H, m
11	6.37 t (3.6)	5.84 s	3.99 d (4.1)
12	2.66 ddd (17.6, 4.7, 3.0), α 2.10 br d (17.6), β		1.57—1.62 m 1.87—1.91 m
13	2.95 br s	3.57 d (4.7)	3.03 br s
14	1.62 d (11.0), α 1.67 dd (11.0, 4.9), β	2.31 d (11.5), α 2.04 dd (11.5, 4.7), β	2.47 dd (17.3, 4.9), α 1.99 d (17.3), β
17	5.41 t (1.1) 5.89 t (1.1)	5.65 s 6.09 s	5.51 s 5.99 s
18	0.89 s	0.94 s	0.84 s
19	0.90 s	0.95 s	0.84 s
20	1.14 s	1.19 s	1.13 s
OH			4.83 d (6.3)

a) Coupling constants (*J* in Hz) are given in parentheses.

Table 2. ^{13}C-NMR Data of **1**—**3**, **6** and **8** (100 MHz, $CDCl_3$)

C	1	2	3	6	8
1	78.5	39.2	75.8	81.8	77.9
2	28.9	18.8	28.6	25.0	28.9
3	39.72[a]	41.6	40.4	39.2	40.4
4	33.3	33.9	33.03	33.2	33.2
5	43.2	42.4	51.8	43.5	51.4
6	18.0	17.1	18.6	17.9	18.8
7	24.3	23.6	32.98	24.2	32.4
8	50.0	53.5	130.7	50.0	128.7
9	149.0	176.1	138.4	148.6	140.2
10	44.7	40.4	45.3	43.3	44.6
11	120.3	121.1	45.7	119.1	39.3
12	36.4	197.3	32.5	36.5	32.7
13	36.7	53.2	37.6	36.5	39.0
14	39.67[a]	45.6	41.0	39.7	44.7
15	203.9	201.8	204.9	203.6	80.4
16	151.9	139.9	150.8	151.4	159.6
17	115.3	120.0	116.7	115.9	107.1
18	31.9	32.4	33.1	31.9	33.3
19	21.4	21.9	21.7	21.5	21.9
20	17.5	23.0	15.1	18.8	15.2
OAc				21.9	
				170.4	

a) May be interchanged.

rived from nardiin (**8**).[9] Thus the absolute structure of **1** was established to be *ent*-1β-hydroxy-9(11),16-kauradien-15-one.

The IR spectrum of **2**, the molecular formula of which was found to be $C_{20}H_{26}O_2$ (obs. *m*/*z* 298.1923) by HR-EI-MS, confirmed the presence of a carbonyl group (1726, 1675 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra (Tables 1, 2) were similar to those of **1**, indicating that compound **2** is a kaurane-type diterpenoid. The ^{13}C-NMR spectrum of **2** showed the presence of two ketone carbons (δ 197.3, 201.8), a trisubstituted double bond (δ 121.1 d, 176.1 s), and an *exo*-methylene (δ 120.0 t, 139.9 s) as well as three methyls, six methylenes, two methines, and three quaternary carbons. The detailed analysis of ^{1}H–^{1}H COSY, HMQC, and HMBC spectra of **2** indicated that the structure is 9(11),16-kauradiene-12,15-dione. NOEs were observed between i) H-5 and H-1β, H-6β, ii) H-18 and H-3α, H-3β, H-5, H-6β, iii) H-19 and H-3α, H-6α, H-20, and iv) H-20 and H-19, H-17 in the NOESY spectrum. From the above spectral evidence, the stereostructure of **2** was established as shown in the drawing.

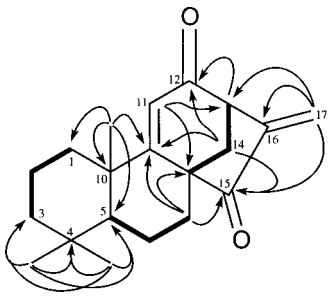

Fig. 2. ^{1}H–^{1}H COSY (Bold Lines) and HMBC (Arrows) of **2**

The EI-MS spectrum of jungermannenone A (**3**) confirmed a molecular ion peak at *m*/*z* 300 $[M]^+$, and its HR-EI-MS showed the molecular formula to be $C_{20}H_{28}O_2$ (obs. *m*/*z* 300.2088). Its IR spectrum displayed the presence of a hydroxy (3463 cm^{-1}) and a carbonyl group (1709 cm^{-1}). The ^{1}H-NMR spectrum (Table 1) of **3** showed the presence of three tertiary methyls (δ 0.84 6H, 1.13), a methine proton (δ 3.47 ddd) bearing a hydroxy group, and an *exo*-methylene (δ 5.51, 5.99 each s). The ^{13}C-NMR (Table 2) and distortionless enhancement by polarization transfer (DEPT) spectra indicated the presence of a ketone carbon (δ 204.9), an *exo*-methylene (δ 116.7 t, 150.8 s), two quaternary sp^2 carbons (δ 130.7, 138.4), and a methine (δ 75.8)-bearing a hydroxy group as well as three methyls, six methylenes, three methines, and two quaternary carbons.

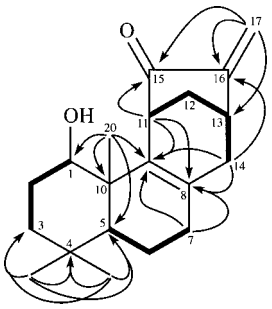

Fig. 3. ^{1}H–^{1}H COSY (Bold Lines) and HMBC (Arrows) of **3**

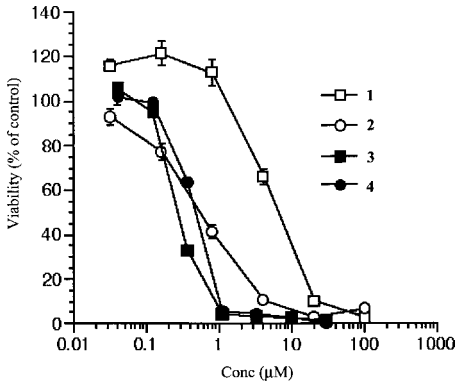

Fig. 4. Cytotoxicity of Compounds **1**—**4** in HL-60 Cells

The cells (1×10^4 cells) were seeded onto 96-well plates, and treated with the chemicals at the indicated concentrations for 24 h, and then the viability was determined using a cell counting kit. Data are expressed as mean±S.D. ($n=4$).

The reduction of **3** with $LiAlH_4$ gave a diol **9** that newly displayed the methine proton (δ 4.55) bearing a hydroxy group on the ^{1}H-NMR spectrum. The analysis of ^{1}H–^{1}H COSY of **3** clarified the presence of three partial structures, and the HMBC spectrum led to the structure as a rearranged kaurane-type diterpenoid, as shown in Fig. 3. In the NOESY spectrum of **3**, NOEs were observed between i) H-1β and H-2β, H-3β, H-5, ii) H-5 and H-1β, H-3β, H-6β, iii) H-20 and H-6α, H-2α, H-11, and iv) H-13 and H-14α, H-14β. Accordingly, the stereochemistry of the hydroxy group at C-1 was clarified to have the α-configuration. The NOESY spectrum of **9** also displayed NOEs between i) H-20 and H-2α, H-19, H-12α, ii) H-15 and H-11, H-12β, iii) H-18 and H-19, H-5, H-3α, H-3β, H-6β, and iv) H-19 and H-18, H-20, H-2α, H-3α. On the basis of the above spectral data, the configuration of the hydroxy group at C-15 of **9** was determined to be β. Accordingly, the structure of jungermannenone A (**3**) was established as shown in the drawing.

Since the present species contains *ent*-kaurane-type diterpenoids as the main components, compounds **2** and **3** are also presumed to be the same *ent*-kaurane series as compounds **1**, **4**, and **5**.

Jungermannia species contain numerous diterpenoids such as *ent*-kaurene-, clerodane-, and labdane-type diterpenoids.[6] The present species contained *ent*-kaurenes as the main components and new rearranged *ent*-kaurene-type diterpenoids.

Antitumor agents induce apoptosis in some cancer cells.[10] Apoptosis-inducing compounds are promising candidates for cancer chemotherapy. We have already reported that treatment with *ent*-11α-hydroxy-16-kauren-15-one (**4**) in a human leukemia cell line (HL-60 cells) induced apoptosis and the enone group played a pivotal role in the ability of **4** to induce apoptosis.[11,12] Therefore we next investigated the cytotoxicity of compounds **1**—**3** with the enone group against HL-60 cells by colorimetric 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8) assay. As shown in Fig. 4, each compound exhibited cytotoxicity against HL-60 cells in a dose-dependent manner. The cytotoxicity was different among the compounds. IC_{50} values of compounds **1**, **2**, **3**, and **4** were 7.0, 0.59, 0.28, and 0.49 μM, respectively. Interestingly, treatment with all of the compounds caused proteolysis of poly(ADP-ribose) polymerase, a sign of activation of apoptotic machinery, whereas the feature of cell death induced by treatment with compounds **1** and **2** was necrosis (data not shown). Treatment with compound **3** induced apoptosis (data not shown). These data indicate that these diterpenes might be useful tools for investigation of the mechanism of cell death. The detailed mechanism of the differences is now under investigation.

Experimental

Melting points were measured on a Yanagimoto micromelting points apparatus without correction. Optical rotations were measured on a Jasco DIP-1000 polarimeter. IR spectra were recorded on a Jasco FT/IR-5300 infrared spectrophotometer. UV spectra were recorded on a Shimadzu UV-1650PC UV–visible spectrophotometer. CD spectra were recorded on a Jasco J-725 spectropolarimeter. The ^{1}H- and ^{13}C-NMR spectra were measured on Varian Unity-600 (^{1}H, 600 MHz, ^{13}C, 150 MHz) and Jeol Eclipse-400 (^{1}H, 400 MHz, ^{13}C, 100 MHz) instruments. Chemical shift values are expressed in δ (ppm) downfield from tetramethylsilane as an internal standard (^{1}H-NMR), and in δ 77.03 (ppm) from $CDCl_3$ as a standard (^{13}C-NMR). Mass spectra were obtained on a Jeol JMS AX-500 instrument. TLC was carried out using silica gel $60F_{254}$ plates (Merck). Column chromatography was performed on Silica-gel 60 (Merck, 230—400 and 35—70 mesh), Sephadex LH-20 (Amersham Pharmacia Biotech, sol. CH_2Cl_2–MeOH 1 : 1) and Lobar® (LiChroprep, Merck) columns. TLC plates were examined under UV (254 nm) light and by spraying with 30% H_2SO_4 or Godin reagent,[13] followed by heating.

Plant Material An unidentified *Jungermannia* species (NZ-49) was collected in Jackson River, New Zealand, in 2000, and identified by J. E. B. A voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

Extraction and Isolation The ether extract (8.6 g) of *Jungermannia* species was divided into nine fractions by column chromatography (CC) on silica gel (35—70 mesh) using an *n*-hexane–EtOAc gradient solvent system. Jungermannenone A (**3**, 199 mg) was isolated from fr. 4 by CC on Sephadex LH-20, silica gel, and Lobar® (LiChroprep® Si-60, CH_2Cl_2–Et_2O 49 : 1).

Fraction 5 was rechromatographed on Sephadex LH-20, silica gel, Lobar®, and preparative HPLC to give *ent*-1β-hydroxy-9(11),16-kauradien-15-one (**1**, 130 mg), *ent*-9(11),16-kauradiene-12,15-dione (**2**, 45 mg), *ent*-11α-hydroxy-16-kauren-15-one (**4**, 11 mg), and (16*R*)-*ent*-11α-hydroxykauran-15-one (**5**, 6 mg).

ent-1β-Hydroxy-9(11),16-kauradien-15-one (**1**): mp 138—140 °C. $[\alpha]_D^{20}$ +238.7° (c=6.05, $CHCl_3$). FT-IR cm^{-1}: 3487, 1721, 1644. UV λ_{max} (MeOH) nm (log ε): 351 (2.53), 231 (3.95), 212 (3.91) ($c=1.89\times10^{-4}$). CD (MeOH): $\Delta\varepsilon_{352\,nm}$ +3.1, $\Delta\varepsilon_{268\,nm}$ −3.3, $\Delta\varepsilon_{209\,nm}$ +27.8 ($c=1.89\times10^{-4}$). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. HR-EI-MS *m/z*: 300.2090 (Calcd for $C_{20}H_{28}O_2$: 300.2089). EI-MS *m/z* (int.): 300 $[M]^+$ (33), 282 (7), 267 (15), 241 (60), 213 (12), 201 (100), 187 (20), 173 (19), 145 (18), 129 (12), 117 (11), 105 (32), 91 (16), 81 (12), 55 (9), 41 (9).

ent-9(11),16-Kauradiene-12,15-dione (**2**): mp 114—116 °C. $[\alpha]_D^{20}$ +386.6° (c=0.41, $CHCl_3$). FT-IR cm^{-1}: 1726, 1675, 1640. UV λ_{max} (MeOH) nm (log ε): 334 (1.50), 257 (3.82), 217 (4.22) ($c=9.56\times10^{-5}$). CD (MeOH): $\Delta\varepsilon_{314\,nm}$ +6.7, $\Delta\varepsilon_{281\,nm}$ +12.6, $\Delta\varepsilon_{233\,nm}$ −33.9 ($c=9.56\times10^{-5}$). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. HR-EI-MS *m/z*: 298.1923 (Calcd for $C_{20}H_{26}O_2$: 298.1933). EI-MS *m/z* (int.): 298 $[M]^+$ (100), 283 (50), 255 (7), 241 (10), 229 (27), 213 (23), 201 (19), 187 (21), 174 (43), 160 (12), 145 (7), 129 (9), 115 (8), 105 (10), 91 (14), 69 (18), 55 (11), 41 (11).

Jungermannenone A (**3**): mp 105—106 °C. $[\alpha]_D^{20}$ −265.5° (c=1.97,

$CHCl_3$). FT-IR cm^{-1}: 3463, 1709, 1644. UV λ_{max} (MeOH) nm (log ε): 341 (2.89), 270 (3.31), 233 (4.00) ($c=1.04\times10^{-4}$). CD (MeOH): $\Delta\varepsilon_{344\,nm}$ −5.2, $\Delta\varepsilon_{275\,nm}$ +4.3 ($c=1.04\times10^{-4}$). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. HR-EI-MS *m/z*: 300.2088 (Calcd for $C_{20}H_{28}O_2$: 300.2089). EI-MS *m/z* (int.): 300 $[M]^+$ (53), 282 (12), 267 (16), 241 (61), 213 (13), 201 (100), 187 (20), 173 (19), 157 (9), 145 (18), 129 (14), 117 (12), 105 (30), 91 (16), 81 (11), 55 (10), 41 (11).

Acetylation of 1 Compound **1** (9 mg) was added to pyridine (1 ml) and Ac_2O (1 ml), and kept at room temperature overnight, and then worked up as usual to give a monoacetate **6** (8 mg).

ent-1β-Acetoxy-9(11),16-kauradien-15-one (**6**): $[\alpha]_D^{19}$ −7.8° ($c=0.86$, $CHCl_3$). FT-IR cm^{-1}: 1738, 1726, 1238. ^{1}H-NMR (600 MHz, $CDCl_3$): δ: 0.91 (6H, s, H-18, H-19), 1.24 (3H, s, H-20), 1.30—1.36 (2H, m, H-3), 1.52 (1H, m, H-2α), 1.55—1.59 (3H, m, H-5, H-6α, H-14α), 1.67 (1H, dd, $J=11.0$, 4.9 Hz, H-14β), 1.75 (1H, m H-2β), 1.72—1.81 (2H, m, H-7), 2.05 (3H, s, –CO$\underline{CH_3}$), 2.07 (1H, br d, $J=17.9$ Hz, H-12β), 2.24 (1H, m, H-6β), 2.59 (1H, ddd, $J=17.9$, 4.7, 3.0 Hz, H-12α), 2.93 (1H, br s, H-13), 4.70 (1H, dd, $J=11.5$, 4.7 Hz, H-1), 5.41 (1H, t, $J=1.1$ Hz, H-17), 5.46 (1H, t, $J=3.6$ Hz, H-11), 5.90 (1H, t, $J=1.1$ Hz, H-17). ^{13}C-NMR: Table 2. HR-EI-MS *m/z*: 342.2191 (Calcd for $C_{22}H_{30}O_3$: 342.2195). EI-MS *m/z* (int.): 342 $[M]^+$ (42), 299 (100), 282 (65), 267 (51), 241 (22), 226 (13), 213 (18), 201 (40), 187 (26), 173 (15), 157 (11), 145 (14), 129 (15), 117 (12), 99 (18), 81 (24), 55 (11), 43 (31).

Reduction of 3 To a suspension of $LiAlH_4$ (12 mg) in dry Et_2O (1 ml) was added compound **3** (23 mg) in dry Et_2O (2 ml) and stirred for 1 h at room temperature. Work-up as usual gave the diol **9** (19 mg).

Diol **9**: mp 85—86 °C. $[\alpha]_D^{17}$ −122.4° ($c=0.42$, $CHCl_3$). FT-IR cm^{-1}: 3360. ^{1}H-NMR (600 MHz, $CDCl_3$): δ: 0.84 (3H, s, H-19), 0.86 (3H, s, H-18), 1.07 (3H, s, H-20), 1.11 (1H, dd, $J=12.4$, 1.6 Hz, H-5), 1.31 (1H, ddd, $J=13.7$, 13.7, 4.4 Hz, H-3β), 1.38 (1H, m, H-6α), 1.39 (1H, d, $J=12.1$ Hz, H-12α), 1.44 (1H, dt, $J=13.5$, 3.0 Hz, H-3α), 1.56 (1H, m, H-2β), 1.59 (1H, m, H-6β), 1.67 (1H, dt, $J=11.0$, 4.9 Hz, H-12β), 1.77 (1H, m, H-7α), 1.79 (1H, m, H-2α), 1.92 (1H, br d, $J=16.5$ Hz, H-14β), 1.93 (1H, m, H-7β), 2.31 (1H, dd, $J=16.8$, 4.9 Hz, H-14α), 2.49 (br s, OH), 2.74 (1H, br s, H-13), 3.12 (br s, OH), 3.44 (1H, t, $J=4.9$ Hz, H-11), 3.61 (1H, dd, $J=11.8$, 3.8 Hz, H-1), 4.55 (1H, br s, H-15), 5.11 (1H, s, H-17), 5.13 (1H, s, H-17). ^{13}C-NMR: Table 2. HR-EI-MS *m/z*: 302.2246 (Calcd for $C_{20}H_{30}O_2$: 302.2245). EI-MS *m/z* (int.): 302 $[M]^+$ (100), 284 (88), 269 (67), 243 (59), 228 (21), 215 (25), 203 (35), 185 (96), 173 (17), 159 (20), 143 (43), 131 (34), 117 (28), 105 (47), 91 (43), 81 (34), 69 (18), 55 (25), 41 (25).

Biological Assay. Cell Culture Human myeloid leukemia HL-60 cells were cultured to the exponential growth phase in RPMI 1640 supplemented with 10% (v/v) fetal calf serum in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay Cytotoxicity against HL-60 cells was assessed as follows: 4×10^4 cells seeded onto 96-well plates were incubated with compounds at the indicated concentrations at 37 °C for 24 h. Cell viability was determined using the colorimetric 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8) method using cell counting kit-8 according to the manufacturer's instructions (Wako Pure Chemicals, Ltd., Osaka, Japan).

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