Cytotoxic and Apoptosis-Inducing *ent*-Kaurane-Type Diterpenoids from the Japanese Liverwort *Jungermannia truncata* Nees

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Five new *ent*-kaurane-type diterpenoids and a new gymnomitrane (=barbatane)-type sesquiterpenoid have been isolated from the Japanese liverwort *Jungermannia truncata* Nees, together with twelve previously known *ent*-kaurane-type diterpenoids. The structures of the new compounds were elucidated by two-dimensional (2D) NMR experiments and chemical reaction. Some of the isolated compounds showed cytotoxicity against human leukemia cell lines and induced apoptosis.

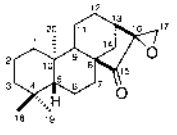
Key words *Jungermannia truncata*; liverwort; gymnomitrane (=barbatane)-type; *ent*-kaurane-type; cytotoxic activity; apoptosis

Most of the Hepaticae (=liverworts) possess characteristic cellular oil bodies which are mainly composed of lipophilic terpenoids and aromatic compounds. We are searching for new biologically active natural products from the rich liverwort flora in Japan and southern hemisphere as lead compounds for pharmaceutical or agricultural drugs. Recently we found that some liverworts produced sesqui- and diterpenoids and cyclic bis-bibenzyls possessing antimicrobial, antifungal, 5-lipoxygenase, cyclooxygenase inhibitor, neurotrophic, muscle relaxing or fish-killing activity.[1—3)]

The genus *Jungermannia* L. belonging to the Jungermanniaceae (Jungermanniales) produces various types of sesqui- and diterpenoids.[4)] *Jungermannia* species are not only morphologically but also chemically interesting since the same species of certain *Jungermannia* collected from geographically different places occasionally display different chemical components. We have studied the chemical constituents of *J. truncata* Nees collected in Kochi prefecture to isolate five new *ent*-kaurane-type diterpenoids (**1**—**5**) and a new gymnomitrane (=barbatane)-type sesquiterpenoid (**6**), along with twelve known *ent*-kaurane-type diterpenoids (**7**—**18**). Here we report the structure determination of the new terpenoids and their cytotoxic and apoptosis-inducing activity.

A combination of column chromatography and prep. HPLC of the ether extract of *J. truncata* resulted in the isolation of five new *ent*-kaurane-type diterpenoids (**1**—**5**) and a new gymnomitrane-type sesquiterpenoid **6** together with twelve *ent*-kaurane-type diterpenoids, *ent*-11α-hydroxy-16-kaurene (**7**),[5)] *ent*-15α-hydroxy-16-kaurene (**8**),[6)] *ent*-11α,15α-dihydroxy-16-kaurene (**9**),[7)] *ent*-16-kauren-15-one (**10**),[6)] *ent*-11α-hydroxy-16-kauren-15-one (**11**),[7)] *ent*-3α-hydorxy-16-kauren-15-one (**12**),[8)] *ent*-14α-hydroxy-16-kauren-15-one (**13**),[9)] rostronol F (**14**),[10)] (16*R*)-*ent*-kauran-15-one (**15**),[6)] (16*R*)-*ent*-7β-hydroxykauran-15-one (**16**),[8)] (16*R*)-*ent*-11α-hydroxykauran-15-one (**17**),[7)] and 16,17-dihydrorostronol F (**18**).[10)] Additionally, a small amount of the crude extract was analyzed by gas chromatography-mass spectrometry (GC-MS) to detect the presence of five terpene hydrocarbons, α-pinene, β-sabinene, β-himachalene, β-barbatene and 16-kaurene. The known compounds were identified by comparison of their spectral data with authentic samples, reference data and/or X-ray crystallographic analysis.

The IR spectrum of **1** showed the presence of a carbonyl group (1741 cm^{-1}) and its electron-impact mass spectrometry (EI-MS) showed the molecular ion at *m/z* 302 $[M]^+$. The high-resolution EI-MS (HR-EI-MS) of **1** showed the molecular formula $C_{20}H_{30}O_2$, indicating six degrees of unsaturation. The ^{1}H-NMR spectrum (Table 1) showed the presence of three tertiary methyls and a methylene proton (δ 3.01, 3.14 each d) bearing an oxygen atom. The ^{13}C-NMR spectrum (Table 2) displayed twenty carbon signals including a ketone carbon (δ 218.5) and a quaternary carbon (δ 63.3) bearing an oxygen atom. Since the IR spectrum showed no hydroxyl absorption band, compound **1** was suggested to have an epoxy group. These spectral data suggested that this compound was a tetracyclic compound with an epoxide. The ^{1}H–^{1}H correlated spectroscopy (COSY) spectrum confirmed the presence of four partial structures, (i) –CH_2(17)–, (ii) –CH(9)–CH_2(11)–CH_2(12)–CH(13)–CH_2(14)–, (iii) –CH_2(1)–CH_2(2)–CH_2(3)–, and (iv) –CH_2(7)–CH_2(6)–



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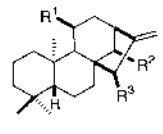

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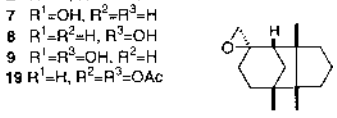
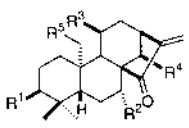
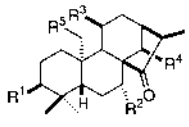


20

2 R^1=H, R^2=R^3=OH
7 R^1=OH, R^2=R^3=H
8 R^1=R^2=H, R^3=OH
9 R^1=R^3=OH, R^2=H
19 R^1=H, R^2=R^3=OAc



3 R^1=R^2=R^4=H, R^3=OH, R^5=OAc
4 R^1=R^5=H, R^2=R^4=OH, R^3=OAc
10 R^1=R^2=R^3=R^4=R^5=H
11 R^1=R^2=R^4=R^5=H, R^3=OH
12 R^1=OH, R^2=R^3=R^4=R^5=H
13 R^1=R^2=R^3=R^5=H, R^4=OH
14 R^1=H, R^2=R^4=R^5=OH, R^3=OAc



5 R^1=OH, R^2=R^3=R^4=R^5=H
15 R^1=R^2=R^3=R^4=R^5=H
16 R^1=R^3=R^4=R^5=H, R^2=OH
17 R^1=R^2=R^4=R^5=H, R^3=OH
18 R^1=H, R^2=R^4=R^5=OH, R^3=OAc

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

H	**1**	**2**	**3**
1	1.77—1.87 m, α	0.86 m, α	2.18 br d (12.6), α
	0.78 ddd (13.2, 13.2, 3.6),[a] β	1.81 m, β	0.93 m, β
2	1.42 m	1.36—1.44 m	1.45—1.55 2H, m
	1.58—1.63 m	1.57—1.64 m	
3	1.39 m, α	1.36—1.44 m, α	1.23 ddd (13.5, 13.5, 3.8)
	1.15 ddd (13.2, 13.2, 3.8), β	1.14 ddd (13.5, 13.5, 4.1), β	1.45—1.55 m
5	0.94 dd (12.1, 2.2)	0.88 m	1.19 dd (13.7, 1.9)
6	1.29—1.37 m, α	1.36—1.44 m	1.32 qd (13.7, 3.6), α
	1.66 m, β	1.57—1.64 m	1.68 d quit.(13.7, 2.5), β
7	1.29—1.37 m	1.33 m, α	1.43 m, α
	1.92 ddd (14.0, 14.0, 3.8), β	2.10 dd (10.7, 3.0), β	2.14 ddd (14.0, 14.0, 4.9), β
9	1.20 br s	1.52—1.56 m	1.53 br s
11	1.54—1.59 m	1.36—1.44 m	4.29 d (4.9)
	1.77—1.87 m	1.52—1.56 m	
12	1.77—1.87 2H, m	1.85 dddd (7.1, 7.1, 6.0, 2.5), α	2.31 ddd (14.3, 4.9, 3.3), α
		1.57—1.64 m	1.87 br d (14.3), β
13	2.32 br s	2.68 br s	3.04 br s
14	2.61 d (13.2), α	4.26 s	2.22 d, (12.1), α
	1.54—1.59 m, β		1.45—1.55 m, β
15		4.21 br s	
17	3.01 d (6.9)	5.10 d (2.7)	5.29 s
	3.14 d (6.9)	5.25 s	5.89 t (1.1)
18	0.88 s	0.87 s	0.94 s
19	0.83 s	0.81 s	0.89 s
20	1.13 s	0.99 s	4.30 d (12.4)
			4.74 d (12.4)
$CO\underline{CH_3}$			2.10 s

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

Table 2. ^{13}C-NMR Data of **1**—**5** and **7** ($CDCl_3$)

C	**1**[a]	**2**[c]	**3**[a]	**4**[c]	**5**[a]	**7**[c]
1	39.4	40.4	34.3	39.4	32.3	39.9
2	18.3[b]	19.5	18.0	18.4	25.0	18.6
3	41.8	41.9	41.3	41.3	75.9	41.9
4	33.19	33.26	33.0	33.3	37.5	33.4
5	55.2	55.6	55.8	53.4	48.1	56.1
6	18.2[b]	18.7	18.3	27.6	18.4	20.1
7	33.24	29.9	33.6	75.1	34.0	41.0
8	52.3	50.9	50.1	60.1	52.6	43.0
9	52.6	48.6	63.9	61.7	52.0	65.1
10	40.1	39.0	41.4	39.1	39.5	38.1
11	18.7	17.8	66.0	67.2	18.0	67.1
12	26.7	33.30	39.9	37.3	24.8	43.3
13	34.2	48.8	36.8	44.5	34.9	42.3
14	34.0	75.3	37.8	74.6	37.4	39.6
15	218.5	79.3	209.4	206.7	224.7	49.0
16	63.3	156.3	150.0	148.2	47.7	156.8
17	54.5	107.8	113.1	116.6	10.1	105.2
18	33.5	33.6	34.0	33.6	28.3	33.7
19	21.5	21.6	22.4	21.6	21.9	21.8
20	17.8	17.7	63.2	18.3	17.4	17.2
OAc			21.1	21.4		
			170.7	169.3		

a) Measured by 150 MHz. *b*) Can be interchanged. *c*) Measured by 100 MHz.

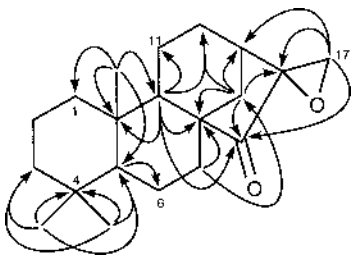

Fig. 1. Long-Range ^{1}H–^{13}C Correlations of **1**

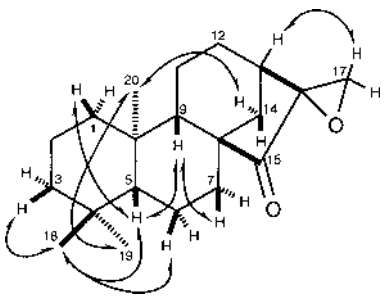

Fig. 2. NOE Correlations of **1**

CH(5)–. The connectivity of each partial structure was clarified by the heteronuclear multiple quantum coherence (HMQC) and the heteronuclear multiple bond correlation (HMBC) spectra as shown in Fig. 1. Accordingly, the structure of **1** was suggested to be kaurane-type diterpenoid with the epoxide at C-16,17. The stereochemistry of **1** was confirmed by the nuclear Overhauser and exchange spectroscopy (NOESY) as shown in Fig. 2. However, the stereochemistry of the epoxide remained to be clarified. The absolute configuration of **1** except the epoxide has been supported by the negative Cotton effect (320 nm)[11] of **1** and co-existence of the same *ent*-kaurene diterpenoids **8**—**18** in the present liverwort. Thus, the structure of **1** was established as *ent*-16,17-epoxykauran-15-one.

The EI-MS of **2** showed *m*/*z* 304 $[M]^+$ and its molecular

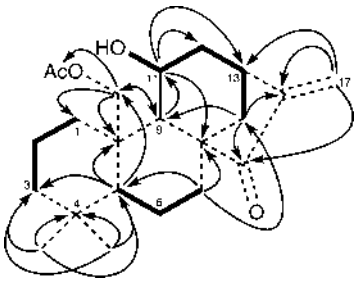

Fig. 3. ^{1}H–^{1}H (Bold Lines) and Long-Range ^{1}H–^{13}C (Arrows) Correlations of **3**

formula was found to be $C_{20}H_{32}O_2$ by HR-EI-MS. The IR spectrum confirmed the presence of a hydroxyl group (3420 cm^{-1}). The ^{1}H-NMR spectrum (Table 1) of **2** was similar to that of *ent*-15α-hydroxy-16-kaurene (**7**),[5] except for the presence of another methine proton bearing a hydroxyl group, suggesting that compound **2** is an *ent*-kaurane-type diterpenoid. Acetylation of **2** gave diacetate **19** (δ_H 2.06, 2.18 each s) indicating the presence of two hydroxyl groups. The ^{13}C-NMR (Table 2) and distortionless enhancement by polarization transfer (DEPT) spectra of **2** showed the presence of an *exo*-methylene, two methines bearing the hydroxyl groups, together with three methyls, seven methylenes, three methines and three quaternary carbons, respectively. The detailed analysis of ^{1}H–^{1}H COSY, HMQC and HMBC spectra of **2** led to the structure of 14,15-dihydroxy-16-kaurene. In addition, the NOESY showed NOEs between (i) H-20 and H-1α, H-7α, H-12α, H-19, (ii) H-19 and H-7α, H-18, (iii) H-14 and H-7α, H-12α, H-20, and (iv) H-15 and H-7α, H-7β. Thus, the stereochemistry of both hydroxyl groups at C-14 and C-15 possesses β-orientation. The absolute configuration was believed to be the same as those of compounds **8**—**18**. Consequently, the structure of **2** was believed to be *ent*-14α,15α-dihydroxy-16-kaurene.

The IR spectrum of **3** showed the presence of a hydroxyl (3420 cm^{-1}) and a carbonyl group (1730 cm^{-1}). The ^{1}H-NMR spectrum (Table 1) indicated an acetyl group (δ 2.10 s), *exo*-methylene protons (δ 5.29 s, 5.89 t), a methine proton (δ 4.29 d) bearing a hydroxyl group and two tertiary methyls. The ^{13}C-NMR (Table 2) and DEPT spectra showed the presence of a ketone carbon (δ 209.4), a methylene (δ 63.2) and a methine (δ 66.0) bearing oxygen atoms and a carboxyl carbon (δ 170.1) originated from the acetoxyl group as well as three methyls, seven methylenes, three methines and three quaternary carbons. The molecular formula of **3** was supported as $C_{22}H_{32}O_4$ by the analysis of the chemical ionization-MS (CI-MS), *m/z* 389 $[M+C_2H_5]^+$, and ^{13}C-NMR spectra. Furthermore, the ^{1}H- and ^{13}C-NMR spectra of **3** were similar to those of *ent*-kaurane-type diterpenoids **8**—**18**. The detailed analysis of ^{1}H–^{1}H COSY, HMQC and HMBC spectra as shown in Fig. 3 led to the structure of 20-acetoxy-11-hydoxy-16-kauren-15-one **3**, the stereochemistry of which was clarified by the NOESY in C_6D_6. It showed NOEs between (i) H-18 and H-5, H-6β, (ii) H-5 and H-1β, H-7β, H-9, (iii) H-19 and H-20, (iv) H-20 and H-14α, H-19, and (v) H-11 and H-1α, H-12α, H-12β. The CD spectrum of **3** indicated first negative, second positive and third negative Cotton effects (see experiments). The first negative Cotton effect (λ_{max} 342 nm) corresponding to an enone system was the same as that of *ent*-kaurenes **12** (λ_{max} 352 nm) and **13** (λ_{max} 336 nm). Thus, the absolute configuration of **3** was established as *ent*-20-acetoxy-11α-hydoxy-16-kauren-15-one.

Table 3. ^{1}H-NMR Data of **4** and **5** (600 MHz, $CDCl_3$)

H	**4**	**5**
1	1.86 m, α	1.19—1.25 m
	0.91 m, β	1.51 dt (14.3, 4.1)
2	1.60 m, α	1.95 dddd (14.3, 14.3, 4.1, 2.5), α
	1.49 d quint. (14.3, 3.8),[a] β	1.52—1.58 m
3	1.44 br d (14.3), α	3.40 t (2.7)
	1.16 ddd (14.3, 14.3, 4.4), β	
5	1.00 dd (12.9, 1.9)	1.40 dd (12.8, 1.9)
6	1.72 q (12.9), α	1.28—1.35 m
	2.03 ddd (12.9, 4.1, 1.9), β	1.52—1.58 m
7	4.39 dt (12.9, 4.1)	1.28—1.35 m, α
		1.88 ddd (13.7, 13.7, 4.4), β
9	1.41 br s	1.20 br s
11	5.07 d (4.9)	1.19—1.25 m
		1.59—1.72 m
12	2.21 ddd (15.1, 4.9, 3.0), α	1.59—1.72 2H, m
	2.06 br d (15.1), β	
13	3.06 br s	2.42 br s
14	4.92 s	2.46 d (12.1), α
		1.28—1.35 m, β
16		2.22 quint. (6.9)
17	5.38 s	1.10 d (6.9)
	6.11 s	
18	0.93 s	0.95 s
19	0.87 s	0.84 s
20	1.06 s	1.09 s
$CO\underline{CH_3}$	1.85 s	
OH	2.78 br s	

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

The IR and ^{13}C-NMR of **4** indicated the presence of two secondary hydroxy groups (3320 cm^{-1}, δ 74.6, 75.1) and an acetoxyl group (1735, 1240 cm^{-1}, δ 21.4 q, 169.3 s). The ^{1}H- and ^{13}C-NMR spectra (Tables 2, 3) of **4** were similar to those of rostronol F (**14**),[10] except for the presence of the methyl proton at C-20. The detailed analysis of ^{1}H–^{1}H COSY, HMQC and HMBC spectra of **4** led to the structure of 11-acetoxy-7,14-dihydroxy-16-kauren-15-one. The NOESY showed NOEs between (i) H-5 and H-3β, H-6β, H-9, (ii) H-7β and H-5, H-6β, H-9, (iii) H-11α and H-1α, H-12α, H-12β, H-20, (iv) H-14α and H-12α, H-20, (v) H-18 and H-3β, H-5, H-6β, (vi) H-19 and H-3α, H-6α, H-20, (vii) H-20 and H-2α, H-6α, H-12α, H-14α, H-19. The absolute configuration of **4** was confirmed have *ent*-kaurene-type by the CD spectrum (see experiments). Thus, the structure of **4** was established as *ent*-11α-acetoxy-7β,14α-dihydroxy-16-kauren-15-one.

The EI-MS spectrum of **5** showed *m/z* 304 $[M]^+$ and its molecular formula was confirmed as $C_{20}H_{32}O_2$ by HR-EI-MS. The IR and ^{13}C-NMR (Table 2) spectra indicated the presence of the secondary hydroxyl (3430 cm^{-1}, δ 75.9 d) and carbonyl groups (1725 cm^{-1}, δ 224.7 s). The ^{1}H-NMR spectrum (Table 3) of **5** showed a secondary methyl (δ 1.10), three tertiary methyls (δ 0.95, 0.84, 1.09) and a methine proton (δ 3.40 t) bearing a hydroxyl group. Since these spectral data of **5** were similar to those of *ent*-kauranes **16** and **17**, compound **5** was also suggested to be *ent*-kauran-15-one type diterpenoid. The ^{1}H–^{1}H COSY, HMQC and HMBC spectra of **5** as shown in Fig. 4 clarified that this compound is

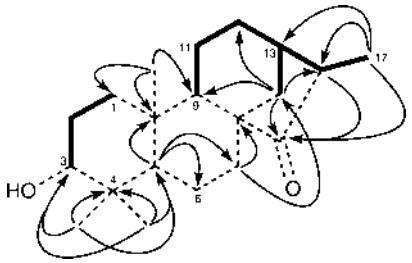

Fig. 4. ^{1}H–^{1}H (Bold Lines) and Long-Range ^{1}H–^{13}C (Arrows) Correlations of **5**

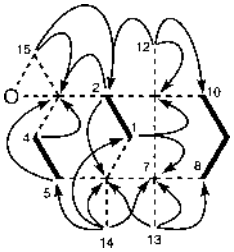

Fig. 5. ^{1}H–^{1}H (Bold Lines) and Long-Range ^{1}H–^{13}C (Arrows) Correlations of **6**

Table 4. ^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR Data of **6**

	^{13}C	^{1}H
1	43.8	1.99 ddd (11.8, 4.7, 2.7),[a] α 1.72 d (11.8), β
2	53.5	1.09 d (5.2)
3	60.2	
4	27.9	2.43 ddd (15.7, 12.6, 8.0), α 1.22—1.29 m, β
5	35.9	1.69 br ddd (14.0, 6.3, 2.6), α 1.62 ddd (14.0, 12.6, 8.0), β
6	42.6	
7	54.5	
8	36.0	1.81—1.88 m, α 1.15 m, β
9	27.6	1.82—1.88 2H, m
10	36.4	2.08 m, α 1.22—1.29 m, β
11	54.4	
12	27.5	1.02 s
13	23.3	0.91 s
14	24.7	0.89 s
15	54.1	2.64 d (4.7) 2.66 d (4.7)

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

3-hydroxykauran-15-one. The NOEs were observed between (i) H-3α and H-2α, H-18, H-19, (ii) H-20 and H-2α, H-14α, H-19, (iii) H-19 and H-2α, H-18, H-20, (iv) H-17 and H-9, (v) H-5 and H-9, H-18, and (vi) H-16 and H-13, H-14β. The CD spectrum of **5** showed first negative (310 nm), second positive (279 nm) and third negative (204 nm) Cotton effects. It has been known that the Cotton effect of (16*R*)-*ent*-kauran-15-one (**15**), which is similar to compound **5**, showed a negative Cotton effect (314 nm).[11] Thus, the structure of **5** was established as (16*R*)-*ent*-3α-hydroxykauran-15-one.

The molecular formula of **6** was confirmed as $C_{15}H_{24}O$ (*m*/*z* 220.1830 $[M]^{+}$) by HR-EI-MS. The ^{1}H-NMR spectrum (Table 4) showed the signals of three tertiary methyls (δ 0.89, 0.91, 1.02) and the isolated methylene protons (δ 2.64, 2.66 each d). The ^{13}C-NMR spectrum (Table 4) of **6** indicated the presence of the quaternary carbon (δ 60.2) and methylene (δ 54.1) bearing oxygen atoms, as well as three methyls, six methylenes, a methine and three quaternary carbons. Since the IR spectrum showed neither hydroxyl nor carboxyl absorption band, compound **6** was suggested to possess epoxy oxygen. The ^{1}H–^{1}H COSY showed the presence of three partial segments, (i) –CH_2(8)–CH_2(9)–CH_2(10)–, (ii) –CH_2(4)–CH_2(5)– and (iii) –CH(2)–CH_2(1)–. The HMBC spectrum of **6** showed the long-range ^{1}H–^{13}C correlations as in Fig. 5, indicating that the structure of **6** might be gymnomitrane (=barbatane)-type sesquiterpenoid with an epoxy ring. This plane structure was the same as compound **20** which was reported as a reaction product.[12] However, the ^{1}H-NMR spectrum of **6** could not be compared with that of **20** because of the different solvents used for measurement of each NMR spectrum. The stereochemistry was clarified by the NOESY which showed NOEs, (i) H-12 and H-1β, H-2, H-10β, H-13, (ii) H-13 and H-1β, H-8β, H-12, H-14, (iii) H-14 and H-1β, H-5β, H-13, and (iv) H-4α and H-8α, H-10α, respectively. However, the stereochemistry of the epoxy ring remained to be clarified even by the NOESY. Thus, the structure of **6** was elucidated as 3(15)-epoxygymnomitrane.

The kaurane-type diterpenoids of *J. truncata* collected in Tokushima, Japan and Malaysia have already been reported by our group[13] and Buchanan *et al.*, respectively.[14] The present species collected in Kochi, Japan also produced *ent*-kaurane-type diterpenoids as the major components. Thus, we reconfirmed that *J. truncata* is one of the rich sources of *ent*-kaurane-type diterpenoid in *Jungermannia* species, and there is no chemical difference between the same species collected in different locations.

Some highly oxygenated kaurane-type diterpenoids have been known to possess strong bitterness and antitumor activity.[15—17] The cytotoxic activity in human leukemia cell lines (HL-60 cells) was examined for compounds **9**, **11** and **16**—**18** by colorimetric 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disufophenyl)-2*H*-tetrazolium monosodium salt (WST-8) assay. IC_{50} value of compound **11** was estimated to be 0.82 μM. However, compounds **9** and **16**—**18** without an enone system in the molecule did not show cytotoxicity even at 10 μM (data not shown). It is suggested that the presence of an enone system may play an important role in the cytotoxicity of **11**. Since apoptosis-inducing compounds are potential candidates as antitumor agents, the induction of apoptosis by compound **11** in HL-60 cells was tested. Apoptotic cells have several typical features such as DNA fragmentation into nucleosomal fragments, accumulation of cells with sub-G1 DNA content and appearance of nuclear condensation.[18,19] Compound **11** induced DNA fragmentation in a dose-dependent manner as shown in Fig. 6A. Moreover, DNA fragmentation appeared 6 h after treatment, and the most marked induction was observed at 12 h (data not shown). Accumulation of cells with sub-G1 DNA content was also observed 12 h after compound **11** treatment (Fig. 6B). In addition, nuclear condensations were observed in compound **11**-treated HL-60 cells (Fig. 7). These results suggested that compound **11** is a potent inducer of apoptosis in these cells. This is the first report of the ability of compound **11** to induce apoptosis in HL-60 cells. Although further

structure-activity relationship studies will be necessary, an *ent*-kaurane-type diterpenoid, such as compound **11**, might be a leading product of an antitumor agent.

Experimental

Optical rotations were measured on a JASCO DIP-1000 polarimeter with $CHCl_3$. IR spectra were recorded on a JASCO FT/IR-5300 infrared spectrophotometer. 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 δ 77.03 (ppm) from $CDCl_3$ as a standard (^{13}C-NMR) unless otherwise stated. Mass spectra (EI, CI, HR-EI-MS) were obtained on a JEOL JMS AX-500 instrument. X-ray crystallographic analysis was carried out on a Mac Science DIP-2020 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), reverse phase silica gel (Cosmosil $140C_{18}$, Nacalai), Sephadex^{TM} LH-20 (Amersham Pharmacia Biotech, sol. CH_2Cl_2–MeOH 1 : 1) and Lobar (LiChroprep Si 60 or RP-18, Merck) columns. TLC plates were visualized under UV (254 nm) light and by spraying with 10% H_2SO_4 or Godin reagent,[20] followed by heating.

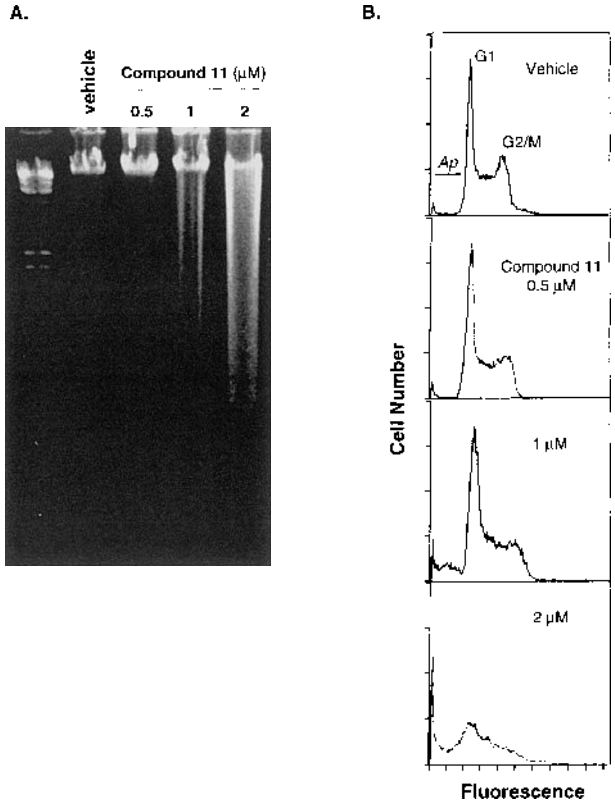

Fig. 6. DNA Fragmentation (A) and Accumulation of Cells with Sub-G1 DNA Content (B) in Compound **11**-Treated HL-60 Cells

HL-60 cells (5×10^5 cells/well in 6-well plate) were treated with compound **11** at the indicated concentrations for 12 h, and then induction of apoptosis was estimated. (A) Formation of DNA fragmentation was determined by agarose gel electrophoresis and stained with ethidium bromide. (B) Accumulation of cells with sub-G1 DNA content. Cell distribution according to their DNA content was measured by PI incorporation. The position of apoptotic cells (Ap) is indicated.

Plant Material *Jungermannia truncata* Nees (F82701) was collected in Aki-shi, Kochi pref. Japan and was identified by Dr. M. Mizutani (The Hattori Botanical Laboratory, Miyazaki, Japan). A voucher specimen was deposited at the Institute of Pharmacognosy, Tokushima Bunri University.

Extraction and Isolation The ether extract (4.6 g) of *J. truncata* (95.3 g) was divided into ten fractions by column chromatography (CC) on silica gel (35—70 mesh) using an *n*-hexane–EtOAc gradient solvent system. Fraction 5 was rechromatographed on Sephadex^{TM} LH-20, silica gel, medium pressure liquid chromatography (MPLC) (LiChroprep Si 60 or RP-18) and finally purified by preparative HPLC to give *ent*-16,17-epoxykauran-15-one (**1**, 3.2 mg), 3(15)-epoxygymnomitrane (**6**, 3.0 mg), *ent*-11α-hydroxy-16-kaurene (**7**, 3.5 mg),[5] *ent*-15α-hydroxy-16-kaurene (**8**, 346.3 mg),[6] *ent*-16-kauren-15-one (**10**, 91.9 mg),[6] and (16*R*)-*ent*-kauran-15-one (**15**, 10.6 mg).[6]

Fraction 7 was divided into four fractions (7-1—7-4) by CC on Sephadex^{TM} LH-20. Fraction 7-1 was rechromatographed on MPLC (Lobar column Si 60) and purified by preparative HPLC (Nucleosil 50-5, *n*-hex.–Et_2O 1 : 1) to yield (16*R*)-*ent*-3α-hydroxykauran-15-one (**5**, 3.5 mg), *ent*-11α,15α-dihydroxy-16-kaurene (**9**, 29.0 mg),[7] *ent*-3α-hydorxy-16-kauren-15-one (**12**, 6.5 mg),[8] (16*R*)-*ent*-7β-hydroxykauran-15-one (**16**, 7.0 mg).[8] Fraction 7-3 was purified by preparative HPLC (Nucleosil 50-5, *n*-hex.–EtOAc 17 : 3) to afford (16*R*)-*ent*-11α-hydroxykauran-15-one (**17**, 6.0 mg), the structure of which has been established by X-ray crystallographic analysis.[7] Fraction 7-4 was also purified by preparative HPLC (CHEMCOSORB 5-ODS, MeOH) to give *ent*-14α-hydroxy-16-kauren-15-one (**13**, 13.7 mg).[9]

Fraction 8 was chromatographed on Sephadex^{TM} LH-20, silica gel, MPLC (LiChroprep Si 60) and finally purified by preparative HPLC (CHEMCOSORB 5-ODS, CH_3CN) to give *ent*-14α,15α-dihydroxy-16-kaurene (**2**, 8.3 mg) and *ent*-11α-hydroxy-16-kauren-15-one (**11**, 162.0 mg).[7]

Fraction 9 was repeatedly chromatographed on Sephadex^{TM} LH-20, MPLC (Lobar Si 60), silica gel, reverse phase silica gel (Cosmosil $75C_{18}$, CH_3CN) and finally purified preparative HPLC (Nucleosil 50-5, *n*-hex.–EtOAc 1 : 1) to yield *ent*-20-acetoxy-11α-hydoxy-16-kauren-15-one (**3**, 4.4 mg), *ent*-11α-acetoxy-7β,14α-dihydroxy-16-kauren-15-one (**4**, 6.2 mg), rostronol F (**14**, 20.6 mg),[10] and 16,17-dihydrorostronol F (**18**, 9.3 mg).[10]

Ent-16,17-epoxykauran-15-one (**1**): Oil. $[\alpha]_D^{23}$ −109.0° (c=0.28). FT-IR cm^{-1}: 1741 (C=O). CD (EtOH): $\Delta\varepsilon_{320\,nm}$ −0.73 ($c=3.0\times10^{-4}$). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. HR-EI-MS: obs. 302.2254 $C_{20}H_{30}O_2$ requires

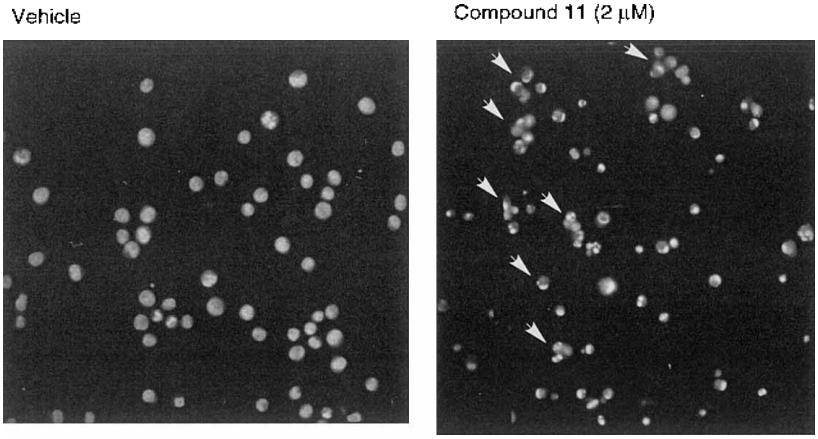

Fig. 7. Fluorescence Microscopic Evaluation of Compound **11**-Treated HL-60 Cells

HL-60 cells (5×10^5 cells/well in 6-well plate) were treated with or without compound **11** for 12 h. The cells were stained with Hoechst 33342 and then examined by fluorescence microscopy. Typical formation of chromatin condensation is indicated by arrowheads.

302.2246. EI-MS *m/z* (int.): 302 $[M]^+$ (100), 287 (59), 269 (15), 259 (14), 241 (8), 228 (10), 217 (8), 189 (10), 166 (11), 148 (12), 137 (38), 123 (42), 109 (23), 95 (31), 81 (31), 69 (29), 55 (21), 44 (86).

Ent-14α,15α-dihydroxy-16-kaurene (**2**): Amorphous. $[\alpha]_D^{22}$ −48.5° (c=2.89). FT-IR cm^{-1}: 3398 (OH). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. HR-EI-MS: obs. 304.2401 $C_{20}H_{32}O_2$ requires 304.2402. EI-MS m/z (int.): 304 $[M]^+$ (28), 286 (100), 271 (32), 253 (16), 230 (13), 215 (20), 201 (36), 189 (18), 162 (21), 149 (30), 137 (62), 123 (43), 109 (31), 95 (36), 81 (36), 69 (35), 55 (29), 44 (31).

Ent-20-acetoxy-11α-hydoxy-16-kauren-15-one (**3**): Oil. $[\alpha]_D^{20}$ −94.0° (c=0.41). FT-IR cm^{-1}: 3420, 1730, 1230. UV: log ε_{236} 3.34 (c=1.47×10^{-4}). CD (EtOH): $\Delta\varepsilon_{342\,nm}$ −0.68, $\Delta\varepsilon_{240\,nm}$ 0.99, $\Delta\varepsilon_{211\,nm}$ −5.65 (c=3.97×10^{-4}). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. ^{1}H-NMR (C_6D_6, 600 MHz): δ: 0.57 (1H, br t, J=13.2 Hz, H-1β), 0.74 (3H, s, H-18), 0.77 (3H, s, H-19), 0.89 (1H, dd, J=12.3, 1.9 Hz, H-5), 0.93—1.02 (2H, m, H-3, H-6α), 1.19 (1H, br d like, H-14β), 1.22—1.29 (3H, m, H-2, H-3, H-7α), 1.36 (1H, br d like, H-6β), 1.50 (1H, m, H-2), 1.52 (1H, br s, H-9), 1.58 (1H, m, H-12), 1.60 (3H, s, –CO$\underline{CH_3}$), 1.92 (1H, d, J=10.4 Hz, H-1α), 1.94 (1H, d, J=11.8 Hz, H-14α), 2.08 (1H, ddd, J=14.0, 4.4, 3.3 Hz, H-12), 2.34 (1H, ddd, J=14.0, 14.0, 5.2 Hz, H-7β), 2.65 (1H, br s, H-13), 4.03 (1H, d, J=4.4 Hz, H-11), 4.07 (1H, d, J=12.6 Hz, H-20), 4.79 (1H, d, J=12.6 Hz, H-20), 4.96 (1H, t, J=1.1 Hz, H-17), 6.02 (1H, s, H-17). CI-MS (CH_4): *m/z* 389 $[M+C_2H_5]^+$, (*iso*-butane): *m/z* 359 $[M-1]^+$. EI-MS *m/z* (int.): 342 $[M-H_2O]^+$ (2), 300 (8), 287 (100), 269 (7), 231 (6), 217 (18), 205 (10), 191 (19), 173 (7), 146 (9), 119 (11), 91 (13), 69 (18), 55 (11), 43 (20).

Ent-11α-acetoxy-7β,14α-dihydroxy-16-kauren-15-one (**4**): Oil. $[\alpha]_D^{19}$ +21.3° (c=0.41). FT-IR cm^{-1}: 3320, 1735, 1240. UV: log ε_{233} 3.74 (c=1.65×10^{-4}). CD (MeOH): $\Delta\varepsilon_{317\,nm}$ −0.24, $\Delta\varepsilon_{235\,nm}$ −4.96, $\Delta\varepsilon_{207\,nm}$ −2.80 (c=3.19×10^{-4}). ^{1}H- and ^{13}C-NMR: Tables 3 and 2. CI-MS (*iso*-butane): *m/z* 377 $[M+1]^+$. EI-MS *m/z* (int.): 316 $[M-AcOH]^+$ (100), 298 (12), 283 (14), 275 (7), 255 (7), 229 (7), 213 (7), 203 (6), 192 (7), 174 (40), 161 (32), 147 (7), 138 (26), 132 (22), 123 (90), 109 (47), 95 (15), 81 (22), 69 (28), 55 (19), 43 (33).

(16*R*)-*Ent*-3α-hydroxykauran-15-one (**5**): Oil. $[\alpha]_D^{19}$ −45.1° (c=0.96). FT-IR cm^{-1}: 3430, 1725. CD (MeOH): $\Delta\varepsilon_{310\,nm}$ −0.40, $\Delta\varepsilon_{279\,nm}$ +0.10, $\Delta\varepsilon_{204\,nm}$ −0.73 (c=7.80×10^{-4}). ^{1}H- and ^{13}C-NMR: Tables 3 and 2. HR-EI-MS: obs. 304.2398 $C_{20}H_{32}O_2$ requires 304.2402. EI-MS *m/z* (int.): 304 $[M]^+$ (26), 286 (100), 271 (49), 253 (14), 246 (35), 243 (35), 228 (35), 213 (34), 173 (11), 161 (13), 146 (21), 136 (69), 121 (53), 107 (36), 93 (35), 79 (24), 67 (17), 55 (23), 40 (33).

3(15)-Epoxygymnomitrane (**6**): Oil. $[\alpha]_D^{21}$ −4.8° (c=0.83). FT-IR cm^{-1}: 1464, 1383, 1302. ^{1}H- and ^{13}C-NMR: Tables 3 and 2. HR-EI-MS: obs. 220.1830 $C_{15}H_{24}O$ requires 220.1827. EI-MS *m/z* (int.): 220 $[M]^+$ (10), 205 (12), 189 (11), 149 (9), 124 (67), 95 (100), 81 (62), 55 (44), 41 (26).

Acetylation of 2 Compound **2** (5 mg) was added to pyridine (ml) and Ac_2O (ml), and kept at room temperature for overnight, then worked up as usual to give diacetate **19** (4 mg).

Ent-14α,15α-dihydroxy-16-kaurene (**19**): Oil. $[\alpha]_D^{21}$ −34.2° (c=3.54). FT-IR cm^{-1}: 1740, 1236. ^{1}H-NMR: δ: 0.73 (1H, dd, J=11.7, 1.8 Hz), 0.81 (3H, s), 0.84 (3H, s), 0.83—0.91 (m), 1.09 (3H, s), 1.08—1.18 (m), 1.25—1.51 (m), 1.56—1.68 (m), 1.80—1.88 (m), 1.98 (1H, m), 2.06 (3H, s), 2.18 (3H, s), 2.70 (1H, br s), 5.01 (2H, br s), 5.45 (1H, s), 5.48 (1H, t, J=2.2 Hz). HR-EI-MS obs. *m/z* 388.2634 $C_{24}H_{36}O_4$ requires 388.2613. EI-MS *m/z* (int.): 388 $[M]^+$ (3), 346 (16), 328 (83), 313 (12), 286 (100), 268 (67), 253 (49), 225 (9), 199 (14), 183 (15), 144 (31), 123 (24), 109 (20), 95 (22), 81 (24), 69 (29), 60 (37), 43 (62).

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 The 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 by the colorimetric 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disufophenyl)-2*H*-tetrazolium, monosodium salt (WST-8) using counting kit-8 according to the manufacturer's instructions (Wako Pure Chemicals, Ltd., Osaka, Japan).

DNA Fragmentation Assay Cells were harvested by centrifugation (1500 g) and washed with PBS. The washed cells were lysed with a lysis buffer [10 mM Tris/HCl, pH 8.0, 10 mM EDTA, 0.5% (w/v) SDS and 0.1% (w/v) RNase A] and incubated for 60 min at 50 °C. The lysate was incubated for an additional 60 min at 50 °C with 1 mg/ml proteinase K. DNA extraction was carried out and the purified samples were electrophoresed on 1.8% (w/v) agarose gel. After electrophoresis, the DNA was visualized by ethidium bromide staining.

Flow Cytometry Analysis To measure the loss of DNA, cells were centrifuged, fixed with cold (−20 °C) 70% (v/v) ethanol and stained with propidium iodide (PI), then analyzed by flow cytometry. Cells with sub-G1 PI incorporation were considered as apoptotic cells.[16]

Nuclear Condensation To analyze the change in chromatin structure, cells were collected by centrifugation, fixed with 1% glutaraldehyde, stained for 15 min at room temperature with Hoechst33342 and mounted on glass slides. Chromatin structures were examined by fluorescence microscopy.

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