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ent-Kaurane-type diterpenoids produced by cell culture of the liverwort *Jungermannia subulata*

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Abstract

An *ent*-Kaurane-type diterpenoid, *ent*-kaurane-3,15-dione has been isolated from cell cultures of the liverwort *Jungermannia subulata*. Its structure was established by spectroscopic methods.

Keywords: *Jungermannia subulata*; Jungermanniales; Hepaticae; *ent*-Kaurane; Diterpenoid; Cell culture

1. Introduction

The difficulty of acquiring large amounts of liverworts is due to the fact that many of them are spread over wide areas and occur only in small populations. A promising way out of this impasse is the use of in vitro culture of liverworts. Liverwort cultures offer essentially the same advantages as in vitro cultures of higher plants, including the possibility of obtaining sufficient amounts of secondary metabolites to study their biosyntheses. In the course of our investigation of secondary metabolites from in vitro cultures of the liverworts (Tazaki, Adam & Becker, 1995; Tazaki, Zapp & Becker, 1995; Tazaki, Nabeta, Okuyama & Becker, 1995; Tazaki, Soutome, Nabeta, Okuyama & Becker, 1996; Tazaki, Soutome, Iwasaki, Nabeta & Arigoni, 1997; Tazaki, Nabeta & Becker, 1998), we examined *Jungermannia subulata* (Jungermanniaceae). An earlier work on *J. subulata* demonstrated the growth behavior of the cells (Ohta & Hirose, 1982; Ohta, Ishikawa, Abe, Katoh & Hirose, 1981) and the production of *ent*-kaurene (**2**) by cell suspension culture (Ohta, Kato & Takeda, 1990). In this paper we discuss the structure of the newly isolated *ent*-kaurane-3,15-dione (**1**) together with three known *ent*-kaurane-type diterpenoids, and the comparison of their amounts between intact plant and calli.

2. Results and discussion

In vitro cultures of the liverwort *J. subulata* were initiated from spores; callus induction was achieved on MSK-medium with 4% glucose. The ether extracts of *J. subulata* were chromatographed on silica gel and further purified by HPLC on a Si-60 column to give a new diterpenoid **1**, together with *ent*-kaurene (**2**) and its known derivatives (**3** and **4**). The spectral data of known compounds **2**–**4** were identical with those of the authentic samples (Fukuyama, Toyota & Asakawa, 1988; Langenbahn, Burkhardt & Becker, 1993). The structures of **3** and **4** were also confirmed by the oxidation of **3** to **4** with PCC, and by the reduction of **4** to **3** $NaBH_4$.

ent-Kaurane-3,15-dione (**1**) (23.6 mg from 19.2 g air-dried cells) was obtained as crystals (mp 176–178°C),

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Table 1
^{1}H and ^{13}C NMR spectral data of compound **1** (500 MHz, $CDCl_3$)

C		H	
1	38.2	α	1.97 *ddd, J* = 5.8, 7.2, 13.3 Hz
		β	1.40 *m*
2	33.8	α	2.49 *m*
		β	2.48 *m*
3	217.7		
4	47.1		
5	53.5		1.54 *dd, J* = 2.2, 12.1 Hz
6	33.3	α	1.38 *m*
		β	1.84 *dt, J* = 3.2, 13.3 Hz
7	20.1	α	1.61 *m*
		β	1.43 *dd, J* = 3.2, 15.2 Hz
8	52.4		
9	51.1		1.19 *m*
10	39.1		
11	18.4	α	1.30 *m*
		β	1.58 *m*
12	24.5	α	1.68 *dt, J* = 3.4, 10.7 Hz
		β	1.68
13	34.4		2.47 *m*
14	38.6	α	1.39 *m*
		β	2.41 *m*
15	224.4		
16	47.7		2.26 *dq, J* = 6.8, 6.9 Hz
17	10.0		1.12 *d, J* = 6.9 Hz
18	20.8		1.04 *s*
19	27.2		1.10 *s*
20	17.6		1.13 *s*

$[\alpha]_D^{23} = -157.2^\circ$. The molecular formula was determined by HR-FABMS as $C_{20}H_{30}O_2$ (*m/z* 303.2363 $[M+H]^+$; cal. for 303.2324). The IR spectrum suggested the presence of cyclohexanone (1750 cm^{-1}) and cyclopentenone (1705 cm^{-1}). The ^{1}H NMR spectrum displayed three singlet methyls and one doublet methyl with 18 other protons. The ^{13}C NMR spectrum showed the signals of four methyls, seven methylenes, four methines and five quaternary carbons including two carbonyl carbons (217.7 and 224.4 ppm). The above spectral data indicated that compound **1** was a tetracyclic diterpenoid. The ^{1}H and ^{13}C NMR signals (Table 1) of **1** were assigned based on ^{1}H-^{1}H COSY, DEPT, NOESY, HMQC, and HMBC experiments. The HMBC spectrum of **1** indicated it was a kaurane-type diterpenoid with two carbonyl groups at C-3 and C-15. The relative stereochemistry of **1** was revealed by a NOESY spectrum in which NOEs were observed between H-1β and H-9, H-1α and H-11β, H-5 and H-9, H-6α and Me-19, H-12β and Me-17, H-14α and Me-20, H-14β and H-16. These observations indicated that **1** has the same stereochemistry as *ent*-kaurene (**2**). Conclusive evidence of the structure of **1** was given by X-ray crystallographic analysis, whose ORTEP drawing is shown in Fig. 1. Thus, **1** was established as *ent*-kaurane-3,15-dione.

ent-Kaurane-type diterpenoids are widely distributed

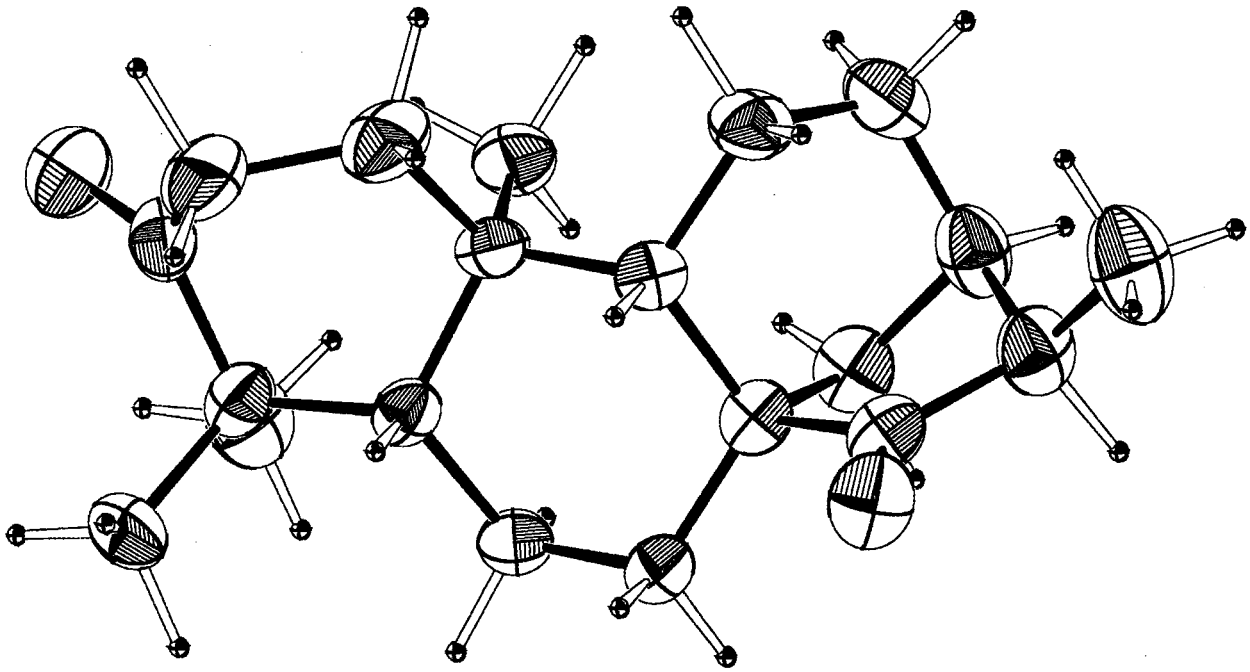
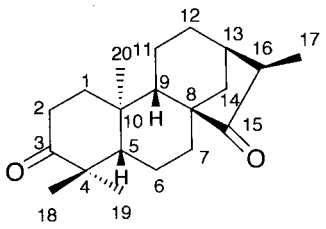


Fig. 1. ORTEP drawing of **1**.

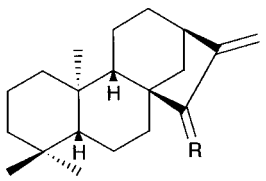
Table 2
The amounts of compounds **1**, **2**, **3** and **4** in calli, suspension cells and gametophytes of *Jungermannia subulata* (mg/g DW)

Compound	Calli	Suspension cells	Gametophytes
1	8.0	2.1	17.6
2	1.8	2.9	2.2
3	2.3	0.6	1.5
4	2.9	3.2	2.8

in liverworts (Asakawa, 1995). Compounds **2**–**4** have been detected in the MeOH extract of *Porella densifolia* (Asakawa, Takikawa, Ueda, Tori & Kumar, 1987). *J. infusca, Nardia scalaris* and *N. succulenta* also produce **3** and **4** (Langenbahn et al., 1993; Toyota, Nagashima & Asakawa, 1989). An earlier work on *J. subulata* demonstrated the production of *ent*-kaurene (**2**) and lunularic acid by cell suspension culture (Abe & Ohta, 1983; Ohta et al., 1990). The calli and suspension cells of *J. subulata* enabled the production of sufficient amounts of plant material for chemical analysis (Table 2). Almost identical gas chromatograms were obtained for samples from gametophytes of the intact liverwort, calli, and cultured cells. Production of *ent*-kaurene and its derivatives in cultured cells of *J. subulata* will greatly support research on the biosynthesis of diterpenoids in liverworts.



1



2 : R = H_2
3 : R = α-H, β-OH
4 : R = O

3. Experimental

3.1. General

^{1}H NMR: 500 MHz in $CDCl_3$; ^{13}C NMR: 125 MHz in $CDCl_3$, solvent peaks as the int. standard. IR: KBr pellet, UV and optical rotations: EtOH and $CHCl_3$, respectively. Optical rotations were measured in EtOH.

3.2. Plant material

An intact plant of *J. subulata* was collected in July 1995 at Mt. Porosiri (altitude 500 m) Hokkaido, Japan and identified by Dr. Tatsuwo Furuki. A voucher specimen is deposited at the Department of Bioresource Science, Obihiro University of Agriculture and Veterinary Medicine. A callus culture of *J. subulata* was induced from the surface-sterilized mature spores of field material. The mature capsules were surface sterilized with 70% EtOH for 1 min followed by 10 min with 1% sodium hypochlorite. The capsules were washed with sterile water twice, and then transferred to Erlenmeyer flasks (100 ml) containing 30 ml of Gamborg B5 agar medium. After 8 weeks, developing calli were transferred to test tubes (30 ml) with 8 ml solid MSK-4 (pH 5.75) medium, containing 40 g/l glucose. The callus cultures were kept under constant illumination (2000 lux) at 25°C and subcultured on fresh medium every 4 weeks.

3.3. GC quantification of compounds *1–4* in J. subulata

Cells (50 mg dry wt) grown as suspension cultures on MSK-4, calli (50 mg dry wt) grown on MSK-4 agar medium and gametophytes (17 mg dry wt) grown in petri dishes with Hyponex nutrient solution were extracted with ca 5 vol (v/w) of MeOH. The MeOH extracts were concd to dryness and then passed through disposable Si-gel cartridge columns with 10 ml of Et_2O. Each Et_2O solution was analyzed by GC on an Rtx-1 (60 m × 0.25 mm i.d. fused silica); carrier gas He (1.17 ml/min); initial temp. at 150°C was elevated to 250°C (3.0°C/min, hold time 60 min); detection, FID; **1** (R_t: 59.9 min), **2** (R_t: 38.8 min), **3** (R_t: 57.6 min), and **4** (R_t: 47.4 min). The amounts of **1**–**4** were determined by co-chromatography with known amounts of **1**–**4**.

3.4. Extraction and isolation

Powdered dry calli (19.2 g) were extracted with Et_2O and MeOH. The combined Et_2O and MeOH extracts (1101.5 mg) were separated into seven fractions by vacuum liquid chromatography (VLC) on silica gel (5.4 × 2.0 cm i.d., *n*-hexane–EtOAc stepwise). Separation of fr. 1 (95% *n*-hexane, 478.5 mg) with VLC (5.4 × 2.0 cm i.d., *n*-hexane–EtOAc stepwise) and HPLC (*n*-hexane–EtOAc, 49:1 and 4:1) yielded **1** (23.6 mg), **2** (11.0 mg), **3** (10.3 mg), and **4** (9.9 mg), and separation of fr. 2 (90% *n*-hexane, 179.5 mg) with HPLC (*n*-hexane–EtOAc, 4:1) yielded **1** (9.2 mg). The spectral data of known compounds **2**–**4** were identical with those of authentic samples (Fukuyama et al., 1988; Langenbahn et al., 1993).

3.5. ent-Kaurane-3,15-dione (*1*)

Colorless needles (from *n*-hexane–EtOAc), mp 176.0–178.0°C; $[\alpha]_D$ −157.2° (c 0.176, $CHCl_3$); FAB

HR-MS; $[M+H]^+$ 303.2363 (cal. for $C_{20}H_{31}O_2$ 303.2324) EIMS *m/z* (rel. int.): 302 ($[M]^+$, 35), 287 (63), 259 (16), 244 (92), 229 (35), 201 (57), 187 (14), 173 (13), 159 (18), 137 (27), 121 (38), 107 (64), 91 (70), 79 (73), 67 (57), 55 (74), 41 (100); UV λ_{max} nm ($\log_\epsilon$): 233 (2.37); IR^{ν}_{max} cm^{-1}: 2926, 1750, 1705, 1452, 1385, 1101; ^1H- and ^{13}C-NMR: see Table 1.

3.6. Oxidation of **3**

Compound **3** (5.7 mg) was oxidized with PCC (40 mg) in CH_2Cl_2 (0.5 ml) at r.t. for 1 h. The reaction mixture was filtered through Si-gel (500 mg) washed with ether (5 ml). The resultant solution was evapd in vacuo to afford compound **4** (1.1 mg).

3.7. Reduction of **4**

Compound **4** (1.8 mg) was reduced by $NaBH_4$ (5 mg) in MeOH (0.5 ml) at r.t. for 1 h. Both 10 μl of AcOH and 5 ml of H_2O were added into the reaction mixture, and then extracted with ether (5 ml × 3). The ether extract was washed with 5% $NaHCO_3$ and saturated NaClaq, and dried (Na_2SO_4). The resulting solution was evapd in vacuo to afford compound **3** (0.8 mg).

3.8. X-ray crystal analysis for **1**

Crystal data: $C_{20}H_{30}O_2$, orthorhombic $P2_12_12_1$, $a = 6.707$ (1), $b = 11.928$ (3), $c = 20.932$ (3) Å^3, $z = 4$, $Dc = 1.200$ g cm^{-1}, λ (MoKα) = 0.71073 Å, $\mu = 0.70$ cm^{-1}, $F(000) = 664$, $R = 0.053$ for 1291 unique reflections with $|F_o| > 2\sigma(|F_o|)$. The structure was solved by the direct method using MULTAN 78. Hydrogen atoms were located from a difference Fourier syntheses.

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