Sesquiterpenoids in Cultured Cells of Liverwort, *Calypogeia granulata* INOUE

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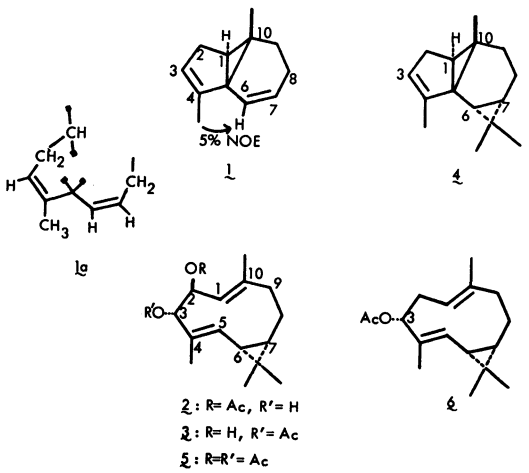
Synopsis. Three new sesquiterpenoids, trinoranastreptene, 2-acetoxy-3-hydroxybicyclogermacrene and 3-acetoxy-2-hydroxybicyclogermacrene, were isolated from cultured cells of *Calypogeia granulata* INOUE. Their structures have been established by spectroscopic analysis.

Recentry, we reported the production of volatile sesquiterpenoids by cultured cells from liverwort of *Calypogeia granulata* INOUE.[1] The result was that the production of essential oil in cultured cells, intact plants and redifferentiated plants was similar. Furthermore, we reported the isolation and structural determination of two new trinor-sesquiterpenoids, 3,10-dihydro-1,4-dimethylazulene and 3,7-dimethylindene-6-carbaldehyde, and biosynthetic studies of trinor-sesquiterpenoids, 1,4-dimethylazulene and 3,7-dimethylindene-6-carbaldehyde, using cultured cells instead of intact plants.[2] The isolation of three new sesquiterpenoids and the identification of eight sesquiterpenoids from the same cultured cells are reported.

The fresh cultured cells for 20 d were steam-distilled to obtain the essential oil (0.3% of fresh weight) as blue oil. The essential oil was subjected to SiO_2 column chromatography using hexane, $CHCl_3$ and Et_2O to give three fractions, I, II, and III. Sesquiterpene hydrocarbons from Fr. I were identified as β-elemene, bicycloelemene, eremophilene, ledene, alloaromadendrene and anastreptene[3] by IR, MS, and GC-retention time of authentic samples. 3-Acetoxy- and 3-hydroxybicyclogermacrene were isolated from Fr. II and identified with authentic data.[4] Three new sesquiterpenoids, trinoranastreptene (**1**), 2-acetoxy-3-hydroxybicyclogermacrene (**2**), and 3-acetoxy-2-hydroxybicyclogermacrene (**3**) were isolated from Fr. I and III, and their structures are described below.

Trinoranastreptene (**1**) was isolated from Fr. I by preparative GC. The molecular formula $C_{12}H_{16}$ of **1** was determined by the appearence of a molecular ion peak at m/z 160 in its mass spectrum. The 360 MHz ^{1}H NMR spectrum indicated signals due to a tertiary methyl at δ 0.93, olefinic methyl at δ 1.73, allylic CH_2 at δ 1.95 and 2.42, and three olefinic protons at δ 5.17, 5.49, and 6.12. In the decoupling experiments of **1**, irradiation of 4-CH_3 collapsed signals of 3-H and 2-H_A to sharp peaks and a multiplet of 2-H_B to a broad double doublet. Also, irradiation of 2-H_B converted two broad doublets of 3-H and 2-H_A into two broad singlets. Further, irradiation of the signal due to 6-H collapsed a double double doublet of 7-H to double doublet. In the NOE experiment of **1**, a 5% NOE was observed on 6-H upon irradiation of 4-CH_3. These results indicate that trinoranastreptene has a partial structure **1a**. The comparison of the 360 MHz NMR of **1** and **4** was quite similar except for the signals of two olefinic protons in **1** and two methyl groups and two protons on the cyclopropane ring in **4**. Also, 1-H of **1** appeared at low field of δ 1.98 relative to 1-H of **4** at δ 1.40. It was represented that 1-H of **4** shifted to high field by anisotropic effect of cyclopropane moiety. These results, together with biogentic consideration showed that the new trinor-sesquiterpene is most favourably represented by formula **1**.

2-Acetoxy-3-hydroxybicyclogermacrene (**2**) and 3-acetoxy-2-hydroxybicyclogermacrene (**3**) were isolated from fraction III by flash chromatography on SiO_2 eluted with hexane–EtOAc (3 : 1). Their molecular weight by high resolution mass spectra indicated the molecular formula to be $C_{17}H_{26}O_3$. Their IR spectra showed a hydroxyl absorption band (**2**: 3450; **3**: 3450 cm^{-1}) an ester absorption band (**2**: 1725; **3**: 1720 cm^{-1}) and a trisubstituted double bond absorption band (**2** and **3**: 1660 and 840 cm^{-1}). The ^{1}H NMR spectra of **2** and **3** indicated signals of two protons on the cyclopropane ring (**2**: δ 0.73 and 1.34; **3**: 0.73 and 1.34), *gem*-dimethyl group (**2**: δ 1.09 and 1.12; **3**: 1.05 and 1.10), two olefinic methyl groups (**2**: δ 1.66 and 1.72; **3**: 1.58 and 1.75), acetoxy methyl group (**2**: δ 2.13; **3**: 2.11) two methine protons (**2**: δ 4.45 and 5.42; **3**: 4.49 and 5.08) adjacent to oxygen and two olefinic protons (**2**: δ 5.10 and 5.23; **3**: 4.90 and 5.08). Acetylation of **2** and **3**, respectively yielded the same diacetate **5** identical in IR and ^{1}H NMR spectra. The ^{1}H NMR spectral signals of **3** closely resembled the corresponding signals in the spectrum of 3-acetoxybicyclogermacrene (**6**) isolated from the same cultured cells, with exception of signal of one proton on a carbon bearing a hydroxyl group at C-2, showing that this compound possessed the same bicyclogermacrene-type sesquiterpene skeleton. The location of acetoxyl and hydroxyl groups in **2** was suggested to be at C-2 and C-3 by spin decoupling experiments. When the proton attached to acetoxyl group at δ 5.42 was irradiated, proton on carbon bearing hydroxyl group (3-H) at δ 4.45 and olefinic proton (1-H) at δ 5.23 collapsed to



broad singlet, respectively. From the above results, the structures of two new sesquiterpenes were established to be **2** and **3**.

The relative stereostructure was determined by the following way. In the NOE experiments of **2**, a 12% NOE was observed on 6-H upon irradiation of 4-CH_3. Also, an 11% NOE was observed on 3-H upon irradiation of 4-CH_3. This result indicates that the hydroxyl group at C-3 and cyclopropane ring have a *cis* relation. The fact that 2-H is coupled to 3-H and 1-H with $J_{2,3}=3.0$ and $J_{1,2}=8.5$ Hz indicates that 2-H and 3-H have a *trans* relation based on inspection of Dreiding models.[5]

Experimental

IR spectra were measured with a Hitachi EPI-G2 spectrometer, ^{1}H NMR spectra with a JEOL FX-100 (100 MHz) or a Nicolet NT-360 (360 MHz) spectrometer in deuteriochloroform solution containing tetramethylsilane as an internal standard, low resolution mass spectra with a Hitachi RMU-6 and high resolution mass spectra with a JEOL 01SG-2, with direct inlet system operating at 70 eV. An analytical GC was performed with a Hitachi 163 type apparatus equipped with a Thermon 600 T glass capillary column, and preparative GC with a Varians model 920 fitted with an aluminum column (10 ft × 3/8 in) packed with 5% Thermon 1000 on Chromosorb W. The gas chromatograph was operated at a bath temperature of 180 °C, He gas was used as the carrier gas. Kieselgel 60 (E. Merck, Darmstadt) was used for column chromatography. Thin-layer chromatography (TLC) was carried out on Kieselgel GF_{254} (E. Merck, Darmstadt) in 0.25 mm thickness.

Isolation. The gametophyte cells developed from spores were cultured for 20 d in twenty 500 ml culture flasks, and the filtered cells (500 g) were steam-distilled to give 1.5 g of a blue oil (2—3.3% of dry weight). The essential oil was subjected to column chromatography on SiO_2. Elution with hexane gave sesquiterpene hydrocarbons (F-I) and 1,4-dimethylazulene. Elution with $CHCl_3$ yielded 3-acetoxybicyclogermacrene and 3-hydroxybicyclogermacrene (F-II). Elution with Et_2O yielded crude 2-acetoxy-3-hydroxybicyclogermacrene and 3-acetoxy-2-hydroxybicyclogermacrene (F-III).

Trinoranastreptene (**1**) was isolated from F-1 by preparative GC, as a colorless oil, ^{1}H NMR δ 0.93 (3H, s, 10-CH_3), 1.73 (3H, br. s, 4-CH_3), 2.00 (1H, br. dd, J=6.4 and 8.5 Hz, 1-H), 1.94 (1H, br. dd, J=6.4 and 17.2 Hz, 2-H_A), 2.42 (1H, m, 2-H_B), 5.16 (1H, br. s, 3-H), 5.49 (1H, ddd, J=2.5, 6.4 and 9.3 Hz, 7-H), and 6.12 (1H, d, J=9.3 Hz, 6-H); MS m/z (%) 160 (42, M^+, $C_{12}H_{16}$), 145 [100, $(M-CH_3)^+$], 130 (21).

2-Acetoxy-3-hydroxybicyclogermacrene (2) and 3-Acetoxy-2-hydroxybicyclogermacrene (3). The crude fraction (F-III) was purified by column chromatography on SiO_2. Elution with hexane–EtOAc (3 : 1) gave **2** (60 mg), colorless viscous oil, $[\alpha]_D^{22}$ −10.3° (c 0.8, $CHCl_3$); IR (film) 3450, 1725, 1660, and 840 cm^{-1}; ^{1}H NMR δ 0.73 (1H, ddd, J=3.0, 9.0, and 12.5 Hz, 7-H), 1.09 and 1.12 (3H each, s, *gem*-dimethyl), 1.34 (1H, dd, J=8.5 and 12.5Hz, 6-H), 1.66 and 1.72 (3H each, br. s, 4-CH_3 and 10-CH_3), 2.48 (1H, m, 9-H_A), 4.45 (1H, d, J=3.0 Hz, 3-H), 5.10 (1H, br. d, J=8.5 Hz, 5-H), 5.23 (1H, br. d, J=8.5 Hz, 1-H), and 5.42 (1H, dd, J=3.0 and 8.5 Hz, 2-H); MS m/z (%) 278 (trace, M^+), 218 [21, $(M-60)^+$], 137 (51), and 109 (100). Found: m/z 278.1908. Calcd for $C_{17}H_{26}O_3$: M, 278.1925.

Successive elution with the same solvent yielded **3** (45 mg), as a colorless viscous oil, $[\alpha]_D^{22}$ +63.2° (c 1.2, $CHCl_3$); IR (film) 3450, 1720, 1660, and 840 cm^{-1}; ^{1}H NMR δ 0.73 (1H, ddd, J=3.0, 9.0, and 12.5 Hz, 7-H), 1.05 and 1.10 (3H each,s, *gem*-dimethyl), 1.34 (1H, dd, J=8.5 and 12.5 Hz, 6-H), 1.58 and 1.75 (3H each, br. s, 4-CH_3 and 10-CH_3), 2.49 (1H, m, 9-H_A), 4.49 (1H, dd, J=3.0 and 8.5 Hz, 2-H), 4.90 (1H, br. d, J=8.5 Hz, 1-H), 5.08 (1H, br. d, J=8.5 Hz, 5-H), and 5.26 (1H, d, J=3.0 Hz, 3-H); MS m/z (%) 278 (trace, M^+), 218 [35, $(M-60)^+$], 152 (100), 109 (95). Found: m/z 278.1903. Calcd for $C_{17}H_{26}O_3$: M, 278.1925.

Acetylation of 2-Acetoxy-3-hydroxybicyclogermacrene (2). A solution of **2** (10 mg) in dry pyridine (1 ml) and Ac_2O (0.5 ml) was kept at room temperature for 1 d. The reaction mixture was treated in the usual way to give a residue, which was chromatographed on SiO_2. Elution with hexane–EtOAc (10 : 1) gave diacetate **5** (10 mg).

Acetylation of 3-Acetoxy-2-hydroxybicyclogermacrene (3). This compound (5 mg) was treated with Ac_2O (0.5 ml) and dry pyridine (1 ml) at room temperature for 1 d and then worked up in the usual way. The resulting residue was chromatographed on SiO_2 using hexane–EtOAc (10 : 1) to give diacetate **5** (6 mg), colorless viscous oil, IR (film) 1735, 1645 and 850 cm^{-1}; ^{1}H NMR δ 1.06 and 1.27 (3H each, s, *gem*-dimethyl), 1.66 and 1.77 (3H each, br. s, 4-CH_3 and 10-CH_3), 2.05 and 2.09 (3H each, s, -$COCH_3$), 4.96 (1H, br. d, J=8.5 Hz, 5-H), 5.09 (1H, br. d, J=8.5 Hz, 1-H), 5.39 (1H, d, J=3.0 Hz, 3-H), and 5.52 (1H, dd, J=3.0 and 8.5 Hz, 2-H); MS m/z (%) 320 (1, M^+, $C_{19}H_{28}O_4$), 278 [7, $(M-42)^+$], 218 [24, $(M-60)^+$], 152 (57), 109 (50), and 43 (100).

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