3,10-Dihydro-1,4-dimethylazulene, a Labile Biosynthetic Intermediate Isolated from Cultured Cells of Liverwort *Calypogeia granulata* Inoue

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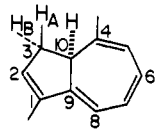
Abstract: The cell culture of the liverwort *Calypogeia granulata* Inoue has yielded a blue oily distillate, the GC profile of which is very similar to profiles of the blue distillate obtained from intact plants or redifferentiated plants. This shows that the cell culture method can be used with advantage in studying secondary metabolites, especially of plant and other sources which are difficult to collect. The cell culture distillate, however, contained an unstable and characteristic peak, which upon careful purification and spectroscopic measurements under N_2 turned out to be 3,10-dihydro-1,4-dimethylazulene, a new dihydroazulene skeleton. Biosynthetic studies employing ^{13}C-labeled acetate and difference ^{13}C NMR techniques showed the terpenoid origin of this dihydroazulene. The biosynthetic route leading to 3,7-dimethylindene-5-carboxaldehyde has also been clarified by ^{13}C-labeling studies; this is a trinorsesquiterpene which has undergone a skeletal rearrangement. The cell culture technique offers a convenient and efficient method for studying the production and biosynthesis of secondary metabolites.

The liverworts are located taxonomically between higher plants and algae and are characterized by the presence of oil bodies within the cells. They have given rise to numerous new terpenoids (including antipodes),[1] some of which exhibit interesting biological activities.[2] The liverworts, however, are frequently very small and, moreover, several species often grow together. Since these aspects render the large-scale collection of single species impossible, studies on the suspension culture of the liverwort *Calypogeia granulata* Inoue (full-grown capsules collected in April 1976 from Seryo near Kyoto, Japan) have been initiated as a means of isolating compounds and studying their biosyntheses.[3] In the following we report the isolation and characterization of the labile and unknown 3,10-dihydro-1,4-dimethylazulene (**1**), the biosynthetic precursor of 1,4-dimethylazulene (**2**) and 3,7-dimethylidene-5-carboxaldehyde (**3**); the results of ^{13}C-incorporation studies employing cultured cells which show their sesquiterpenoid origin are also reported.



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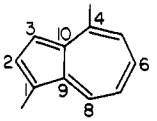

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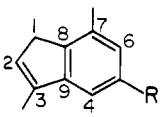

3 R=CHO

4 R=COOMe

The gametophyte cells developed from spores were cultured for 20 days, and filtered cells were steam-distilled to give a blue oil (2–3.3% of dry weight).[3] Silica gel chromatography of the blue oil and spectroscopic studies of various components led to the identification of 1,4-dimethylazulene (**2**)[4] (major component, ca. 50% of oil), the unknown 3,7-dimethylindene-5-carboxaldehyde (**3**) (see below), nine known sesquiterpenes, and three new sesqui- and trinorsesquiterpenes.[5]

The gas chromatography (GC) profile of the blue oil was very similar to profiles of the blue distillate obtained from intact plants or redifferentiated plantlets except for the presence of a minor peak in the oil from cultured cells; preliminary GC–MS (M^+, m/z 158) studies of this peak suggested that it may be a dihydroazulene. Since this component was too unstable to be collected by preparative GC, it was isolated by rapid flash chromatography, neutral SiO_2, hexane elution, N_2 atmosphere, and subsequent preparative HPLC. Although removal of solvent under N_2 converted the compound into blue 1,4-dimethylazulene and a colorless polymer, it can be stored in hexane under N_2 at -20 °C. The ultraviolet spectrum (Figure 1) [211 nm (sh), 228 (ϵ 12 675), 234 (25 575), and 312 (5 375)] indicated this compound had a conjugated tetraene system. The 360-MHz ^1H NMR spectrum (Figure 2) showed the presence of two olefinic methyl groups at δ 1.78 and 1.82, allylic methylene group at δ 2.67 and 2.79 (3-$H_{A,B}$), methine group at δ 2.57 (10-H), and five olefinic protons at δ 5.94 (2-H and 8-H), 6.00 (5-H), 6.34 (6-H), and 6.56 (7-H). Decoupling experiments of **1** indicated that 7-H was coupled to 8-H with J = 5.6 Hz and 6-H with J = 11.2 Hz, and that 6-H was also coupled to 5-H with 5.9 Hz. Furthermore, the methylene group (3-$H_{A,B}$) was coupled to the methyl group (1-CH_3), to the olefinic proton (2-H), and to the methine group (10-H). Its ready oxidation to 1,4-dimethylazulene, the presence of two olefinic Me signals, and the very intense optical activity ($[\alpha]_D$ +1165°), suggesting a strongly distorted, inherently chiral chromophore, uniquely define the structure of this unstable compound as 3,10-dihydro-1,4-dimethylazulene (**1**) which possesses the hitherto unknown and labile dihydroazulene nucleus.[6] Several dihydroazulenoids have been isolated from plants, i.e., 3,6-dihydro-,[7] 5,6-dihydro-,[7] and 6,7-dihydroazulenoids,[8] all of which are fulvene-type azulenoids. It should be noted that, as judged from the GC profile, the intact plants or redifferentiated plantlets contain, if any, only trace amounts of the dihydroazulene, and that the quantity of **1** in cultured cells builds up to an isolatable maximum around day 20 of the culture.

3,7-Dimethylindene-5-carboxaldehyde (**3**) was obtained as a colorless oil having the molecular formula $C_{12}H_{12}O$. The infrared spectrum showed bands due to the formyl group at 1690 cm^{-1} and the olefinic group at 1600 cm^{-1}. The formyl group was further supported by ^1H (δ 10.00) and ^{13}C NMR (δ 192.7) spectra.

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(2) Asakawa, Y. *J. Hattori Bot. Lab.* **1981**, *50*, 123.

(3) Takeda, R.; Katoh, K. *Planta* **1981**, *151*, 525.

(4) Meuche, D.; Huneck, S. *Chem. Ber.* **1969**, *102*, 2493.

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(6) Theoretical calculations of the CD curve (Figure 1) based on the π framework approximation lead to an *S* configuration (as shown in **1**): Professor N. Harada, Tohoku University, private communications.

(7) Bertelli, D. J.; Crabtree, J. H. *Tetrahedron* **1968**, *24*, 2079.

(8) Vokac, K.; Samek, Z.; Herout, V.; Sorm, F. *Collect. Czech. Chem. Commun.* **1970**, 1296.

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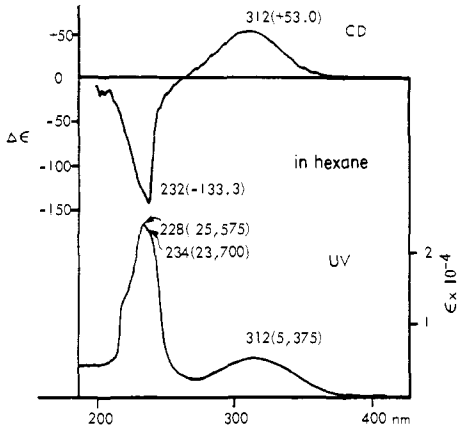


Figure 1. UV and CD of **1** in hexane.

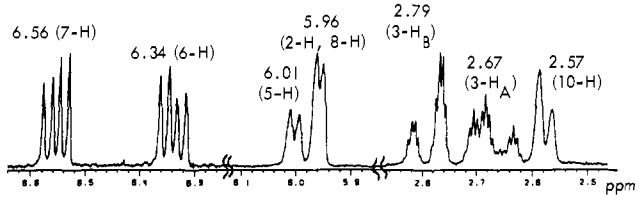
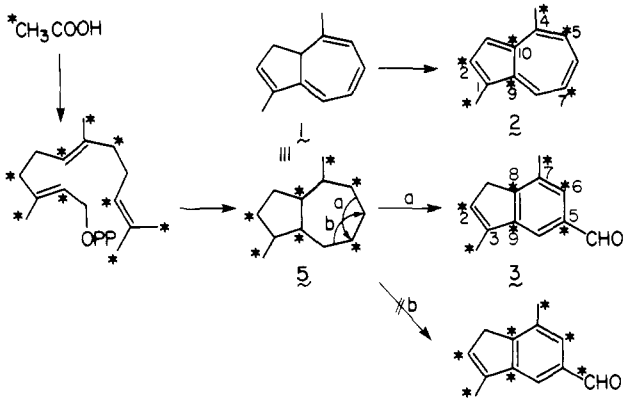


Figure 2. 360-MHz ^1H NMR spectrum of **1** in $CDCl_3$.

Scheme I. Incorporation of ^{13}C from [2-^{13}C]-Labeled Acetate into Compounds **1**-**3**



Moreover, the ^1H NMR spectrum showed signals due to two olefinic methyl group at δ 2.16 and 2.40, an olefinic proton at δ 6.28 (2-H), an allylic methylene at δ 3.24 (1-$H_{A,B}$), and two aromatic protons at δ 7.53 (6-H) and 7.65 (4-H). The results of decoupling experiments of **3** indicated that the olefinic methyl group at C-3 was coupled to the olefinic proton at C-2 and to the allylic methylene group at C-1. Also, the aromatic 4-H was coupled to 6-H with J = 2.0 Hz (meta coupling). The ultraviolet spectrum [277 nm (ϵ 1610), 261 (8330), 236 (40 600), and 208 (14 250)] suggested that it may be an indene derivative. Irradiation of 3-CH_3 led to the observation of a 7% NOE of the 4-H signal; the aldehyde carbon is coupled to both the 7.53 (6-H) and 7.65 ppm (4-H) protons, $3J_{C,H}$ = 5.9 Hz, as deduced by long-range selective proton decoupling, and this places the CHO at C-6. These data together with a comparison of the NMR spectrum of 3,5-dimethyl-5-methoxycarbonylidene (**4**)[9] isolated from the same genus *Calypogeia tricomanis* established the structure as shown in **3**.

3,7-Dimethylindene-5-carboxaldehyde (**3**) is considered to be derived from hypothetical precursor **5** via route a or b as shown in Scheme I. Incorporation of [2-^{13}C]-labeled acetate into **3** via mevalonic acid, farnesyl pyrophosphate, and hypothetical precursor

(9) Meuche, D.; Huneck, S. *Chem. Ber.* **1966**, *99*, 2669.

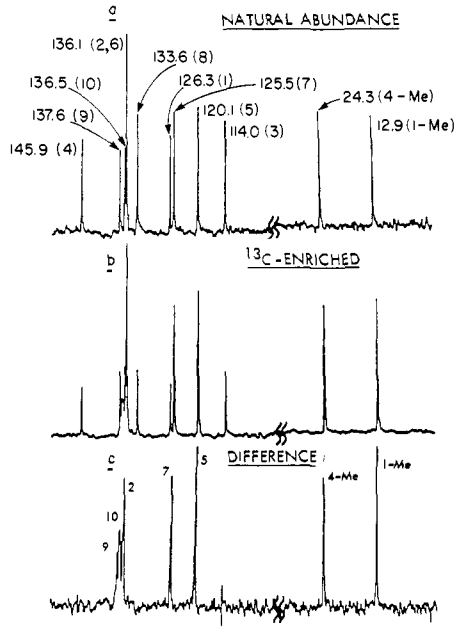


Figure 3. ^{13}C NMR of (a) **2** (assignments shown in parentheses), (b) **2** biosynthesized from [2-^{13}C]acetate, (c) difference spectrum showing enriched carbons.

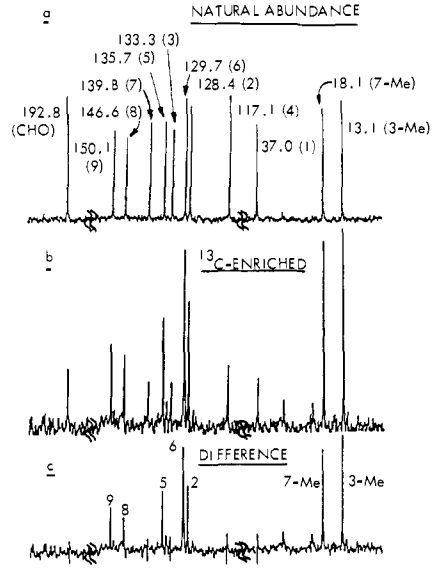


Figure 4. ^{13}C NMR of (a) **3**, natural abundance spectrum; (b) **3** biosynthesized from [2-^{13}C]acetate; (c) difference spectrum showing enriched carbons.

Table I. Carbon-13 Chemical Shifts of 2 and 3

C atom	2	3	C atom	2	3
1(3)-CH_3	12.9	(13.1)	C-5	120.1	135.7
4(7)-CH_3	24.3	(18.1)	C-6	136.1	129.7
C-1	126.3	37.0	C-7	125.5	139.8
C-2	136.1	128.4	C-8	133.6	146.6
C-3	114.0	133.3	C-9	137.6	150.1
C-4	145.9	117.1	C-10	136.5	
			CHO		192.8

as shown in Scheme I would result in labeling at the asterisked carbons. Cultured cells were grown in MSK-4 medium[3] for 30 days which contained 5 mM [2-^{13}C]acetate instead of 2% glucose, and the dimethylazulene **2** and indene aldehyde **3** were isolated by ether extraction of fresh material, SiO_2 chromatography, and elution with hexane (for **2**) and $CHCL_3$ (for **3**). Assignments of the ^{13}C NMR peaks of **2** and **3** shown in Figures 3a and 4a are based on selective off-resonance decoupling and long-range se-

lective proton-decoupling experiments which clarified $^3J_{C,H}$ coupling constants involving quaternary carbons 1, 4, 9, and 10 in **2**, and 3, 7, 8, and 9 in **3** (Table I). Figure 4 shows the ^{13}C-enriched, the natural abundance, and difference spectra of **3**. The latter spectrum clearly shows that carbons 2, 5, 6, 8, 9, 3-CH_3, and 7-CH_3 (not carbonyl carbon) were derived from the methyl carbon of [2-^{13}C]acetate. Namely, since it is C-6 and not the aldehyde carbon which is labeled, 3,7-dimethylindene-5-carboxaldehyde is formed as the hypothetical trinorsesquiterpenoid intermediate by route a (and not route b) as shown in Scheme I.

A similar difference spectrum of azulene **2** clearly shows the enriched carbons 2, 5, 7, 9, 10, 1-CH_3, and 4-CH_3, which in turn follow the terpenoid biogenetic route (Scheme I) via optically active dihydroazulene **1**. The results given above demonstrate that cell cultures offer a convenient and efficient method for studying the production and biosynthesis of secondary metabolites, including labile intermediates, which otherwise would be extremely difficult or impossible to detect.

Experimental Section

Infrared (IR) spectra were recorded on Nicolet Nic-7002-Cl or a Hitachi Model 285 spectrophotometer. Ultraviolet (UV) spectra were recorded on a Shimadzu Model UV-210-A double-beam spectrophotometer. Optical rotation was measured on a Perkin-Elmer Model 141 polarimeter using a 10-cm microcell. Circular dichroism (CD) spectra were measured on a JASCO J-20 spectrometer. ^{1}H NMR spectra were recorded on a JEOL-FX 100 (100 MHz) or a Nicolet Model 293 (360 MHz) spectrometer. ^{13}C NMR spectra were recorded on a JEOL-FX 100 (25.0 MHz) spectrometer. Low-resolution mass spectra are measured on a Hitachi RMU-6 mass spectrometer. GC-MS was measured with the direct combination of GLC [Hitachi K-53 type apparatus, glass capillary column (0.25 mm × 45 m) coated with Thermon 600 T] and Hitachi RMU-6 mass spectrometers. High-resolution mass spectra were recorded on a JEOL 01SG-2. HPLC was performed with a Waters apparatus fitted with a Licrosorb 60 column (Merck, 8 mm × 30 cm). Kieselgel 60 (E. Merck, Darmstadt) and silica CC-7 special (Mallinckrodt) were used for column chromatography. Thin layer chromatography (TLC) was carried out on precoated silica plates (60 F-254).

Tissue Culture of *Calypogeia granulata* Inoue. Fully grown capsules were collected in April 1976 from Seryo near Kyoto, Japan. They were soaked in a 0.1% benzalkonium chloride solution (Wako Pure Chemical Industries, Osaka, Japan) for 10 min, sterilized in a 1% sodium hypochlorite solution for 5 min, and washed three times with sterile distilled water. The spores within the capsules were aseptically taken out by opening the capsules with a needle, and sown on MSK-3 medium.[10] In order to induce callus, leafy gametophytes which had developed from spores on MSK-3 medium were transferred onto MSK-4 medium.[3] After 4 weeks, calli were induced from the gametophytes and transferred onto MSK-4 medium to which 2% glucose, but no 2,4-D (2,4-dichlorophenoxyacetic acid), had been added, and successively subcultured for 2 years at intervals of 4 weeks, then used for suspension culture. A suspension culture was started from a callus culture with an inoculum of 5 g fresh weight and was propagated routinely in a flat, oblong 700-mL flask containing 500 mL of MSK-4 medium with 2% glucose but without 2,4-D by inoculating 20 mL of cell suspension; subcultures were carried out every 20 days for more than 5 years. The culture flasks were continously illuminated with fluorescent lamps (8000 lux). Aeration and agitation to keep the culture is suspension were done by passing air through the culture (250 mL/min).

Chromatography of the Essential Oil. The fresh cultured cells (200 g) for 20 days were steam-distilled to give 600 mg of a blue oil. The essential oil was subjected to flash column chromatography on neutral SiO_2 (CC-7). The colorless dihydroazulene **1** was eluted with hexane and just preceded the major blue product, 1,4-dimethylazulene (**2**, 320 mg). 3,10-Dihydro-1,4-dimethylazulene (**1**, 5 mg) was purified by preparative HPLC on Licrosorb 60 (E. Merck) using hexane (2.4 mL/min) as eluate. Removal of solvent under N_2 converted dihydroazulene **1** into the blue 1,4-dimethylazulene and colorless polymer; however, the IR, NMR, CD, and UV spectra, and $[\alpha]_D$ value of **1**, can be measured in $CDCl_3$ or hexane under N_2 at room temperature.[11] The fraction eluted with $CHCl_3$ contained crude 3,7-dimethylindene-5-carboxaldehyde (**3**, 35 mg) that was rechromatographed on silica gel (Kieselgel 60) using 6% ethyl acetate in hexane as eluant.

3,10-Dihydro-1,4-dimethylazulene (1): colorless unstable oil; $[\alpha]_D^{22}$ +1165° (*c* 0.51, hexane); CD (Figure 1) $\Delta\epsilon_{312}$ +53.0, $\Delta\epsilon_{232}$ −133.3 (*c* 0.14, hexane); UV (Figure 1) (hexane) 211 nm (sh), 228 (ϵ 25 575), 234 (23 700), 312 (5375); IR ($CDCl_3$) 3025, 2960, 2935, 2860, 1438, 1224, 1215, 1210, 785, 738, 672 cm^{-1}; ^{1}H NMR ($CDCl_3$, 360 MHz) δ 1.78 (3 H, br s, 4-CH_3), 1.82 (3 H, $J_{1\text{-Me},2}$ = 2.5, $J_{1\text{-Me},3\text{-H}_{A,B}}$ = 2.5 Hz, 1-CH_3), 2.57 (1 H, br d, *J* = 8.4 Hz, 10-H), 2.67 (1 H, $J_{3\text{-H}_{A,B}}$ = 18.7, $J_{3,10}$ = 8.4, $J_{3,2}$ = 2.6, $J_{3,1\text{-Me}}$ = 2.5 Hz, 3-H_A), 2.79 (1 H, $J_{3\text{-H}_{A,B}}$ = 18.7, $J_{3,10}$ = 2.3, $J_{3,2}$ = 2.3, $J_{3,1\text{-Me}}$ = 2.5 Hz, 3-H_B), 5.96 (1 H, br s, 2-H), 5.96 (1 H, d, *J* = 5.6 Hz, 8-H), 6.01 (1 H, d, *J* = 5.9 Hz, 5-H), 6.34 (1 H, d of d, $J_{6,5}$ = 5.9, $J_{6,7}$ = 11.2 Hz, 6-H), 6.56 (1 H, d of d, $J_{7,6}$ = 11.2, $J_{7,8}$ = 5.6 Hz, 7-H); MS *m/z* 158 (35%, M$^+$ $C_{12}H_{14}$), 143 (100%, M − CH_3), 128 (65%, M − 30).

3,7-Dimethylindene-5-carboxaldehyde (3): colorless viscous oil; UV (cyclohexane) 208 nm (ϵ 14 250), 236 (40 600), 261 (8330), 277 (1610); IR (film) 2910, 1690, 1600, 1585, 1449, 1380, 1355, 1320, 1255, 1175, 1150, 1110, 1010, 870 cm^{-1}; ^{1}H NMR ($CDCl_3$, 100 MHz) δ 2.16 (3 H, br s, 3-CH_3), 2.40 (3 H, s, 7-CH_3), 3.24 (2 H, br s, 1-$H_{A,B}$), 6.28 (1 H, br s, 2-H), 7.53 (1 H, d, *J* = 2.0 Hz, 6-H), 7.65 (1 H, d, *J* = 2.0 Hz, 4-H), 10.00 (1 H, s, CHO); ^{13}C NMR ($CDCl_3$, 25.0 MHz) δ 13.1 (3-CH_3), 18.1 (7-CH_3), 37.0 (C-1), 117.1 (C-4), 128.4 (C-2), 129.7 (C-6), 133.3 (C-3), 135.7 (C-5), 139.8 (C-7), 146.6 (C-8), 150.1 (C-9), 192.8 (CHO); MS *m/z* 172.1350 (M$^+$; calcd for $C_{12}H_{12}O$, 172.1354), 143 (31%, M − CHO), 129 (100%).

Incorporation of ^{13}C-Labeled Acetate into 2 and 3. The cultured cells were transferred into MSK-4 medium containing 5 mM [2-^{13}C]-labeled acetate instead of 2% glucose and cultured for 30 days in six culture flasks. The culture liquid was filtered to obtain the cells (70 g) that were extracted with ether at room temperature. Ether was evaporated under reduced pressue to obtain a black green oil (1 g). The extract (1 g) was subjected to column chromatography over silica gel (Kieselgel 60). The blue band eluted with hexane gave pure 1,4-dimethylazulene (68 mg). Elution with $CHCl_3$ yielded crude 3,7-dimethylindene-5-carboxaldehyde, which was rechromatographed on silica gel (Kieselgel 60). Elution with 6% ethyl acetate in hexane gave **3** (6 mg). The IR, UV, MS, and ^{1}H NMR spectra of **2** were found to be identical with those of known 1,4-dimethylazulene.

1,4-Dimethylazulene (2): blue oil; ^{1}H NMR ($CDCl_3$, 100 MHz) δ 2.62 (3 H, br s, 1-CH_3), 2.80 (3 H, s, 4-CH_3), 6.95 (1 H, d, *J* = 9.7 Hz, 5-H), 6.95 (1 H, t, *J* = 9.8 Hz, 7-H), 7.28 (1 H, d, *J* = 3.7 Hz, 3-H), 7.38 (1 H, t, *J* = 9.8 Hz, 6-H), 7.62 (1 H, br d, *J* = 3.7 Hz, 2-H), 8.17 (1 H, d, *J* = 9.8 Hz, 8-H); ^{13}C NMR ($CDCl_3$, 25.0 MHz) δ 12.9 (1-C-H_3), 24.3 (4-CH_3), 114.0 (C-3), 120.1 (C-5), 125.5 (C-7), 126.3 (C-1), 133.6 (C-8), 136.1 (C-2), 136.1 (C-6), 136.5 (C-10), 137.6 (C-9), 145.9 (C-4).

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Registry No. 1, 85337-23-1; **3**, 85337-24-2.

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(11) The CD and UV spectra and $[\alpha]_D$ value were measured in hexane solution which was collected by preparative HPLC and concentrated to various concentrations by N_2. After the hexane solution was replaced with $CDCl_3$, the ^{1}H NMR and IR spectra were measured.