New Bisabolane Sesquiterpene from the Mycelia of *Amanita virgineoides*

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A bisabolane type of sesquiterpene was isolated from the culture medium of *Amanita virgineoides* Bas, together with two known compounds, including lignoren, and harziandion. The structure of the new compound “virgineol” was established using a combination of different NMR spectroscopy techniques, whereas the chemical structure of the known compound was identified as lignoren, and the structure of harziandion was confirmed by X-ray crystallographic analysis.

Key words *Amanita virgineoides*; fungus; culture medium; harziandion; virgineol

Fungi are generally well known to produce interesting organic compounds with diverse skeletal structures and pharmacological activities, such as fomanosin,¹⁾ hirsutene,²⁾ illudins S³⁾ and M,⁴⁾ and marasmic acid.⁵⁾ Compounds belonging to these structural classes are particularly unique to fungi and have been isolated only rarely as the components of other plant materials. In our continuing search for unique compounds from fungi, we collected several different types of fungi from the Nagasaki prefecture for analysis. Of the fungi collected, we were particularly interested in *Amanita virgineoides* Bas (Amanitaceae) because, to the best of our knowledge, only two reports have appeared in the literature to date concerning the components contained within this fungus.^{6,7)} The genus Amanitaceae has been reported to contain particularly toxic species as well as the edible *Amanita hemibapha*. Although *A. virgineoides* is known to be inedible, information pertaining to its toxicity remains scarce and poorly understood.

Results and Discussion

Pure spores isolated from the gill of *A. virgineoides* were cultured to provide a liquid medium (45 L). The medium was purified by column chromatography on a Diaion resin. The aqueous fractions were extracted with CHCl₃, and the CHCl₃ layer gave a residue that was purified by a Chromatorex octadecyl silyl (ODS) column to give compound **1** (10.1 mg).

In an alternative method, the liquid medium (45 L) was extracted with EtOAc. The organic extracts gave a brown gum, which was purified by an ODS column to give compounds **2** (11.8 mg) and **3** (2.7 mg).

Compound **1** was isolated as a yellow gum. The molecular formula was deduced to be C₁₆H₂₈O₇ by high resolution-fast atom bombardment-mass spectroscopy (HR-FAB-MS), with an [M+Na]⁺ mass ion observed at *m/z* 355.1761. The ¹³C-NMR and heteronuclear single quantum coherence (HSQC) spectra showed 16 carbon signals that were attributed to three methyl, one methoxy methyl, four methylene, four methine, and four quaternary carbons on the basis of their shifts. Based on the ¹H- and ¹³C-NMR spectra data shown in Table 1, one of sixteen carbons should be methoxy methyl group. Except for this methyl carbon, compound **1** consisted of fifteen carbons, hydrogen and oxygen. These data therefore suggested that compound **1** supposed to be a sesquiterpene. As shown in Fig. 1, the ¹H–¹H correlation spectroscopy (COSY) spectrum

of **1** revealed the successive connectivity of C-2, C-3, C-4, C-5, and C-6. In the heteronuclear multiple-bond correlation (HMBC) spectrum of **1**, the resonance at δ_{H} 1.23 corresponding to the protons on the C-7 methyl group correlated with the signals at C-1 (δ_{C} 72.2), C-2 (δ_{C} 34.7), and C-6 (δ_{C} 74.8). These correlations indicated the presence of a methyl cyclohexane moiety in the structure of **1**. The HMBC spectrum of **1** also revealed that the oxymethylene proton signals of H-9a correlated with C-4 (δ_{C} 46.1), C-8 (δ_{C} 86.6), C-10 (δ_{C} 110.9), and C-11 (δ_{C} 107.6). Moreover, the presence of an isobutyl moiety was suggested by the correlations of proton resonances of the methyl groups at C-14 (δ_{H} 0.95) and C-15 (δ_{H} 0.98) to C-12 (δ_{C} 38.3) together with C-13 (δ_{C} 24.0). The location of the isobutyl moiety at C-11 was deduced from the HMBC correlation between H-12 (δ_{H} 1.59) and C-11 (δ_{C} 107.6). On the basis of these NMR experiments, the basic carbon connectivity of structure of **1** was deduced to be that of a bisabolane skeleton. Given that the degree of unsaturation in **1** was three, compound **1** should be composed of three different ring systems.

Table 1. ¹H- and ¹³C-NMR Data together with the HMBC Relationships for **1** in Methanol-*d*₄ (500 MHz for ¹H-NMR and 125 MHz for ¹³C-NMR)

Position	δ_{C}	δ_{H} (<i>J</i> in Hz)	HMBC (H to C)
1	72.2	—	
2a	34.7	1.50, ddd (14.0, 7.5, 5.0)	1, 3, 4, 6
2b		1.75–1.69, m	
3a	20.3	1.80–1.74, m	4
3b		1.63, ddd (12.5, 8.0, 5.0)	
4	46.1	2.35, m	8
5	82.8	4.32, dd (7.5, 5.5)	1, 3, 6, 8
6	74.8	3.46, br d (5.5)	2, 4, 5
7	25.7	1.23, s	1, 2, 6
8	86.6		
9a	70.8	4.04, d (10.0)	8, 10, 11
9b		3.47, d (10.0)	
10	110.9	—	
11	107.6	—	
12	38.3	1.59, d (6.5)	11, 13, 14, 15
13	24.0	2.07, m	
14	25.1	0.95, d (7.0)	12, 13, 15
15	23.5	0.98, d (6.5)	12, 13, 14
16	48.1	3.20, s	11

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Fig. 1. The ^1H - ^1H COSY and HMBC Correlations for Compound **1**

The chemical shifts of C-5 (δ_{C} 82.8) and C-10 (δ_{C} 110.9) suggested that these carbons were linked to an oxygen atom in the form of a furan ring moiety, which was in agreement with the HMBC correlation observed between H-9 and C-11. Moreover, the HMBC correlation between the protons of the methoxy group at C-16 (δ_{H} 3.20) and C-9 (δ_{C} 70.8) revealed that the methoxy group was located at C-11. The ^{13}C chemical shifts of C-1, C-5, C-6, C-8, and C-10 indicated that they were directly connected their own hydroxy group. This result was confirmed by deuterium-induced isotope shifts (modified Pfeiffer method) in the ^{13}C -NMR spectrum.⁸⁾

Nuclear Overhauser effect spectroscopy (NOESY) correlations between H-9a (δ_{H} 4.04) and H-3a (δ_{H} 1.74–1.80), and H-9a and H-2b (δ_{H} 1.50) suggested that the cyclic systems possessed a *cis*-configuration. The ^1H - ^1H COSY spectrum of **1** revealed a W-coupling between H-4 and H-6 through the C-6–C-5–C-4 bonds, indicating that the juncture of ring A and ring B must also be fixed in the *cis* configuration with the 6-hydroxyl group configured as α . In addition, the NOESY spectrum showed correlations between both of the H-3 methylene protons and H-4, H-2b (δ_{H} 1.69–1.75) and H-7, H-6 and H-5, H-6 and H-7, and H-5 and H-4. These NOE correlations indicated that the C-7 methyl group was configured as α . The configuration of C-11 was deduced from the NOE correlations between H-16 (δ_{H} 3.20) and H-9b (δ_{H} 3.47), H-12 and H-9b, and H-16 and H-12. Thus, the structure of **1** was elucidated as shown in Fig. 3 and subsequently named virgineol.

Compound **2** was obtained as a pale yellow gum. Electron impact (EI) MS measurements showed an $[\text{M}+\text{H}]^+$ molecular ion peak at m/z 223. The ^{13}C -NMR and HSQC spectra showed 15 carbon signals that were attributed to five methyl, four methylene, three methine, and three quaternary carbons, indicating the molecular formula of compound **2** to be $\text{C}_{15}\text{H}_{26}\text{O}$.

The HMBC correlations between CH_3 -8 (δ_{H} 1.04) and C-7 (δ_{C} 44.2), CH_3 -9 (δ_{H} 1.26) and C-6 (δ_{C} 81.5), CH_3 -10 (δ_{H} 1.17) and C-2 (δ_{C} 75.1), CH_3 -15 (δ_{H} 1.62) and C-14 (δ_{C} 131.8), and CH_3 -16 (δ_{H} 1.69) and C-14 (δ_{C} 131.8) showed that the five methyl groups were located at C-7, C-6, C-2, and C-14, respectively. In addition, the ^1H - ^1H COSY and HMBC correlations revealed the construction of the carbon skeleton of **2**. The calculated degree of unsaturation in compound **2** was

Fig. 2. Selected Deuterium-Induced Isotope Effects in the ^{13}C -NMR Spectrum of Compound **1**

two, and the downfield shifting of the C-2 and C-6 signals in its ^{13}C -NMR spectrum, suggested that these carbons were linked by an ether bond. A comparison of the data for **2** with data from the literature^{9,10)} revealed that **2** was lignoren.

Compound **3** was obtained as colorless prisms. The molecular formula was deduced to be $\text{C}_{20}\text{H}_{28}\text{O}_2$ by HR-FAB-MS, which showed an $[\text{M}+\text{H}]^+$ mass ion at m/z 301.2189. The ^{13}C -NMR and HSQC spectra showed twenty carbon signals that were attributed to five methyl, five methylene, three methine, five quaternary, and two carbonyl carbons.

A thorough analysis of the COSY, HSQC, and HMBC spectral data proved unsuccessful in allowing for the structure of compound **3** to be determined absolutely. The gross and relative structure of **3** was therefore confirmed by X-ray crystallographic analysis, as well as by comparison of its NMR data with data from the literature references, which indicated that compound **3** was harziandione.^{11–13)}

Experimental

General Experimental Procedures Optical rotations were measured on a DIP-370 digital polarimeter (JASCO, Tokyo, Japan). IR spectra were recorded on FT/IR-400 spectrophotometer (JASCO), and melting point was recorded on a MP-3 micro melting point apparatus (Yanaco, Kyoto, Japan). ^1H - (500 MHz) and ^{13}C - (125 MHz) NMR spectra were recorded on a Unity plus 500 spectrometer (Varian, Palo Alto, CA, U.S.A.). The chemical shifts have been given as δ values relative to the solvent peaks (*i.e.* methanol- d_4 : δ_{H} 3.30, δ_{C} 49.0; and CDCl_3 : δ_{H} 7.24, δ_{C} 77.0). HR-EI-MS and HR-FAB-MS data were obtained on a JMS 700N spectrometer (JEOL) in the positive mode. HR-FAB-MS used *m*-nitrobenzyl alcohol or glycerol as the matrix. Diaion HP20SS (Mitsubishi Chemical Co., Tokyo, Japan) and Chromatorex ODS (100–200 mesh; Fuji Silysia Chemical Ltd., Tokyo, Japan) were used for column chromatography. Preparative HPLC was performed on a PU-986 intelligent Prep. pump (JASCO), a RI-930 intelligent RI detector (JASCO), and Cosmosil 5C₁₈-AR-II column (20×250 mm, Nacalai Tesque Inc., Japan). TLC was carried out on precoated Silica gel 60 F₂₅₄ (0.2 mm; Merck, Darmstadt, Germany) and RP-18 F₂₅₄S (0.2 mm; Merck) plates, and spots were visualized by spraying with 5% H_2SO_4 aq. solution

Fig. 3. The Structures of Compounds 1, 2, and 3

Fig. 4. The Key NOE Correlations for Compound 1

followed by heating. Single crystal X-ray structure analysis was performed using Varimax Saturn724 (Rigaku, Tokyo, Japan).

Fungal Material The fruiting body of *A. virgineoides* was collected from Isahaya city, Nagasaki prefecture under the authentication of the Nagasaki Prefectural Fungi Association.

Extraction and Isolation Carefully selected pure spores isolated from the gill of *A. virgineoides* were cultured for 28 d at 25°C on a medium composed of glucose (1.0%), peptone (0.5%), yeast extract (0.3%), KH_2PO_4 (0.3%), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1%). Following a cultivation period of 28 d, the medium was separated from the mycelium by filtration.

The filtrate was purified on a Diaion column eluted with acetone– H_2O (0:1 to 1:0 gradient) to remove any unwanted components from the culture medium, such as sugars.

In the first of two possible methods, the combined eluents from the column were mixed with CHCl_3 . The resulting organic layer (15.2 g) was passed through a Chromatorex ODS column eluted with MeOH– H_2O (0:1 to 1:0 gradient) to give eight fractions. Fraction 3 (829.3 mg) was passed through a Chromatorex ODS column eluted with MeOH– H_2O (0:1 to 6:4 gradient) to give four fractions. Fraction 3-3 was passed through a Diaion HP-20 column eluted with MeOH– H_2O (0:1 to 1:1 gradient) to give three fractions. Fraction 3-3-1 (96.5 mg) was passed through a Diaion column eluted with MeOH– H_2O (1:0 to 8:2 gradient) to give compound 1 (10.1 mg).

In the second method, the combined eluents from the column were mixed with EtOAc and the EtOAc layer (1.8 g)

was passed through a Chromatorex ODS column eluted with MeOH– H_2O (9:1) to afford five fractions. Fraction 1 was passed through a Chromatorex ODS column eluted with MeOH/ H_2O (7:3) to afford three fractions. Fraction 1-2 (28.6 mg) was purified by reversed phase HPLC eluting with 100% MeOH to give compounds 2 (6.4 mg) and 3 (9.2 mg).

Virgineol (1): Yellow gum. $[\alpha]_D^{17.0} -17.5$ ($c=0.3$, MeOH). IR $\nu_{\text{max}} \text{ cm}^{-1}$ 3406, 2957, 1087. HR-FAB-MS m/z 355.1725 (Calcd for $\text{C}_{16}\text{H}_{28}\text{O}_7\text{Na}$, 355.1725). FAB-MS m/z 371 $[\text{M}+\text{K}]^+$, 355 $[\text{M}+\text{Na}]^+$, 323 $[\text{M}-\text{MeOH}+\text{Na}]^+$, 301 $[\text{M}-\text{OMe}]^+$. ^1H -NMR (500 MHz, methanol- d_4) and ^{13}C -NMR (125 MHz, methanol- d_4) data are shown in Table 1.

Lignoren (2): Pale yellow gum. $[\alpha]_D^{22.0} -6.2$ ($c=0.3$, MeOH). EI-MS m/z 223 $[\text{M}+\text{H}]^+$.

Harziandione (3): Colorless prisms. $[\alpha]_D^{22.0} +104.8$ ($c=0.1$, MeOH). mp 172–174°C. IR $\nu_{\text{max}} \text{ cm}^{-1}$ 2934, 1733, 1699. HR-FAB-MS m/z 301.2189 (Calcd for $\text{C}_{20}\text{H}_{29}\text{O}_2$, 301.2167). FAB-MS m/z 301 $[\text{M}+\text{H}]^+$.

X-Ray Crystal Analysis of Structure 3 (Harziandione): Colorless block crystal (0.210×0.190×0.060 mm). Crystal data: $\text{C}_{20}\text{H}_{29}\text{O}_2$, $M=300.44$. Orthorhombic, space group: $P2_12_12_1$, $a=8.6995(12) \text{ \AA}$, $b=11.258(2) \text{ \AA}$, $c=16.903(3) \text{ \AA}$, $V=1655.5(4) \text{ \AA}^3$, $Z=4$, $D=1.205 \text{ g/cm}^3$, $F(000)=656.00$, $\mu (\text{MoK}\alpha)=0.754 \text{ cm}^{-1}$, R factor=0.0343, $wR2$ factor=0.0854, Flack parameter: $\chi=0.000$; Rigaku Saturn 724 diffractometer, MoK α radiation ($\lambda=0.71075 \text{ \AA}$) multi-layer mirror monochromated.

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