New Triterpenoids from *Tricholoma saponaceum*

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Received May 7, 2002; accepted August 26, 2002

Six new triterpenes, saponaceolides E (1), F (2), and G (3), and saponaceoic acids I (4), II (5), and III (6) were isolated from the fruiting body of the fungus *Tricholoma saponaceum*, and their structures were elucidated on the base of extensive NMR experiments. Compounds 1 and 2 exhibited inhibitor activity against B16 and L929 cells.

Key words *Tricholoma saponaceum*; saponaceolide; saponaceoic acid; Tricholomataceae; lanostane

As part of our ongoing study of biologically active compounds from higher mushrooms,[1] we have investigated *Tricholoma saponaceum* collected in Nagano prefecture, Japan. *T. saponaceum* (Tricholomataceae) is an edible mushroom with bitter taste, it mainly grows under broadleaf and coniferous trees.[2] De Bermardi *et al.*[3] have reported the isolation of four unique triterpenoids, saponaceolides A—D from Italian *T. saponaceum*. The 70% EtOH extract of the fruiting bodies afforded novel triterpenes, saponaceolides E (**1**), F (**2**), and G (**3**), and lanostane triterpenes, saponaceoic acids I (**4**), II (**5**), and III (**6**), along with known compounds, saponaceolides A (**7**), B (**8**),[3] trematenolic acid (**9**),[4] and steroids, 5α,8α-epodioxy-(22*E*,24*R*)-ergosta-7,9(11),22-triene-3β,5α,6β-triol (**10**), 5α,8α-epodioxy-(22*E*,24*R*)-ergosta-6,22-diene-3β-ol (**11**), 3β-hydroxy-(22*E*,24*R*)-ergosta-5,8,22-trien-7-one (**12**), (22*E*,24*R*)-ergosta-7,22-diene-3β,5α,6β-triol (**13**),[5] 5α,8α-epodioxy-(22*E*,24*R*)-ergosta-6,22-diene-3β-ol 3-*O*-β-D-glucopyranoside (**14**), 5α,8α-epodioxy-(22*E*,24*R*)-ergosta-6,9(11),22-triene-3β-ol 3-*O*-β-D-glucopyranoside (**15**).[6] This paper describes experimental evidence that has led to the structural and stereochemical assignments of **1**—**6**.

Saponaceolide E (**1**) was obtained as an amorphous powder, and its IR spectrum showed hydroxyl (3420 cm^{-1}) and carboxyl (1745 cm^{-1}) absorptions. The molecular formula $C_{30}H_{46}O_7$ for **1** obtained from the high resolution (HR)-FAB-MS data [*m*/*z* 541.3114, M+Na]$^+$ indicated eight equivalents of unsaturation, one of which accounted for a carboxyl group. The NMR data indicated that **1** was analogous to saponaceolide A (**7**).[3] The full assignments of protons and carbons could be completed by the detailed analysis of distortionless enhancement by polarization transfer (DEPT) and two dimensional (2D) NMR, including ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H-detected multiple quantum coherence spectrum (HMQC), ^{1}H-detected heteronuclear multiple bond correlation spectrum (HMBC), and rotating frame nuclear Overhauser and exchange spectroscopy (ROESY) experiment (Table 1). NMR spectral comparison of **1** with that of **7** showed that the difference occurred for the chemical shifts of protons and carbons at the C-7—C-11 and C-15 positions. The upfield shift of H-8 (δ 6.73) was brought about by the disappearance of the anisotropic effect of the C=O group at C-15, implying a *Z* configuration of the double bond at C-8(9). However, the configuration of the hydroxy group at C-10 was undecided. The ^{13}C-NMR shifts for the remaining carbons of **1** were in good agreement with those of **7**. Thus, the structure of saponaceolide E was formulated as shown for **1**.

Saponaceolide F (**2**) showed a [M($C_{30}H_{44}O_7$)+Na]$^+$ ion peak at *m*/*z* 539.2982, differing from **7** by 2H. The ^{1}H-NMR spectrum of **2** exhibited the same features as **7**, the only differences being the appearance of one olefinic proton at δ 5.21 (t, J=7.6 Hz), and the coupling patterns (J=12.8 Hz) and chemical shifts (δ 4.43, 4.65) of H_2-15′. These couplings implied that C-15′ was connected to a quaternary carbon instead of a methine carbon (C-9′ at δ 36.3) in **7**. The ^{13}C-NMR spectrum of **2** evidently exhibited one additional double bond appearing at δ 135.1 (s) and 123.5 (d), and a shift in the carbon for C-15′ (δ 60.4) on comparison of the NMR data of **2** with **7**. Analysis of COSY and HMQC spectra revealed connectivitites from the proton at δ 5.21 to H-11′ (δ 1.58, 2.14), to H-2 (δ 1.22), to H-3 (δ 0.99, 1.64), and to H-4 (δ 1.90, 2.22), indicating the presence of a double bond at the C-9′ and C-10′ positions. The relative stereochemistry of the double bond was fixed as *Z* through NOE correlation be-

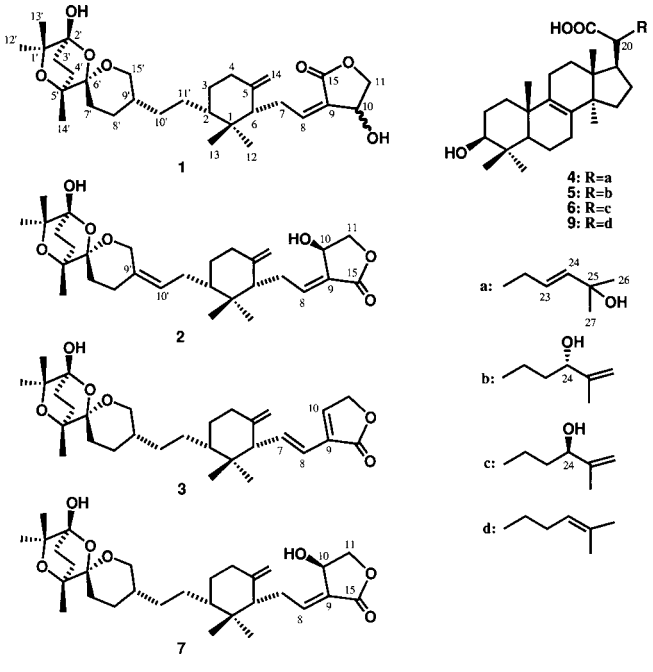

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Table 1. NMR Spectral Data for **1**—**3**, **7** (in Pyridine-d_5, 125 and 600 MHz)

Positions	**1**		**2**		**3**		**7**	
1	39.9		39.8		39.4		40.0	
2	48.1	1.05	48.6	1.22	47.9	1.10	48.4	1.09
3	30.3	1.03, 1.70	30.3	0.99, 1.64	29.1	1.12, 1.70	30.3	1.05, 1.73
4	37.5	1.91, 2.30	37.7	1.90, 2.22	36.5	2.08, 2.38	37.8	1.95, 2.28
5	149.0		148.6		149.8		148.7	
6	54.2	1.88	54.0	1.98	59.1	2.52 d (10.3)	54.1	1.99
7	24.9	3.17, 3.19	26.3	2.80, 2.83	136.6	7.29 dd (15.9, 10.3)	26.4	2.80, 2.83
8	148.8	6.73 dt (1.4, 7.1)	147.2	7.16 dt (1.8, 6.9)	122.0	6.25 d (15.9)	147.3	7.17 dt (1.6, 6.9)
9	129.7		130.1		128.9		130.1	
10	69.1	5.07 ddd (6.2, 3.4, 1.4)	66.2	5.34 ddd (6, 2.1, 1.8)	145.0	7.27 br s	66.2	5.36 ddd (6, 2.2, 1.6)
11	74.3	4.41 dd (9.7, 3.4)	75.6	4.51 dd (9.8, 2.1)	70.3	4.77 br s (2H)	75.6	4.51 dd (9.9, 2.2)
		4.54 dd (9.7, 6.2)		4.60 dd (9.8, 6)				4.62 dd (9.9, 6.0)
12	26.4	0.59 s	26.7	0.52 s	27.6	0.76 s	26.7	0.54 s
13	15.0	1.03 s	15.1	1.01 s	15.4	0.99 s	15.2	1.04 s
14	108.0	4.75, 4.92	108.9	4.88, 4.90	108.6	4.80, 4.96	108.8	4.88, 4.90
15	170.0		170.7		172.8		170.8	
1′	78.1		78.4		78.1		78.4	
2′	97.1		97.4		97.1		97.2	
3′	29.3	2.27 (2H)	29.6	2.28 (2H)	29.3	2.26 (2H)	29.6	2.25 (2H)
4′	29.5	1.81, 2.39	29.8	1.81, 2.37	29.5	1.81, 2.40	29.8	1.81, 2.38
5′	72.6		72.7		72.6		72.8	
6′	101.1		101.8		101.1		101.2	
7′	30.0	1.62, 2.23	32.1	1.90, 2.24	30.0	1.66, 2.23	30.3	1.62, 2.22
8′	25.4	1.62, 1.72	28.8	2.25, 2.40	25.4	1.64, 1.74	25.7	1.64, 1.70
9′	35.9	1.50	135.1		36.0	1.49	36.3	1.50
10′	32.0	0.96, 1.17	123.5	5.21 t (7.6)	31.8	1.00, 1.22	32.3	0.98, 1.18
11′	27.7	0.74, 1.50	28.9	1.58, 2.14	27.3	0.79, 1.53	28.1	0.74, 1.54
12′	26.6	1.58 s	26.9	1.58 s	26.6	1.59 s	27.0	1.58 s
13′	23.9	1.52 s	24.3	1.52 s	24.0	1.53 s	24.3	1.53 s
14′	21.4	1.27 s	21.7	1.23 s	21.5	1.28 s	21.5	1.27 s
15′	65.9	3.72 dd (11.2, 6)	60.4	4.43 d (12.8)	66.0	3.77 dd (11.3, 6)	66.2	3.71 dd (11.1, 5.8)
		3.87 dd (11.2, 11)		4.65 d (12.8)		3.89 t (11.3)		3.87 t (11.1)

tween H-8′ (δ 2.25)/H-10′ (δ 5.21) obtained in the ROSEY spectrum of **2**. The remainder of the structure was proposed by detailed analysis of ^{1}H- and ^{13}C-NMR data aided with 2D NMR, including COSY, HMQC, HMBC, and ROESY experiments. Hence, the structure of saponaceolide F were determined to be **2**.

The HR-FAB-MS for saponaceolide G (**3**) showed a molecular-related ion at *m/z* 523.3039 $[M(C_{30}H_{44}O_6)+Na]^+$, differing from **1** by H_2O. The ^{1}H-NMR spectrum of **3** exhibited the presence of three olefinic protons at δ 6.25 (d, J=15.9 Hz), 7.27 (br s) and 7.29 (dd, J=15.9, 10.3 Hz). These olefinic protons correlated to carbons at δ 122.0, 145.0, and 136.6 in the HMQC spectrum, suggesting the presence of disubstituted and trisubstituted double bonds. The full assignment of protons and carbons was completed by DEPT and 2D NMR including COSY, HMQC, HMBC, and ROESY experiments, as shown in Table 1. Namely, the HMBC experiment defined two double bonds located at the C-7 and C-9 positions. The stereochemistry of the C-7–C-8 double bond was confirmed to be *E* by the chemical shifts and coupling constants. Thus, the structure of saponaceolide G was formulated to be as shown for **3**.

Saponaceoic acid I (**4**) showed a molecular-related ion peak at *m/z* 495.3455 $[M+Na]^+$ in the HR-FAB-MS, corresponding to the molecular formula $C_{30}H_{48}O_4$. The ^{13}C-NMR spectrum for **4** closely resembles tramentenolic acid (**9**),[4] that is, seven methyl groups, one oxymethine at δ 78.2, and one carboxylic acid at δ 178.3. However, both compounds differed in the side chain (C-22–C-27), which in **4** is composed of two tertiary methyls at δ 31.0 and 30.9, and one each of methylene at δ 36.2, tertiary carbinol at δ 69.9, and a double bond at δ 141.9 (d) and 123.1 (d). The two olefinic protons at δ 6.12 (1H, d, J=15.4 Hz) and 6.22 (1H, ddd, J= 15.4, 8.1, 6.2 Hz) newly appeared in the ^{1}H-NMR spectrum, indicating the presence of an *E*-substituted double bond. The HMBC long-range correlations from H_3-26 (δ 1.49) and H_3-27 (δ 1.49) to C-24 (δ 141.9) and C-25 (δ 69.9) established the presence of a double bond at C-23(24) and the hydroxy group at the C-25 position. Thus, the structure of saponaceoic acid I was determined to be 3β,25-dihydroxylanosta-8,23*E*-dien-21-oic acid.

Saponaceoic acids II (**5**) and III (**6**) showed the same molecular-related ion peak at *m/z* 495 $[M(C_{30}H_{48}O_4)+Na]^+$ in the FAB-MS. The ^{13}C-NMR data for **5** also differed from **4** in the side chain (C-22–C-27). The side chain of **5** is composed of one tertiary methyl at δ 18.0, and two methylenes at δ 34.3 and 29.8, one secondary carbinol at δ 75.8, and one double bond at δ 150.0 (s) and δ 110.8 (t). These ^{1}H-NMR signals, correlated by HMQC spectra, revealed the presence of one vinylic methyl at δ 1.91 (s), an *exo*-methylene group due to two characteristic broad singlets at δ 4.94 and 5.22, and one oxymethine at δ 4.49 (1H, t, J=5.8 Hz). These functions were combined by the HMBC experiment, and the structure of saponaceoic acid II was determined to be 3,24-dihydroxylanosta-8,25-dien-21-oic acid. The configuration at C-24 of **5** was assigned as *S* on the basis of diagnostic chemical shifts of the C-24 (δ_C 75.8, δ_H 4.49), C-26 (δ_C 110.8, δ_H 4.94, 5.22), C-27 (δ_C 18.0, δ_H 1.91) positions in the NMR

Table 2. NMR Spectral Data for **4**—**6**, **9** (in Pyridine-d_5, 125 and 600 MHz)

Positions	**4**		**5**		**6**		**9**	
1	36.4	1.17, 1.62	36.4	1.13, 1.64	36.5	1.10, 1.52	36.1	1.18, 1.58
2	29.0	1.83, 1.85	28.9	1.83, 1.85	29.0	1.83, 1.85	28.7	1.82, 1.86
3	78.2	3.42 dd (11.5, 4.5)	78.2	3.43 t (8.0)	78.3	3.43 t (8.0)	78.0	3.43 dd (11.5, 4.5)
4	39.8		39.8		39.9		39.5	
5	51.1	1.17	51.1	1.24	51.2	1.20	50.9	1.17
6	19.1	1.55, 1.74	19.0	1.60, 1.81	19.1	1.60, 1.79	18.7	1.54, 1.74
7	27.6	2.06 (2H)	27.1	2.06 (2H)	27.2	2.06 (2H)	26.6	2.08 (2H)
8	134.0		134.0		134.0		134.0	
9	134.3		134.3		134.3		134.6	
10	37.7		37.7		37.7		37.4	
11	21.6	2.04 (2H)	21.6	1.98 (2H)	21.7	2.02 (2H)	21.3	1.98 (2H)
12	29.7	1.95, 2.10	29.9	1.95, 2.08	29.6	1.91, 2.02	29.4	1.93, 2.00
13	45.2		45.1		45.2		44.9	
14	50.1		50.1		50.2		49.9	
15	31.2	1.28, 1.70	31.2	1.20, 1.70	31.3	1.15, 1.70	30.9	1.25, 1.69
16	27.1	1.46, 2.03	27.7	1.50, 2.03	27.8	1.42, 2.05	27.5	1.46, 2.00
17	47.7	2.45	48.1	2.46	48.2	2.48	47.7	2.45
18	16.7	1.07 s	16.7	1.07 s	16.8	1.07 s	16.3	1.07 s
19	19.8	0.99 s	19.7	1.00 s	19.8	1.00 s	19.4	1.00 s
20	50.1	2.70	49.6	2.68	50.2	2.73	49.0	2.66
21	178.3		178.3		178.3		178.6	
22	36.2	2.54, 2.62	29.8	1.95, 2.14	29.8	2.17, 2.50	33.3	1.76, 1.92
23	123.1	6.22 ddd (15.4, 8.1, 6.2)	34.3	2.08, 2.14	34.3	2.07, 2.14	26.7	2.28, 2.37
24	141.9	6.12 d (15.4)	75.8	4.49 t (5.8)	75.2	4.53 t (5.3)	124.9	5.32 m
25	69.9		150.0		150.0		131.6	
26	30.9	1.49 s	110.8	4.94, 5.22	110.3	4.94, 5.33	25.8	1.61 s
27	31.0	1.49 s	18.0	1.91 s	18.8	1.90 s	17.7	1.66 s
28	29.0	1.07 s	28.9	1.07 s	29.0	1.07 s	28.6	1.07 s
29	16.8	1.02 s	16.8	1.01 s	16.9	1.01 s	16.4	1.00 s
30	24.8	1.24 s	24.8	1.24 s	24.9	1.24 s	24.5	1.24 s

spectrum.[7] The ^{13}C-NMR spectral comparison of **6** with that of **5** showed that **6** differs slightly from **5** in the side chain, in which the chemical shifts for C-24 (δ 75.2), C-26 (δ 110.3), and C-27 (δ 18.8) were indicative of the 24*R* configuration in **6**.[7] Therefore, the structure of saponaceoic acid III was determined to be 3β,24*R*-dihydroxylanosta-8,25-dien-21-oic acid.

Biological evaluation of saponaceolides E (**1**) and F (**2**) was conducted with B16 and L929 cells.[8,9] Compounds **1** and **2** exhibited the same activity of 10 μg/ml and 1 μg/ml with IC_{50} values, respectively.

Experimental

General Experimental Procedures Optical rotations were taken on a JASCO DIP-360 polarimeter. IR spectra were recorded on a Hitachi IR-27G, and NMR spectra were run on Varian UNITY 600 or JEOL GSX-400 spectrometers in C_5D_5N solution, using tetramethylsilane (TMS) as an internal standard. NMR experiments included COSY, HMQC, HMBC, DEPT, and ROESY pulse sequences. Coupling constants (*J* values) are given in Hz. FAB-MS (Xe gun, 10 kV, triethylene glycol as the matrix) were measured on a JEOL JMS-HX-100 mass spectrometer. Si gel 60 (230—400 mesh, Merck) and Si gel 60F-254 (Merck) were used for column chromatography and TLC, respectively.

Plant Material The fruiting body of *Tricholoma saponaceum* was collected at Nagano, Japan, in Autumn 2000. A specimen (TB 2085) has been deposited at the Herbarium of the Department of Pharmacognosy, Tokushima Bunri University, Tokushima, Japan.

Extraction and Isolation The fresh fruit bodies (1.15 kg) of *T. saponaceum* were extracted with 70% EtOH at room temperature for 6 weeks. The ethanolic extract was partitioned between EtOAc and H_2O. The EtOAc soluble portion (7.1 g) was repeatedly subjected to silica gel column chromatography with $[(CH_3)_2CH]_2O$–MeOH (50 : 1—25 : 6) to afford six fractions (frs. 1—6). Fraction 2 was further subjected to silica gel column chromatography with hexane–$[(CH_3)_2CH]_2O$ (1 : 1) and purified by preparative HPLC (ODS, 75% MeOH) to afford trametenolic acid (**9**, 15 mg), and compounds **10** (3.5 mg) and **11** (5.4 mg). Fraction 3 was further subjected to silica gel column chromatography with $[(CH_3)_2CH]_2O$–MeOH (50 : 1—25 : 2) to give four fractions (frs. 3.1—4). Fraction 3-2 was purified by preparative HPLC (ODS, 75% MeOH) to afford saponaceolides E (**1**, 15.3 mg), F (**2**, 6 mg), G (**3**, 3.5 mg), A (**7**, 150 mg), and B (**8**, 2.5 mg). Fraction 3-4 was purified by preparative HPLC (ODS, 80—95% MeOH) to afford saponaceoic acids I (**4**, 2.5 mg), II (**5**, 2.5 mg), III (**6**, 2.5 mg) and compound **12** (3.5 mg). Fraction 5 was further subjected to silica gel column chromatography with $[(CH_3)_2CH]_2O$–MeOH–H_2O (25 : 2 : 0.1—25 : 6 : 0.1), and purified by preparative HPLC (ODS, 83—88% MeOH) to afford compounds **13** (4.2 mg), **14** (19.5 mg), and **15** (8.4 mg).

Saponaceolide E (**1**): Amorphous powder; $[\alpha]_D^{25}$ +15.4° (*c*=0.8, MeOH); IR (KBr) ν_{max} 3420, 1745, 1670, 1080 cm^{-1}; FAB-MS *m/z* 517 $[M-H]^-$; HR-FAB-MS *m/z* 541.3114 (Calcd for $C_{30}H_{46}O_7$+Na, 541.3141); ^{1}H- and ^{13}C-NMR see Table 1.

Saponaceolide F (**2**): Amorphous powder; $[\alpha]_D^{25}$ +26.8° (*c*=0.3, MeOH); IR (KBr) ν_{max} 3450, 1740, 1050 cm^{-1}; FAB-MS *m/z* 513 $[M-H]^-$; HR-FAB-MS *m/z* 539.2982 (Calcd for $C_{30}H_{44}O_7$+Na, 539.2985); ^{1}H- and ^{13}C-NMR see Table 1.

Saponaceolide G (**3**): Amorphous powder; $[\alpha]_D^{25}$ +27.7° (*c*=0.3, MeOH); IR (KBr) ν_{max} 3450, 1750, 1050 cm^{-1}; FAB-MS *m/z* 499 $[M-H]^-$; HR-FAB-MS *m/z* 523.3039 (Calcd for $C_{30}H_{44}O_6$+Na, 523.3036); ^{1}H- and ^{13}C-NMR see Table 1.

Saponaceoic Acid I (**4**): Amorphous powder; $[\alpha]_D^{25}$ +10.0° (*c*=0.2, MeOH); IR (film) cm^{-1} 3400 (br), 1700 (br), 1060; FAB-MS *m/z* 471 $[M-H]^-$; HR-FAB-MS *m/z* 495.3455 (Calcd for $C_{30}H_{48}O_4$+Na, 495.3450); ^{1}H- and ^{13}C-NMR see Table 2.

Saponaceoic Acid II (**5**): Amorphous powder; $[\alpha]_D^{25}$ +3.8° (*c*=0.2, MeOH); IR (film) cm^{-1} 3420 (br), 1700 (br), 1060; FAB-MS *m/z* 471 $[M-H]^-$; HR-FAB-MS *m/z* 495.3432 (Calcd for $C_{30}H_{48}O_4$+Na, 495.3450); ^{1}H- and ^{13}C-NMR see Table 2.

Saponaceoic Acid III (**6**): Amorphous powder; $[\alpha]_D^{25}$ −5.0° (*c*=0.2, MeOH); IR (film) cm^{-1} 3400 (br), 1700 (br), 1080; FAB-MS *m/z* 471 $[M-H]^-$; HR-FAB-MS *m/z* 495.3422 (Calcd for $C_{30}H_{48}O_4$+Na, 495.3450); ^{1}H- and ^{13}C-NMR see Table 2.

Saponaceolide A (**7**): Colorless needles, mp 147—150 °C; $[\alpha]_D^{25}$ +73.4° (*c*=0.9, $CHCl_3$), lit. +78.1° ($CHCl_3$); IR (KBr) ν_{max} 3420, 1745, 1670, 1080 cm^{-1}; FAB-MS *m/z* 517 $[M-H]^-$; HR-FAB-MS *m/z* 541.3124 (Calcd

for $C_{30}H_{46}O_7$+Na, 541.3141); ^{1}H- and ^{13}C-NMR see Table 1.

Medium Complete E-MEM medium (Nissui Pharmaceutical Co., Ltd., Japan) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml anfoterin B and 0.3 mg/ml L-glutamin was used throughout the study.

Cells The cancer cells B16 and L929 were maintained in the E-MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, U.S.A.). Cells were cultured in the medium at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air throughout the study.

Cytotoxic Assay B16 and L929 cells in the exponential growth phase were plated in 96-well flat-bottom microplates at a density of 1×10^3 cells per 100 μl in each well with various concentrations of test compounds. After 96 h culturing, cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Test compounds **1** and **2** dissolved in dimethyl sulfoxide (DMSO) were used.

Acknowledgments We are grateful to Mr. T. Ohashi, mycologist, Tokushima Prefectural Tomioka Nishi High School, for confirming the identity of the fungus.

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