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Omphaloprenol A: a new bioactive polyisoprenepolyol isolated from the mycelium of poisonous mushroom *Omphalotus japonicus*

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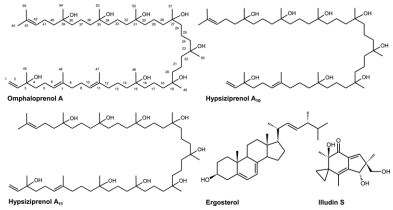
ABSTRACT

Mushrooms of the *Omphalotus* genus are known to be rich in secondary metabolites. In the quest for new bioactive compounds, we analyzed the compounds isolated from the mycelium of the poisonous mushroom *Omphalotus japonicus*. As a result, a new polyisoprenepolyol, which was named omphaloprenol A, was identified, along with known substances such as hypsiziprenol A_{10} and A_{11}, illudin S, and ergosterol. The chemical structure of omphaloprenol A was elucidated by nuclear magnetic resonance and infrared spectroscopies and mass spectrometry, and its bioactivity was investigated. Omphaloprenol A showed growth promoting activity against the root of lettuce seeds and cytotoxicity against HL60 cells. To the best of our knowledge, this is the first report on the isolation of a polyisoprenepolyol compound from Omphalotaceae mushrooms.

Graphical Abstract



Omphaloprenol A, hypsiziprenol A_{10}, A_{11}, ergosterol, and illudin S were isolated from the mycelium culture of poisonous mushroom *Omphalotus japonicus*.

Keywords: polyisoprenepolyol, mushroom, natural products, *Omphalotus japonicus*, cytotoxicity

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Omphalotus japonicus (*Omphalotus japonicus* [Kawam.] Kirchm. and O.K. Mill., *O. guepiniformis*, *Lampteromyces japonicus*, and *Tsukiyotake* in Japanese) is a poisonous luminescent mushroom native to Japan (Kasahara and Itou 2009). The substance causing its toxicity is an illudane-type sesquiterpene, that is, illusin S (Tada *et al.* 1964; Kasahara and Itou 2009; Matsumoto *et al.* 1965; McMorris and Anchel 1965; Harada and Nakanishi 1970). Other related compounds including dihydroilludin S and neoilludins A and B were previously isolated from *Omphalotus japonicus* (Ichihara *et al.* 1969; Kuramoto, Tsukihara and Ono 1999). Illudin S is well known as a potent cytotoxic substance with application as a lead compound in the development of anticancer drugs (Kelner *et al.* 1987). Especially, irofulven, one of the semisynthetic analogs of illudin S, improved significantly the therapeutic index and was studied for practical use as an anticancer drug (McMorris *et al.* 1990, 1996, 1997; Kelner *et al.* 1995; MacDonald *et al.* 1997). In mushrooms of the *Omphalotus* genus, variety types of secondary metabolites such as nematocidal peptides omphalotins (Sterner *et al.* 1997; Buchel *et al.* 1998), the luminescent substance lampteroflavin (Uyakul, Isobe and Goto 1990), and around 20 sesquiterpenes including illudane, protoilludane, illudalane, fomannosane, and africanane derivatives (Wawrzyn *et al.* 2012) were isolated in previous studies. Furthermore, secondary metabolites from the fruiting body of *O. japonicus* have been recently studied in detail, leading to the isolation of numerous new sesquiterpenoids, that is, neoilludins C, 4-*O*-methylneoilludin A and B, 5-hydroxydichomitol, and tsukiyols A-C (Aoki *et al.* 2020). In addition, the cytotoxic steroid 3β,5α,9α-trihydroxyergosta-7,22-diene-6-one was isolated (Aoki *et al.* 2020). Taken together, these works demonstrate the potential of mushrooms of the *Omphalotus* genus as a source of secondary metabolites, and new bioactive compounds can be expected to be discovered. Accordingly, in this study, we focused on 1 particular mushroom of the *Omphalotus* genus, that is, *O. japonicus*, to investigate the isolation of new metabolites from the mycelium culture on brown rice medium. As a result, we isolated 3 polyisoprenepolyols, namely, the new compound omphaloprenol A (**1**) and hypsiziprenol A_{10} (**2**) and A_{11} (**3**) (Sawabe *et al.* 1996, 1999) from the mycelial culture of *O. japonicus* using silica gel chromatography, solid-phase extraction (SPE), and octa decyl silyl high-performance liquid chromatography (ODS HPLC). The chemical structure of the new compound **1** was elucidated by nuclear magnetic resonance (NMR) and infrared (IR) spectroscopies and mass spectrometry (MS). The known compounds ergosterol (**4**) and illudin S (**5**; Tada *et al.* 1964; Matsumoto *et al.* 1965; Kasahara and Itou 2009) were also isolated. The chemical structures of compounds **1**-**5** are shown in Figure 1. In previous studies, hypsiziprenols, a type of polyisoprenepolyols, were isolated from the edible Lyophyllaceae mushroom *Hypsizygus marmoreus* (*Bunashimeji* in Japanese; Sawabe *et al.* 1996, 1999), and gymnopilin and gymnoprenols were isolated from the Strophariaceae hallucinogenic mushroom *Gymnopilus junonius* (*O-waraitake* in Japanese; Aoyagi *et al.* 1983; Nozoe *et al.* 1983, 1984; Kim, Choi and Lee 2012). However, to the best of our knowledge, the isolation of polyisoprenepolyols from Omphalotaceae mushrooms has not been reported to date. Interestingly, omphaloprenol A showed growth promoting activity against the root of lettuce seeds and cytotoxicity against HL60 cells. Although hypsiziprenol A_{10} and A_{11} exhibited the same level of cytotoxicity as omphaloprenol A, no significant growth promotion against lettuce seeds was observed for the hypsiziprenol compounds. Meanwhile, illudin S showed potent cytotoxicity against HL60 cells and strong growth inhibition against the root and the hypocotyl of lettuce seeds.

Results and discussion

In a thin layer chromatography (TLC) analysis performed to compare the EtOAc fraction from MeOH extracts of the fruiting body and the brown rice mycelium culture of *O. japonicus*, we observed different spots originated polyisoprenepolyols at R_f = 0.14 (**1**) and 0.24 (**2, 3**) only in the mycelium culture (Figure 2), which indicated the presence of polyisoprenepolyols in the mycelium culture that were absent in the fruiting body. Therefore, we tackled the isolation of such compounds. In the process of isolation, compounds **4** and **5** were also isolated.

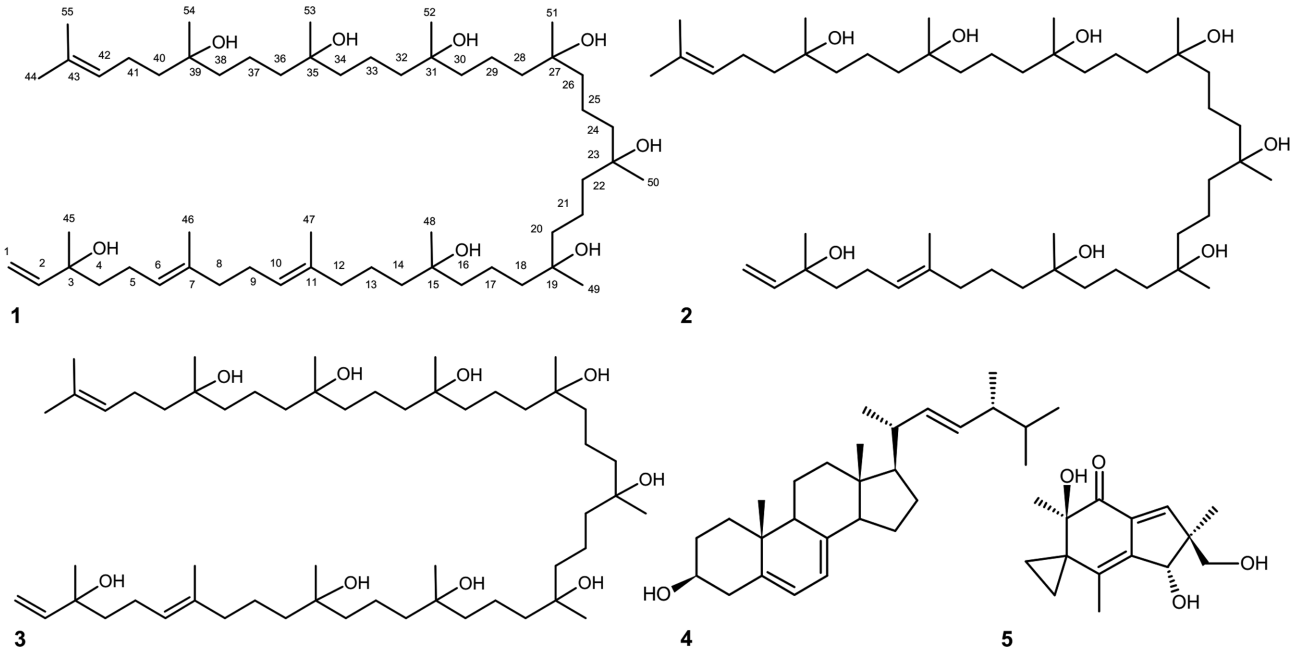

Figure 1. Chemical structures of compounds **1**-**5**.

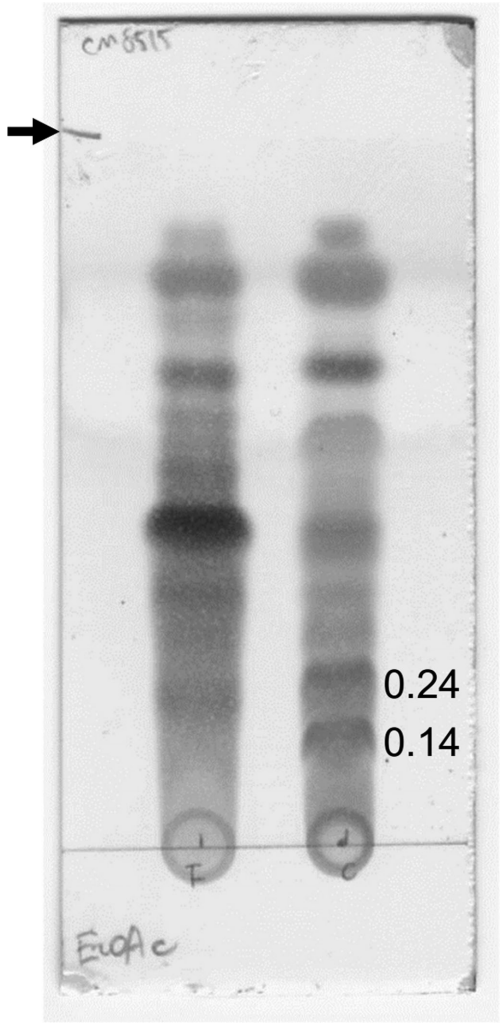

Figure 2. Thin layer chromatography analysis of the EtOAc fraction of the fruiting body (left side) and mycelium culture (right side) of lettuce seeds. The spot was colored by spraying H_2SO_4 containing 10% vanillin. The black arrow indicates the solvent front.

Omphaloprenol A (**1**) was obtained as a colorless amorphous solid. A high-resolution electrospray ionization MS (HR-ESI-MS) analysis revealed a protonated molecule ion peak at *m/z* 893.7808, which is consistent with the molecular formula $C_{55}H_{104}O_8$ (Figure S1) and the presence of 4 degrees of unsaturation in the molecule. The ^{13}C NMR spectrum exhibited peaks at δ_C 111.7, 124.3, 124.4, 124.5, 131.8, 134.9, 135.4, and 145.0 ppm, which suggest the presence of 4 double bonds. Therefore, an acyclic structure can be proposed for this compound. Despite some overlapping, signals attributable to 8 methyl, 4 allylic methyl, and 8 oxygenated sp^3 quaternary carbon atoms, along with various resonances ascribable to methylene groups, could be ascertained in the ^{13}C NMR spectrum (Figure S2). The ^1H NMR spectrum showed signals at δ_H 1.14 and 1.16 ppm attributable to 8 singlet methyl groups, 4 singlet allylic methyl groups at δ_H 1.57, 1.58, 1.61, and 1.67 ppm, 22 methylene groups at δ_H 1.31-1.51 ppm, 5 allylic methylene groups at δ_H 1.91-2.10 ppm, and 6 olefin protons at δ_H 5.04, 5.07, 5.11, 5.20, and 5.90 ppm from their chemical shifts (Figure S3). These spectral characteristics were similar to those of bionectin F isolated from the endophytic fungus *Bionectria* sp. (Yang *et al.* 2019). However, the α-terminal part of **1** contains a double bond, as can be extracted from the coupling constants (*J*) of the resonances at δ_H 5.04 (1H, dd, $J = 10.6$, 1.2 Hz), 5.20 (1H, dd, $J = 17.4$, 1.2 Hz), and 5.90 (1H, dd, $J = 10.6$, 17.4 Hz) ppm, instead of an OH group in bionectin F. Therefore, a polyisoprenepolyol structure can be suggested for compound **1**, such as that of the hypsiziprenols previously reported from the edible mushroom *H. marmoreus*.

Correlations of 2D NMR (double quantum filtered correlation spectroscopy [DQF-COSY], heteronuclear multiple quantum correlation [HMQC], and heteronuclear multiple bond correlation [HMBC]) also supported that **1** is a polyisoprenepolyol (Figures S4-S6). In DQF-COSY observed the correlation at H-1/H-2,/H-5/H-6, H-9/H-10, and H-41/H-42. HMBC observed the correlation at H-44/C-42, C-43, C-55; H-45/C-2, C-3; H-46/C-6, C-7, C-8; H-47/C-10, C-11, C-12; H-55/C-42, C-43, C-44; methyl at δ_H 1.14 and 1.16/oxygenated sp^3 quaternary carbon at δ_C 72.8. However, most of the peaks overlapped in 1D NMR, so 2D NMR correlations other than those mentioned above were unclear.

In the EI-MS analysis, fragment peaks at *m/z* 69, 109, 135, 177, 203, 245, 275, 313, 343, 399, 411, 467, 479, 535, 547, and 615 were observed (Figure S7), among which those at *m/z* 69, 135, and 203 can be assigned to the cleavage at the allylic positions, indicating the position of internal double bonds at C-6, C-10, and C-42 (Yang *et al.* 2019). The other fragments of **1** were attributed to cleavage of the vinyl position of the quaternary carbon of each isoprene unit, as shown in Figure 3. As can be extracted from the comparison with a previous study (Nishida *et al.* 1992), all the internal double bonds exhibited *E*-configuration according to the chemical shifts of the allylic methyl carbon atoms at δ_C 15.9, 16.0, and 17.7 ppm in the ^{13}C NMR spectrum. From the NMR spectra, all the carbons and protons in omphaloprenol A (**1**) were assigned as shown in Table 1.

Next, the bioactivity of compounds **1**-**5** was evaluated. The results of activity against germinated lettuce seeds are summarized in Figure 4. Omphaloprenol A (**1**) exhibited growth promoting activity against the root of germinated lettuce seeds, affording a growth rate of 132.87% ± 9.98% (mean ± SE) for an initial concentration of 100 ppm (v/w). Meanwhile, a weak growth promotion against the root of lettuce seeds was observed for compound **4** (118.9% ± 5.53%), whereas **5** showed potent inhibition against both the root and hypocotyl of lettuce seeds (21.45% ± 1.77% and 49.11% ± 2.44%, respectively). Furthermore, the cytotoxic activity of compounds **1**-**3** and **5** against HL60 cells was investigated (the cytotoxicity of compound **4** could not be assessed because it decomposed during storage). The following cytotoxicity values were observed: $IC_{50} = 3.7$ μM (**1**), 3.8 μM (**2**), 3.4 μM (**3**), and 15.2 nM (**5**) (Table 2). Although **1** exhibited growth promotion against the root of lettuce seeds, it did not show any growth promoting activity against cancer cells.

Experimental

General

NMR measurements were performed using a JNM ECX-600 (^1H NMR: 600 MHz, ^{13}C NMR: 150 MHz, JEOL Ltd., Tokyo, Japan) spectrometer. IR spectra were recorded on an FT-710 (HORIBA Ltd., Kyoto, Japan) spectrometer. HR-ESI-MS analysis was conducted on a Synapt G2 (Waters Corporation, MA, USA) instrument. HPLC was performed using a SHIMADZU LC workstation CLASS LC-10 (Shimadzu Corporation, Kyoto, Japan) and an ODS column (Inertsil ODS-3, 10 × 250 mm, GL Science Inc., Tokyo, Japan). Silica gel 60 (Merck KGaA, Darmstadt, Germany) was used for the column chromatography, TLC plates were prepared with silica gel F_{254} (Merck, KGaA, Darmstadt, Germany), and SPE was per-

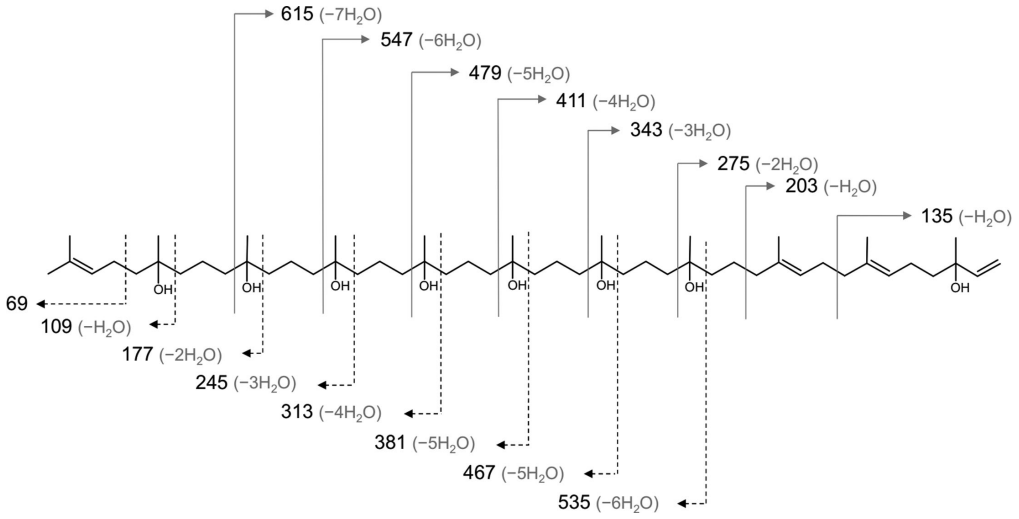

Figure 3. Assignment of the EI-MS fragmentation of omphaloprenol A.

Table 1. ^{1}H (700 MHz) and ^{13}C NMR (150 MHz) data for compound **1** (in $CDCl_3$)

	^{1}H	^{13}C		^{1}H	^{13}C
1	5.04 (1H, dd, J = 10.6, 1.2 Hz)	111.7	28	1.31-1.51 (2H)	41.6-42.5
	5.20 (1H, dd, J = 17.4, 1.2 Hz)		29	1.31-1.51 (2H)	18.1-18.2
2	5.90 (1H, dd, J = 17.4, 10.6 Hz)	145.0	30	1.31-1.51 (2H)	41.6-42.5
3		73.51	31		72.8
4	1.31-1.51 (2H)	41.6-42.5	32	1.31-1.51 (2H)	41.6-42.5
5	1.91-2.10 (2H)	26.3	33	1.31-1.51 (2H)	18.1-18.2
6	5.11 (1H, m)	124.3-124.5	34	1.31-1.51 (2H)	41.6-42.5
7		134.9-135.4	35		72.8
8	1.91-2.10 (2H)	39.6-40.0	36	1.31-1.51 (2H)	41.6-42.5
9	1.91-2.10 (2H)	39.6-40.0	37	1.31-1.51 (2H)	18.1-18.2
10	5.11 (1H, brt, m)	124.3-124.5	38	1.31-1.51 (2H)	41.6-42.5
11		134.9-135.4	39		72.8
12	1.91-2.10 (2H)	22.7-22.8	40	1.31-1.51 (2H)	41.6-42.5
13	1.31-1.51 (2H)	18.1-18.2	41	1.91-2.10 (2H)	22.7-22.8
14	1.31-1.51 (2H)	41.6-42.5	42	5.07 (1H, brtd, J = 7.1, 1.0 Hz)	124.3-124.5
15		72.8	43		131.8
16	1.31-1.51 (2H)	41.6-42.5	44	1.67 (3H, s)	25.7
17	1.31-1.51 (2H)	18.1-18.2	45	1.26 (3H, s)	27.7
18	1.31-1.51 (2H)	41.6-42.5	46	1.57-1.58 (3H, s)	15.9-16.0
19		72.8	47	1.57-1.58 (3H, s)	15.9-16.0
20	1.31-1.51 (2H)	41.6-42.5	48	1.14-1.16 (3H, s)	26.8-27.1
21	1.31-1.51 (2H)	18.1-18.2	49	1.14-1.16 (3H, s)	26.8-27.1
22	1.31-1.51 (2H)	41.6-42.5	50	1.14-1.16 (3H, s)	26.8-27.1
23		72.8	51	1.14-1.16 (3H, s)	26.8-27.1
24	1.31-1.51 (2H)	41.6-42.5	52	1.14-1.16 (3H, s)	26.8-27.1
25	1.31-1.51 (2H)	18.1-18.2	53	1.14-1.16 (3H, s)	26.8-27.1
26	1.31-1.51 (2H)	41.6-42.5	54	1.14-1.16 (3H, s)	26.8-27.1
27		72.8	55	1.61 (3H, s)	17.7

formed with Waters Sep-Pak Vac 35cc C_{18}-10g (Waters Corporation, MA, USA). Ergosterol (Tokyo Kasei, Tokyo, Japan) was used for the structure determination with NMR of **4**.

Fungal isolation

Mycelium was isolated from the fruiting body of *O. japonicus* collected in Yamagata University Research Forest, Yamagata prefecture, Japan (2016). The fruiting body was identified by D. A. and mycelium was identified by BEX Co., Ltd. Japan, using a DNA analysis of the 18S rDNA regions. Culture collection (YUOJ0825) was deposited in the Laboratory of Bioorganic Chemistry, Faculty of Agriculture, Yamagata University.

Fermentation

A potato dextrose agar (PDA) culture (15 days) was inoculated in 4 samples of brown rice medium (120 g of brown rice and 210 mL

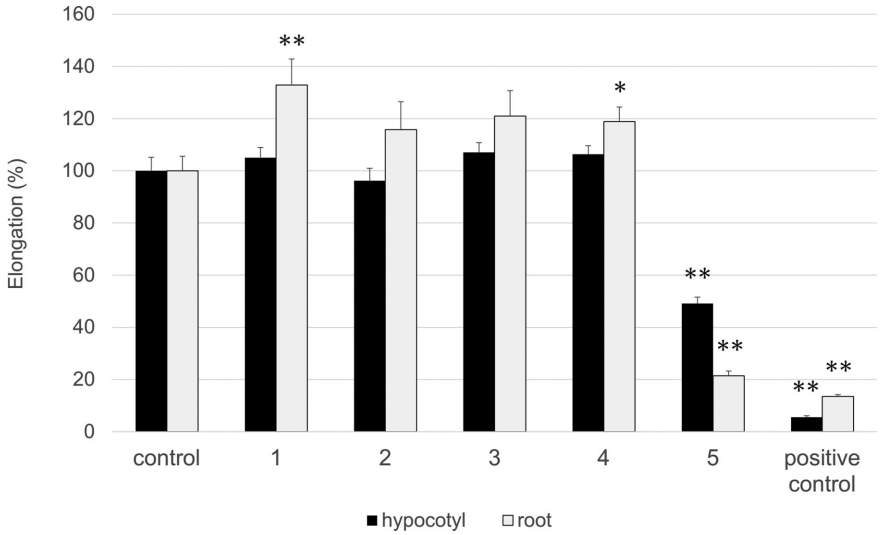

Figure 4. Growth rate of compounds **1**-**5** against the root or hypocotyl of germinated lettuce seeds. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as a positive control. The results are displayed as the mean ± SE ($n = 20$). The Welch's *t*-test was used for the statistical analysis ($^{*}P < .05$; $^{**}P < .01$).

Table 2. Cytotoxicity against HL60 cells of compounds **1**-**3** and **5**

Compounds	IC_{50} [a]
1	3.7 μM
2	3.8 μM
3	3.4 μM
5	15.2 nM

[a]IC_{50} refers to the concentration that caused 50% inhibition of HL60 cells.

of H_2O in 500 mL Erlenmeyer flasks) and allowed to stand for 40 days at 25 °C in the dark.

TLC analysis

An EtOAc fraction of the fruiting body of *O. japonicus* was prepared following the previously reported protocol for the EtOAc fraction of the mycelium culture (Aoki *et al.* 2020), which was also prepared to provide comparison. Both EtOAc fractions were subjected to TLC analysis (80 × 30 mm) using $CHCl_3$/MeOH = 85/15. The developed plate was air-dried and colored by spraying H_2SO_4 containing 10% vanillin.

Extraction and isolation

The brown rice medium culture was extracted with MeOH 3 times, and the solvent was then concentrated in an evaporator. The residue (192.4 g) was suspended with H_2O and partitioned with hexane, EtOAc, and BuOH. The EtOAc fraction (1.4 g) was subjected to silica gel chromatography using $CHCl_3$/MeOH (85/15, 7/3, 1/1, and 0/1) as a stepwise eluent, and the fractions RE-1 (515.0 mg), RE-2 (300.1 mg), and RE-3 (218.6 mg) were obtained. RE-1 was subjected to silica gel chromatography eluted with hexane/EtOAc (7/3, 3/2, 1/1, and 2/3) in a stepwise manner, affording RE-1-8 to 10 (14.3 mg). RE-1-8 to 10 was then subjected to silica gel chromatography using hexane/EtOAc (7/3 and 3/2) as a stepwise eluent, and 3.8 mg of RE-1-8 to 10-6 (**4**) was isolated. RE-2 was subjected to ODS Sep-Pak eluted with MeOH/H_2O (1/3, 1/1, 3/1, and 1/0) stepwisely, affording RE-2-1 (71.6 mg). The RE-2-1 fraction was then subjected to ODS HPLC using MeOH/H_2O (25:75) as an eluent, and **5** (8.1 mg) was obtained. RE-3 was subjected to ODS Sep-Pak eluted with MeOH/H_2O (1/3, 1/1, 3/1, and 1/0) in a stepwise manner to obtain RE-3-4 (142.5 mg). RE-3-4 was subjected to ODS HPLC using a gradient elution system of MeOH/H_2O (0-15 min at 85/15 to 100/0 and 15-40 min at 100/0) to give RE-3-4-A (t_R = 14.4, 73.5 mg) and RE-3-4-B (t_R = 18.9 min, 14.1 mg, compound **1**). RE-3-4-A was subjected to ODS HPLC using MeOH/H_2O (80/20) to give **2** (7.1 mg) and **3** (26.9 mg) with t_R = 45.8 and 48.4 min, respectively.

Bioassay using germinated lettuce seeds

The biological activity of the compounds was evaluated using germinated lettuce (*Lactuca sativa*) seeds with Melbourne MT (Tohoku Seed Co. Ltd., Tochigi, Japan). Lettuce seeds were germinated with H_2O for 1 day 25 °C under dark condition. Meanwhile, 1 mL of MeOH solution containing 100 ppm of each compound was placed on a filter paper in a Petri dish (40 mm i.d.). After being air-dried, the samples were soaked in 1 mL of H_2O containing 0.1% Tween 80 (Kanto chemical Co. Inc., Tokyo, Japan). Two Petri dishes were prepared for each compound. To each Petri dish, 10 of germinated lettuce seeds were added and allowed to stand for 4 days at 25 °C in the dark. The length of the hypocotyl and the root of the seeds was then measured using a ruler. 2,4-Dichlorophenoxyacetic acid (Kanto chemical Co. Inc., Tokyo, Japan) was used as a positive control, which was prepared using only H_2O containing 0.1% Tween 80 after air-drying 1 mL of MeOH.

Cytotoxicity against HL60 cells

Human acute promyelocytic leukemia HL60 cells (RCB0041, RIKEN BioResource Center, Tsukuba, Japan) were seeded in a 96-well plate at a density of 1×10^5 cells/mL, and then treated with each compound dissolved in MeOH using a 96-well microplate at 37 °C under a humidified, 5% CO_2 atmosphere for 2 days in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Co., St. Louis, MO, USA), 50 units/mL of penicillin-50 μg/mL of streptomycin (Gibco,

Thermo Fisher Scientific Inc., Waltham, MA, USA). Then, the samples were incubated for 4 h with 0.5 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Dojindo Laboratories, Kumamoto, Japan), and the resulting MTT–Formazan product was dissolved by adding isopropanol containing 0.04 M HCl. The absorbance was recorded at 560 nm using a microplate reader (Tecan, Männedorf, Switzerland). For each experiment, the viability was calculated as the absorbance of the treated cells in comparison with that of the control cells. The positive control camptothecin showed an IC_{50} value of 35.7 nM (Aoki *et al*. 2020).

Omphaloprenol A: Colorless amorphous solid;$[\alpha]_{20}^{D} = +17.8$ (c 0.1, MeOH); IR (NaCl) $\nu_{max} = 3365\ cm^{-1}$ (Figure S8); HR-ESI-MS *m/z*: 893.7808 $[M + H]^+$ (calcd for $C_{55}H_{97}O_8$: 893.7806; NMR spectroscopy data is shown in Table 1.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

S.A. and T.M. designed this study. S.A., T.A., T.O., K.K., A.D., and Y.I. were performed sampling of the fruiting body, analysis, and experiments.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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