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Note

Functional-Food Constituents in the Fruiting Bodies of *Stropharia rugosoannulata*

Jing WU,[1] Keiji FUSHIMI,[1] Shinji TOKUYAMA,[2] Masato OHNO,[3] Takaaki MIWA,[3] Tomoyuki KOYAMA,[3] Kazunaga YAZAWA,[3] Kaoru NAGAI,[4] Tetsuo MATSUMOTO,[5] Hirofumi HIRAI,[2] and Hirokazu KAWAGISHI[1,2,†]

[1]*Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[2]*Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[3]*Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan*
[4]*Department of Epigenetic Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi 409-3898, Japan*
[5]*Gunma Prefectural Forestry Experiment Station, 2935 Arai, Shinto-mura, Kitagunma-gun, Gunma 370-3503, Japan*

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Nine compounds (1–9) were isolated from the fruiting bodies of *Stropharia rugosoannulata*. Compounds 1–5, 8, and 9 suppressed the formation of osteoclast. Compounds 2 and 5 showed anti-fungal activity, and their MIC were 250 μM and 500 μM respectively. Compounds 2–6 showed inhibitory effects on thapsigargin toxicity.

Key words: *Stropharia rugosoannulata*; mushroom; osteoclast-forming suppression; anti-fungal; endoplasmic reticulum stress-suppression

In order to develop new functional foods from mushrooms, we are screening for biological activities such as osteoclast-forming suppression, anti-fungal effects, and endoplasmic reticulum stress-suppression of extracts of various eatable mushrooms. During screening, we found activities in an extract of the fruiting bodies of *Stropharia rugosoannulata*, and we tried to isolate the active principles from the mushroom. The mushroom, *S. rugosoannulata* (saketsubatake in Japanese, and wine-cap stropharia in English), belongs to the family Strophariaceae. It is widespread in northern temperate zones throughout the world. It is edible and is cultivated for food.

Bone homeostasis during remodeling is maintained by osteoclastic bone resorption and osteoblastic bone formation.[1)] Osteoclasts play a role in bone resorption, and abnormal acceleration of osteoclast formation is one the causes of osteoporosis.

Systemic candidosis is a major deep-seated mycosis in patients with immunosuppressive conditions. An inhibitory effect on the hyphal outgrowth of *Candida albicans* is drawing keen attention, because the clinically invasive form of *C. albicans* is the hyphal form.[2)]

Endoplasmic reticulum (ER) stress induces apoptotic pathways with signaling between ER and the mitochondria. By triggering apoptosis in neural cells, this stress is a major cause of degenerative diseases such as Alzheimer disease.[3,4)]

Hence, materials that show suppressive effects on osteoclast formation, fungal hyphal outgrowth, and ER stress are potential drugs or functional foods. Here we describe the isolation, structural identification, and biological activities of these compounds.

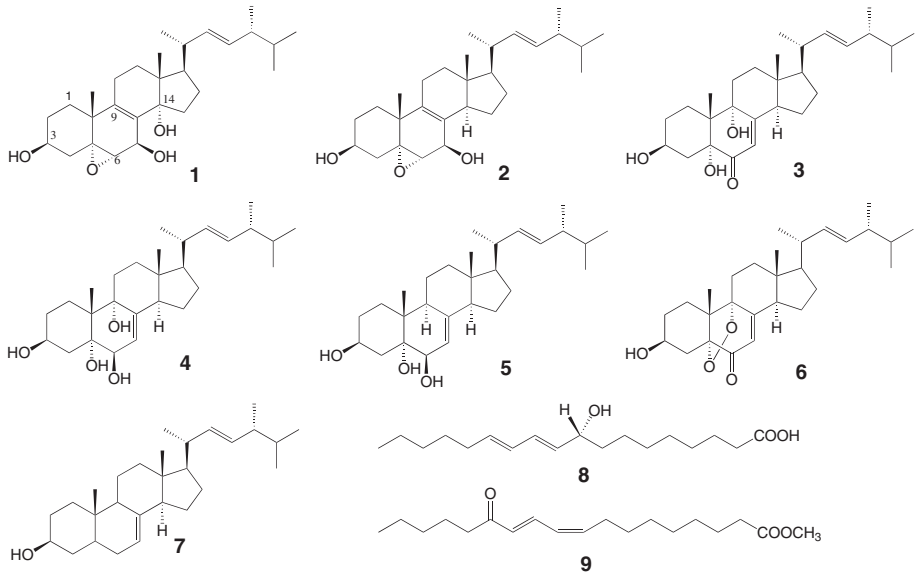
Fresh fruiting bodies of *S. rugosoannulata* were extracted with EtOH and then with acetone. After the solutions were combined and concentrated, they were partitioned between hexane and H_2O, $CHCl_3$ and H_2O, and then EtOAc and H_2O. The hexane-soluble part was fractionated by repeated chromatography and/or recrystallization. Seven compounds (**1**–**7**) were purified. On the other hand, two compounds (**8** and **9**) were isolated from the $CHCl_3$-souble part. All the compounds have been reported previously, and were identified by comparison of their spectroscopic data with data reported previously (Scheme 1).[5–10)]

Compounds **1**–**5**, **8**, and **9** suppressed the formation of osteoclasts dose-dependently (Fig. 1). The oxidized fatty acids (**8** and **9**) showed weak activity. Among the sterols, **1** to **4** showed cytotoxicity at high concentrations, but **5** showed suppressed activity without any cytotoxicity, even at 25 μg/mL (55 μM). This suggests that the hydroxyl group at C-9 or the epoxy at C-5 and C-6 in the sterol skeleton strengthened cytotoxicity.

Compounds **2** and **5** showed anti-fungal activity. Their MICs were 250 μM and 500 μM respectively. A comparison of the structures between **1** and **2** and between **4** and **5** indicates that the hydroxyl group at C-14 or C-9 weakened activity.

In a protective activity assay against ER stress-dependent cell death caused by tunicamycin (TM) or thapsigargin (TG), none of the compounds showed an inhibitory effect on tunicamycin toxicity, but compounds **2**–**6** showed inhibitory effects on TG toxicity dose-dependently (Fig. 2). TM is an inhibitor of *N*-glycosylation to glycoproteins in the ER, and causes protein-misfolding there. TG, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, also in-

† To whom correspondence should be addressed. Tel/Fax: +81-54-238-4885; E-mail: achkawa@ipc.shizuoka.ac.jp



Scheme 1.

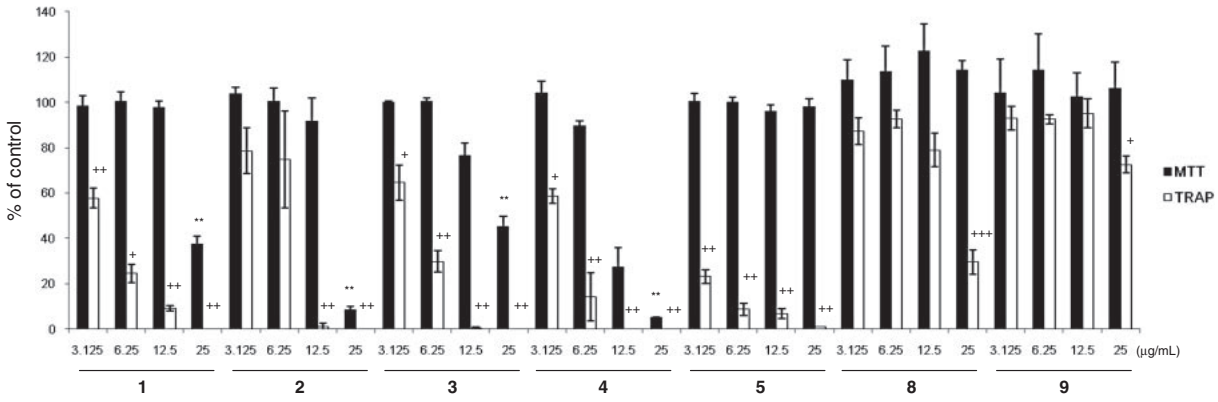


Fig. 1. Inhibitiotory Activity of **1**–**5**, **8**, and **9** against Osteoclast Formation.

Solid and hollow columns indicate cell viability and osteoclast formation respectively. TRAP-positive multinucleated cells that had more than three nuclei were counted. Cell viability was determined by MTT assay. Data are mean ± SE of two cultures ($^{*}p < 0.05$, $^{***}p < 0.005$ *vs.* control (MTT activity); $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.005$ *vs.* control (bone formation rate) by Student's *t*-test).

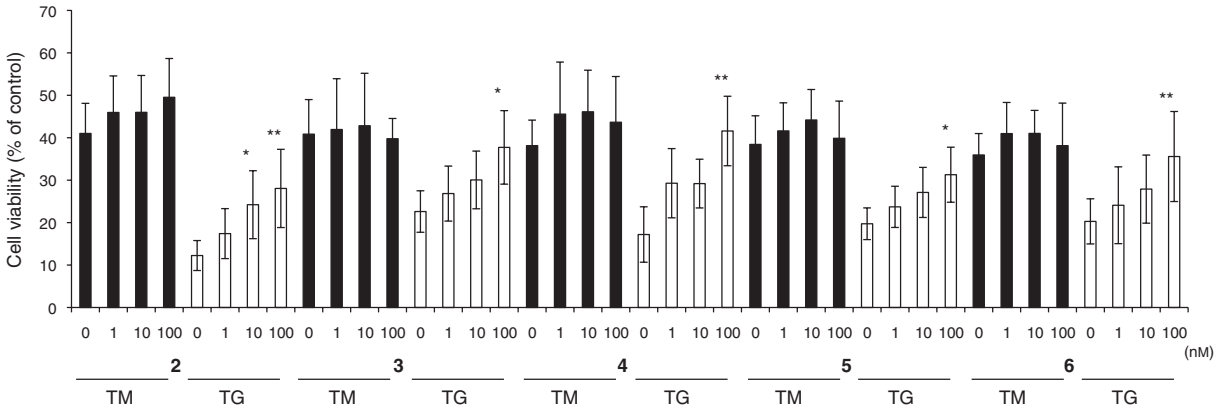


Fig. 2. Protective Effects of **2**–**6** on ER Stress-Induced Cell Death.

Cell viability was analyzed by MTT assay, and values are presented as mean ± SD of the relative percentage of surviving cells as compared to the untreated cells (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$, non-paired *t*-test.

duces ER stress by disrupting the homeostatic balance of the Ca^{2+} concentration in the ER. This suggests that **2–6** can protect neuronal cells by attenuating the ER stress caused by the Ca^{2+}-ATPase inhibitor. This also indicates that the oxygen atom(s) at C-5 and C-6 in the sterol skeleton is necessary for the suppression of TG toxicity, and that a hydroxyl group at C-14 in the skeleton contributes to deactivation.

Compound **2** has been isolated from the mushroom *Amanita subjunquillea*. It showed cytotoxicity against four human tumor cells *in vitro*.[6] Compound **3** was cytotoxic against HeLa cells.[7] Although enantiomer of **8** showed fungitoxic activity,[11] this is the first report describing the biological activity of **8**.

Although these compounds are known and the details of the mechanisms of the effects of the compounds remain unsolved, the above activities were first found in this study. These results should provide useful information for the development of functional foods having osteoclast-forming suppression, anti-fungal effects, and ER stress-suppression activities.

Experimental

General experiments. ^{1}H NMR spectra (one- and two-dimensional) were recorded on a Jeol lambda-500 spectrometer at 500 MHz, and ^{13}C NMR spectra were recorded on the same instrument at 125 MHz (Jeol, Tokyo, Japan). ESIMS spectra were measured on a JMS-T100LC mass spectrometer (Jeol, Tokyo, Japan). Specific rotation values were measured using a Jasco DIP-1000 polarimeter. HPLC separations were performed with a Jasco Gulliver system using reverse-phase HPLC columns (Capcell Pak C18 AQ, Shiseido, Tokyo, Japan; Wakosil-II 5C18 HG Hrep, Wako, Osaka, Japan). Silica gel plates (Merck F_{254}) and silica gel 60N (Merck 100–200 mesh) were used for analytical TLC and for flash column chromatography respectively (Merck, Darmstadt, Germany).

Fungal strain and culture conditions. The strain of *S. rugosoannulata* was isolated from a fruiting body collected in Gunma Prefecture, Japan, in August 2000. Voucher material has been deposited in Gunma Prefectural Forestry Experiment Station, Japan. The culture medium was prepared by mixing ground flakes of Japanese oak and fresh rice bran at a weight ratio of 8:2 adjusted to 65% moisture. The medium (2.5 kg) was packed in a polypropylene bag and autoclaved. The pre-cultured mycelium was inoculated into each bag of the medium. After cultivation in the dark at 25 °C at 65% humidity for 5 months, each set of two bags was transplanted into a plastic planter filled with bark compost. Fruiting body induction and further cultivation were done in a greenhouse without control of temperature, humidity, or light for about 2 months.

Extraction and isolation. Fresh fruiting bodies of *S. rugosoannulata* (20.0 kg) were extracted with EtOH (42 L, 3 times) and then with acetone (20 L, 3 times). After the solutions were combined and concentrated under reduced pressure, the concentrate was partitioned between hexane and H_2O, then $CHCl_3$ and H_2O, and then EtOAc and H_2O. The hexane-soluble part (109.5 g) was fractionated by silica gel flash column chromatography (hexane; 70%, 50%, 20% hexane/CH_2Cl_2; CH_2Cl_2; 90%, 80%, 70%, 40% CH_2Cl_2/EtOAc; EtOAc; 80%, 70%, 50%, 30% EtOAc/MeOH; and MeOH, 1.5 L each) to obtain 28 fractions (fractions 1 to 28). Fractions 19 (1.832 g) and 24 (1.807 g) were further separated by silica gel flash column chromatography (EtOAc; 90%, 80%, 70%, 60%, 50%, 30%, 20%, 10% EtOAc/MeOH; and MeOH, 0.5 L each) to give 11 and eight fractions (fractions 19-1 to 19-11 and 24-1 to 24-8) respectively. Fraction 19-4 was further separated by reverse-phase HPLC (Wakosil-II 5C18, 90% MeOH) to afford **1** (2.9 mg) and **2** (6.9 mg). Fractions 24-2 and 24-3 were separated by reverse-phase HPLC (Wakosil-II 5C18, 90% MeOH) to afford **3** (3.1 mg) and **4** (7.2 mg), and **5** (32.7 mg) respectively. Fraction 18 (38.7 mg) was further separated by reverse-phase HPLC (Capcell Pak C18, 80% MeOH) to afford **6** (4.1 mg). Compound **7** (2.1 mg) was obtained from fraction 11 (7.319 g) by repeated recrystallization. On the other hand, the $CHCl_3$-soluble part (50.2 g) was fractionated by silica gel flash column chromatography (CH_2Cl_2; 90%, 80%, 70%, 50% CH_2Cl_2/EtOAc; EtOAc; 80%, 50% EtOAc/MeOH; and MeOH, 1.5 L each) to obtain 14 fractions (fractions 1 to 14). Fractions 6 (3.790 g) and 8 (1.187 g) were further separated by silica gel flash column chromatography (CH_2Cl_2; 95%, 90%, 85%, 80%, 70%, 60% CH_2Cl_2/EtOAc; EtOAc; and MeOH, 0.5 L each) to give eight and nine fractions (fractions 6-1 to 6-8 and 8-1 to 8-9) respectively. Fraction 8-4 (749.4 mg) was further separated by reverse-phase HPLC (Wakosil-II 5C18, 75% MeOH) to obtain **8** (22.3 mg). Compound **9** (16.3 mg) was obtained from fraction 6-3 (358.6 mg) by reverse-phase HPLC (Wakosil-II 5C18, 75% MeOH).

Bioassay 1: suppression of the formation of osteoclasts. Stromal/osteoblastic cells, UAMS-32, were cultured in an α-minimal essential medium (α-MEM) (ICN Biomedicals, Barcelona, Spain) containing 10% fetal bovine serum (FBS) for 1 week. The cells were detached from the culture dishes using trypsin-EDTA, suspended in α-MEM containing 10% FBS, and used for the co-culture as osteoblastic cells. Femoral and tibiae bone marrow cells were collected from 5-week-old mice that had been killed by cervical dislocation, as described previously.[12] The tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavities were flushed by slowly injecting a medium with a 26-gauge needle. Osteoblastic cells and bone marrow cells collected were washed and used in co-culture. Osteoclasts were prepared by a co-culture system, as previously described.[13] The osteoblastic cells (1.0×10^4 cells/well) were co-cultured with bone marrow cells (2.0×10^7 cells/well) in α-MEM containing 10% FBS in 96-well plates (Corning, NY, USA). The culture volume was made up to 200 µL per well with α-MEM supplemented with 10% FBS in the presence of 10 nM 1α, 25-dihydroxyvitamin D_3 (Biomol, PA, USA), and 10 µM prostaglandin E_2, with and without a sample. All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Three-quarters of the medium was changed after co-culturing for 3 d. After cultivation, the adhering cells were fixed with 10% formaldehyde in 10 mM phosphate-buffered saline (pH 7.4) for 20 min. After treatment with 95% ethanol for 1 min, the well surface was dried and treated with TRAP staining solution: 0.1 M sodium acetate buffer pH 5.0 containing 50 mM sodium tartrate, 0.1 mg/mL of naphthol AS-MX phosphate (Sigma, MO, USA), and 1 mg/mL of fast red violet LB salt (Sigma) for 30 min. TRAP-positive multinucleated cells were then counted under a microscope. Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) assay. After culture, the cells were treated with 1 mg/mL of MTT for 2 h, and then precipitated dye was solubilized into dimethylsulfoxide and the absorbance at 570 nm was measured.

Bioassay 2: anti-fungal assay.[2] The inhibitory effect on hyphal outgrowth of *Candida albicans* was measured using 96 well-microplates. Saline-washed blastospores (1×10^2) of *C. albicans* were inoculated in 0.2 mL of Eagle's minimum essential medium (EMEM) containing serially diluted test samples in DMSO, and cultured at 37 °C at 60% relative humidity under 5% CO_2. Controls were similarly set up with an equivalent quantity of the solvent (final conc. 1.0%). Hyphal development from the yeast cells was examined microscopically 24 h after incubation.

Bioassay 3: anti-ER stress. Cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Cell protection assay on ER stress by MTT assay was performed as follows: Neuro2a cells were cultured in 96-well plates at a cell density of 5,000 cells/well. After 1 d of cultivation, the cells were cultured in D-MEM without FBS, and 0.5 µg/mL of tunicamycin (or 20 nM thapsigargin) and varying concentrations of the compounds were applied to the medium. The cells were incubated for 24 h, and then viability was measured by MTT assay, as described previously.[14] Briefly, 0.25 mg/mL of MTT in D-MEM without FBS was added onto the cells and this was incubated for 2 h. Incubation was terminated by the addition of 20% SDS v/w and 50% dimethylformamide v/v in water. The absorbance at 570 nm of the reaction mixture was measured with a microplate reader (Molecular Devices, CA, USA).

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