

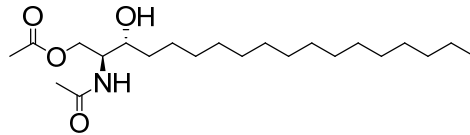
A novel sphingosine with osteoclast-forming suppressing activity, from the edible mushroom *Grifola gargal*

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| メタデータ | 言語: eng 出版者: 公開日: 2013-10-23 キーワード (Ja): キーワード (En): 作成者: Choi, Jae-Hoon, Yoshida, Miyuki, Suzuki, Tomohiro, Harada, Etsuko, Kawade, Mitsuo, Yazawa, Kazunaga, Nishimoto, Sogo, Hirai, Hirofumi, Kawagishi, Hirokazu メールアドレス: 所属: |
| URL | http://hdl.handle.net/10297/7450 |

1 Graphical abstract

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(H. Kawagishi)

Abstract

A novel sphingosine, 1,2-diacetylsphingosine (**1**), was isolated from the edible mushroom *Grifola gargar*. The structure of **1** was determined by the interpretation of spectroscopic data. Compound **1** suppressed the formation of osteoclast without toxicity.

Keywords: mushroom; *Grifola gargar*; osteoclast-forming suppressing; sphingosine; structure determination; structure-activity relationship

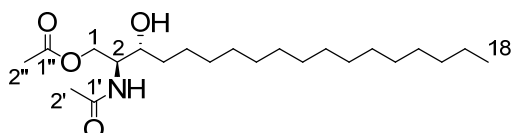
1. Introduction

Osteoporosis is a serious health problem that predominantly affects postmenopausal women and aged people and leads to a high risk of fracture. An imbalance between bone formation mediated by osteoblasts and resorption mediated by osteoclasts is related to metabolic bone diseases, such as osteoporosis and osteopetrosis.^{1,2} Therefore, substances that can suppress the formation of osteoclasts are candidates for drugs or functional foods to cure or prevent osteoporosis. We have reported the isolation of novel osteoclast-forming suppressing compounds, gargalols A to C, and some known steroids, from the edible mushroom *Grifola gargal*.³ *G. gargal* is an edible mushroom with a characteristic almond flavor, collected and eaten by native people of southern Argentina and Chile. The species has only been reported from the Nothofagus-dominated forests of the area. Nutraceutical properties and pharmacological potential of the mushroom have been studied; aqueous extracts of the mushroom showed the anti-oxidant and anti-inflammatory effects and the methanol extracts displayed a free radical scavenging activity.⁴ Commercial production of the mushroom has started in 2009 in Japan.⁵ During further search for osteoclast-formation suppressing compounds from the extracts of *G. gargal*, we obtained an active compound from the mushroom. Here we describe the isolation, structure determination, biological activity

of the compound from the mushroom, and structure-activity relationship among the compound and its analogues.

2. Results and discussion

The powder of dried fruiting bodies of *Grifola gargal* was successively extracted with hexane, EtOAc and then EtOH. Since EtOAc soluble fraction showed the strong suppressing activity against the formation of osteoclast, this fraction was repeatedly subjected to column chromatography, being guided by the result of the bioassay. As a consequence, novel compound **1** was purified.



Compound **1** was isolated as a white powder showing a specific rotation of $[\alpha]_D^{27} +7.5^\circ$ (c 0.34, MeOH). The IR spectrum showed absorption bands at 3269, 2915, 2848, 1738, 1649, 1557 and 1466 cm^{-1} . Its molecular formula was determined as $\text{C}_{22}\text{H}_{43}\text{NO}_4$ by HRESIMS m/z 408.3063 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_{22}\text{H}_{43}\text{NNaO}_4$, 408.3090), indicating the presence of two degrees of unsaturation in the molecule.

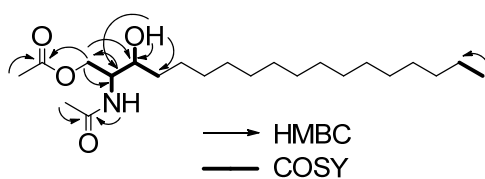


Fig. 1. COSY and HMBC correlations of **1**.

Table 1 ^1H and ^{13}C NMR data for **1** (in CDCl_3)

| Position | 1 | |
|----------|---|--|
| | δ_{H} (multiplicity, J in Hz) | δ_{C} |
| 1 | 4.18 (dd, 11.6, 3.7), 4.33 (dd, 11.6, 6.4) | 63.1 |
| 2 | 4.11 (m) | 53.0 |
| 3 | 3.62 (m) | 72.6 |
| 4 | 1.48 (m) | 34.0 |
| 5-17 | 1.23-1.29 (m) | 22.7, 26.0, 29.3, 29.5, 29.6, 29.7 ^a , 31.9 |
| 18 | 0.86 (t, 6.9) | 14.1 |
| 1' | - | 170.2 |
| 2' | 2.00 (s) | 23.4 |
| 1'' | - | 171.3 |
| 2'' | 2.06 (s) | 20.9 |

^a The signal was much higher than the others.

The DEPT experiment and the molecular formula indicated the presence of 3 methylys, 15 methylenes, 2 methines, and 2 quaternary carbons. The chemical shifts of the two quaternary carbons (δ_{C} 170.2, 171.3) and the HMBC correlations [$\text{H}2''/\text{C}1''$; $\text{H}2'/\text{C}1'$; $\text{NH}(\delta$ 5.92, d, $J = 8.5)/\text{C}1'$] indicated that the unsaturation of the compound was due to acetamido- and acetoxy-groups. The 3-hydroxyoctadecyl acetate moiety was elucidated by the COSY (bold line in Fig. 1) and the HMBC correlations ($\text{H}1/\text{C}2$,

H1/C3, H1/C1'', H18/C17, HO-C3 (δ 2.37, d, $J=6.1$ Hz) /C2, HO-C3/C3, HO-C3/C4, H2''/C1''). The connection of the other part, the acetamido-group, with the moiety was suggested by the molecular formula, the HMBC correlations [H2'/C1', NH(δ 5.92, d, $J = 8.5$)/C1'], and the COSY correlation (H2/NH). All the data allowed us to conclude that the plane structure of **1** was 2-acetamido-3-hydroxyoctadecyl acetate. The complete assignment of all the protons and carbons accomplished by DEPT, HMQC, COSY, and HMBC experiments was shown in Table 1 and Fig. 1. The confirmation of the plane structure and determination of the absolute configuration of **1** were done by comparison of spectroscopic data of each per-acetate (**2**) obtained by acetylation of **1** and commercially available *N*-acetyl-D-erythro-sphingosine (**3**). All the data of each per-acetate (**2**) including their specific rotation are identical with each other; acetate of **1** (**2**), $[\alpha]_D^{29} +11$ (c 0.10, CHCl₃); acetate of **3** (**2**), $[\alpha]_D^{29} +12$ (c 0.13, CHCl₃).

Compounds **1** and its analogues (**2** to **4**) were evaluated by the osteoclast-forming assay. The assay is based on the principle that osteoclast-like multinucleated cells can be formed *in vitro* from co-cultures of mouse bone marrow cells and osteoblastic cells by treatment with osteotropic factors. By adding suppressive agents, the formation of osteoclast is inhibited during the differentiation. As shown in Fig. 2, all the compounds inhibited osteoclast formation dose-dependently and **1**, **3** and **4** showed even at lower

concentration (0.8 $\mu\text{g/mL}$). However, **3** and **4** showed cytotoxicity at 3.1 $\mu\text{g/mL}$ and above. On the other hand, **1** inhibited osteoclast formation by 92% at 25 $\mu\text{g/mL}$ with no cytotoxicity. These results led to the conclusion that hydroxyl group at position of C3 in the sphingosine moiety was indispensable for the activity of the compounds and **1** obtained from the mushroom *Grifola gargar* was the best compound for our purpose among the compounds tested.

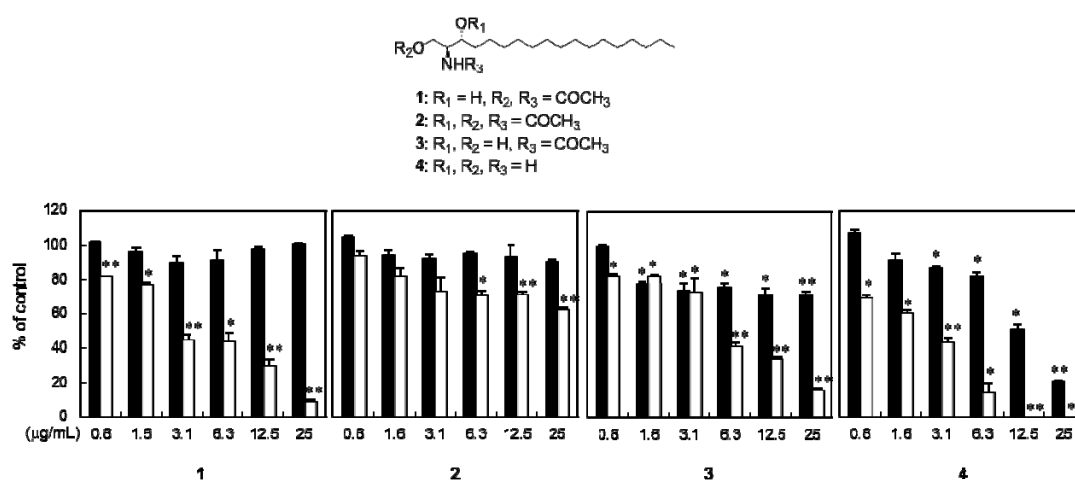


Fig. 2. Inhibition of osteoclast formation by **1** and its analogues (**2** to **4**). Closed and open columns indicate cell viability and osteoclast formation, respectively. TRAP-positive multinucleated cells that had more than three nuclei were counted. Cell viability was determined by MTT assay. Data are the mean \pm SE of two cultures (* $P < 0.01$, ** $P < 0.001$ vs control using Student's *t*-test).

3. Conclusion

A novel compound **1** was isolated from an edible mushroom *Grifola gargar*. The

structure of **1** was determined by the interpretation of spectroscopic data and chemical induction. The structure-activity relationship for osteoclast-forming suppressing activity was considered using **1** and its analogues. As a result, compound **1** was the best compound that inhibited osteoclast formation without cytotoxicity.

4. Experimental

4.1. General

N-Acetyl-D-erythro-sphingosine (**3**) was purchased from EMD Biosciences, Inc. (San Diego, CA). D-Erythro-dihydrosphingosine (**4**) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY). ¹H NMR spectra (one- and two-dimensional) were recorded by a Jeol lambda-500 spectrometer at 500 MHz, while ¹³C NMR spectra were recorded by the same instrument at 125 MHz. The HRESIMS data were measured by a JMS-T100LC mass spectrometer. A Jasco grating infrared spectrophotometer was used to record the IR spectra and the specific rotation values were measured by a Jasco DIP-1000 polarimeter. HPLC separation was performed with a Jasco Gulliver system, using reverse-phase HPLC columns (Develosil C30-UG-15/30 and Develosil C30-UG-5, Nomura Chemical, Japan). A silica gel plate (Merck F₂₅₄) and silica gel 60 N (Kanto Chemical, Japan) were respectively used for analytical TLC and flash column

chromatography.

4.2. Fungal materials

A voucher specimen of the organism is located in Iwade Research Institute of Mycology.

4.3. Extraction and isolation

The powder of dried fruiting bodies of *G. gargal* (8.78 kg) was successively extracted with hexane, EtOAc and then EtOH (5 L, two times). After removing the solvent under reduced pressure, the EtOAc-soluble part (90.1 g) was fractionated by silica gel flash column chromatography (hexane/EtOAc 9:1; CH₂Cl₂; CH₂Cl₂/acetone 8:2; acetone; EtOH and MeOH/H₂O 9:1, 2 L each) to obtain 28 fractions. Fraction 18 (3.6 g) was further separated by a reverse-phase HPLC (Develosil C30-UG-15/30, 90% MeOH) affording 57 fractions. Fraction 18-28 (34.4 mg) was further separated by recrystallization with CH₂Cl₂ and MeOH to afford compound **1** (3.4 mg).

4.3.1. *Compound 1*. White powder; mp 120-121°C; $[\alpha]_D^{27} +7.5^\circ$ (c 0.34, MeOH); IR (neat) 3269, 2915, 2848, 1738, 1649, 1557 and 1466 cm⁻¹; ¹H and ¹³C NMR, see Table

1; ESIMS m/z 408 $[M+Na]^+$; HRESIMS m/z 408.3063 $[M+Na]^+$ (calcd. for $C_{22}H_{43}NNaO_4$, 408.3090).

4.4. Preparation of acetates of **1** and *N*-acetyl-D-erythro-sphingosine

One mg of compound **1** or 2.5 mg of *N*-acetyl-D-erythro-sphingosine (**3**) was dissolved in 0.1 mL anhydrous pyridine in a 4 mL vial, and acetic anhydride (0.1 mL) was added to the solution. After leaving to stand the reaction mixture at room temperature for 24 h, the mixture was added with distilled water and then partitioned between $CHCl_3$ and water. After washing of the $CHCl_3$ layer with 1 N HCl, saturated $NaHCO_3$, and saturated NaCl, the organic phase was dried with anhydrous $MgSO_4$. After removing the solvent under reduced pressure, the residue was purified by a reverse-phase HPLC (Develosil C30-UG-5, 90% MeOH) to give acetate of **1** (**2**; 1.0 mg) or acetate of *N*-acetyl-D-erythro-sphingosine (**2**; 1.2 mg).

4.4.1 Compound 2. White powder; mp 96-97°C; 1H NMR (500 MHz, in $CDCl_3$) δ 4.88 (1H, dd, $J = 13.4, 5.3$ Hz, H2), 4.37 (1H, m, H3), 4.23 (1H, dd, $J = 11.6, 6.1$ Hz, H1a), 4.04 (1H, dd, $J = 11.6, 4.0$ Hz, H1b), 2.05 (3H, s, H2''), 2.03 (3H, s, -O-COMe), 1.98 (3H, s, H2'), 1.58 (2H, m, H4), 1.23-1.26 (36H, m, H5-H17), 0.84 (3H, t, $J = 6.3$ Hz,

H18); ESIMS m/z 450 [M+Na]⁺; HRESIMS m/z 450.3166 [M+Na]⁺ (calcd for C₂₄H₄₅NaNO₅, 450.3195).

4.5. Bioassay

The stromal/osteoblastic cells, UAMS-32, were cultured in an α -minimal essential medium (α -MEM) (ICN Biomedicals, Inc.) containing 10% fetal bovine serum (FBS) for a week. The cells were detached from the culture dishes by using trypsin-EDTA, suspended in α -MEM containing 10% FBS and used for the co-culture as osteoblastic cells. Bone marrow cells were isolated from mice as described previously.⁶ Femoral and tibiae bone marrow cells were collected from 5-week-old mice which had been killed by cervical dislocation. 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 media with a 26-gauge needle. The osteoblastic cells and bone marrow cells collected were washed and used in the co-culture. Osteoclasts were prepared from a co-culture system as previously described.⁷ 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 Inc.). The culture volume was made up to 200 μ L per well with α -MEM supplemented with 10% FBS in the presence of 10^{-8} M

$1\alpha,25(\text{OH})_2\text{D}_3$ (Biomol) and 10^{-6} M PGE_2 , with or without a sample. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. Three-quarter of medium was changed after co-culture for 3 days. After the cultivation, the adherent cells were fixed with 10% formaldehyde in 10 mM phosphate-buffered saline (pH 7.4) for 20 min. After being treated with 95% ethanol for 1 min, the well surface was dried and treated with the TRAP staining solution [0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/mL naphthol AS-MX phosphate (Sigma chemical Co.), and 1 mg/mL fast red violet LB salt (Sigma chemical Co.)] for 30 min. The TRAP-positive multinucleated cells were then counted under a microscope. Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma chemical Co.) assay. After the culture, cells were treated with 1 mg/mL MTT for 2 h, then precipitated dye was solubilized into dimethylsulfoxide, and the absorbance was measured at 570 nm. Data thus collected were analyzed statistically using Student's *t*-test to determine significant difference in the data among the groups. *P* values less than 0.05 were considered significant. The values are expressed as mean \pm SE.

Acknowledgment

This work was partially supported by a Grant-in-Aid for Scientific Research on Innovative Areas “Chemical Biology of Natural Products” from MEXT (Grant Number 24102513).

References and notes

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Figure Legends

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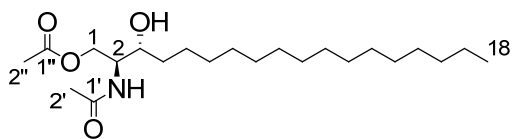
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(* P <0.01, ** P <0.001 vs control using Student's t -test).



Scheme

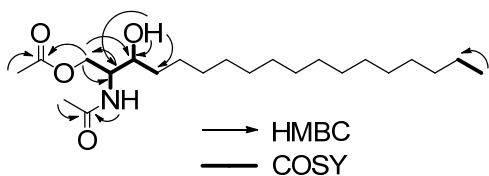


Fig. 1.

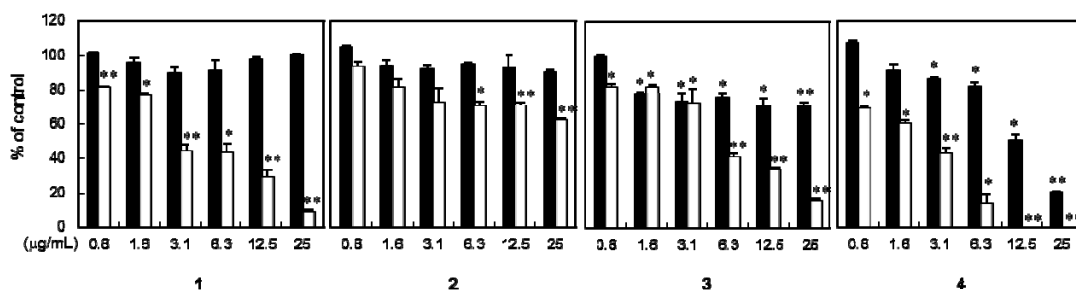
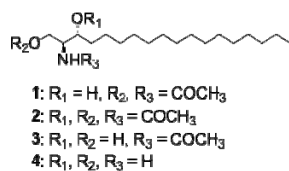


Fig. 2.