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CHAXINE A, AN OSTEOCLAST-FORMING SUPPRESSING SUBSTANCE, FROM THE MUSHROOM *AGROCYBE CHAXINGU*

Hirokazu Kawagishi,[a, b,*] Takuto Akachi,[b] Tetsuhiro Ogawa,[b] Kikuko Masuda,[c] Kohji Yamaguchi,[c] Kazunaga Yazawa,[c] and Mamoru Takahashi[d]

[a] Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

[b] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.

[c] Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7, Konan, Minato-ku, Tokyo, 108-8477, Japan

[d] Kougen Co., Ltd. 3-7-29 Sakuratutumi Suntou-gun, Nagaizumi-cho, 411-0951, Japan

Abstract – A novel compound, chaxine A, (**1**), and a known one (**2**) were isolated from the mushroom *Agrocybe chaxingu*. These compounds suppressed the formation of osteoclast.

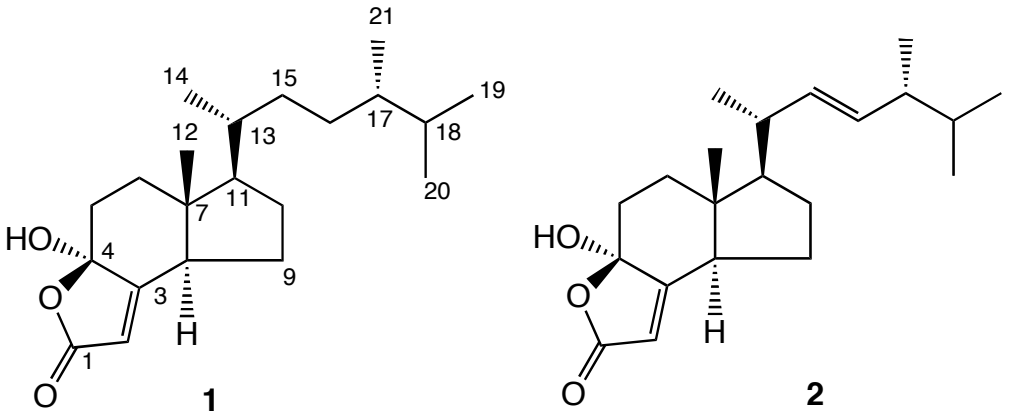
INTRODUCTION

Osteoporosis is caused by an imbalance between bone resorption and bone formation, which results in bone loss and fractures after mineral flux. The hip fracture in senile patients is a very serious problem because it often limits their quality of life. Osteoclasts are multinucleated, giant cells that are primarily responsible for bone resorption. The most characteristic feature of osteoclasts is the presence of ruffled borders and clear zone. Osteoclast-like multinucleated cells can be differentiated *in vitro* from co-cultures of mouse bone marrow cells and osteoblastic cells by treatment with osteotropic factors, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and prostaglandin E_2 (PGE_2).[1] During screening for

osteoclast-formation suppressing effects of the extracts of various mushrooms by using the assay, we found very strong activity in the extract of the mushroom *Agrocybe chaxingu*. Therefore, an attempt was made to isolate the active principles from the mushroom and to determine their structures. This mushroom grows in dry and died boles of broadleaf such as grease tea plant and poplar, and exists only in mountainous areas in South China.

RESULTS AND DISCUSSION

Powder of the dried fruiting bodies of *Agrocybe chaxingu* was extracted with $CHCl_3$, EtOAc and then EtOH. The $CHCl_3$-soluble fraction only showed the suppressing activity. After repeated chromatography of the fraction, compounds (**1**) and (**2**) were purified as the active principles.



FABMS of chaxine A (**1**) showed the [M+H] ion at *m/z* 335. The molecular formula, $C_{21}H_{34}O_3$, of the compound was determined by HRESIMS of the $[M+Na]^+$ ion (data given in experimental). The ^1H- and ^{13}C-NMR data of 1 were similar to those of (17*R*)-4-hydroxy-17-methylincisterol (**2**) (Table 1). [2-4] However, **1** has two sp^3 carbons instead of an olefin in **2**. The plane structure of **1** was confirmed by HMBC correlations (Figure 1). Since the NMR chemical shifts, especially those of C1 to C12, were very similar to those of **2**, relative stereochemistry of **1** is probably same as that of **2**. The absolute configuration of **2** has been already determined. Comparison of the $[\alpha]_D$ of **1** (+133°, c = 0.10, MeOH) with that of **2** (+130°, c = 0.69, MeOH) allowed us to deduce that the absolute configuration of **1** was as proposed. However, further confirmation is necessary to determine the stereochemistry.

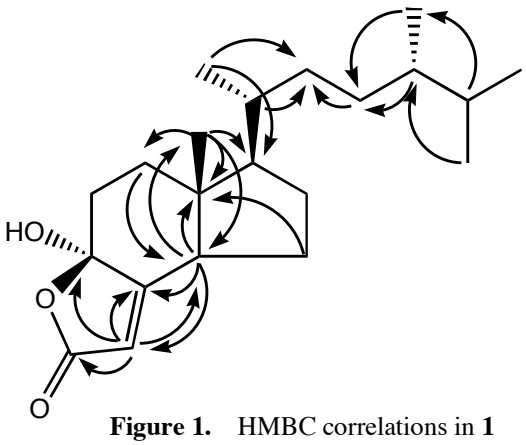


Figure 1. HMBC correlations in **1**

Table 1. NMR data for **1** and **2** ($CDCl_3$)[a]

Position	**1**		**2**	
	δ_H(multiplicity, *J* in Hz)	δ_C	δ_H(multiplicity, *J* in Hz)	δ_C
1	–	171.0	–	171.0
2	5.61 (s)	112.4	5.57 (d, 1.5)	112.3
3	–	170.9	–	170.6
4	–	105.0	–	104.8
5	1.85 (m), 2.25 (m)	35.3	1.82 (m), 2.28 (m)	35.3
6	1.59 (m), 1.99 (m)	35.2	1.61 (m), 1.95 (m)	35.0
7	–	48.9	–	48.9
8	2.62 (m)	50.3	2.63 (m)	50.3
9	1.50 (m), 1.73 (m)	21.4	1.48 (m). 1.69 (m)	21.4
10	1.45 (m). 2.06 (m)	28.6	1.45 (m), 1.85 (m)	28.8
11	1.45 (m)	55.4	1.47 (m)	55.3
12	0.58 (s)	11.5	0.57 (s)	11.7
13	1.38 (m)	36.2	2.05 (m)	40.1
14	0.92 (d, 6.1)	18.9	1.01 (d, 6.7)	21.0
15	0.97 (m), 1.40 (m)	33.5	5.14 (dd, 15.3, 8.2)	134.6
16	0.96 (m), 1.38 (m)	30.5	5.22 (dd, 15.2, 6.7)	132.9
17	1.20 (m)	39.0	1.84 (m)	42.8
18	1.55 (m)	31.5	1.46 (m)	33.0
19, 20	0.77 (d, 7.0), 0.84 (d, 7.0)	17.6, 20.5	0.80 (d, 7.0), 0.81 (d, 7.0)	19.6, 19.9
21	0.77 (d, 7.0)	15.4	0.92 (d, 6.7)	17.6

[a] These assignments were established by DEPT, HMQC and HMBC experiments.

Compound (**2**) has been reported as an intermediate in the synthesis of (17*R*)-17-methylincisterol.[2] This compound also has been known as an inhibitor of DNA polymerase-α and as a cytotoxic compound against several kinds of tumor cells. [3,4]

Osteoclast differentiation was estimated by TRAP-(+) multinucleated cell formation in osteoblastic cells/bone marrow cells co-culture.[1,5,6] The number of differentiated osteoclast in the absence of the samples was 720.31 ± 17.1 cells/cm^2. The addition of **1** and **2** (1.6 μg/ml, 4.8 μM) reduced the number of TRAP-(+) multinucleated cells to 6.7% and 55.0% without cytotoxicity, respectively (Figure 2). Bone resorption is suppressed by inhibition of osteoclast formation. Therefore, **1** and **2** might be applicable for the prevention of bone diseases such as postmenopausal osteoporosis.

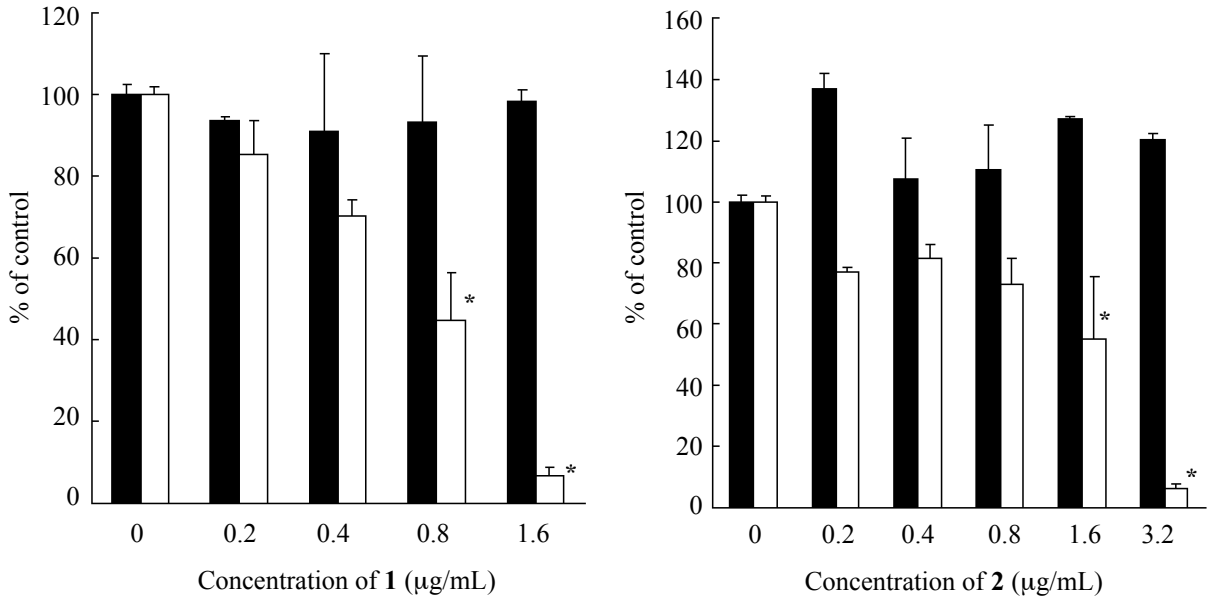


Figure 2. Inhibition of osteoclast formation by **1** and **2**

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 ± S.E of two cultures (*$p<0.05$ vs. Control using Student's t-test).

EXPERIMENTAL

The ^{1}H-NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same instrument at 125 MHz. The FABMS spectra were recorded on a JEOL DX-303HF and the HRESIMS spectra were measured on a JMS-T100LC mass spectrometer. A JASCO grating infrared spectrophotometer was used to record the IR spectra. The $[\alpha]_D$ spectra were measured by using a JASCO DIP-1000 spectropolarimeter. MPLC was done with a YAMAZEN MPLC-system (Japan) and an UltraPack ODS-S50D column (50 x 300 mm, YAMAZEN, Japan). HPLC separations were performed with a JASCO Gulliver system using an ODS column (Grandpack ODS-A S-5 YC, 20 x 300 mm, Masis, Japan). Silica gel plate (Merck F254) and silica gel 60 N (Merck 100-200 mesh) were used for analytical TLC and for flash column chromatography, respectively.

Fungus Materials

Mature fruiting bodies of *Agrocybe chaxingu* were collected in Fujian Sheng, China. Voucher specimens were deposited in the Faculty of Agriculture, Shizuoka University.

Extraction and Isolation

Powder of the dried fruiting bodies of *Agrocybe chaxingu* (1.5 kg) was successively extracted with $CHCl_3$, EtOAc and then EtOH. The $CHCl_3$-soluble part (34.0 g) (EtOAc-soluble part, 3.4 g; EtOH soluble part, 72.8 g) was fractionated by silica gel flash column chromatography (90% n-hexane/EtOAc, 95%, 90% CH_2Cl_2/acetone, 90% CH_2Cl_2/ MeOH, MeOH, each 2 L) to obtain sixteen fractions. Fraction 8 (3.5 g) was further separated by reversed-phase MPLC (95% MeCN) to afford thirty-eight fractions. Finally, compounds (**1**) (1.4 mg) and (**2**) (8.1 mg) were purified from fraction 8-10 (100.2 mg) by reversed-phase HPLC (80% AcCN).

Chaxine A (**1**): colorless oil. IR (neat) ν_{max} cm^{-1}: 3628, 1733; FABMS (matrix, glycerol) *m/z* 335 $(M+H)^+$; HRESIMS *m/z* 357.2409 [calcd for $C_{21}H_{34}NaO_3$ $(M+Na)^+$, 357.2406].

Bioassay

The stromal/osteoblastic cells. UAMS-32, were cultured in α-minimal essential medium (α-MEM) (ICN Biomedicals, Inc.) containing 10 % fetal bovine serum (FBS) for 1 week. The cells were then 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.[5] 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 the 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.[6] 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 μl per well with α-MEM supplemented with 10% FBS, in the presence of 10^{-8} M 1α, $25(OH)_2D_3$ (Biomol) and 10^{-6} M PGE_2, with or without the samples. 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 cell cultures, adherent 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 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. Then TRAP-positive multinucleated cells were 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 culture, cells were treated with 1 mg/ml MTT for 2 h, then precipitated dye was solubilized into dimethylsulfoxide, and the absorbance at 570 nm was measured.
Data were statistically analyzed 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 ± S. E.

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