## Note

# Osteoclast-Forming Suppressive Compounds from Makomotake, Zizania latifolia Infected with Ustilago esculenta

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A novel compound (1) and a known one (2) were isolated from Makomotake, *Zizania latifolia* infected with *Ustilago esculenta*, as osteoclast-forming suppressive substances.

Key words: osteoclast-forming suppressive substance; Makomotake; Zizania latifolia; Ustilago esculenta

Osteoclasts play a role in bone resorption, and abnormal acceleration of osteoclast formation is one the causes of osteoporosis. Therefore, substances which can suppress the formation of osteoclasts are candidates for drugs or functional foods to prevent osteoporosis. During our screening for the osteoclast-formation suppressive effects of extracts of various foodstuffs, we found strong activity in an extract of Makomotake.

The fungus, *Ustilago esculenta*, penetrates into the aquatic perennial grass, *Zizania latifolia*. After penetration, the fungus incites the formation of an edible gall and inhibits inflorescence and seed production in the plant. The gall is called Makomotake in Japanese and used as a foodstuff in Japan, China and other Asian countries.

We attemped to isolate the active principles from Makomotake and succeeded in purifying the compounds.

General experimental procedure. NMR spectra were measured with a Jeol Lambda-500 spectrometer and are given in ppm ( $\delta$ ) downfield from internal TMS. The FABMS data were recorded by a Jeol DX-303HF instrument and the HRESIMS data were measured by a JMS-T100LC mass spectrometer. A Jasco grating infrared spectrophotometer was used to record the IR spectra. The specific rotation values were measured by using a Jasco DIP-1000 spectropolarimeter. MPLC was done with a Yamazen MPLC system (Japan) and an UltraPack Diol-40D column ( $50 \times 300$  mm, Yamazen, Japan). HPLC separation was performed with a Jasco Gulliver system, using an ODS column (Grandpack ODS-A S-5 YC,  $20 \times 300$  mm; Masis, Japan). Silica gel plates (Merck F254) and silica gel 60 N (Merck 100–200 mesh) were respectively used for analytical TLC and flash column chromatography.

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*Extraction and isolation.* Fresh Makomotake (16.7 kg) was extracted with EtOH (221, 3 times) and then with acetone (101, once). After the solutions had been combined and concentrated under reduced pressure, the concentrate was partitioned between  $CH_2Cl_2$  and  $H_2O$ , and then between EtOAc and  $H_2O$ . The residue (58.7 g) obtained after removing  $CH_2Cl_2$  was fractionated by silica gel flash column chromatography (90% hexane/EtOAc,  $CH_2Cl_2$ , 80%  $CH_2Cl_2/acetone$ , 50%  $CH_2Cl_2/MeOH$ , MeOH, each 21) to obtain seventeen fractions. Fraction 11 (1.55 g) was further separated by MPLC (90%, 70%, 50% hexane/EtOAc), and eighteen fractions were obtained. Fraction 11-5 (55.6 mg) was separated by twice-repeated reversed-phase HPLC (99% MeOH) to afford compounds **1** (1.1 mg) and **2** (2.7 mg).

*Bioassay.* The stromal/osteoblastic cells, UAMS-32, were cultured in an  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) (ICN Biomedicals) containing 10% fetal bovine serum (FBS) for 1 week. The cells were then detached from the culture dishes by using trypsin-EDTA, suspended in  $\alpha$ -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 which had been killed by cervical dislocation as

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#### Osteoclast-Forming Suppressive Compounds from Makomotake

Table 1.	<sup>1</sup> H- and <sup>13</sup> C-NMR	Data for 1 a	nd 2 (in $CDCl_3$ ) <sup>a</sup>
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Position	1		2		
	$\delta_{\rm H}$ (multiplicity, J in Hz)	$\delta_{\mathrm{C}}$	$\delta_{\rm H}$ (multiplicity, J in Hz)	$\delta_{\rm C}$	
1	1.60 (m)	34.7	1.60 (m), 2.06 (m)	38.1	
2	1.40 (m), 1.88 (m)	27.5	2.31 (m), 2.39 (m)	37.4	
3	_	100.2	_	211.3	
4	1.49 (m), 1.97 (m)	27.9	2.31 (m), 2.58 (m)	37.0	
5	2.41 (dd, 12.0, 2.7)	55.1	2.58 (m)	57.5	
6	_	211.8	_	209.1	
7	1.95 (m), 2.28 (dd, 13.2, 4.5)	46.8	1.98 (dd, 12.8,12.8), 2.37 (dd, 12.8, 4.6)	46.6	
8	1.76 (m)	38.0	1.84 (m)	38.0	
9	1.30 (m)	53.6	1.31 (m)	53.5	
10		41.1	_	41.3	
11	1.30 (m), 1.58 (m)	21.5	1.42 (m), 1.63 (m)	21.7	
12	1.20 (m), 2.01 (m)	39.5	1.20 (m), 2.04 (m)	39.4	
13	_	43.0	_	43.0	
14	1.20 (m)	56.7	1.23 (m)	56.6	
15	1.05 (m), 1.52 (m)	24.0	1.08 (m), 1.53 (m)	24.0	
16	1.25 (m), 1.86 (m)	28.1	1.25 (m), 1.85 (m)	28.0	
17	1.13 (m)	56.0	1.14 (m)	56.0	
18	0.64 (s)	11.9	0.69 (s)	12.0	
19	0.72 (s)	12.6	0.94 (s)	12.6	
20	1.35 (m)	36.1	1.35 (m)	36.0	
21	0.90 (d, 6.4)	18.7	0.91 (d, 6.4)	18.7	
22	1.00 (m), 1.30 (m)	33.9	1.00 (m), 1.31 (m)	33.8	
23	1.13 (m)	26.1	1.15 (m)	26.1	
24	0.90 (m)	45.8	0.90 (m)	45.8	
25	1.65 (m)	29.1	1.65 (m)	29.1	
26, 27	0.79 (d, 6.7), 0.81 (d, 6.7)	19.0, 19.8	0.79 (d, 6.7), 0.82 (d, 6.7)	19.0, 19.8	
28	1.25 (m)	23.1	1.25 (m)	23.1	
29	0.83 (t, 9.8)	12.0	0.83 (t, 7.6)	12.0	
3-OMe	3.10 (s), 3.20 (s)	47.4, 47.7			

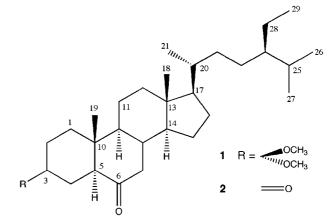
<sup>a</sup>These assignments were established by DEPT, HMQC and HMBC experiments.

described previously.<sup>1)</sup> 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. The osteoblastic cells and bone marrow cells collected were washed and used in the co-culture. The osteoclasts were prepared from a co-culture system as previously described.<sup>2)</sup> The osteoblastic cells  $(1.0 \times 10^4)$ cells/well) were co-cultured with the bone marrow cells  $(2.0 \times 10^7 \text{ cells/well})$  in  $\alpha$ -MEM containing 10% FBS in 96-well plates (Corning). The culture volume was made up to 200  $\mu$ l per well with  $\alpha$ -MEM supplemented with 10% FBS in the presence of  $10^{-8}$  M 1 $\alpha$ , 25dihydroxyvitamin D<sub>3</sub> (Biomol) and 10<sup>-6</sup> M prostaglandin E<sub>2</sub>, with or without a sample.<sup>3)</sup> All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2 in air. Three-quarters of the medium was changed after co-culturing for 3 days.

After the cultivation, the adhering 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 of naphthol AS-MX phosphate (Sigma Chemical Co.), and 1 mg/ml of 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 at 570 nm was measured.



Compound 1 was isolated as white crystals. FABMS of 1 showed a molecular ion peak at m/z 475 (M + H)<sup>+</sup>. Its molecular formula was determined as C<sub>31</sub>H<sub>54</sub>O<sub>3</sub> by HRESIMS [m/z 497.3962 (calcd. for C<sub>31</sub>H<sub>54</sub>NaO<sub>3</sub>

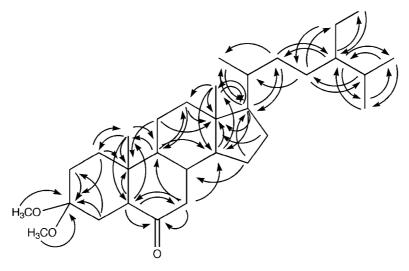


Fig. 1. HMBC Correlations in 1.

 $(M + Na)^+$ , 497.3971)], indicating the presence of five degrees of unsaturation in the molecule. A band at 1714 cm<sup>-1</sup> was observed in the IR spectrum. The <sup>1</sup>Hand <sup>13</sup>C-NMR data for 1 were very similar for those of 2 (Table 1). However, **1** had an sp<sup>3</sup> quarternary carbon ( $\delta_{\rm C}$ 100.2) attached to two methoxy groups [ $\delta_H$  3.10 (s), 3.20 (s);  $\delta_{\rm C}$  47.2, 47.4) instead of the carbonyl group in 2 (Table 1). The complete planar structure of 1 was confirmed by HMBC correlations (Fig. 1). The absolute configuration of the compound was deduced as proposed by comparing its specific rotation,  $[\alpha]_D + 75^\circ$  (c = 0.11, CHCl<sub>3</sub>), with that of **2**,  $[\alpha]_D + 27^\circ$  (c = 0.19, CHCl<sub>3</sub>).<sup>4)</sup> However, further confirmation of the stereochemistry is necessary. Compound 1 was not an artifact, because HPLC and NMR analyses indicated that the EtOH extract of this mushroom also contained this compound.

Compound **2** has been isolated as an antimutagenic constituent from the thorns of *Gleditsia sinebsis*, and the full assignments of all the <sup>1</sup>H- and <sup>13</sup>C-NMR signals and determination of the stereochemistry were made by various spectral data.<sup>4,5)</sup>

Compounds 1 and 2 were evaluated in an osteoclastforming suppression assay. The addition of 1 ( $25 \mu g/ml$ , 53  $\mu$ M) and 2 ( $25 \mu g/ml$ , 58  $\mu$ M) reduced the respective number of TRAP-(+) multinucleated cells to 49% and 19% without cytotoxicity.

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