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Osteoclast-forming suppressing compounds, gargalols A, B, and C, from the edible mushroom *Grifola gargal*

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ABSTRACT

Three novel sterols, gargalols A–C (**1**–**3**), and four known ones were isolated from the edible mushroom *Grifola gargal*. The structures of **1**–**7** were determined or identified by the interpretation of spectroscopic data. Compounds **1**–**5** suppressed the formation of osteoclast without toxicity.

1. Introduction

Osteoporosis is a serious health problem that predominantly affects postmenopausal women and aged people leading to a high risk of fracture. Osteoclasts are derived from the monocyte/macrophage cell lineage and are specialized cells responsible for the breakdown of bone.[1] The progressive bone loss is due to both an increase in osteoclastic bone resorption and a decrease in osteoblastic bone formation.[2] Therefore, substances, which can suppress the formation of osteoclasts are candidates for therapy or can be used as supplements or functional foods to prevent osteoporosis. Recently, beneficial effects of natural products and their derivatives that affect the process of bone remodeling, in particular bone resorption, have been reported. For example, two sterols were isolated as suppressive compounds from the edible mushroom *Leccinum extremiorientale* by us.[3] Earlier we have reported the isolation of novel osteoclast-forming suppressing compounds, chaxines A–C and some steroids, from the edible mushroom *Agrocybe chaxingu*.[4–6]

During further screening for the osteoclast-formation suppressing effects of the extracts of various mushrooms, we found strong activity in the extract of the mushroom *Grifola gargal*, and tried to isolate the active principles from the mushroom.

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.[7] Commercial production of the mushroom has just started in Japan.[8] Here we describe the isolation, structural determination, and biological activity of compounds from the mushroom.

2. Results and discussion

The dried fruiting bodies of *G. gargal* were extracted with hexane, EtOAc and then with EtOH subsequently. 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, three novel compounds (**1**–**3**) and four known ones were purified.

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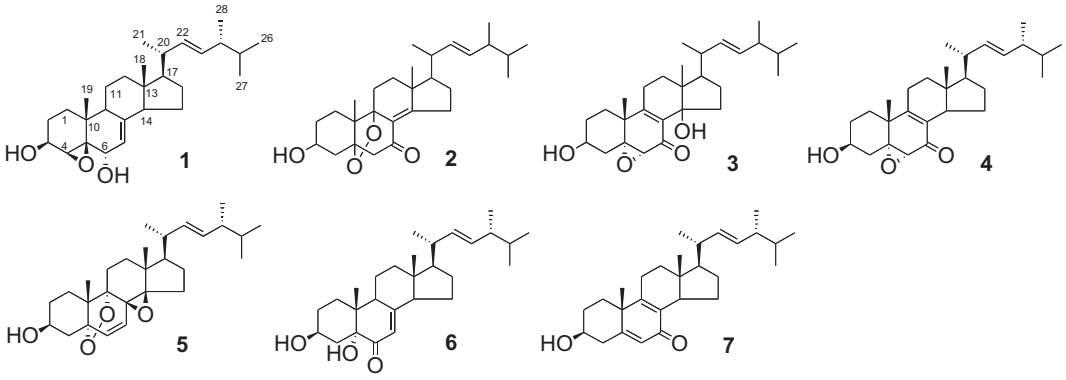

Gargalol A (**1**) was purified as a white powder. Its molecular formula was determined as $C_{28}H_{44}O_3$ by HRESIMS *m*/*z* 451.3156 $[M+Na]^+$ (calcd for $C_{28}H_{44}NaO_3$, 451.3188), indicating the presence of seven degrees of unsaturation in the molecule. The structure of **1** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMBC, and HMQC. The DEPT experiment indicated the presence of six methyls, six methylenes, twelve methines, and four quaternary carbons. In the NMR spectra of **1**, typical signals of a sterol, such as a side-chain olefine (C-22, δ_H 5.14, dd, J=15.3, 6.7 Hz; δ_C 135.3: C-23, δ_H 5.20, dd, J=15.3, 8.5 Hz; δ_C 132.2), a hydroxymethine (C-3, δ_H 4.06, m; δ_C 62.7), four doublet methyls (C-21, δ_H 1.00, d, J=6.4 Hz; δ_C 21.0: C-26, 27, δ_H 0.80, d, J=6.8 Hz, 0.82, d, J=6.8 Hz; δ_C 19.6, 19.9: C-28, δ_H 0.89, d, J=6.4 Hz; δ_C 17.6), and two singlet methyls (C-18, δ_H 0.57, s; δ_C 12.2: C-19, δ_H 1.04, s; δ_C 21.1) were observed. The complete assignment of the protons and carbons and the HMBC correlations were summarized in Table 1. The structure including relative stereochemistry of **1** was confirmed by X-ray crystallography analysis (Fig. 1). In addition, the absolute configuration of **1** was determined by circular dichroic exciton chirality method using its dinaphthoate (λ_{max} ($\Delta\varepsilon$); 242 (+20.4), 230 (−12.6) nm).[9] As a result, the structure of **1** was determined to be 4β,5β-epoxy-(22*E*)-ergosta-7,22-dien-3β,6α-diol.

Gargalol B (**2**) was isolated as a white powder. Its molecular formula was determined as $C_{28}H_{42}O_4$ by HRESIMS *m*/*z* 441.2983 $[M-H]^-$ (calcd for $C_{28}H_{41}O_4$, 441.3005) and the degree of unsaturation of the compound was eight. The NMR data of **2** were similar to those of **1** (Table 1), suggesting this compound was also a sterol. The structure elucidation was accomplished in the same manner as **1** (Table 1). The DEPT experiment indicated the presence of six methyls, eight methylenes, seven methines, and seven quaternary carbons. The molecular formula, the HMBC correlations (Fig. 2 and Table 1) and the chemical shifts indicated the presence of a hydroxyl (δ_H 3.93, m; δ_C 66.0), an enone (δ_C 197.8, 126.5, 172.4), and a peroxide (δ_C 85.9, 85.8) at the positions of C-3, C-7/8/14, and C-5/C-9. As a result, the planar structure of **2** was determined as shown. However, since any significant NOE was not observed, the stereochemistry of **2** could not be determined.

Gargalol C (**3**) was purified as a white powder. Its molecular formula was determined as $C_{28}H_{42}O_4$ by HRESIMS *m*/*z* 465.2877 $[M+Na]^+$ (calcd for $C_{28}H_{42}NaO_4$, 465.2890) and the degree of unsaturation of the compound was eight. The NMR data of **3** were similar to those of **1** and **2** (Table 1). The molecular formula, the HMBC correlations (Fig. 2 and Table 1) and the chemical shifts indicated the presence of two hydroxyls (C-3, δ_H 3.97, m; δ_C 68.7: C-14, δ_C 80.8), an epoxide (C-5, δ_C 65.6: C-6, δ_H 3.34, s; δ_C 62.3), and an enone (C-7, δ_C 200.2; C-8, δ_C 133.0; C-9, δ_C 157.0). Since an NOE was observed between H-6 and H-19 in the NOE difference and NOESY experiments, the relative configuration at C-5, C-6, and C-19 was determined as shown. However the stereochemistry of the other parts remains unknown.

Compound **4** was purified as a white powder. Its molecular formula was determined as $C_{28}H_{42}O_3$ by HRESIMS *m*/*z* 425.3029 $[M-H]^-$ (calcd for $C_{28}H_{41}O_3$, 425.3056). The ^1H and ^{13}C NMR data of **4** were very similar to those of **3** (Table 1). The comparison of the molecular formula of **4** with that of **3** indicates that **4** is a dehydroxylated form of **3**. The position of the missing hydroxy was elucidated by the chemical shift of position 14 (δ_H 2.11; δ_C 48.8) and the HMBC correlations (Table 1). Compound **4** has been previously reported as a product of thermal rearrangement of ergosterol peroxide but its spectroscopic data have not been given in the report.[10] This is the first report of isolation of **4** from a natural source. The absolute configuration of **4** was determined by comparison of its specific rotation value with that reported previously:[10] compound **4**, $[\alpha]_D^{27}$ +43.0 (*c* 1.10, $CHCl_3$); reported data, $[\alpha]_D^{25}$ +40.6 (*c* 1.10, $CHCl_3$). As a result, the structure of **4** was determined as shown.

The data of NMR, MS, IR and specific rotation of **5** were identical with those of the compound that had been isolated from two kinds of mushrooms, *Pleurotus eryngii* and *Panellus serotinus* and whose stereochemistry had been determined as 3β-ol, 5α,9α-epidioxy, 8α,14α-epoxy, by interpretation of the NOESY data.[11] However, X-ray crystallography analysis on a *p*-bromobenzoate of **5** in this study indicated that **5** was 5α,9α-epidioxy-8β,14β-epoxy-(22*E*)-ergosta-6,22-dien-3β-ol (Fig. 1).

Based on the comparison of the spectroscopic data for **6** and **7** with those reported in previous papers,[12,13] **6** and **7** were identified as 3β,5α-dihydroxy-(22*E*)-ergosta-7,22-diene-6-one and 3β-hydroxy-(22*E*)-ergosta-5,8,22-triene-7-one, respectively.

Compounds **1**–**7** were evaluated in 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. 3, **2** and **4** inhibited osteoclast formation at lower concentration (0.78 μg/mL) than the other compounds, and **5** exhibited the activity dose-dependently and the activity was the strongest among all the compounds. On the other hand, compounds **6** and **7** significantly showed cytotoxicity even at 1.56 μg/mL (data not shown). The structure–activity relationship and the mode of action of the compounds remain unsolved.

3. Experimental

3.1. General

^1H 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 HRESIMS spectra were measured on a JMS-T100LC mass

Table 1
^{1}H and ^{13}C NMR data for **1**–**4** (in $CDCl_3$)

Position	**1**			**2**		
	δ_H (multiplicity, *J* in Hz)	δ_C	HMBC correlation	δ_H (multiplicity, *J* in Hz)	δ_C	HMBC correlation
1	1.23 (m), 1.42 (m)	25.0	C-2, 3, 5, 9, 10	1.42 (m), 2.04 (m)	28.2	C-2, 3, 5, 10
2	1.44 (m), 1.62 (m)	26.5	C-1, 3, 4, 10	1.45 (m), 1.99 (m)	31.4	C-3, 10
3	4.06 (m)	62.7	C-1	3.93 (m)	66.0	—
4	3.76 (m)	58.0	C-2, 3, 5, 6	1.45 (m), 2.13 (m)	37.3	C-2, 3, 5, 10
5	—	70.2	—	—	85.9	—
6	4.61 (br s)	65.2	C-4, 5, 7, 8	2.46 (d, 19.2), 2.56 (d, 19.2)	50.4	C-4, 5, 7, 8, 10
7	5.19 (m)	119.1	C-5, 14	—	197.8	—
8	—	142.2	—	—	126.5	—
9	2.17 (m)	41.7	C-7, 8, 10, 11, 14, 19	—	85.8	—
10	—	35.6	—	—	51.4	—
11	1.41(m), 1.52 (m)	22.6	C-8, 9, 12, 13	1.93 (m), 1.98 (m)	23.6	C-8, 9, 10, 12, 13
12	1.27 (m), 2.02 (m)	39.1	C-9, 11, 13, 14, 17, 18	1.45 (m), 1.96 (m)	33.0	C-13, 14, 17
13	—	43.9	—	—	46.0	—
14	1.88 (m)	55.1	C-7, 8, 13, 15, 18	—	172.4	—
15	1.43(m), 1.53 (m)	22.3	C-8, 13, 14, 16	2.74 (dd, 21.5, 8.9) 2.98 (ddd, 21.5, 9.2, 9.2)	31.0	C-8, 13, 14, 16, 17
16	1.27 (m), 1.74 (m)	28.1	C-13, 15, 17	1.45 (m), 1.79 (m)	27.7	C-13, 14, 17
17	1.27 (m)	55.9	C-13, 16, 18, 22	1.33 (m)	54.8	C-12, 13, 16, 20
18	0.57 (s)	12.2	C-12, 13, 14, 17	0.90 (s)	17.6	C-13, 14, 17
19	1.04 (s)	21.1[a]	C-1, 5, 9, 10	1.05 (s)	16.0	C-1, 5, 9, 10
20	2.01 (m)	40.4	C-17, 21, 22, 23	2.13 (m)	38.5	C-17, 22, 23
21	1.00 (d, 6.4)	21.0[a]	C-17, 20, 22	1.02 (d, 6.7)	21.4	C-17, 20, 22
22	5.14 (dd, 15.3, 6.7)	135.3	C-20, 21, 23, 24	5.19 (m)	132.9	C-20, 21, 23, 24
23	5.20 (dd, 15.3, 8.5)	132.2	C-20, 22, 24, 25, 28	5.22 (m)	134.6	C-20, 22, 24, 25
24	1.84 (m)	42.8	C-22, 23, 25, 26, 28	1.85 (m)	42.9	C-22, 23, 25, 26, 27, 28
25	1.45 (m)	33.1	C-23, 24, 26, 27	1.45 (m)	33.3	C-23, 24, 26, 27
26	0.80 (d, 6.8)	19.6	C-24, 25, 27	0.80 (d, 6.7)	20.0	C-24, 25, 27
27	0.82 (d, 6.8)	19.9	C-24, 25, 26	0.82 (d, 6.7)	19.6	C-24, 25, 26
28	0.89 (d, 6.4)	17.6	C-23, 24, 25	0.90 (d, 6.7)	17.5	C-23, 24, 25

Position	**3**			**4**		
	δ_H (multiplicity, *J* in Hz)	δ_C	HMBC correlation	δ_H (multiplicity, *J* in Hz)	δ_C	HMBC correlation
1	1.69 (m), 1.91 (m)	28.9	C-2, 3, 5, 9, 10	19 1.70 (m), 1.84 (m)	30.4	C-3, 5, 9, 10, 19
2	1.69 (m), 2.06 (m)	30.3	C-3, 4, 10	1.70 (m), 2.02 (m)	30.6	C-4, 10
3	3.97 (m)	68.7	—	3.93 (m)	68.4	C-2, 4
4	1.52 (m), 2.27 (m)	38.3	C-2, 3, 5, 6, 10	1.49 (m), 2.24 (m)	38.2	C-3, 5, 6, 10
5	—	65.6	—	—	64.5	—
6	3.34 (s)	62.3	C-4, 5, 7, 8	3.25 (s)	62.4	C-4, 5, 7, 10
7	—	200.2	—	—	196.7	—
8	—	133.0	—	—	128.7	—
9	—	157.0	—	—	158.0	—
10	—	40.7	—	—	40.5	—
11	2.10 (m), 2.14 (m)	23.0	C-8, 9, 12, 13	2.21 (m), 2.23 (m)	25.6	C-8, 9, 13
12	1.47 (m), 1.69 (m)	29.8	C-9, 11, 13, 18	1.45 (m), 1.96 (m)	35.6	C-9, 11, 13, 14, 17, 18
13	—	44.8	—	—	42.1	—
14	—	80.8	—	2.11 (m)	48.8	C-8, 9, 13, 15
15	1.74 (m), 1.89 (m)	35.4	C-8, 13, 14, 16, 17	1.80 (m), 1.95 (m)	24.3	C-8, 14
16	1.38 (m), 1.47 (m)	26.0	C-13, 14, 15, 17, 20	1.33 (m), 1.74 (m)	29.4	C-13, 14, 15, 17
17	1.44 (m)	44.8	C-16, 22	1.10 (m)	53.3	C-12, 13, 14, 18, 20, 21, 22
18	0.93 (s)	16.4	C-12, 13, 14, 17	0.55 (s)	11.5	C-12, 13, 14, 17
19	1.21 (s)	23.0	C-1, 5, 9, 10	1.21 (s)	24.1	C-5, 9, 10
20	2.06 (m)	39.6	C-22, 23	1.99 (m)	40.3	—
21	1.01 (d, 6.5)	21.0	C-17, 20, 22	0.99 (d, 7.3)	21.0	C-17, 20, 22
22	5.11 (dd, 15.3, 6.7)	134.8	C-20, 21, 23, 24	5.12 (dd, 15.9, 7.3)	135.3	C-17, 20, 21, 24
23	5.20 (dd, 15.3, 7.6)	132.8	C-20, 22, 24, 25, 28	5.19 (dd, 15.9, 7.3)	132.2	C-20, 24, 25, 28
24	1.83 (m)	42.8	C-22, 23, 25, 26, 27, 28	1.80 (m)	42.8	C-22, 23, 26, 27
25	1.45 (m)	33.1	C-23, 24, 26, 27, 28	1.44 (m)	33.0	C-23, 24, 26, 27
26	0.79 (d, 7.0)	19.6	C-24, 25, 27	0.78 (d, 7.3)	19.6	C-24, 25, 27
27	0.81 (d, 7.0)	19.9	C-24, 25, 26	0.79 (d, 7.3)	19.9	C-24, 25, 26
28	0.88 (d, 7.0)	17.5	C-23, 24, 25	0.88 (d, 7.3)	17.5	C-23, 24, 25

[a] Interchangeable.

spectrometer. CD spectrum was recorded on a JASCO J-820 spectropolarimeter. A JASCO grating infrared spectrophotometer was used to record the IR spectra. The specific rotation values were measured by using a JASCO DIP-1000 polarimeter. HPLC separations were performed with a JASCO Gulliver system using reverse-phase HPLC columns (Develosil C30-UG-15/30, Nomura chemical Co., Ltd., Japan; Wakosil-II 5C18 HG Hrep, Wako, Japan; Capcell PAK C18 AQ, Shiseido, Japan; COSMOSIL Cholester water, Nacalai tesque, Japan; Phenomenex Luna PFP (2), Shimadzu GLC Ltd., Japan) and a normal-phase HPLC column (Senshu PAK AQ, Senshu scientific Co., Ltd., Japan). Silica gel plate (Merck F_{254}) and silica gel 60N (Merck 100–200 mesh) were used for analytical TLC and for flash column chromatography, respectively.

3.2. Fungus materials

A voucher specimen of the organism is located in Iwadekingaku laboratory.

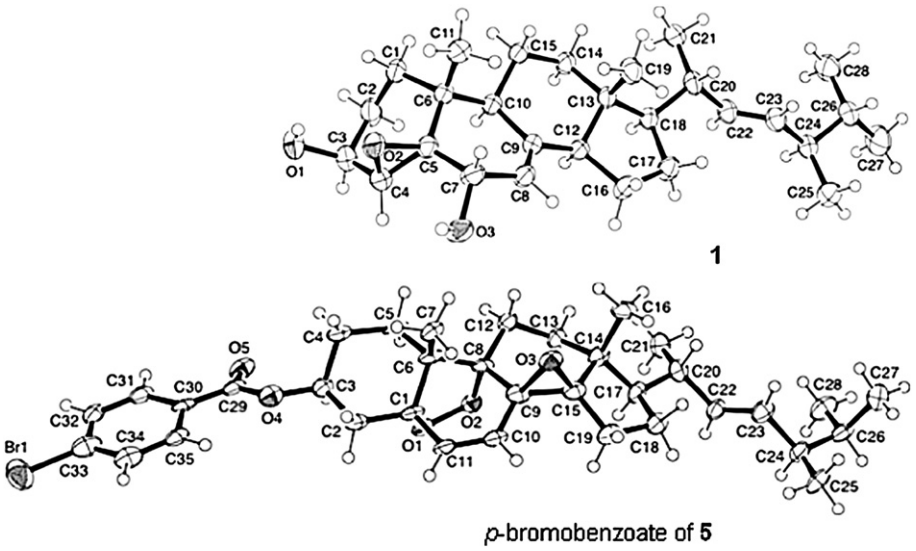

Fig. 1. ORTEP drawings of **1** and *p*-bromobenzoate of **5** with ellipsoids at the 50% probability level. Hydrogen atoms are shown as small spheres of arbitrary radii.

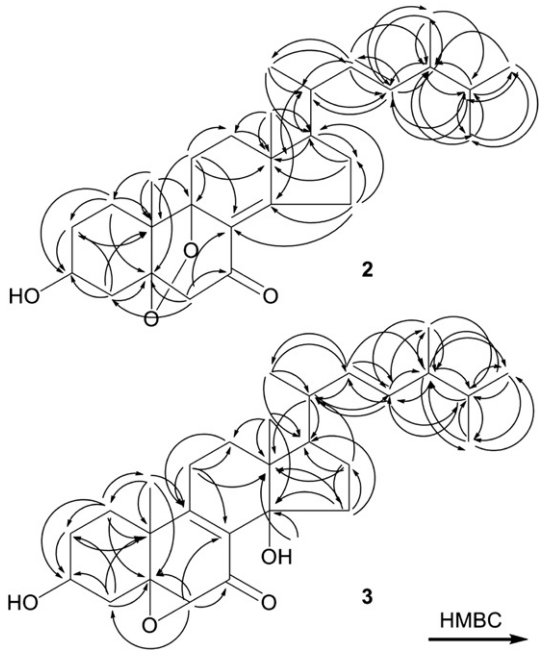

Fig. 2. 2D NMR correlations of **2** and **3**.

3.3. Extraction and isolation

Powder of the air-dried fruiting bodies of *G. gargal* (8.78 kg) was successively extracted with hexane (5 L, twice), EtOAc (5 L, twice) and then EtOH (5 L, twice). The EtOAc-soluble part (90.1 g) was fractionated by silica gel flash column chromatography (hexane/EtOAc 9:1; CH_2Cl_2; CH_2Cl_2/acetone 8:2; acetone; EtOH and MeOH/H_2O 9:1, 2.0 L each) to obtain 28 fractions (fractions 1–28). Fraction 17 (296 mg) was separated by reverse-phase HPLC (Wakosil-II 5C18 HG Hrep, 95% MeOH) to afford compound **5** (11.1 mg). Fraction 18 (3.6 g) was further separated by reverse-phase HPLC (Develosil C30-UG-15/30, 90% MeOH), affording 57 fractions (fractions 18-1 to 18-57). Compounds **1** (2.3 mg), **4** (13.9 mg), and **7** (2.0 mg) were obtained from fraction 18-49 (75.7 mg) by normal-phase HPLC (Senshu Pak AQ, EtOAc/$CHCl_3$ 1:9). Compound **2** (2.9 mg) was purified from fraction 18-33 (46.5 mg) by normal-phase HPLC (Senshu PAK AQ, $CHCl_3$). Fraction 18-21 (16.1 mg) was further separated by reverse-phase HPLC (Capcell PAK C18 AQ, 80% MeOH) to obtain compound **3** (2.9 mg). Fractions 18–45 (53.4 mg) was separated by normal-phase HPLC (Senshu Pak AQ, EtOAc/$CHCl_3$ 1:9) and reverse-phase HPLC (COSMOSIL Cholester water, 90% MeOH) to give compound **6** (4.0 mg).

*3.3.1. Gargalol A (**1**).* White powder; $[\alpha]_D^{28}$ +19 (*c* 0.29, $CHCl_3$); mp 190–192 °C; IR (neat): 3398 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 451 $[M+Na]^+$; HRESIMS *m*/*z* 451.3158 $[M+Na]^+$ (calcd for $C_{28}H_{44}NaO_3$, 451.3188).

*3.3.2. Gargalol B (**2**).* White powder; $[\alpha]_D^{27}$ +47 (*c* 0.26, $CHCl_3$); mp 59–60 °C; IR (neat): 3348, 1683 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 441 $[M-H]^-$; HRESIMS *m*/*z* 441.2983 $[M-H]^-$ (calcd for $C_{28}H_{41}O_4$, 441.3005).

*3.3.3. Gargalol C (**3**).* White powder; $[\alpha]_D^{24}$ +118 (*c* 0.11, $CHCl_3$); mp 194–196 °C; IR (neat): 3435, 1658 cm^{-1}; ^1H and ^{13}C NMR, see Table

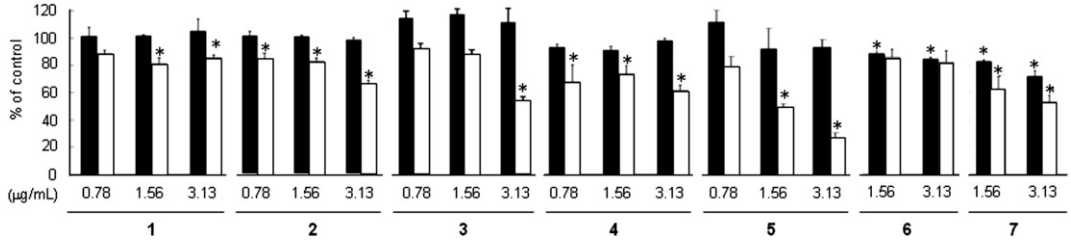

Fig. 3. Inhibitory activity of **1**–**7** against osteoclast formation. Closed and open columns indicate cell viability and osteoclast formation, respectively. TRAP-positive multinucleated cells that had more than three nuclei were counted. Cell viability was determined by MIT assay. Data are the mean±SE of two cultures (*$P<0.05$ vs control using Student's *t*-test).

1; ESIMS m/z 465 $[M+Na]^+$; HRESIMS m/z 465.2877 $[M+Na]^+$ (calcd for $C_{28}H_{42}NaO_4$, 465.2890).

3.3.4. Compound ***4***. White powder; $[\alpha]_D^{27}$ +43.0 (c 1.10, $CHCl_3$); mp 206–207 °C; IR (neat): 3392, 1653 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS m/z 425 $[M-H]^-$; HRESIMS m/z 425.3029 $[M-H]^-$ (calcd for $C_{28}H_{41}O_3$, 425.3056).

3.3.5. Compound ***5***. White powder; $[\alpha]_D^{27}$ +4.5 (c 1.10, $CHCl_3$); mp 152–154 °C; IR (neat): 3425 cm^{-1}; ESIMS m/z 465 $[M+Na]^+$; HRESIMS m/z 465.2961 $[M+Na]^+$ (calcd for $C_{28}H_{42}NaO_4$, 465.2981); ^1H NMR (500 MHz, $CDCl_3$): δ 0.80 (H-26), 0.82 (H-27), 0.90 (H-28), 0.91 (H-18), 0.98 (H-21), 1.11 (H-19), 1.35 (H-1a), 1.44 (H-16a), 1.45 (H-12a, H-25), 1.46 (H-17), 1.50 (H-2a), 1.53 (H-11a), 1.56 (H-12b), 1.64 (H-4a), 1.65 (H-15a), 1.67 (H-16b), 1.70 (H-11b), 1.84 (H-24), 1.88 (H-1b), 1.92 (H-2b), 2.00 (H-15b), 2.11 (H-20), 2.20 (H-4b), 3.99 (H-3), 5.15 (H-22), 5.21 (H-23), 5.53 (H-7), 5.85 (H-6); ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.5 (C-18), 15.6 (C-19), 17.6 (C-28), 19.6 (C-26), 19.7 (C-11), 19.9 (C-27), 21.0 (C-21), 26.5 (C-15), 27.2 (C-16), 27.6 (C-1), 30.8 (C-2), 33.1 (C-25), 33.3 (C-12), 35.6 (C-4), 39.1 (C-20), 40.2 (C-13), 42.8 (C-24), 50.5 (C-10), 55.7 (C-17), 63.8 (C-8), 66.0 (C-3), 75.2 (C-14), 85.8 (C-5), 86.8 (C-9), 128.7 (C-7), 132.8 (C-23), 134.9 (C-22), 135.6 (C-6).

3.4. Preparation of dinaphthoate of 1

Compound **1** (1.0 mg, 2.3 μmol) was stirred with 2-naphthoyl chloride (8.1 mg, 42.5 μmol) and 4-*N*,*N*-dimethylaminopyridine (2.5 mg, 20.5 μmol) in pyridine (50 μL) at 65 °C for 3 days. The resulting mixture was evaporated to dryness under reduced pressure and then separated by reverse-phase HPLC (Phenomenex Luna PFP (2), 99% CH_3CN) to give a dinaphthoate of **1** (0.41 mg). Dinaphthoate of **1**. CD (0.00222 M, CH_3CN) λ_{max} ($\Delta\varepsilon$) 242 (+20.4), 230 (−12.6) nm; ^1H NMR (500 MHz, in $CDCl_3$): δ 7.49–8.62 (14H, aromatic naphthoate protons), 6.15 (1H, br, s, H-6), 5.59 (1H, m, H-3), 5.13–5.28 (3H, m, H-7, 22, 23), 4.00 (1H, d, 4.0, H-4), 2.37 (1H, m, H-9), 1.22–2.10 (17H, m, H-1, 2, 11, 12, 14, 15, 16, 17, 20, 24, 25), 1.27 (3H, s, H-19), 1.03 (3H, d, 6.7, H-21), 0.90 (3H, d, 7.0, H-28), 0.82 (3H, d, 7.0, H-27), 0.81 (3H, d, 7.0, H-26), 0.63 (3H, s, H-18); ESIMS m/z 759 $[M+Na]^+$.

3.5. Preparation of *p*-bromobenzoate of 5

Compound **5** (2.0 mg) was dissolved in 0.5 mL anhydrous pyridine in a 4 mL vial, and *p*-bromobenzoyl chloride (10.4 mg) was added to the solution. After stirring at 50 °C for 2 days, the reaction mixture was evaporated to dryness under reduced pressure and then separated by normal-phase HPLC (Senshu PAK AQ, hexane/$CHCl_3$ 8:2) to give a *p*-bromobenzoate (1.2 mg) of **5**.

p-Bromobenzoate of **5**. ESIMS m/z 647 $[M+Na]^+$; HRESIMS m/z 647.2328 $[M+Na]^+$ (calcd for $C_{35}H_{45}BrNaO_5$, 647.2348). ^1H NMR (500 MHz, in $CDCl_3$): δ 0.80 (3H, d, J=6.7 Hz, H-26), 0.81 (3H, d, J=7.0 Hz, H-27), 0.89 (3H, d, J=6.7 Hz, H-28), 0.91 (3H, s, H-18), 0.98 (3H, d, J=6.6 Hz, H-21), 1.14 (3H, s, H-19), 1.35 (1H, m, H-1), 1.45–1.60 (8H, m, H-2, H-11, H-12, H-16, H-17, H-25), 1.65 (1H, m, H-15), 1.70 (1H, m, H-11), 1.84 (1H, m, H-24), 1.88 (1H, m, H-1), 1.92 (1H, m, H-2), 2.00 (1H, m, H-15), 2.11 (1H, m, H-20), 2.20 (1H, m, H-4), 5.17 (1H, dd, J=15.4, 8.4 Hz, H-22), 5.24 (1H, dd, J=15.4, 7.7 Hz, H-23), 5.29 (1H, m, H-3), 5.56 (1H, d, J=9.5 Hz, H-7), 5.87 (1H, d, J=9.5 Hz, H-6), 7.56 (2H, d, J=8.5 Hz, COC_6H_4–*p*-Br), 7.86 (2H, d, J=8.5 Hz, COC_6H_4–*p*-Br).

3.6. X-ray crystallography analysis

Crystal data for **1**: $C_{28}H_{44}O_3$, M=428.63, orthorhombic, a=7.71614(18) Å, b=8.53412(18) Å, c=37.6177(8) Å, V=2477.14(9) $Å^3$, T=193 K, space group $P2_12_12_1$, Z=4, λ=1.54187 Å, μ(Cu Kα)=0.558 mm^{-1}, F(000)=944. The size of the crystal used for measurements was 0.60×0.25×0.05 mm. Diffraction data were collected on a Rigaku *R*-AXIS-RAPID diffractometer with imaging plate detector. 45,484 reflections were collected in the range $4.70<\theta<68.25$, of which 2639 unique (R_{int}=0.0424) reflections. The structure was refined by full-matrix least-squares procedure on F^2 values using all unique reflections. The final R indices were $R(F)$=0.0348, $wR(F^2)$=0.0960 (2512 reflections with $I>2\sigma(I)$) with goodness-of fit=1.028.

p-Bromobenzoate of **5**: $C_{35}H_{45}BrO_5$, M=625.62, monoclinic, a=6.4110(10) Å, b=9.373(2) Å, c=26.057(6) Å, b=95.832(12)°, V=1557.7(5) $Å^3$, T=95 K, space group $P2_1$, Z=2, λ=0.8000 Å, $\mu(\lambda=0.80)$=1.798 mm^{-1}, F(000)=660. The size of the crystal used for measurements was 0.10×0.05×0.01 mm. Diffraction data were collected at PF-AR NW12A beamline (Tsukuba, Japan), with ADSC Quantum 210r CCD detector. 11,915 reflections were collected in the range $1.76<\theta<25.01$, of which 3561 unique (R_{int}=0.096) reflections. The structure was refined by full-matrix least-squares procedure on F^2 values using all unique reflections. The final R indices were $R(F)$=0.0831, $wR(F^2)$=0.1612 (all reflections) with goodness-of fit=1.068.

Crystallographic data for **1** and *p*-bromobenzoate of **5** have been deposited at The Cambridge Crystallographic Data Centre and allocated the deposition number, CCDC 791830 and 791831, respectively. The data can be obtained free of charge via www.ccdc.cam.ac.uk/products/csd/request.

3.7. 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 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.[14] 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 media with a 26-gauge needle. The osteoblastic cells and bone marrow cells collected and washed to be used in the co-culture subsequently. Osteoclasts were prepared from a co-culture system as previously described.[15] 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 $1\alpha,25(OH)_2D_3$ (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 dimethyl sulfoxide, and the absorbance was measured at 570 nm.

3.8. Statistical analysis

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±SE.

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