

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

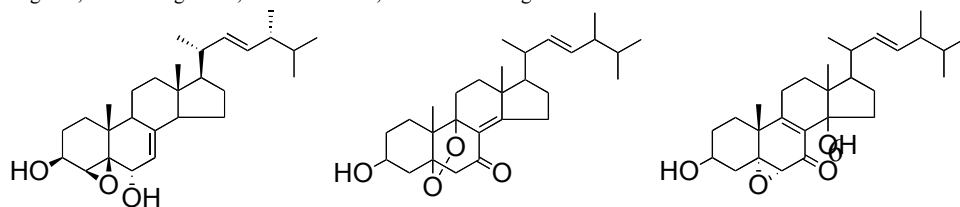
著者	Wu Jing, Choi Jae-Hoon, Yoshida Miyuki, Hirai Hirofumi, Harada Etsuko, Masuda Kikuko, Koyama Tomoyuki, Yazawa Kazunaga, Noguchi Keiichi, Nagasawa Kazuo, Kawagishi Hirokazu
journal or publication title	Tetrahedron
volume	67
number	35
page range	6576-6581
year	2011-09-02
出版者	Elsevier
権利	Copyright (C) 2011 Elsevier Ltd. All rights reserved.
URL	<a href="http://hdl.handle.net/10297/6697">http://hdl.handle.net/10297/6697</a>

doi: 10.1016/j.tet.2011.05.091

# 1 Graphical abstract

2 Osteoclast-forming suppressing compounds, gargalols A, B and C, from the edible mushroom *Grifola gargal*  
3 Jing Wu, Jae-Hoon Choi, Miyuki Yosida, Etsuko Harada, Kikuko Masuda, Tomoyuki Koyama, Kazunaga Yazawa, Keiichi  
4 Noguchi, Kazuo Nagasawa, Hirofumi Hirai, Hirokazu Kawagishi \*

5



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

3  
4 Jing Wu<sup>a,†</sup>, Jae-Hoon Choi<sup>a,†</sup>, Miyuki Yoshida<sup>a</sup>, Hirofumi Hirai<sup>a</sup>, Etsuko Harada<sup>b</sup>,  
5 Kikuko Masuda<sup>c</sup>, Tomoyuki Koyama<sup>c</sup>, Kazunaga Yazawa<sup>c</sup>, Keiichi Noguchi<sup>d</sup>, Kazuo  
6 Nagasawa<sup>e</sup>, Hirokazu Kawagishi<sup>a,f,\*</sup>

7  
8 <sup>a</sup> *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka*  
9 *University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

10 <sup>b</sup> *Iwadekingaku Laboratory, Tsu 514-0012, Japan,*

11 <sup>c</sup> *Graduate School of Marine Science and Technology, Tokyo University of Marine*  
12 *Science and Technology, 4-5-7, Konan, Minato-ku, Tokyo 108-8477, Japan,*

13 <sup>d</sup> *Instrumentation Analysis Center, Tokyo University of Agriculture and Technology,*  
14 *2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan*

15 <sup>e</sup> *Department of Biotechnology and Life Science, Tokyo University of Agriculture and*  
16 *Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan*

17 <sup>f</sup> *Graduate School of Science and Technology, Shizuoka University, 836 Ohya,*  
18 *Suruga-ku, Shizuoka 422-8529, Japan*

1 \* Corresponding author. E-mail address: achkawa@ipc.shizuoka.ac.jp (H. Kawagishi)

2 † These authors contributed equally to this work.

3

1 **ABSTRACT**

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

6

## 1 **1. Introduction**

2

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

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

1           *G. gargal* is an edible mushroom with a characteristic almond flavor, collected and  
2 eaten by native people of southern Argentina and Chile. The species has only been  
3 reported from the *Nothofagus*-dominated forests of the area. Nutraceutical properties  
4 and pharmacological potential of the mushroom have been studied; aqueous extracts of  
5 the mushroom showed the anti-oxidant and anti-inflammatory effects and the methanol  
6 extracts displayed a free radical scavenging activity.<sup>7</sup> Commercial production of the  
7 mushroom has just started in Japan.<sup>8</sup> Here we describe the isolation, structural  
8 determination, and biological activity of compounds from the mushroom.

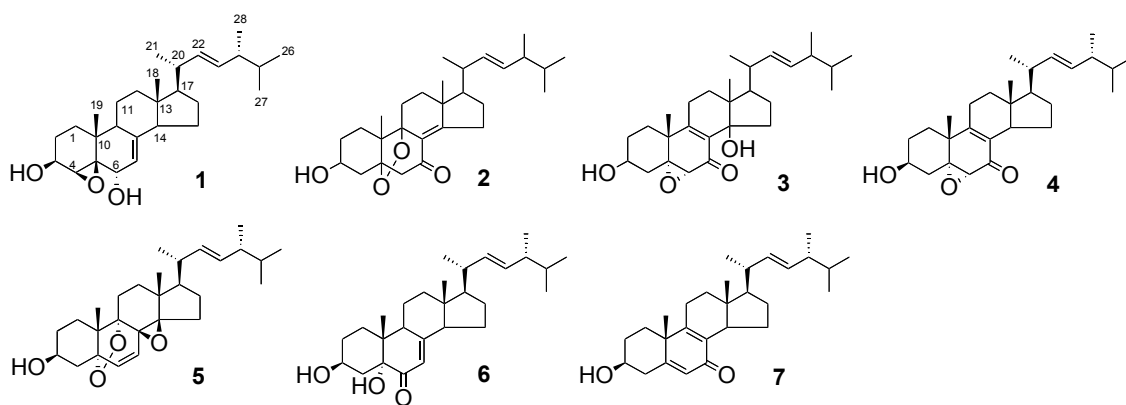
9

## 10   **2. Results and discussion**

11

12           The dried fruiting bodies of *G. gargal* were extracted with hexane, EtOAc and then  
13 with EtOH subsequently. Since EtOAc soluble fraction showed the strong suppressing  
14 activity against the formation of osteoclast, this fraction was repeatedly subjected to  
15 column chromatography, being guided by the result of the bioassay. As a consequence,  
16 three novel compounds (**1-3**) and four known ones were purified.

17



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,  $\delta_H$  5.14, dd,

$J=15.3, 6.7$  Hz;  $\delta_C$  135.3: C-23,  $\delta_H$  5.20, dd,  $J=15.3, 8.5$  Hz;  $\delta_C$  132.2), a

hydroxymethine (C-3,  $\delta_H$  4.06, m;  $\delta_C$  62.7), four doublet methyls (C-21,  $\delta_H$  1.00, d,

$J=6.4$  Hz;  $\delta_C$  21.0: C-26, 27,  $\delta_H$  0.80, d,  $J=6.8$  Hz, 0.82, d,  $J=6.8$  Hz;  $\delta_C$  19.6, 19.9:

C-28,  $\delta_H$  0.89, d,  $J=6.4$  Hz;  $\delta_C$  17.6), and two singlet methyls (C-18,  $\delta_H$  0.57, s;  $\delta_C$  12.2:

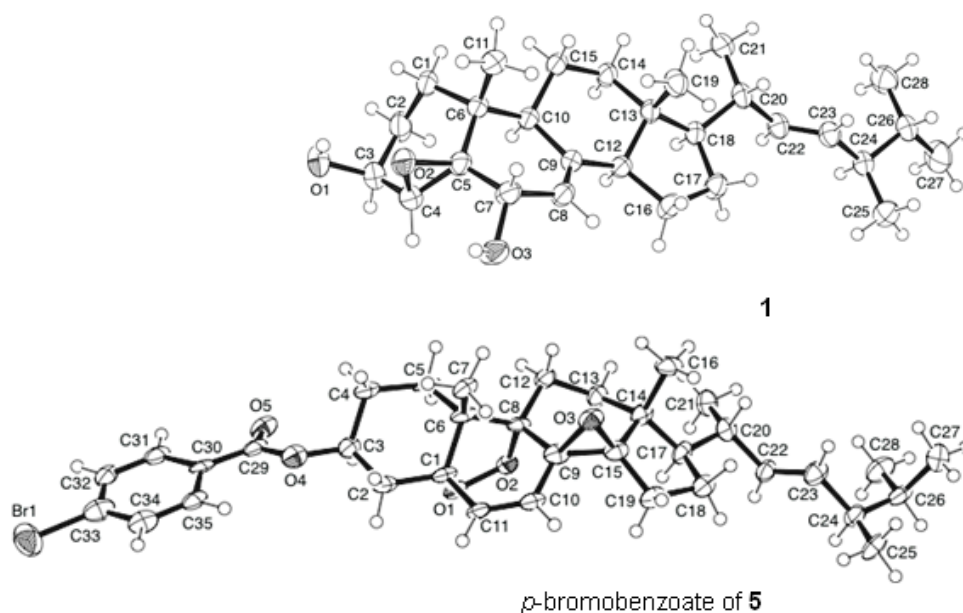
C-19,  $\delta_H$  1.04, s;  $\delta_C$  21.1) were observed. The complete assignment of the protons and

carbons and the HMBC correlations were summarized in Table 1. The structure



1 including relative stereochemistry of **1** was confirmed by X-ray crystallography analysis  
2 (Fig. 1). In addition, the absolute configuration of **1** was determined by circular dichroic  
3 exciton chirality method using its dinaphthoate ( $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) ; 242 (+20.4), 230 (-12.6)  
4 nm).<sup>9</sup> As a result, the structure of **1** was determined to be  
5 4 $\beta$ ,5 $\beta$ -epoxy-(22*E*)-ergosta-7,22-dien-3 $\beta$ ,6 $\alpha$ -diol.

6

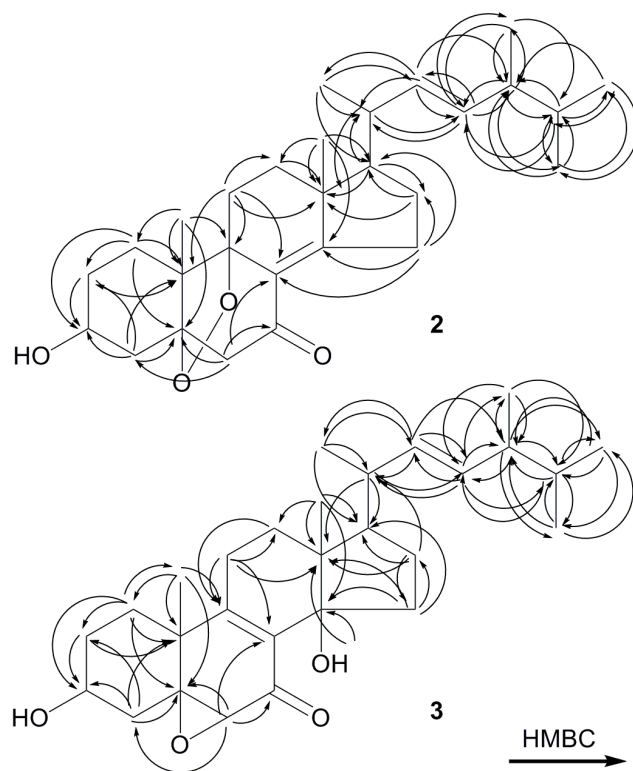


**Figure 1.** ORTEP drawings of **1** and *p*-bromobenzoate of **5** with ellipsoids at the 50% probability level. Hydrogen atoms are shown as small spheres of arbitrary radii.

7

8 Gargalol B (**2**) was isolated as a white powder. Its molecular formula was  
9 determined as C<sub>28</sub>H<sub>42</sub>O<sub>4</sub> by HRESIMS  $m/z$  441.2983 [M-H]<sup>-</sup> (calcd for C<sub>28</sub>H<sub>41</sub>O<sub>4</sub>,  
10 441.3005) and the degree of unsaturation of the compound was eight. The NMR data of  
11 **2** were similar to those of **1** (Table 1), suggesting this compound was also a sterol. The

1 structure elucidation was accomplished in the same manner as **1** (Table 1). The DEPT  
2 experiment indicated the presence of six methyls, eight methylenes, seven methines and  
3 seven quaternary carbons. The molecular formula, the HMBC correlations (Fig. 2 and  
4 Table 1) and the chemical shifts indicated the presence of a hydroxyl ( $\delta_{\text{H}}$  3.93, m;  $\delta_{\text{C}}$   
5 66.0), an enone ( $\delta_{\text{C}}$  197.8, 126.5, 172.4), and a peroxide ( $\delta_{\text{C}}$  85.9, 85.8) at the positions  
6 of C-3, C-7/8/14, and C-5/C-9. As a result, the planar structure of **2** was determined as  
7 shown. However, since any significant NOE was not observed, the stereochemistry of **2**  
8 could not be determined.



9

**Figure 2.** 2D NMR Correlations of **2** and **3**.

1 Gargalol C (**3**) was purified as a white powder. Its molecular formula was  
2 determined as C<sub>28</sub>H<sub>42</sub>O<sub>4</sub> by HRESIMS *m/z* 465.2877 [M+Na]<sup>+</sup> (calcd for C<sub>28</sub>H<sub>42</sub>NaO<sub>4</sub>,  
3 465.2890) and the degree of unsaturation of the compound was eight. The NMR data of  
4 **3** were similar to those of **1** and **2** (Table 1). Judging from the molecular formula, the  
5 HMBC correlations (Fig. 2 and Table 1) and the chemical shifts, the presence of two  
6 hydroxyls (C-3, δ<sub>H</sub> 3.97, m; δ<sub>C</sub> 68.7; C-14, δ<sub>C</sub> 80.8), an epoxide (C-5, δ<sub>C</sub> 65.6; C-6, δ<sub>H</sub>  
7 3.34, s; δ<sub>C</sub> 62.3) and an enone (C-7, δ<sub>C</sub> 200.2; C-8, δ<sub>C</sub> 133.0; C-9, δ<sub>C</sub> 157.0). Since an  
8 NOE was observed between H-6 and H-19 in the NOE difference and NOESY  
9 experiments, the relative configuration at C-5, C-6 and C-19 was determined as shown.  
10 However the stereochemistry of the other parts remains unknown.

11 Compound **4** was purified as a white powder. Its molecular formula was  
12 determined as C<sub>28</sub>H<sub>42</sub>O<sub>3</sub> by HRESIMS *m/z* 425.3029 [M-H]<sup>-</sup> (calcd for C<sub>28</sub>H<sub>41</sub>O<sub>3</sub>,  
13 425.3056). The <sup>1</sup>H and <sup>13</sup>C NMR data of **4** were very similar to those of **3** (Table 1).  
14 The comparison of the molecular formula of **4** with that of **3** indicates that **4** is a  
15 dehydroxylated form of **3**. The position of the missing hydroxy was elucidated by the  
16 chemical shift of position 14 (δ<sub>H</sub> 2.11; δ<sub>C</sub> 48.8) and the HMBC correlations (Table 1).  
17 Compound **4** has been previously reported as a product of thermal rearrangement of  
18 ergosterol peroxide but its spectroscopic data have not been given in the report.<sup>10</sup> This is

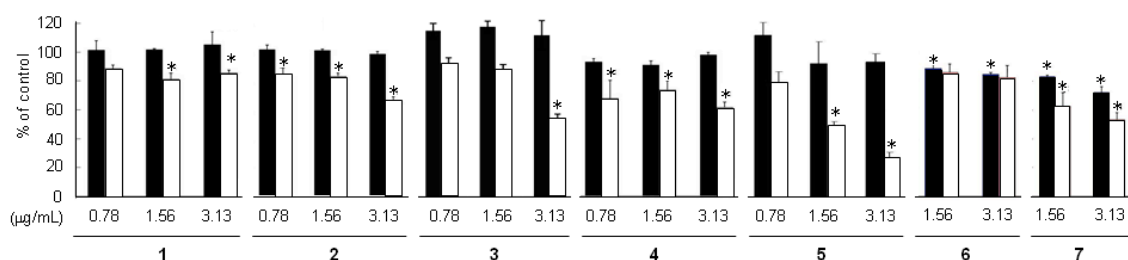
1 the first report of isolation of **4** from a natural source. The absolute configuration of **4**  
2 was determined by comparison of its specific rotation value with that reported  
3 previously:<sup>10</sup> **4**,  $[\alpha]_D^{27} +43.0$  (*c* 1.10, CHCl<sub>3</sub>); reported data,  $[\alpha]_D^{25} +40.6$  (*c* 1.10, CHCl<sub>3</sub>).  
4 As a result, the structure of **4** was determined as shown.

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

11 Based on the comparison of the spectroscopic data for **6** and **7** with those reported  
12 in previous papers,<sup>12,13</sup> **6** and **7** were identified as  
13  $3\beta,5\alpha$ -dihydroxy-(*22E*)-ergosta-7,22-diene-6-one and  
14  $3\beta$ -hydroxy-(*22E*)-ergosta-5,8,22-triene-7-one, respectively.

15 Compounds **1-7** were evaluated in the osteoclast-forming assay. The assay is based  
16 on the principle that osteoclast-like multinucleated cells can be formed *in vitro* from  
17 co-cultures of mouse bone marrow cells and osteoblastic cells by treatment with  
18 osteotropic factors. By adding suppressive agents, the formation of osteoclast is

1 inhibited during the differentiation. As shown in Fig. 3, **2** and **4** inhibited osteoclast  
2 formation at lower concentration (0.78  $\mu\text{g/mL}$ ) than the other compounds, and **5**  
3 exhibited the activity dose-dependently and the activity was the strongest among all the  
4 compounds. On the other hand, compounds **6** and **7** significantly showed cytotoxicity  
5 even at 1.56  $\mu\text{g/mL}$  (data not shown). The structure-activity relationship and the mode  
6 of action of the compounds remain unsolved.



**Figure 3.** Inhibitory activity of **1** to **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 MTT assay. Data are the mean  $\pm$  SE of two cultures (\* $P < 0.05$  vs control using Student's t-test).

7

8

### 9 **3. Experimental**

10

#### 11 **3.1. General**

12

$^1\text{H}$  NMR spectra (one- and two-dimensional) were recorded on a JEOL

13

lambda-500 spectrometer at 500 MHz, while  $^{13}\text{C}$  NMR spectra were recorded on the

14

same instrument at 125 MHz. The HRESIMS spectra were measured on a JMS-T100LC

15

mass spectrometer. CD spectrum was recorded on a JASCO J-820 spectropolarimeter.

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

10

### 11 **3.2. Fungus materials**

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

13

### 14 **3.3. Extraction and Isolation**

15 Powder of the air-dried fruiting bodies of *G. gargal* (8.78 kg) was successively  
16 extracted with hexane (5 L, twice), EtOAc (5 L, twice) and then EtOH (5 L, twice). The  
17 EtOAc-soluble part (90.1 g) was fractionated by silica gel flash column chromatography  
18 (hexane/EtOAc 9:1; CH<sub>2</sub>Cl<sub>2</sub>; CH<sub>2</sub>Cl<sub>2</sub>/acetone 8:2; acetone; EtOH and MeOH/H<sub>2</sub>O 9:1,

1 2.0 L each) to obtain 28 fractions (fractions 1 to 28). Fraction 17 (296 mg) was  
2 separated by reverse-phase HPLC (Wakosil-II 5C18 HG Hrep, 95% MeOH) to afford  
3 compound **5** (11.1 mg). Fraction 18 (3.6 g) was further separated by reverse-phase  
4 HPLC (Develosil C30-UG-15/30, 90% MeOH), affording 57 fractions (fractions 18-1 to  
5 18-57). Compounds **1** (2.3 mg), **4** (13.9 mg) and **7** (2.0 mg) were obtained from fraction  
6 18-49 (75.7 mg) by normal-phase HPLC (Senshu Pak AQ, EtOAc/CHCl<sub>3</sub> 1:9).  
7 Compound **2** (2.9 mg) was purified from fraction 18-33 (46.5 mg) by normal-phase  
8 HPLC (Senshu PAK AQ, CHCl<sub>3</sub>). Fraction 18-21 (16.1 mg) was further separated by  
9 reverse-phase HPLC (Capcell PAK C18 AQ, 80% MeOH) to obtain compound **3** (2.9  
10 mg). Fraction 18-45 (53.4 mg) was separated by normal-phase HPLC (Senshu Pak AQ,  
11 EtOAc/CHCl<sub>3</sub> 1:9) and reverse-phase HPLC (COSMOSIL Cholester water, 90%  
12 MeOH) to give compound **6** (4.0 mg).

13

14 *3.3.1. Gargalol A (I)*. White powder;  $[\alpha]_D^{28} +19$  (*c* 0.29, CHCl<sub>3</sub>); mp 190-192°C; IR  
15 (neat): 3398 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR, see Table 1; ESIMS *m/z* 451 [M+Na]<sup>+</sup>; HRESIMS  
16 *m/z* 451.3158 [M+Na]<sup>+</sup> (calcd for C<sub>28</sub>H<sub>44</sub>NaO<sub>3</sub>, 451.3188).

17

1 3.3.2. *Gargalol B (2)*. White powder;  $[\alpha]_D^{27} +47$  (*c* 0.26, CHCl<sub>3</sub>); mp 59-60°C; IR (neat):  
2 3348, 1683 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR, see Table 1; ESIMS *m/z* 441 [M-H]<sup>-</sup>; HRESIMS *m/z*  
3 441.2983 [M-H]<sup>-</sup> (calcd for C<sub>28</sub>H<sub>41</sub>O<sub>4</sub>, 441.3005).  
4  
5 3.3.3. *Gargalol C (3)*. White powder;  $[\alpha]_D^{24} +118$  (*c* 0.11, CHCl<sub>3</sub>); mp 194-196°C; IR  
6 (neat): 3435, 1658 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR, see Table 1; ESIMS *m/z* 465 [M+Na]<sup>+</sup>;  
7 HRESIMS *m/z* 465.2877 [M+Na]<sup>+</sup> (calcd for C<sub>28</sub>H<sub>42</sub>NaO<sub>4</sub>, 465.2890).  
8  
9 3.3.4. *Compound 4*. White powder;  $[\alpha]_D^{27} +43.0$  (*c* 1.10, CHCl<sub>3</sub>); mp 206-207°C; IR  
10 (neat): 3392, 1653 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR, see Table 1; ESIMS *m/z* 425 [M-H]<sup>-</sup>;  
11 HRESIMS *m/z* 425.3029 [M-H]<sup>-</sup> (calcd for C<sub>28</sub>H<sub>41</sub>O<sub>3</sub>, 425.3056).  
12  
13 3.3.5. *Compound 5*. White powder;  $[\alpha]_D^{27} +4.5$  (*c* 1.10, CHCl<sub>3</sub>); mp 152-154°C; IR  
14 (neat): 3425 cm<sup>-1</sup>; ESIMS *m/z* 465 [M+Na]<sup>+</sup>; HRESIMS *m/z* 465.2961 [M+Na]<sup>+</sup> (calcd  
15 for C<sub>28</sub>H<sub>42</sub>NaO<sub>4</sub>, 465.2981); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 0.80 (H-26), 0.82 (H-27),  
16 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  
17 (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  
18 (H-15a), 1.67 (H-16b), 1.70 (H-11b), 1.84 (H-24), 1.88 (H-1b), 1.92 (H-2b), 2.00



1 (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),  
2 5.85 (H-6);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  15.5 (C-18), 15.6 (C-19), 17.6 (C-28), 19.6  
3 (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  
4 (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  
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  
6 (C-7), 132.8 (C-23), 134.9 (C-22), 135.6 (C-6).

7

#### 8 **3.4. Preparation of dinaphthoate of 1**

9 Compound **1** (1.0 mg, 2.3  $\mu\text{mol}$ ) was stirred with 2-naphthoyl chloride (8.1 mg,  
10 42.5  $\mu\text{mol}$ ) and 4-*N,N*-dimethylaminopyridine (2.5 mg, 20.5  $\mu\text{mol}$ ) in pyridine (50  $\mu\text{L}$ )  
11 at 65°C for 3 days. The resulting mixture was evaporated to dryness under reduced  
12 pressure and then separated by reverse-phase HPLC (Phenomenex Luna PFP (2), 99%  
13  $\text{CH}_3\text{CN}$ ) to give a dinaphthoate of **1** (0.41 mg). Dinaphthoate of **1**. CD (0.00222 M,  
14  $\text{CH}_3\text{CN}$ )  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) 242 (+20.4), 230 (-12.6) nm;  $^1\text{H}$  NMR (500 MHz, in  $\text{CDCl}_3$ )  $\delta$   
15 7.49-8.62 (14H, aromatic naphthoate protons), 6.15 (1H, br, s, H-6), 5.59 (1H, m, H-3),  
16 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  
17 (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,

1 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  
2 (3H, s, H-18); ESIMS  $m/z$  759 [M+Na]<sup>+</sup>

3

#### 4 **3.5. Preparation of *p*-bromobenzoate of **5****

5 Compound **5** (2.0 mg) was dissolved in 0.5 mL anhydrous pyridine in a 4 mL vial,  
6 and *p*-bromobenzoyl chloride (10.4 mg) was added to the solution. After stirring at  
7 50°C for 2 days, the reaction mixture was evaporated to dryness under reduced pressure  
8 and then separated by normal-phase HPLC (Senshu PAK AQ, hexane/CHCl<sub>3</sub> 8:2) to  
9 give a *p*-bromobenzoate (1.2 mg) of **5**.

10 *p*-Bromobenzoate of **5**. ESIMS  $m/z$  647 [M+Na]<sup>+</sup>; HRESIMS  $m/z$  647.2328 [M+Na]<sup>+</sup>  
11 (calcd for C<sub>35</sub>H<sub>45</sub>BrNaO<sub>5</sub>, 647.2348). <sup>1</sup>H NMR (500 MHz, in CDCl<sub>3</sub>): δ 0.80 (3H, d,  $J$ =  
12 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,  
13 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),  
14 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,  
15 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,  
16 H-15), 2.11 (1H, m, H-20), 2.20 (1H, m, H-4), 5.17 (1H, dd,  $J$ = 15.4 Hz, 8.4 Hz, H-22),  
17 5.24 (1H, dd,  $J$ = 15.4 Hz, 7.7 Hz, H-23), 5.29 (1H, m, H-3), 5.56 (1H, d,  $J$ = 9.5, H-7),

1 5.87 (1H, d,  $J = 9.5$ , H-6), 7.56 (2H, d,  $J = 8.5$  Hz, COC<sub>6</sub>H<sub>4</sub>-*p*-Br), 7.86 (2H, d,  $J =$   
2 8.5 Hz, COC<sub>6</sub>H<sub>4</sub>-*p*-Br).

3

#### 4 **3.6. X-ray crystallography analysis**

5 Crystal data for **1**: C<sub>28</sub>H<sub>44</sub>O<sub>3</sub>,  $M = 428.63$ , orthorhombic,  $a = 7.71614(18)$  Å,  $b =$   
6  $8.53412(18)$  Å,  $c = 37.6177(8)$  Å,  $V = 2477.14(9)$  Å<sup>3</sup>,  $T = 193$  K, space group  $P2_12_12_1$ ,  
7  $Z = 4$ ,  $\lambda = 1.54187$  Å,  $\mu(\text{CuK}\alpha) = 0.558$  mm<sup>-1</sup>,  $F(000) = 944$ . The size of the crystal  
8 used for measurements was 0.60 X 0.25 X 0.05 mm. Diffraction data were collected on  
9 a Rigaku *R*-AXIS-RAPID diffractometer with imaging plate detector. 45484 reflections  
10 were collected in the range  $4.70 < \theta < 68.25$ , of which 2639 unique ( $R_{\text{int}} = 0.0424$ )  
11 reflections. The structure was refined by full-matrix least-squares procedure on  $F^2$   
12 values using all unique reflections. The final  $R$  indices were  $R(F) = 0.0348$ ,  $wR(F^2) =$   
13  $0.0960$  (2512 reflections with  $I > 2\sigma(I)$ ) with goodness-of fit = 1.028.

14 *p*-Bromobenzoate of **5**: C<sub>35</sub>H<sub>45</sub>BrO<sub>5</sub>,  $M = 625.62$ , monoclinic,  $a = 6.4110(10)$  Å,  $b$   
15  $= 9.373(2)$  Å,  $c = 26.057(6)$  Å,  $\beta = 95.832(12)^\circ$ ,  $V = 1557.7(5)$  Å<sup>3</sup>,  $T = 95$  K, space  
16 group  $P2_1$ ,  $Z = 2$ ,  $\lambda = 0.8000$  Å,  $\mu(\lambda = 0.80) = 1.798$  mm<sup>-1</sup>,  $F(000) = 660$ . The size of  
17 the crystal used for measurements was 0.10 X 0.05 X 0.01 mm. Diffraction data were  
18 collected at PF-AR NW12A beamline (Tsukuba, Japan), with ADSC Quantum 210r

1 CCD detector. 11915 reflections were collected in the range  $1.76 < \theta < 25.01$ , of which  
2 3561 unique ( $R_{\text{int}} = 0.096$ ) reflections. The structure was refined by full-matrix  
3 least-squares procedure on  $F^2$  values using all unique reflections. The final  $R$  indices  
4 were  $R(F) = 0.0831$ ,  $wR(F^2) = 0.1612$  (all reflections) with goodness-of fit = 1.068.  
5 Crystallographic data for **1** and *p*-bromobenzoate of **5** have been deposited at The  
6 Cambridge Crystallographic Data Centre and allocated the deposition number, CCDC  
7 791830 and 791831, respectively. The data can be obtained free of charge via  
8 [www.ccdc.cam.ac.uk/products/csd/request](http://www.ccdc.cam.ac.uk/products/csd/request).

9

### 10 **3.7. Bioassay**

11 The stromal/osteoblastic cells, UAMS-32, were cultured in  $\alpha$ -minimal essential  
12 medium ( $\alpha$ -MEM) (ICN Biomedicals, Inc.) containing 10% fetal bovine serum (FBS)  
13 for a week. The cells were detached from the culture dishes by using trypsin-EDTA,  
14 suspended in  $\alpha$ -MEM containing 10% FBS and used for the co-culture as osteoblastic  
15 cells. Bone marrow cells were isolated from mice as described previously.<sup>14</sup> Femoral  
16 and tibiae bone marrow cells were collected from 5-week-old mice which had been  
17 killed by cervical dislocation. The tibiae and femora were removed and dissected free of  
18 adhering tissues. The bone ends were removed and the marrow cavities were flushed by

1 slowly injecting media with a 26-gauge needle. The osteoblastic cells and bone marrow  
2 cells collected and washed to be used in the co-culture subsequently. Osteoclasts were  
3 prepared from a co-culture system as previously described.<sup>15</sup> The osteoblastic cells ( $1.0$   
4  $\times 10^4$  cells/well) were co-cultured with bone marrow cells ( $2.0 \times 10^7$  cells/well) in  
5  $\alpha$ -MEM containing 10% FBS in 96-well plates (Corning Inc.). The culture volume was  
6 made up to 200  $\mu$ L per well with  $\alpha$ -MEM supplemented with 10% FBS in the presence  
7 of  $10^{-8}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  (Biomol) and  $10^{-6}$  M  $\text{PGE}_2$ , with or without a sample. All  
8 cultures were maintained at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$  in air.  
9 Three-quarter of medium was changed after co-culture for 3 days. After the cultivation,  
10 the adherent cells were fixed with 10% formaldehyde in 10 mM phosphate-buffered  
11 saline (pH 7.4) for 20 min. After being treated with 95% ethanol for 1 min, the well  
12 surface was dried and treated with the TRAP staining solution [0.1 M sodium acetate  
13 buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/mL naphthol AS-MX  
14 phosphate (Sigma chemical Co.), and 1 mg/mL fast red violet LB salt (Sigma chemical  
15 Co.)] for 30 min. The TRAP-positive multinucleated cells were then counted under a  
16 microscope. Cell viability was evaluated using a  
17 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma chemical  
18 Co.) assay. After the culture, cells were treated with 1 mg/mL MTT for 2 hours, then

1 precipitated dye was solubilized into dimethyl sulfoxide, and the absorbance was  
2 measured at 570 nm.

3

#### 4 **3.8. Statistical Analysis**

5 Data thus collected were analyzed statistically using Student's *t*-test to determine  
6 significant difference in the data among the groups. *P* values less than 0.05 were  
7 considered significant. The values are expressed as mean  $\pm$  SE.

8

#### 9 **Acknowledgment**

10 We thank V. K. Deo (Shizuoka University) and M. Hasimoto (Hirosaki University)  
11 for valuable discussion.

12

#### 13 **References**

14

- 15 1. Massey, H. M.; Flanagan, A. M. *Br. J. Haematol.* **1999**, *106*, 167-170.
- 16 2. Teitelbaum, S. L. *Science* **2000**, *289*, 1504-1508.
- 17 3. Choi, J.-H.; Ozawa, N.; Masuda, K.; Koyama, T.; Yazawa, K.; Kawagishi, H. *Int.*  
18 *J. Med. Mushr.* **2010**, *12*, 401-406.

- 1 4. Kawagishi, H.; Akachi, T.; Ogawa, T.; Masuda, K.; Yamaguchi, K.; Yazawa, K.;  
2 Takahashi, M. *Heterocycles* **2006**, *69*, 253-258.
- 3 5. Choi, J.-H.; Ogawa, A.; Abe, N.; Masuda, K.; Koyama, T.; Yazawa, K.;  
4 Kawagishi, H. *Tetrahedron* **2009**, *65*, 9850–9853.
- 5 6. Choi, J.-H.; Abe, N.; Kodani, S.; Masuda, K.; Koyama, T.; Yazawa, K.;  
6 Takahashi, T.; Kawagishi, H. *Int. J. Med. Mushr.* **2010**, *12*, 151-155.
- 7 7. Schmeda-Hirschmann, G.; Razmilic, I.; Gutierrez, M. I.; Loyola, J. I. *Econ. Bot.*  
8 **1999**, *53*, 177-187.
- 9 8. Kawade, M.; Harada, E.; Nishioka, H.; Meguro, S. *Mushr. Sci. Biotechnol.* **2009**,  
10 *17*, 75-79 (in Japanese).
- 11 9. Harada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy-Exciton Coupling in*  
12 *Organic Stereochemistry*; University Science Books: Mill Valley, CA, 1983
- 13 10. Bergmann, W.; Meyers, M. B. *Justus Liebigs Ann. Chem.* **1959**, *620*, 46-62.
- 14 11. Yaoita, Y.; Yoshihara, Y.; Kakuda, R.; Machida, K.; Kikuchi, M. *Chem. Pharm.*  
15 *Bull.* **2002**, *50*, 551-553.
- 16 12. Ishizuka, T.; Yaoita, Y.; Kikuchi, M. *Chem. Pharm. Bull.* **1997**, *45*, 1756-1760.
- 17 13. Yaoita, Y.; Endo, M.; Tani, Y.; Machida, K.; Amemiya, K. *Chem. Pharm. Bull.*  
18 **1999**, *47*, 847-851.

- 1 14. Wani, M. R.; Fuller, K.; Kim, N. S.; Choi, Y.; Chambers, T. *Endocrinology* **1999**,
- 2 *140*, 1927-1935.
- 3 15. Takami M.; Woo, JT; Nagai, K. *Cell Tissue Res.* **1999**, *298*, 327-334.



Table 1 <sup>1</sup>H and <sup>13</sup>C NMR data for **1-4** (in CDCl<sub>3</sub>)

Position	<b>1</b>			<b>2</b>		
	$\delta_{\text{H}}$ (multiplicity, <i>J</i> in Hz)	$\delta_{\text{C}}$	HMBC correlation	$\delta_{\text{H}}$ (multiplicity, <i>J</i> in Hz)	$\delta_{\text{C}}$	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*	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*	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	<b>3</b>			<b>4</b>		
	$\delta_{\text{H}}$ (multiplicity, $J$ in Hz)	$\delta_{\text{C}}$	HMBC correlation	$\delta_{\text{H}}$ (multiplicity, $J$ in Hz)	$\delta_{\text{C}}$	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

\* Interchangeable