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GANOMASTENOLS A, B, C AND D, CADINENE SESQUITERPENES, FROM *GANODERMA MASTOPORUM**

MASAO HIROTANI, CHIEKO INO, AKIKO HATANO, HIROAKI TAKAYANAGI and TSUTOMU FURUYA

School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

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Key Word Index—*Ganoderma mastoporum*; Polyporaceae; Basidiomycetes; higher fungi; sesquiterpenes; cadinene derivatives; ganomastenols A–D.

Abstract—Four new cadinene sesquiterpenes, named ganomastenols A–D, have been isolated from the cultured mycelia and its broth of *Ganoderma mastoporum*. Their structures were elucidated by spectral data.

INTRODUCTION

During our study of fungi of the Polyporaceae family, we have reported the isolation and structural elucidation of new compounds from cultured mycelia and fruit bodies of several species in *Ganoderma* genus and other genera [1–7]. In the Polyporaceae there is a suggestion that the *Ganoderma* genus should be separated from Polyporaceae to establish a separate *Ganoderma* family. We have isolated several ganoderic acid derivatives as new compounds from *Ganoderma lucidum* and its related species [3–7].

We have investigated the cultured mycelia and its broth for the presence of ganoderic acid derivatives in the context of chemotaxonomy. This investigation led to the isolation of four new cadinene derivatives. In this paper we report the isolation and structural elucidation of these four novel compounds **1**–**4**.

Cadinene derivatives are sesquiterpenoids widely distributed in the plant kingdom. In the fungal kingdom, several cadinanes have been isolated from fungi in the Agaricaceae [8, 9] and Geoglosaceae [10]. This is the first time that cadinene derivatives have been obtained from the fungi of the *Ganoderma* genus.

RESULTS AND DISCUSSION

The extract of the cultured mycelia and its broth of *G. mastoporum* was subjected to silica gel column chromatography and separated into six fractions as described in the Experimental. Repeated reverse-phase HPLC afforded compounds **1**, **3** and **4** from fraction 3 and compound **2** from fraction 5 using acetonitrile in 2% acetic acid as solvent system. Compound **1**, ganomastenol A, was isolated as a white powder and recrystallized from methanol–water. It had the molecular formula $C_{15}H_{24}O_3$ by HR- and FAB-mass spectrometry. The IR spectrum showed absorption at 3425 cm^{-1} indicating the presence of a hydroxy group. Its ^{13}C NMR spectrum (Table 1) recorded the presence of three methyl carbons at δ19.9, 20.1 and 22.1, three methine carbons bearing an oxygen atom at 71.6, 79.0 and 79.1, one olefinic methine carbon at 127.3, one olefinic methylene carbon at 103.2, two quaternary olefinic carbons at 139.4 and 152.2, four methine carbons at 28.0, 43.1, 44.9 and 52.7 and one methylene carbon at 37.2.

With the help of ^{13}C–^1H COSY and ^1H–^1H COSY, carbon connectivities were determined. The methine carbon signal at δ79.0 was determined to carry an oxygen on the basis of the chemical shift value, and it had connectivity with another oxygenated methine carbon at 79.1 by ^1H–^1H COSY. On examination of the ^1H–^1H COSY spectrum, carbon connectivities were observed as follows. The methine carbon at δ79.0 had a connectivity with the methine carbon at δ52.7 which had connections with methine carbons at 44.9 and 28.0. In the ^1H–^1H COSY spectrum, two methyl protons at 1.00 and 1.09 had couplings with the methine proton at 2.27 with the same coupling constants 7 Hz. The methine proton was attached to the carbon at δ28.0. This coupling pattern indicated the presence of an isopropyl group and connected to the carbon at δ52.7. In this way all the carbon connectivities were established except for the quaternary carbons. To accomplish the connectivities of all the carbons the HMBC spectrum was examined. In this spectrum, the quaternary carbon at δ152.2 had coupling with the proton at δ3.80, which was attached to the oxygenated carbon at δ79.1, and with the methine proton at δ1.89 attached to the carbon at δ43.1. Another quaternary carbon at δ139.4 had coupling with the proton at δ2.24, which was attached to the carbon at δ37.2, and with the olefinic methine proton at δ5.61, which was

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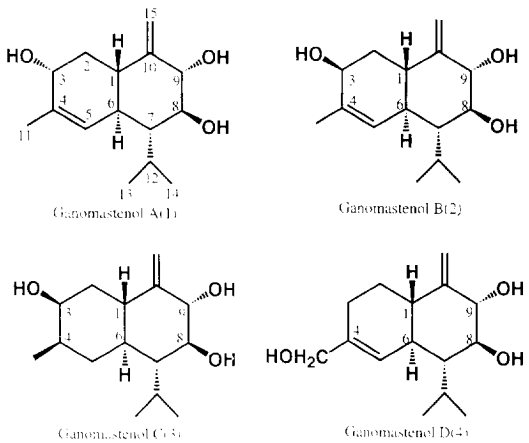

Table 1. ^{13}C NMR spectral data of compounds 1, 2, 3 and 4

C	1	2	3	4
1	43.1	37.7	38.7	43.9
2	37.2	36.2	37.8	26.8
3	71.6	68.7	71.1	27.3
4	139.4	137.1	37.6	139.9
5	127.3	128.6	35.4	125.2
6	44.9	44.9	45.2	44.3
7	52.7	55.2	53.8	52.7
8	79.0	79.2	78.9	79.2
9	79.1	79.2	79.2	79.2
10	152.2	152.8	153.3	153.0
11	20.1	21.8	19.3	67.6
12	28.0	27.7	27.7	27.8
13	19.9	19.9	20.3	19.9
14	22.1	22.0	21.6	22.0
15	103.2	103.1	103.0	103.0

attached to the carbon at δ127.3. These results revealed the structure of compound **1** to be a new cadinene compound.

Naturally occurring cadinenes are subdivided into four classes based on the ring fusion and orientation of the isopropyl group at C-7 [11]. The stereochemistry of the ring junction was confirmed by the coupling constants between H-1 and H-6 (see Table 2). The large coupling constant (11 Hz) and the absence of NOE between H-1 and H-6 indicated a dihedral angle of 180° between them, thus the ring fusion was *trans*. Also, the large coupling constant (11 Hz) between H-6 and H-7 clarified the orientation of the isopropyl, which had the same orientation as that of H-6. This evidence revealed that compound **1** had a cadinene skeleton [12, 13]. The coupling constant (10 Hz) between H-3 and one of the H-2 protons also indicated a dihedral angle of 180°, and the presence of a 9% NOE between H-3 and H-1 (Table 3) indicated that these protons had the same orientation and that the orientation of the C-3 hydroxyl group was the same as that of H-6. This evidence led to the conclusion that compound **1** is *rel*-3α,8β,9α-trihydroxycadina-4,10(15)-diene.

Compound **2**, ganomastenol B, was isolated as needles, and deduced to have the molecular formula $C_{15}H_{24}O_3$ by HR- and FAB-mass spectrometry. The IR spectrum showed absorption at 3320 cm^{-1} indicating the presence of a hydroxy group. In the ^1H and ^{13}C NMR spectra, it showed the same spectral pattern as compound **1** but the chemical shift values were a little different, as shown in Tables 1 and 3.

In the ^{13}C NMR spectrum of compound **2**, the C-3 signal was observed at δ68.7, shifted upfield by about 3 ppm in relation to that of compound **1**. As shown in Table 1, the C-1 and C-2 signals were observed shifted upfield relative to those of compound **1**. These upfield shifts demonstrated the orientation of the hydroxyl group at C-3 to be axial and opposite to that of compound **1**. This result is also supported by the coupling constant between H-3 and H-2 (Table 2). From this evidence the structure of compound **2** was established to be *rel*-3β,8β,9α-trihydroxycadina-4,10(15)-diene.

Compound **3**, ganomastenol C, was obtained as needles and deduced to have the molecular formula $C_{15}H_{26}O_3$ by HR- and FAB-mass spectrometry. The IR spectrum showed an hydroxyl absorption band at 3360 cm^{-1}. From a comparison of the spectral data of **3** with those of **2** the structure of compound **3** was elucidated to be *rel*-3β,8β,9α-trihydroxycadina-10(15)-ene. In the ^{13}C NMR spectrum of compound **3** only two olefinic carbon signals were observed that could be attributed to C-10 and C-15 which suggested the absence of a 4,5-double bond. However, one methylene carbon signal, not observed in **2**, was observed. Except for these differences the spectra were very similar each other. The carbon connectivities were determined by ^{13}C–^1H COSY, ^1H–^1H COSY and NOE experiments. These spectral data confirmed that the structure of compound **3** was *rel*-3β,8β,9α-trihydroxycadina-10(15)-ene.

Compound **4**, gaomastenol D, was obtained as a white powder and deduced to have the molecular formula $C_{15}H_{24}O_3$ by HR- and FAB-mass spectrometry. The structure of compound **4** was elucidated with the help of ^{13}C–^1H and ^1H–^1H COSY spectra. The ^{13}C–^1H NMR spectrum of compound **4** showed two methyl carbon signals, four methylene carbon signals, seven methine carbon signals and two quaternary carbon signals (Table 1). The ^{13}C–^1H COSY spectrum easily revealed the carbon and proton connectivities while the ^1H–^1H COSY spectrum showed the carbon connectivities. The H-12 signal were easily assigned from the coupling with the isopropyl methyls. The H-12 had coupling with H-7 which coupled with H-8 while H-8 coupled with H-9. All the carbon connectivities were deduced in this way. The quaternary carbon connectivities were achieved by HMBC spectrum. The small coupling constant of 1 Hz between H-5 and H-6, and the large coupling constant of 11 Hz between H-1 and H-6 suggested, the *trans*-ring fusion [12, 13]. These investigations elucidated the structure of compound **4** as *rel*-8β,9α-dihydroxy-4-hydroxymethylcadina-4,10(15)-diene.

Table 2. ^{1}H NMR spectral data of compounds **1**, **2**, **3** and **4** (Coupling constants, J in Hz, in parentheses)

	1	2	3	4
1	1.89 *ddd* (12, 11, 2.5)	2.05 *ddd* (11, 11, 2)	2.03 *ddd* (13, 11, 2)	1.78 *ddd* (12, 12, 2.5)
2β	1.48 *ddd* (12, 12, 10)	1.63 *ddd* (13, 11, 4)	1.54 *ddd* (13, 11, 2.5)	1.53 *ddd* (12, 12, 5)
2α	2.24 *ddd* (12, 6, 2.5)	2.00 *ddd* (13, 2, 2)	1.95 *ddd* (13, 4, 2)	2.00 *ddd* (12, 7, 2.5)
3α		3.99 *dd* (4, 2)	3.85 *dd* (4, 2.5)	2.14 *m*
3β	4.16 *dd* (10, 6)	-	-	2.14 *m*
4	-	-	1.47 *m*	-
5	5.61 *dd* (1, 1)	5.72 *dd* (1, 1)	1.13 *ddd* (13, 12, 12)	5.83 *ddd* (1, 1)
	-	-	1.67 *ddd* (13, 3.5, 3)	-
6	1.62 *ddd* (11, 11, 1)	1.48 *ddd* (11, 11, 1)	0.93 *dddd* (13, 12, 11, 3)	1.62 *ddd* (12, 11, 1)
7β	1.24 *ddd* (11, 11, 1)	1.31 *ddd* (11, 11, 1)	1.26 *ddd* (12, 11, 1.5)	1.28 *ddd* (11, 11, 1)
8α	3.12 *dd* (11, 10)	3.14 *dd* (11, 10)	3.07 *dd* (11, 9)	3.15 *dd* (11, 9)
9β	3.80 *ddd* (10, 1.5, 1.5)	3.85 *ddd* (10, 1.5, 1.5)	3.79 *ddd* (9, 1.5, 1.5)	3.82 *ddd* (9, 1.5, 1.5)
11	1.76 *brs*	1.81 *brs*	0.96 *d* (7)	3.94 *brs*
12	2.27 *qqd* (7, 7, 1)	2.31 *qqd* (7, 7, 1)	2.24 *qqd* (7, 7, 1)	2.32 *qqd* (7, 7, 1)
13	1.00 *d* (7)	1.02 *d* (7)	0.98 *d* (7)	1.02 *d* (7)
14	1.09 *d* (7)	1.09 *d* (7)	1.04 *d* (7)	1.09 (7)
15a	4.76 *dd* (3, 1.5)	4.73 *dd* (3, 1.5)	4.70 *dd* (2.5, 1.5)	4.76 *dd* (2.5, 1.5)
15b	5.09 *dd* (3, 1.5)	5.08 *dd* (3, 1.5)	5.06 *dd* (2.5, 1.5)	5.08 *dd* (2.5, 1.5)

The investigation of NOE experiments of compound **1** proposed the relative stereochemistry of **1**. An 8% NOE was observed between H-8 and H-6 and a 5% NOE observed between H-7 and H-9, and the NOEs were absent between H-8 and H-7, and H-8 and H-9. Furthermore, a 12% NOE between H-9 and H-1 was observed. These results indicated the diaxial relationship between H-9 and H-1, as well as H-9 and H-7, H-8 and H-6. The presence of these diaxial relationships suggested the configurations of the C-8 and C-9 hydroxyl groups (8β-hydroxy and 9α-hydroxy) as shown in Fig. 1. Unfortunately the absolute configurations of these four new compounds were not determined.

EXPERIMENTAL

Mps. uncorr. IR spectra: KBr discs. ^{1}H and ^{13}C-NMR: 400 and 100 MHz respectively in CD_3OD; EIMS: 20 eV; HPLC was performed by Senshu pak ODS (10 × 300 mm) coupled with UV detecter and differential refractometer.

Culture conditions. The cultured fungi *Ganoderma mastoporum* used in this study was a generous gift from Dr H. Furukawa (Ohita Mushroom Research Institute). The mycelium was subcultured on a malt agar medium and 10-mm plugs were cut from the culture and seeded with 5 plugs to each Roux flask containing 200 ml soytone medium. The components of the soytone medium were glucose (15 g), soytone (1 g), yeast extract (0.5 g), KH_2PO_4 (0.5 g), NaCl (0.1 g), $MgSO_4 \cdot 7H_2O$(0.05 g), $CaCl_2 \cdot 2H_2O$ (0.11 g) and distilled H_2O (1 l). The dispersed medium in 1 l Roux flasks was autoclaved at 121° for 20 min. The mycelia were cultured statically at 25° in the dark for 52 days.

Extraction and separation. After 52 days culture (49 Roux flasks), the mycelia (1166.9 g) were harvested with nylon cloth and homogenized with 2.3 l MeOH and then allowed to stand for 10 days at room temp. The cultured broth, concd under red. pres. to 1.5 l and adjusted to pH 2 with 2 M HCl, was extracted with EtOAc (1.5 l × 2). The EtOAc layer was dried over Na_2SO_4 and then evapd to dryness to afford 855.4 mg crude extract.

Table 3. ^{1}H NOE difference spectral data for compound **1**

Irradiated proton	Enhanced proton		
1.00 (H_3-13)	3.12 (H_α-8) 10%	2.27 (H-12) 8%	
1.09 (H_3-14)	3.12 (H_α-8) 4%	2.27 (H-12) 10%	
1.24 (H_β-7)	3.80 (H_β-9) 5%	2.27 (H-12) 5%	
1.48 (H_α-2)	2.24 (H_β-2) 19%		
1.62 (H_α-6)	3.12 (H_α-8) 7%		
1.89 (H_β-1)	4.16 (H_β-3) 10%	3.80 (H_β-9) 14%	1.24 (H_β-7) 6%
2.24 (H_β-2)	5.61 (H-5) 10%	4.16 (H_β-3) 9%	1.48 (H_α-2) 21%
2.27 (H-12)	5.61 (H-5) 10%		
3.12 (H_α-8)	1.62 (H_α-6) 8%		
3.80 (H_β-9)	1.89 (H_β-1) 12%	1.24 (H_β-7) 6%	
4.16 (H_β-3)	5.61 (H-5) 8%	2.24 (H_β-2) 6%	1.89 (H_β-1) 9%
4.76 (Ha-15)	2.24 (H_β-2) 8%		
5.08 (Hb-15)	3.12 (H_α-8) 14%		
5.61 (H-5)	2.27 (H-12) 13%	1.76 (H_3-11) 8%	

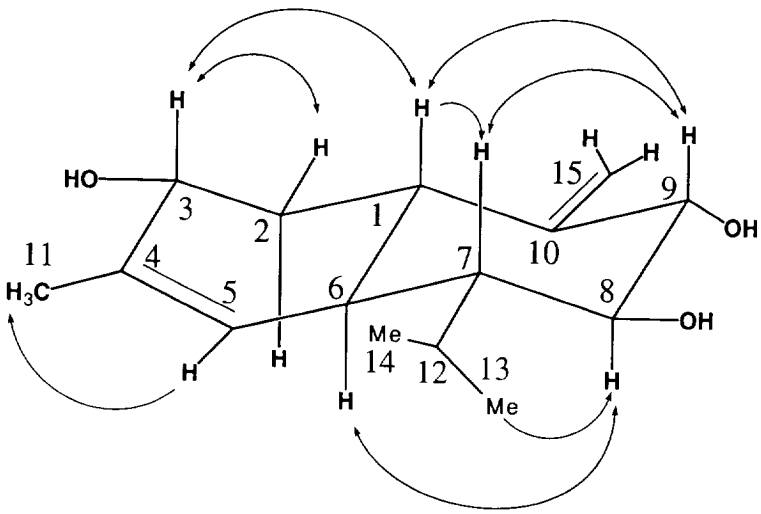

Fig. 1. Stereostructure of ganomastenol A (**1**) based on the NOE experiment.

After 10 days the MeOH-homogenized mycelium was filtered and the residue re-extracted with MeOH (2.3 l × 2) and organic solvent removed under red. pres. The residue was extracted with $CHCl_3$ (500 ml × 2) and the $CHCl_3$ fr. was dried and evapd to dryness (1.03 g). The combined $CHCl_3$ extract of the mycelia and the EtOAc extract of culture broth (1·885) was subjected to a silica gel column (Wako-gel C-200, 210 g) eluted with an EtOAc–*n*-hexane solvent system and separated into 6 frs. Fr. 1 eluted with *n*-hexane (200 ml) and EtOAc–*n*-hexane (1:4; 400 ml); fr. 2 eluted with EtOAc–*n*-hexane (1:4; 200 ml); fr. 3 EtOAc–*n*-hexane (1:1; 500 ml); fr. 4 EtOAc–*n*-hexane (1:1; 100 ml); fr. 5 EtOAc–*n*-hexane (1:1; 940 ml); fr. 6 EtOAc–*n*-hexane (1:1; 200 ml), EtOAc–*n*-hexane (3:2; 800 ml); EtOAc (300 ml) and MeOH (500 ml).

Isolation of compounds **1–4**. Fr. 3 afforded compound **1**, **3** and **4**. Repeated purification of fr. 3 (137.8 mg) by HPLC eluted with 40% MeCN in 2% HOAc solvent flow rate 3.0 ml min^{-1}, yielded two peaks, R_t 11.2 min. and R_t 14.2 min.

The peak R_t 14.2 min afforded compound **3**, recrystallized from MeOH and H_2O as needles (4.5 mg). The peak R_t 11.2 min was further purified by HPLC, eluted with 28% MeCN in 2% HOAc, flow rate 3 ml min^{-1} and afforded compound **1** from the peak R_t 20.9 min and compound **4** from the peak R_t 22.3 min. Recrystallization from MeOH and H_2O of the fraction R_t 20.9 gave compound **1** (12.6 mg) as a white powder. Another fr. R_t 22.3 min gave compound **4** (13.8 mg) as an oil.

Compound **1**. White powder (12.6 mg); FAB-MS *m/z*: 275[M + Na]$^+$; HREI-MS *m/z*: 234.1625 [M–H_2O]$^+$ $C_{15}H_{22}O_2$ requires 234.1620; EIMS *m/z* (rel. int.): 234 [M – H_2O]$^+$ (97), 191 [M – H_2O – isopropyl]$^+$ (49), 173 [M – 2H_2O – isopropyl]$^+$ (100). IR ν_{max}^{KBr} cm^{-1}: 3425 (OH), 2890(CH). ^{13}C and ^{1}H NMR: see Tables 1 and 2.

Compound **4**. Oil (13.8 mg); FAB-MS *m/z*: 275[M + Na]$^+$; HREI-MS *m/z*: 234.1620 [M – H_2O]$^+$

$C_{15}H_{22}O_2$ requires 234.1620: EIMS *m/z* (rel. int.): 234 $[M - H_2O]^+$ (100), 203 $[M - 2H_2O\text{-}H]^+$ (46). ^{13}C and ^1H NMR: see Tables 1 and 2.

Compound **3**. Needles (4.5 mg); mp 155.5–157° (MeOH, H_2O); FAB-MS *m/z*: 277 $[M + Na]^+$; HREI-MS *m/z*: 236.1778 $[M - H_2O]^+$ $C_{15}H_{24}O_2$requires 236.1777; EIMS *m/z* (rel. int.): 236 $[M - H_2O]^+$ (38), 218 $[M - 2H_2O]^+$ (40), 175 $[M - 2H_2O$ – isopronyl] (100), 91 $[M - 2H_2O$ – isopropyl-CH_3-CH_2] (28). IR ν_{max}^{KBr} cm^{-1} 3360 (OH), 2890 (CH). ^{13}C and ^1H NMR; see Tables 1 and 2.

Compound **2**. This product was obtained from fr. 5 by repeated purification with HPLC eluted with 40% MeCN in 2% HOAc, flow rate 3 ml min^{-1}. The peak R_t 12.8 min recrystallized from MeOH and H_2O gave compound **2** as needles (9.1 mg); mp 139–140°; FAB-MS *m/z*: 275 $[M + Na]^+$; HREI-MS *m/z*: 234.1614 $[M - H_2O]^+$ $C_{15}H_{22}O_2$ requires 234.1620; EIMS *m/z* (rel. int.); 234 $[M - H_2O]^+$ (10), 216 $[M - 2H_2O]^+$ (25), 173 $[M - 2H_2O$ – isopropyl-H$]^+$ (70), 145 $[C_{11}H_{13}]^+$ (100). IR ν_{max}^{KBr} cm^{-1}: 3320 (OH), 2880 (CH). ^{13}C and ^1H NMR: see Tables 1 and 2.

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