Geranylgeraniol-Type Diterpenoids, Boletinins A−J, from *Boletinus cavipes* as Inhibitors of Superoxide Anion Generation in Macrophage Cells

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In addition to16-hydroxygeranylgeraniol (**1**) and cavipetin B (**2**), 10 new geranylgeraniol-type diterpenoids, named boletinins A−J (**3**−**12**), were isolated from the fruiting bodies of *Boletinus cavipes*. Compounds **1**−**9** and **11** exhibited inhibitory activities of less than 10% at 25−125 μM in the xanthine oxidase test. A bioassay on superoxide anion ($O_2^{\bullet-}$) generation in macrophage cells revealed that **1** and **4**−**12** suppressed the generation by more than 20% at 25 μM. Compounds **4** and **5** showed inhibitory activities against $O_2^{\bullet-}$ generation of more than 50% at 50 μM and exhibited no or low cytotoxicities against macrophage cells at 25−50 μM, suggesting that **4** and **5** are the most promising candidates for $O_2^{\bullet-}$ generation inhibitors. *O*-Acyl geranylgeraniol derivatives, **2** and **7**−**12**, showed cytotoxicities at 25 μM.

Generation of superoxide anion ($O_2^{\bullet-}$), one of the active oxygens, is essential for the defense of living bodies and for signal transfer in living cells. The overgeneration of $O_2^{\bullet-}$, however, causes oxidative stress-related diseases such as inflammation, hypertension, aging, and cancer.[1] It would therefore be beneficial in maintaining a healthy body to scavenge overgenerated $O_2^{\bullet-}$ or to depress $O_2^{\bullet-}$ generation.[2,3] A large number of natural products have already been isolated and recognized as radical scavengers;[4,5] therefore, we have focused on $O_2^{\bullet-}$ generation inhibitors as candidates for prevention of oxidative stress-related diseases. An African legume, *Cassia spectabilis*, is one of the potent plants from which a new piperidine alkaloid, spectamine A, has been isolated as a possible inhibitor of $O_2^{\bullet-}$ generation.[6] We also conducted screening tests of the extracts from various species of mushrooms, finding in some species remarkable inhibitory activities against $O_2^{\bullet-}$ generation in macrophages. In the present paper, we describe the isolation and characterization of 10 new geranylgeraniol-type diterpenoids from *Boletinus cavipes* (Opat.) Kalchbr (Boletaceae). The inhibitory activities of $O_2^{\bullet-}$ generation of the isolated compounds were evaluated using macrophage cells induced by a tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The cell viabilities and xanthine oxidase (XOD) tests were also carried out to distinguish cytotoxic compounds and $O_2^{\bullet-}$ scavengers, respectively, from inhibitors of $O_2^{\bullet-}$ generation.

Results and Discussion

Bioassay-guided purification from a methanolic extract of *B. cavipes* led to the isolation of 16-hydroxygeranylgeraniol (**1**).[7] The notable inhibitory activity of **1** against $O_2^{\bullet-}$ generation urged us to examine the extract for related compounds, resulting in isolation of cavipetin B (**2**) and 10 new geranylgeraniol-type diterpenoids (**3**−**12**).[8]

The ¹H NMR spectrum of **3** was similar to that of **1**, except for the presence of *trans*-C*H*=C*H* (each 1H, δ 5.59 and 5.52, *J* = 15.6 Hz), =C−C*H₂*−C= (2H, δ 2.69), and C−C−C*H₂*−C−C (2H, δ 1.60) signals (Table 1).[7] In the ¹³C NMR spectrum of **3** (Table 2), an additional signal assignable to an oxygen-bearing carbon was observed other than those at δ 68.9 (CH₂) and 59.4 (CH₂). The additional signal
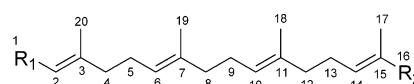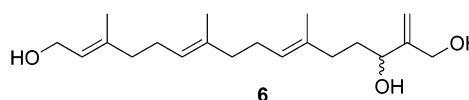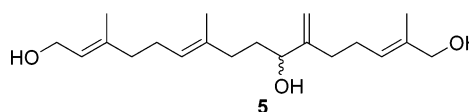

1: R₁=R₂=CH₂OH
2: R₁=R₂=fumaroyloxy-CH₂
7: R₁=CH₂OH, R₂=fumaroyloxy-CH₂
8: R₁=fumaroyloxy-CH₂, R₂=CH₂OH
9: R₁=fumaroyloxy-CH₂, R₂=CHO
10: R₁=fumaroyloxy-CH₂, R₂=COOH
11: R₁=mesaconoyloxy-CH₂, R₂=CHO
12: R₁=mesaconoyloxy-CH₂, R₂=COOH
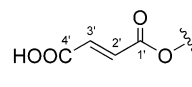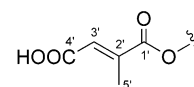
fumaroyloxy group   mesaconoyloxy group
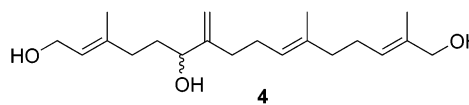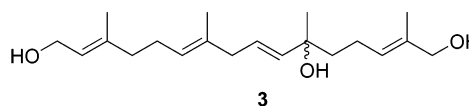
(**δ** 72.9) is quaternary; therefore the position of oxygenation must be C-3, C-7, C-11, or C-15. These observations led to the partial structure (Figure 1a), from which the structure of **3** was elucidated using 2D NMR spectra. The H−H COSY spectrum of **3** demonstrated the correlations between H₂-1 and H-2 and between H-2 and CH₃-20, excluding C-3 from the possible positions of the oxygenated carbon. The correlations between H-14 and H₂-16 and

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Table 1. ^{1}H NMR Data for **3**−**6** (500 MHz, CDCl$_3$)

| position	3	4	5	6
1	4.14 (2H, d, 6.8 Hz)	4.15 (2H, d, 6.9 Hz)	4.12 (2H, d, 6.7 Hz)	4.15 (2H, d, 6.8 Hz)
2	5.39 (1H, t, 6.8 Hz)	5.46 (1H, t, 6.9 Hz)	5.39 (1H, t, 6.7 Hz)	5.41 (1H, t, 6.8 Hz)
4	2.21 (2H, m)	2.09 (2H, m)	2.02 (2H, m)	1.99 (2H, m)
5	2.14 (2H, m)	1.64 (1H, m)	2.15 (2H, m)	2.00 (2H, m)
		1.53 (1H, m)		
6	5.13 (1H, t, 6.3 Hz)	4.05 (1H, d, 7.4 and 5.2 Hz)	5.17 (1H, t, 6.8 Hz)	5.15 (1H, t, 6.7 Hz)
8	2.69 (2H, d, 6.4 Hz)	2.14 (2H, m)	2.02 (2H, m)	1.99 (2H, m)
9	5.59 (1H, dt, 15.6 and 6.4 Hz)	2.19 (2H, m)	1.68 (1H, m)	2.00 (2H, m)
			1.58 (1H, m)	
10	5.52 (1H, d, 15.6 Hz)	5.14 (1H, t, 6.2 Hz)	4.06 (1H, dd, 8.1 and 4.3 Hz)	5.16 (1H, t, 6.7 Hz)
12	1.60 (2H, m)	2.06 (2H, m)	2.21 (2H, m)	2.09 (2H, m)
13	2.13 (2H, m)	2.15 (2H, m)	2.23 (2H, m)	1.76 (1H, m)
				1.72 (1H, m)
14	5.42 (1H, t, 7.3 Hz)	5.35 (1H, t, 6.9 Hz)	5.42 (1H, t, 6.5 Hz)	4.23 (1H, t, 6.6 Hz)
16	3.99 (2H, s)	3.98 (2H, s)	3.99 (2H, s)	4.30 (1H, d, 13.1 Hz)
				4.16 (1H, d, 13.1 Hz)
17	1.67 (3H, s)	1.66 (3H, s)	1.68 (3H, s)	5.13 (1H, s)
				5.10 (1H, s)
18	1.29 (3H, s)	1.61 (3H, s)	5.05 (1H, s)	1.62 (3H, s)
			4.87 (1H, s)	
19	1.67 (3H, s)	5.05 (1H, s)	1.63 (3H, s)	1.60 (3H, s)
		4.89 (1H, s)		
20	1.59 (3H, s)	1.69 (3H, s)	1.66 (3H, s)	1.68 (3H, s)

Table 2. ^{13}C NMR Data for **3**−**12** (125 MHz, CDCl$_3$)

position	3	4	5	6	7	8	9	10	11	12
1	59.4 (CH$_2$)	59.4 (CH$_2$)	59.4 (CH$_2$)	59.5 (CH$_2$)	59.5 (CH$_2$)	62.3 (CH$_2$)	62.4 (CH$_2$)	62.4 (CH$_2$)	62.6 (CH$_2$)	62.6 (CH$_2$)
2	123.8 (CH)	123.7 (CH)	124.2 (CH)	123.5 (CH)	123.0 (CH)	117.7 (CH)	117.6 (CH)	117.8 (CH)	117.8 (CH)	117.9 (CH)
3	139.2 (C)	139.4 (C)	138.9 (C)	139.7 (C)	140.1 (C)	143.3 (C)	143.3 (C)	143.2 (C)	143.1 (C)	143.0 (C)
4	39.3 (CH$_2$)	35.6 (CH$_2$)	39.3 (CH$_2$)	39.5 (CH$_2$)	39.7^a (CH$_2$)	39.7^a (CH$_2$)	39.6 (CH$_2$)	39.5 (CH$_2$)	39.5 (CH$_2$)	39.5 (CH$_2$)
5	26.0 (CH$_2$)	33.4 (CH$_2$)	25.7 (CH$_2$)	26.4 (CH$_2$)	26.7^b (CH$_2$)	26.7^b (CH$_2$)	26.2 (CH$_2$)	26.0 (CH$_2$)	26.2 (CH$_2$)	26.0 (CH$_2$)
6	124.8 (CH)	75.1 (CH)	125.0 (CH)	124.1 (CH)	125.0^c (CH)	124.6^c (CH)	123.7 (CH)	123.6 (CH)	123.7 (CH)	123.6 (CH)
7	134.1 (C)	151.4 (C)	135.5 (C)	135.1 (C)	135.4^d (C)	135.6^d (C)	135.4 (C)	135.6 (C)	135.4 (C)	135.4 (C)
8	42.4 (CH$_2$)	31.3 (CH$_2$)	36.2 (CH$_2$)	39.5 (CH$_2$)	39.4^a (CH$_2$)	39.4^a (CH$_2$)	39.5 (CH$_2$)	39.5 (CH$_2$)	39.6 (CH$_2$)	39.6 (CH$_2$)
9	126.1 (CH)	26.5 (CH$_2$)	33.2 (CH$_2$)	26.2 (CH$_2$)	26.2^b (CH$_2$)	26.2^b (CH$_2$)	26.6 (CH$_2$)	26.6 (CH$_2$)	26.6 (CH$_2$)	26.6 (CH$_2$)
10	138.0 (CH)	124.3 (CH)	75.1 (CH)	125.0 (CH)	123.8^c (CH)	123.5^c (CH)	125.6 (CH)	125.2 (CH)	125.6 (CH)	125.2 (CH)
11	72.9 (C)	135.0 (C)	151.1 (C)	134.6 (C)	134.1^d (C)	134.6^d (C)	133.4 (C)	133.7 (C)	133.4 (C)	133.7 (C)
12	42.3 (CH$_2$)	39.2 (CH$_2$)	31.0 (CH$_2$)	35.9 (CH$_2$)	38.9^a (CH$_2$)	39.2^a (CH$_2$)	38.0 (CH$_2$)	38.0 (CH$_2$)	38.0 (CH$_2$)	38.0 (CH$_2$)
13	22.5 (CH$_2$)	25.8 (CH$_2$)	26.0 (CH$_2$)	34.0 (CH$_2$)	26.1^b (CH$_2$)	26.0^b (CH$_2$)	27.5 (CH$_2$)	27.5 (CH$_2$)	27.5 (CH$_2$)	27.6 (CH$_2$)
14	126.3 (CH)	125.7 (CH)	125.4 (CH)	74.4 (CH)	129.9 (CH)	126.4 (CH)	154.6 (CH)	145.3 (CH)	154.6 (CH)	145.2 (CH)
15	135.0 (C)	135.2 (C)	135.3 (C)	149.9 (C)	129.2^d (C)	134.4^d (C)	139.4 (C)	126.8 (C)	139.4 (C)	126.9 (C)
16	68.9 (CH$_2$)	68.9 (CH$_2$)	68.8 (CH$_2$)	64.2 (CH$_2$)	71.0 (CH$_2$)	69.0 (CH$_2$)	195.4 (CH)	173.3 (C)	195.4 (CH)	173.3 (C)
17	13.7 (CH$_3$)	13.7 (CH$_3$)	13.8 (CH$_3$)	112.3 (CH$_2$)	14.0 (CH$_3$)	13.7 (CH$_3$)	9.2 (CH$_3$)	12.0 (CH$_3$)	9.2 (CH$_3$)	12.0 (CH$_3$)
18	28.2 (CH$_3$)	16.0 (CH$_3$)	109.8 (CH$_2$)	16.0 (CH$_3$)	16.0^e (CH$_3$)	16.1^e (CH$_3$)	15.9 (CH$_3$)	16.0 (CH$_3$)	15.9 (CH$_3$)	16.1 (CH$_3$)
19	16.2 (CH$_3$)	110.1 (CH$_2$)	15.9 (CH$_3$)	16.0 (CH$_3$)	15.9^e (CH$_3$)	16.0^e (CH$_3$)	16.0 (CH$_3$)	15.9 (CH$_3$)	16.0 (CH$_3$)	15.9 (CH$_3$)
20	16.2 (CH$_3$)	16.3 (CH$_3$)	15.9 (CH$_3$)	16.3 (CH$_3$)	16.3 (CH$_3$)	16.5 (CH$_3$)	16.6 (CH$_3$)	16.6 (CH$_3$)	16.6 (CH$_3$)	16.5 (CH$_3$)
1'					164.7 (C)	164.8 (C)	164.7 (C)	164.7 (C)	166.9 (C)	167.0 (C)
2'					132.9 (CH)	132.8 (CH)	132.4 (CH)	132.8 (CH)	146.3 (C)	146.1 (C)
3'					135.0 (CH)	135.4 (CH)	135.7 (CH)	135.4 (CH)	125.6 (CH)	125.8 (CH)
4'					167.3 (C)	168.4 (C)	168.5 (C)	169.7 (C)	169.9 (C)	170.8 (C)
5'									14.6 (CH$_3$)	14.6 (CH$_3$)

$^{a–e}$ Values with the same symbol may be interchanged.
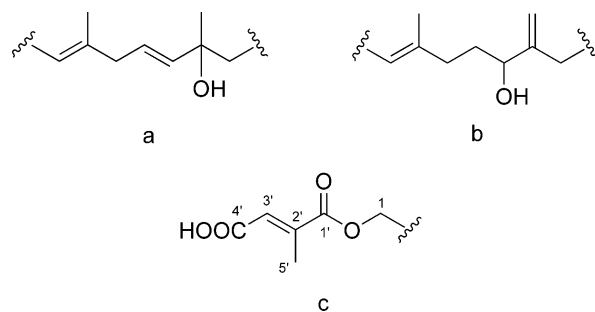
Figure 1. Partial structures of (a) **3**, (b) **4**−**6**, and (c) **11** and **12**.

between H-14 and CH$_3$-17 ruled out C-15. The oxygenated carbon position turned out to be C-11 and not C-7, as proven by the H−H COSY and the HMBC correlations. The HREIMS of **3** showed an ion at *m/z* 286.2274 (C$_{20}$H$_{30}$O, [M − 2H$_2$O]$^+$), which corresponded to the molecular formula of an oxygenated form of **1** (C$_{20}$H$_{34}$O$_2$). Thus, the structure

of **3** was established, and this new diterpenoid was named boletinin A (**3**).

Compound **4** showed an [M]$^+$ ion at *m/z* 322.2518 in the HREIMS, suggesting a molecular formula of C$_{20}$H$_{34}$O$_3$. The ^{1}H NMR spectrum of **4** exhibits signals similar to those of **1** (Table 1). A couple of signals assignable to one of the four −C*H*=C(C*H*$_3$)− groups are absent, while signals due to C=C*H*$_2$ (each 1H, δ 5.05 and 4.89) and −C*H*(OH)− (1H, δ 4.05) were observed in the spectrum of **4**. With these data, the signals at δ 110.1 (C=*C*H$_2$) and δ 75.1 [−*C*H(OH)−] in the ^{13}C NMR spectrum suggested the partial structure shown in Figure 1b (Table 2). The signal assignable to CH$_3$-20 was determined using the H−H COSY spectrum. The correlations from C-4 to CH$_3$-20 and from C-4 to H-6 were observed in the HMBC spectrum of **4**. All these spectral data revealed that the oxygenated position of **4** was located at C-6. We named this new diterpenoid boletinin B (**4**).

The ^{1}H and ^{13}C NMR spectra of **5** were almost identical to those of **4** except for slight differences in the δ values

Table 3. ^{1}H NMR Data for **7**−**10** (500 MHz, CDCl$_3$)

position	7	8	9	10
1	4.18 (2H, d, 6.9 Hz)	4.72 (2H, d, 7.2 Hz)	4.73 (2H, d, 7.0 Hz)	4.73 (2H, d, 7.2 Hz)
2	5.41 (1H, t, 6.9 Hz)	5.37 (1H, t, 7.2 Hz)	5.38 (1H, t, 7.0 Hz)	5.37 (1H, t, 6.9 Hz)
4	1.96 (2H, m)	1.96 (2H, m)	2.06 (2H, m)	1.96 (2H, m)
5	2.01 (2H, m)	1.96 (2H, m)	2.06 (2H, m)	1.96 (2H, m)
6	5.11 (1H, t, 6.9 Hz)	5.08 (1H, t, 6.7 Hz)	5.09 (1H, t, 6.4 Hz)	5.09 (1H, t, 6.3 Hz)
8	1.96 (2H, m)	1.96 (2H, m)	1.98 (2H, t, 8.0 Hz)	1.96 (2H, m)
9	2.01 (2H, m)	1.96 (2H, m)	2.06 (2H, m)	1.96 (2H, m)
10	5.11 (1H, t, 6.9 Hz)	5.11 (1H, t, 6.5 Hz)	5.15 (1H, t, 6.7 Hz)	5.14 (1H, t, 6.4 Hz)
12	1.96 (2H, m)	1.96 (2H, m)	2.16 (2H, t, 7.3 Hz)	2.05 (2H, t, 7.4 Hz)
13	2.01 (2H, m)	1.96 (2H, m)	2.45 (2H, q, 7.3 Hz)	2.29 (2H, q, 7.4 Hz)
14	5.47 (1H, t, 7.0 Hz)	5.39 (1H, t, 7.6 Hz)	6.47 (1H, t, 7.3 Hz)	6.89 (1H, t, 7.4 Hz)
16	4.58 (2H, s)	4.00 (2H, s)	9.38 (1H, s)	
17	1.67 (3H, s)	1.66 (3H, s)	1.74 (3H, s)	1.83 (3H, s)
18	1.60 (3H, s)	1.60 (3H, s)	1.63 (3H, s)	1.61 (3H, s)
19	1.60 (3H, s)	1.60 (3H, s)	1.60 (3H, s)	1.59 (3H, s)
20	1.68 (3H, s)	1.73 (3H, s)	1.73 (3H, s)	1.73 (3H, s)
2′	6.85 (1H, d, 15.8 Hz)	6.84 (1H, d, 15.8 Hz)	6.84 (1H, d, 15.8 Hz)	6.83 (1H, d, 15.8 Hz)
3′	6.92 (1H, d, 15.8 Hz)	6.91 (1H, d, 15.8 Hz)	6.93 (1H, d, 15.8 Hz)	6.92 (1H, d, 15.8 Hz)

(Tables 1 and 2), suggesting that the partial structure shown in Figure 1b is located at a different position in the molecule. A peak at m/z 323.2597 ($C_{20}H_{35}O_3$, [M + H]$^+$) in the HRFABMS of **5** supported this presumption. The H−H COSY spectrum of **5** showed the correlation between H-14 and H$_2$-16. In the HMBC spectrum, correlations from C-12 to H-18 and from C-12 to H-14 were observed. These spectral data showed that the oxygenation site is at C-10. Compound **5**, named boletinin C, is a regioisomer of **4**.

Compound **6** is also a regioisomer of **4** on the basis of the ^{1}H and ^{13}C NMR spectra and the HRFABMS (Tables 1 and 2). The δ values (δ 4.23, 5.13, and 5.10) of −C*H*(OH)C-(=C*H$_2$*)− shifted downfield, compared with those of **4** (δ 4.05, 5.05, and 4.89) and **5** (δ 4.06, 5.05, and 4.87), probably due to the presence of the adjacent −CH$_2$OH group at C-16. The HMBC spectrum showed the correlation from C-16 to H-17, elucidating the structure of **6**, named boletinin D. The absolute configurations of **3**−**6** could not be determined because of their small amounts.

The UV spectrum of **7** showed λ_{max} at 198 nm, while those of **3**−**6** exhibited end absorption only (<190 nm), suggesting the presence of conjugated system(s) in the molecule. Bands observed at 1716, 1297, and 1262 cm^{-1} in the IR spectrum of **7** suggested the presence of an α,β-unsaturated ester. The ^{1}H NMR spectrum of **7** was similar to that of **1** (Table 3), except for the presence of additional doublets (each 1H, δ 6.92 and 6.85, J = 15.8 Hz). In the ^{13}C NMR spectrum of **7** (Table 2), signals were observed at δ 167.3 (C), 164.7 (C), 135.0 (CH), and 132.9 (CH) in addition to those of a 16-geranylgeraniol moiety. These spectroscopic data suggested that the additional moiety consisted of a *trans*-CH=CH and two C=O (ester or amide) groups. The HRFABMS of **7** exhibited a negative [M − H]$^-$ ion at m/z 403.2507, which indicated that **7** is an *O*-fumaroyl derivative of **1**. The 2D NMR spectra confirmed the presence of the fumaroyloxy group. The substituted position, either C(1)-OH or C(16)-OH, was determined to be the latter by the HMBC spectrum, in which the correlation from *C*(=O)→C(16)-*H$_2$* was observed. This new diterpenoid was named boletinin E (**7**).

The spectral data of **8** were almost identical to those of **7** (Tables 2 and 3). The HRFABMS of **8** exhibited a negative [M − H]$^-$ ion at m/z 403.2458, which indicated the same molecular formula as that of **7**, $C_{24}H_{36}O_5$. However, the δ values of C(1)-*H$_2$* and C(16)-*H$_2$* of **8** are δ 4.72 and 4.00, respectively, while those of **7** are δ 4.18 and 4.58, respectively. This observation suggested that the substituted

position of **8** was C(1)-OH, as established by the HMBC spectrum. Compound **8**, named boletinin F, is a regioisomer of **7**.

The ^{13}C and ^{1}H NMR spectra of **9** were similar to those of **8** (Tables 2 and 3), but **9** showed a negative [M − H]$^-$ ion at m/z 401.2327 in the HRFABMS, indicating a molecular formula of $C_{24}H_{34}O_5$. These spectroscopic data suggested that **9** is a dehydrogenated form of **8** ($C_{24}H_{36}O_5$). A singlet (1H, δ 9.38) in the ^{1}H NMR spectrum and a signal (δ 195.4, CH) in the ^{13}C NMR spectrum confirmed the presence of a −CHO group. The position of the fumaroyl group, C(16)-OH, was established using the HMBC spectrum of **9**. The UV spectrum of **9** showed λ_{max} at 200 and 230 nm. The latter would be derived from an α,β-unsaturated aldehyde, since the λ_{max} value for R−CH=C(CH$_3$)−CHO was calculated to be 229 nm on the basis of the Woodward−Feiser rules.[9] This new diterpenoid was named boletinin G (**9**).

Compound **10** exhibited a negative [M − H]$^-$ ion at m/z 417.2270 in the HRFABMS, suggesting that the molecular formula is $C_{24}H_{34}O_6$, which is an oxygenated analogue of **9**. The singlet assignable to −CHO was not observed in the ^{1}H NMR spectrum (Table 3), and the signal of the carbonyl group shifted upfield (δ 173.3, C) in the ^{13}C NMR spectrum (Table 2). These observations indicated that **10** possesses a −COOH group instead of a −CHO group. The structure and substitution at C-1 were confirmed by the 2D NMR spectra of **10**. This new compound was named boletinin H (**10**).

In the ^{13}C and ^{1}H NMR spectra of **11** (Tables 2 and 4), all the signals assignable to the diterpene moiety were almost identical to those of **9**, but C(1)-OH of **11** was substituted by a functional group other than fumaroyl. The doublets due to a *trans*-CH=CH were absent, but two singlets (1H, δ 6.80 and 3H, δ 2.31) were observed in the ^{1}H NMR spectrum. A partial structure of **11** as shown in Figure 1c was suggested by the correlations from C(1)-*H$_2$*←*C*(1′)=O→C(5′)*H$_3$* in the HMBC spectrum. These results indicated that **11** is a 1-*O*-mesaconoyl derivative of **9**, as supported by a peak at m/z 415.2481 ($C_{25}H_{35}O_5$, [M − H]$^-$) in the HRFABMS of **11**, named boletinin I.

The ^{13}C and ^{1}H NMR spectra of **12** were similar to those of **11** (Tables 2 and 4), but a negative [M − H]$^-$ ion peak at m/z 431.2442 in the HRFABMS suggested that **12** is an oxygenated form of **11**. The singlet assignable to −CHO was not observed in the ^{1}H NMR spectrum, and the signal of the carbonyl group shifted upfield (δ 173.3, C) in the ^{13}C NMR spectrum, indicating that **12** possesses a −COOH

Table 4. ^{1}H NMR Data for **11** and **12** (500 MHz, CDCl$_3$)

position	11	12
1	4.72 (2H, d, 7.0 Hz)	4.72 (2H, d, 7.0 Hz)
2	5.38 (1H, t, 7.0 Hz)	5.37 (1H, t, 7.0 Hz)
4	2.05 (2H, m)	2.05 (2H, m)
5	2.05 (2H, m)	2.05 (2H, m)
6	5.10 (1H, t, 6.5 Hz)	5.09 (1H, t, 6.6 Hz)
8	1.98 (2H, m)	1.97 (2H, m)
9	2.05 (2H, m)	2.05 (2H, m)
10	5.15 (1H, t, 6.8 Hz)	5.14 (1H, t, 6.7 Hz)
12	2.16 (2H, t, 7.3 Hz)	2.05 (2H, m)
13	2.45 (2H, q, 7.3 Hz)	2.27 (2H, m)
14	6.47 (1H, t, 7.3 Hz)	6.89 (1H, t, 7.2 Hz)
16	9.38 (1H, s)	
17	1.74 (3H, s)	1.83 (3H, s)
18	1.63 (3H, s)	1.61 (3H, s)
19	1.60 (3H, s)	1.60 (3H, s)
20	1.74 (3H, s)	1.73 (3H, s)
3′	6.80 (1H, s)	6.78 (1H, s)
5′	2.31 (3H, s)	2.30 (3H, s)

Table 5. Inhibitory Effects of **1**–**12** on O$_2$$^{•-}$ Generation in Macrophage Cells

tested compd	macrophage test				XOD test	
	inhibition (%)[a]		viability (%)		inhibition (%)[a]	
	25 μM	50 μM	25 μM	50 μM	25 μM	125 μM
1	84 ± 1	NT[b]	82	6	1 ± 2	9 ± 14
2	6 ± 4	NT[b]	5	0	−1 ± 1	7 ± 7
3	12 ± 7	18 ± 13	98	86	5 ± 6	7 ± 1
4	45 ± 0	74 ± 8	98	84	2 ± 2	5 ± 4
5	39 ± 2	52 ± 9	89	75	0 ± 6	7 ± 1
6	33 ± 8	45 ± 8	92	60	1 ± 4	6 ± 3
7	26 ± 5	NT[b]	16	0	−7 ± 8	6 ± 1
8	22 ± 6	NT[b]	3	0	−2 ± 2	8 ± 1
9	26 ± 7	NT[b]	58	22	0 ± 0	6 ± 13
10	29 ± 5	NT[b]	50	18	8 ± 6	33 ± 6
11	41 ± 4	NT[b]	33	2	1 ± 2	7 ± 9
12	21 ± 7	NT[b]	47	27	−5 ± 6	20 ± 4
quercetin	19 ± 8	28 ± 14	99	101	31 ± 8	72 ± 2

[a] Values are means ± SD ($N = 3$). [b] Not tested.

group. The substitution at C-1 was confirmed by the HMBC spectra of **12**. This new diterpenoid was named boletinin J (**12**).

Ten geranylgeraniol derivatives, including cavipetins A–E, were isolated from the fruiting bodies of *B. cavipes* so far.[7,8] Cyclic diterpenoids, on the other hand, have never been reported as components of *B. cavipes*, suggesting that this fungus lacks the cyclization-catalyzing enzymes in the diterpenoid biosynthetic pathways.

The inhibitory effects of **1**–**12** on O$_2$$^{•-}$ generation in macrophage cells are summarized in Table 5. In the xanthine oxidase (XOD) test, **1**–**9** and **11** exhibited inhibitory activities of less than 10% at 125 μM, while quercetin, a positive control as a radical scavenger, showed an inhibitory activity of 31% at 25 μM and 72% at 125 μM. These results revealed that **1**–**9** and **11** possess no ability to quench O$_2$$^{•-}$. In the macrophage test, where the macrophage cells were induced to generate O$_2$$^{•-}$ by the application of TPA, **1** and **4**–**12** suppressed the generation by more than 20% at 25 μM. Compounds **1**, **4**–**9**, and **11** could be regarded as either O$_2$$^{•-}$ generation suppressers or cytotoxic compounds, considering their inability to quench O$_2$$^{•-}$. We thus measured cell viabilities with regard to the application of **1**–**12**. Compounds **2** and **7**–**12**, which are *O*-acyl geranylgeraniol derivatives, showed cytotoxicities at 25 μM. The application of **1** exhibited a cell viability of 6% at 50 μM, suggesting its cytotoxicity. Compounds **3**–**6** showed no or low cytotoxicities against macrophage cells at 25–50 μM. We conducted the macrophage test of **3**–**6** also at

50 μM, in which **4** and **5** showed prominent inhibitory activities of more than 50%. On the basis of the assay results, **4** and **5** would be the most promising candidates for O$_2$$^{•-}$ generation inhibitors. To the best of our knowledge, this is the first report on geranylgeraniol-type diterpenoids possessing O$_2$$^{•-}$ generation inhibitory activity. Compounds **1**–**12** possess no or low ability to quench O$_2$$^{•-}$, although geranylgeraniol-type diterpenoids, 12,13-dehydrogeranylgeraniol and neogrifolin, have been reported as antioxidants.[10,11] The triene moiety of 12,13-dehydrogeranylgeraniol and the polyphenol moiety of neogrifolin could be responsible for the activities.

Experimental Section

General Experimental Procedures. Optical rotation values were measured with a JASCO DIP1000 polarimeter. Mass spectra were obtained by a JEOL JMS 700 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 FT-NMR spectrometer operating at 500.1 MHz for protons and at 125.8 MHz for carbons, with TMS used as the internal standard. IR spectra were measured with a JASCO IR spectrometer. The ultraviolet spectra were recorded on a Shimadzu UV mini 1240 spectrophotometer. The visible absorptions were measured using a Bio-Rad Model 550.

Fungal Material. Fruiting bodies of *B. cavipes* were collected in Nagano, Japan. Authenticated voucher specimens, FB-33809 (CBM) and FB-33810 (CBM), have been deposited at the Natural History Museum and Institute, Chiba, Japan.

Extraction and Isolation of 1 and 3–6. Fruiting bodies (2.3 kg) of *B. cavipes* collected in September 1996 were extracted using MeOH at room temperature. The extract was filtered and concentrated in vacuo, and the resulting concentrate (59 g) was successively partitioned between *n*-hexane, EtOAc, and H$_2$O. The major inhibitory activity was found in the EtOAc layer in a bioassay using macrophages as described below. Half of the EtOAc layer (6.5 g) was concentrated and subjected to silica gel (Wakogel C-300, 160 g; Wako Pure Chemical Industries) column chromatography using *n*-hexane–EtOAc as the eluent (500 mL/fraction). The *n*-hexane–EtOAc (1:1) eluate was subjected to silica gel (C-300, 55 g) column chromatography using CHCl$_3$–acetone (19:1) as the eluent (20 mL/fraction). Fractions 18–21 were subjected to silica gel (60H, 17 g; Merck) column chromatography using benzene–acetone (9:1) as the eluent (15 mL/fraction). The eluate (fractions 10–15) was subjected to silica gel (60H, 17 g) column chromatography using *n*-hexane–EtOAc (7:3) as the eluent (15 mL/fraction). Fraction 12 was concentrated, giving **1** (6.9 mg). The rest of the EtOAc layer (6.1 g) was partitioned between EtOAc and 5% NaHCO$_3$. The EtOAc layer (1.9 g) was concentrated and subjected to silica gel (C-300, 100 g) column chromatography using benzene–EtOAc as the eluent. The benzene–EtOAc (7:13) eluate was subjected to silica gel (60H, 17 g) column chromatography using *n*-hexane–acetone (4:1) as the eluent (10 mL/fraction). Fractions 25–28 were purified by HPLC with an ODS column (YMC RS-323, 250 × 10 mm) eluting with MeOH–H$_2$O (7:3) at a flow rate of 3.0 mL/min with detection at 210 nm to give **5** (4.7 mg) and **6** (2.5 mg). Fractions 29–35 were purified by HPLC with an ODS column (Nomura Chemical ODS-UG-5, 250 × 8 mm) eluting with MeOH–H$_2$O (13:7) at a flow rate of 3.0 mL/min with detection at 210 nm to give **3** (3.4 mg) and **4** (4.5 mg).

Boletinin A (3): colorless oil; $[\alpha]^{18}_D$ −3.0° (*c* 0.23, CHCl$_3$); UV (CH$_3$CN) λ_{max} <190 nm; IR (film) ν_{max} 3335, 2920, 2856, 1436, 1383, 1005, 965 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 1; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HREIMS *m/z* 286.2274 (calcd for C$_{20}$H$_{30}$O [(M − 2H$_2$O)$^+$], 286.2297).

Boletinin B (4): colorless oil; $[\alpha]^{18}_D$ +0.7° (*c* 0.35, CHCl$_3$); UV (CH$_3$CN) λ_{max} <190 nm; IR (film) ν_{max} 3341, 2922, 1445, 1012, 898 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 1; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HREIMS *m/z* 322.2518 (calcd for C$_{20}$H$_{34}$O$_3$ [M$^+$], 322.2508).

Boletinin C (5): colorless oil; $[\alpha]^{27}_D$ −0.1° (*c* 0.53, CHCl$_3$); UV (CH$_3$CN) λ_{max} <190 nm; IR (film) ν_{max} 3338, 2925, 2857,

1442, 1382, 1063, 1005, 899 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 1; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 323.2597 (calcd for C$_{20}$H$_{35}$O$_3$ [(M + H)$^+$], 323.2586).

Boletinin D (6): colorless oil; [α]^{25}$_D$ −0.7° (*c* 0.18, CHCl$_3$); UV (CH$_3$CN) λ_{max} <190 nm; IR (film) ν_{max} 3345, 2924, 1718, 1667, 1446, 1382, 1253, 1017, 911 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 1; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 323.2560 (calcd for C$_{20}$H$_{35}$O$_3$ [(M + H)$^+$], 323.2587).

Extraction and Isolation of 9 and 11. Fruiting bodies (3.6 kg) of *B. cavipes* collected in September 2002 were extracted using MeOH at room temperature. The extract was filtered and concentrated in vacuo, and the resulting concentrate (160 g) was successively partitioned between *n*-hexane, benzene, CHCl$_3$, and H$_2$O. The benzene layer (14 g) was concentrated and subjected to silica gel (C-300, 280 g) column chromatography using CHCl$_3$−acetone (49:1) as the eluent (200 mL/fraction). Fractions 6−8 were subjected to silica gel (C-300, 85 g) column chromatography using benzene as the eluent (300 mL/fraction), and fractions 1−3 and 5 were purified further. Fraction 5 was subjected to silica gel (60H, 17 g) column chromatography using *n*-hexane−acetone−HOAc (90: 10:1) as the eluent (8 mL/fraction), and the eluate (fractions 20 and 21) was purified by HPLC with an ODS column (YMC RS-323) eluting with MeOH−H$_2$O−HOAc (90:10:0.1) at a flow rate of 3.0 mL/min with detection at 254 nm to give **9** (10.1 mg). Fractions 1−3 were subjected to silica gel (C-300, 63 g) column chromatography using *n*-hexane−EtOAc−HOAc (90: 10:1) as the eluent (50 mL/fraction), and the eluate (fraction 7) was purified by HPLC with an ODS column (YMC RS-323) eluting with MeOH−H$_2$O−HOAc (88:12:0.2) at a flow rate of 3.0 mL/min with detection at 254 nm to give **11** (8.8 mg).

Boletinin G (9): colorless oil; UV (CH$_3$CN) λ_{max} (log ϵ) 200 (4.40), 230 (sh) (4.15) nm; IR (film) ν_{max} 2923, 1716, 1645, 1446, 1384, 1296, 1260, 1165, 980 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 3; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 401.2327 (calcd for C$_{24}$H$_{33}$O$_5$ [(M − H)$^-$], 401.2328).

Boletinin I (11): colorless oil; UV (CH$_3$CN) λ_{max} (log ϵ) 195 (4.50), 224 (sh) (4.42) nm; IR (film) ν_{max} 2924, 1718, 1687, 1645, 1438, 1382, 1251, 1118, 903 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 4; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 415.2481 (calcd for C$_{25}$H$_{35}$O$_5$ [(M − H)$^-$], 415.2484).

Isolation of 2, 7, 8, 10, and 12. The CHCl$_3$ layer (10 g) was concentrated and subjected to silica gel (C-300, 200 g) column chromatography using CHCl$_3$−acetone (19:1) as the eluent (200 mL/fraction), and Fractions 5−6 and 7 were purified further. Fractions 5 and 6 were subjected to silica gel (C-300, 110 g) column chromatography using *n*-hexane−EtOAc−HOAc (100:0:1, 95:5:1, and 90:10:1) as the eluent (100 mL/fraction). The *n*-hexane−EtOAc−HOAc (90:10:1) eluate was purified by HPLC with an ODS column (YMC RS-323) eluting with MeOH−H$_2$O−HOAc (85:15:0.3) at a flow rate of 3.0 mL/min with detection at 254 nm to give **12** (9.2 mg). Fraction 7 was subjected to silica gel (C-300, 100 g) column chromatography using *n*-hexane−EtOAc−HOAc (90:10:1, 85: 15:1, 80:20:1, and 70:30:1) as the eluent. The *n*-hexane−EtOAc−HOAc (80:20:1 and 70:30:1) eluates were subjected to silica gel (60H, 17 g) column chromatography using *n*-hexane−acetone−HOAc (80:20:1) as the eluent (10 mL/fraction). Fractions 11−13 were purified by HPLC with a silica gel column (YMC SH-043-5, 250 × 20 mm) eluting with *n*-hexane−*i*-PrOH−HOAc (96:4:1) at a flow rate of 7.5 mL/min with detection at 254 nm to give **7** (2.5 mg) and **8** (9.7 mg). Fractions 17−22 were concentrated, giving **2** (76.8 mg). The *n*-hexane−EtOAc−HOAc (90:10:1) eluate was subjected to silica gel (60H, 17 g) column chromatography using *n*-hexane−acetone−HOAc (95:5:1) as the eluent (10 mL/fraction). Fractions 47−51 were subjected to ODS (YMC AM120-S50, 33 g) column chromatography using MeOH−H$_2$O−HOAc (75:25:0.1) as the eluent (10 mL/fraction). Fractions 20−32 were purified by HPLC with an ODS column (YMC RS-323) eluting with MeOH−H$_2$O−

HOAc (90:10:0.1) at a flow rate of 3.0 mL/min with detection at 254 nm to give **10** (16.6 mg).

Boletinin E (7): colorless powder; UV (CH$_3$CN) λ_{max} (log ϵ) 198 (4.44) nm; IR (film) ν_{max} 2921, 1716, 1644, 1447, 1385, 1297, 1262, 1163, 980 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 3; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 403.2507 (calcd for C$_{24}$H$_{35}$O$_5$ [(M − H)$^-$], 403.2485).

Boletinin F (8): colorless powder; UV (CH$_3$CN) λ_{max} (log ϵ) 197 (4.59) nm; IR (film) ν_{max} 3445, 2921, 1716, 1644, 1445, 1384, 1296, 1260, 1164, 980 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 3; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 403.2458 (calcd for C$_{24}$H$_{35}$O$_5$ [(M − H)$^-$], 403.2485).

Boletinin H (10): colorless oil; UV (CH$_3$CN) λ_{max} (log ϵ) 203 (5.49) nm; IR (film) ν_{max} 2924, 1698, 1644, 1422, 1385, 1294, 1164, 979 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 3; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 417.2270 (calcd for C$_{24}$H$_{33}$O$_6$ [(M − H)$^-$], 417.2277).

Boletinin J (12): colorless oil; UV (CH$_3$CN) λ_{max} (log ϵ) 197 (4.47), 220 (sh) (4.28) nm; IR (film) ν_{max} 2926, 1720, 1693, 1644, 1425, 1384, 1262, 1238, 1120, 904 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$), see Table 4; ^{13}C NMR (125 MHz, CDCl$_3$), see Table 2; HRFABMS m/z 431.2442 (calcd for C$_{25}$H$_{35}$O$_6$ [(M − H)$^-$], 431.2433).

Macrophage Test and Cell Viability. The macrophage test was performed according to the reported method with modifications.[12,13] Test compound solution dissolved in DMSO (5 mM, 15 μL) was added to Hank's buffer (500 μL). Macrophage cell suspension was prepared from female CD mice that were intraperitoneally injected with 10% peptone solution (1 mL). After 4 days, peritoneal exudate cells were collected, centrifuged for 10 min (1400g) × 3, and diluted with Hank's buffer to 3.0 × 10^7 cells/mL. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) dissolved in DMSO (0.1 mg/mL, 15 μL) was added to Hank's buffer (500 μL). For the macrophage test, the test compound solution (25 μL), the macrophage cell suspension (50 μL), and the TPA solution (25 μL) were successively added to a test tube containing nitro blue tetrazolium (150 μg) in Hank's buffer (50 μL). The tube was incubated at 37 °C for 2 h. The reaction was terminated by placing the tube in a freezer (−20 °C) for 1 h, followed by addition of DMSO (50 μL) and 2% SDS solution (50 μL) to the thawed solution. The visible absorption at 540 nm was measured on a microplate (each 200 μL) after ultrasonication of the solution at 0 °C. Inhibitory effects are expressed by the decreasing ratio of absorbance of the test compound relative to that of a control experiment. Cell viability was assessed by the trypan blue exclusion test. The experiment complied with regulations concerning animal experimentation and the care of experimental animals of the Faculty of Agriculture at Shinshu University.

XOD Test. The XOD test was performed according to the reported method with some modifications.[14] *N*-1-Naphthyl-ethylenediamine dihydrochloride (3.9 mg) was dissolved in hot distilled H$_2$O (300 mL), followed by the addition of sulfanilic acid (260 mg), HOAc (125 mL), and distilled H$_2$O to 500 mL in preparing the color-forming reagent. KH$_2$PO$_4$ (2.21 g), Na$_2$B$_4$O$_7$·10H$_2$O (3.34 g), and EDTA·2Na (46.5 mg) were dissolved in distilled H$_2$O (250 mL) for 65 mM KH$_2$PO$_4$−35 mM Na$_2$B$_4$O$_7$·10H$_2$O buffer (pH 8.2). For the assay, a test compound solution in DMSO (20 μL) and xanthine oxidase solution (200 mU/mL, 20 μL) were added to a test tube containing the KH$_2$PO$_4$−Na$_2$B$_4$O$_7$ buffer (20 μL), hypoxanthine solution (0.5 mM, pH 8.2−8.4, 20 μL), hydroxylamine hydrochloride (10 mM, 10 μL), and distilled H$_2$O (10 μL). After incubation at 37 °C for 30 min, the color-forming reagent (200 μL) was added to the solution, after which the solution was left for 1 h at room temperature. The visible absorption at 540 nm was measured on a microplate (each 200 μL). Inhibitory effects are expressed by the decreasing ratio of absorbance of the test compound relative to that of a control experiment.

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