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Running title: New lanostane triterpenoid from *Hypholoma fasciculare*

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A new lanostane triterpenoid from the mushroom *Hypholoma fasciculare*

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ABSTRACT

A novel compound (**1**) and three known compounds (**2**−**4**) were isolated from the fruiting bodies of *Hypholoma fasciculare*. The structure of **1** was determined by the interpretation of spectroscopic data. Compounds **2**−**4** were identified by comparing the spectra data of known compounds. In the bioassay examining growth inhibitory activity against phytopathogenic bacteria *Clavibacter michiganensis*, *Burkholderia glumae* and *Peptobacterium carotovorum*, compounds **1**, **2** and **4** showed inhibition effects on *C. michiganensis* only.

Keywords: structural determination, mushroom, *Hypholoma fasciculare*, isolation, phytopathogenic bacterial activity

Hypholoma fasciculare (Japanese name: Nigakuritake) is a small, bitter poisonous mushroom of the genus Hypholoma. The mushroom is widely distributed worldwide and grows on the stumps of old trees in tufts (Shiono *et al.* 2004). This mushroom is known to produce diverse compounds including steroids, triterpenoids and sesquiterpenoids, and the toxic components have been identified as lanostane triterpenoids. (Kim *et al.*1997; Ikeda *et al.* 1997; Kubo *et al.* 1985; Suzuki *et al.* 1983; Takahashi *et al.* 1989; Doi *et al.* 1990). Fasciculols A−F inhibited the growth of Chinese cabbage seedlings (Ikeda *et al.* 1997a-c). Fasciculol D also showed antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* (Ikeda *et al.* 1997a). Moreover, fasciculols E and F paralyzed the respiratory center of mice and caused death (Suzuki *et al.* 1983). Fasciculic acids A−C have been isolated as calmodulin inhibitors (Takahashi *et al.* 1989).

Phytopathogenic fungi and bacteria can reduce crop yields and cause extensive damage (Dang *et al.* 2014). Among the phytopathogenic bacteria, most are Gram-negative, however, of some Gram-positive phytopathogens sometimes can also cause significant losses in crop cultivation (Gartemann *et al.* 2003). To control the plant disease rapidly and effectively, one generally achieved way is using synthetic pesticides and antibiotics (Kotan *et al.* 2014). However, these chemicals are associated with undesirable effects on the environment due to their slow biodegradation in the environment and some toxic residues in the degraded products for mammalian health (Barnard *et al.* 1997; Isman *et al.* 2000). Therefore, it is important to look for effective chemicals from natural sources that can be used against phytopathogenic bacteria

without affecting the environment. Although the lanostane triterpenoids from this mushroom have been reported many biological activities, any research based on the aspect mentioned above has not been carried out. In this study, we describe the isolation, structural determination, and anti-phytopathogenic bacterial activity of the compounds.

Results and discussion

The fresh fruiting bodies of *H. fasciculare* were extracted with EtOH and acetone. The solutions were combined and concentrated under reduced pressure. The concentrated extracts were partitioned between *n*-hexane and water, and then EtOAc and water, successively. The water soluble part was dried under reduced pressure, and then extracted with EtOH. The EtOAc soluble part was fractionated by repeated chromatography and four compounds (**1**–**4**) were purified (**Figure 1**).

Compound **1** was obtained as a colorless gum with a molecular formula of $C_{40}H_{67}NO_{10}$ deduced by the molecular ion peak $[M + Na]^+$ at *m/z* 744.4650 (calcd for $C_{40}H_{67}NO_{10}Na$, 744.4657) in the HRESIMS. The structure of **1** was elucidated by interpretation of NMR spectra (**Table 1**) including DEPT, COSY, HMQC, and HMBC (**Figures S1-5**). The ^{13}C NMR, DEPT and HMQC data established the presence of 10 methyls, 12 methylenes, 7 methines and 11 tetrasubsutituted carbons, including two olefinic carbons [δ_C 133.7 and 136.7], two oxygenated tetrasubsutituted carbons [δ_C 71.6 and 73.9], and three carboxy groups [δ_C 171.3, 173.1 and 174.0]. The ^1H NMR showed the presence of signals due to nine methyls [δ_H 0.65 (s), 0.91 (s), 0.91 (s), 1.08 (s), 1.11 (s), 1.13 (s), 1.16 (s), 1.26 (t) and 1.41 (s)], a secondary methyl [δ_H 1.03 (d, J = 6.5 Hz)], and four oxygenated methines [δ_H 3.22 (m), 3.82 (ddd, J = 12.0, 10.1, 4.6 Hz), 4.00 (d, J = 7.8 Hz), and 4.57 (d, J = 10.1 Hz)]. All these data showed that **1** is a lanostane triterpenoid. The lanostane skeleton was elucidated by the HMBC correlations (H-1/C-2, 3, 5, 10; H-3/C-2, 4, 28, 29; H-5/C-3, 4, 6, 7, 10; H-7/C-5, 8, 9, 14; H-11/C-8, 9, 12; H-12/C-9, 13, 14, 18; H-15/C-13, 14, 16, 30; H-17/C-13, 14, 20; H-18/C-13, 14, 17; H-

19/C-1, 5, 9, 10; H-21/C-20, 22; H-22/C-21; H-23/C-22; H-24/C-22, 23, 25, 26; H-26/C-24, 25, 27; H-27/C-24, 25, 26) and the COSY correlations (H-1/H-2, H-2/H-3, H-5/H-6, H-6/H-7, H-11/H-12, H-16/H-17, H-17/H-20, H-23/H-24) (**Figure 2**). Moreover, the HMBC correlations (H-3/C-1') confirmed the presence of the side chain is combined with C-3. The structure of side chain was elucidated by the HMBC correlations (H-2'/C-1', 3', 4', 5'; H-4'/C-2', 3', 5'; H-5'/C-2', 3', 4', 6'; H-7'/C-6', 8'; H-9'/C-8', 10'; H-10'/C-9') (**Figure 2**). As a result, the plane structure of **1** was determined as shown in **Figure 1**, which is similar to fasciculol G (**2**) except an ethoxy group at C-8'. The absolute configuration except for C-3'of **1** was determined by the comparation of NMR chemical shift (Kim *et al.* 2013) and the CD spectrum of **2** (**Figure S6**), however, the absolute configuration at C-3' remains unknown (**Figure 1**).

Since EtOH was used for the extraction and fractionation, there is a possibility that **1** is an artifact. To confirm that **1** is a natural product, the fruiting bodies of *H. fasciculare* were extracted with MeOH and fractionated by middle pressure liquid chromatography not using EtOH. As a result, LC-MS/MS analysis showed the existence of **1** in a fraction (**Figure S7**).

Compound **2** was identified as fasciculol G, which has significant selective cytotoxicity against the SK-MEL-2 cell line of malignant melanoma (IC_{50}＝8.60 μM) (Kim *et al.* 2013) (**Figures S8-12**). Compound **3** was identified as fasciculic acid B, which showed inhibition activity of plant growth (Takahashi *et al.* 1989) (**Figures S13-17**). Compound **4** has been isolated from the mushroom of *Naematoloma sublateritium* as fasciculol B (Bernardi *et al.* 1981) (**Figures S18-22**), which has the activity to inhibit

97 the root elongation of the plant (Ikeda *et al.* 1977c) (**Figure 1**).

98 Compounds **1** to **4** were tested for effect on the growth of *Clavibacter*
99 *michiganensis*, *Burkholderia glumae*, and *Pectobacterium carotovorum*. *C.*
100 *michiganensis*, a Gram-positive plant pathogenic bacterium, is the causal agent of
101 bacterial canker disease of tomato (Davis *et al.* 1984). *B. glumae*, a Gram-negative
102 bacterium, was first described in Japan as the cause of grain rotting and seedling blight
103 on rice (Gartemann *et al.* 2003; Goto *et al.* 1956). *P. carotovorum* causes soft-rot disease
104 in diverse plants (Roh *et al.* 2010). As a result, **1**, **2** and **4** inhibited the growth of *C.*
105 *michiganensis* at 0.1 μmol/paper disc (**Figure 3**, **Table 2**), while all the compounds
106 showed no activity against the growth of *B. glumae* and *P. carotovorum*.

107 A comparison of the structures between **1** to **4** indicated that the carboxy group
108 at C-6' of **3** weakened the inhibition of the growth of *C. michiganensis.* This bacterium
109 is one of the most severe pathogens of tomato (Jacques *et al.* 2012). Control of the
110 bacterium is known to be very difficult (Fatmi *et al*. 2017). In addition, there are very
111 few control measures for gram-positive bacterial plant pathogens. **1**, **2** and **4** could be
112 leading compounds for development of specific antibacterial agents against *C.*
113 *michiganensis.*
114

Experimental

General Experimental procedures

^{1}H NMR spectra (one- and two-dimensional) were recorded on JNM-ECZ500R spectrometer at 500 MHz, and ^{13}C NMR spectra were recorded on the same instrument at 125 MHz (JEOL, Tokyo, Japan). HRESIMS spectra were measured on a JMS-T100LP mass spectrometer (JEOL, Tokyo, Japan). An FT/IR-4100 (Jasco, Tokyo, Japan) instrument was used to record the IR spectra, and the specific rotation values were measured by a Jasco DIP-1000 polarimeter (Jasco, Tokyo, Japan). CD spectra was recorded by J-820 Spectropolarimeter. HPLC separations were performed with a Jasco Chromatography Data Station ChromNAV system using reverse-phase HPLC columns (ODS-P, InertSustain, Tokyo, Japan). Silica gel plate (Merck F254), ODS gel plate (Merck F254), and silica gel 60 N (Kanto Chemical, Tokyo, Japan) were used for analytical TLC and for flash column chromatography. All solvents used throughout the experiments were obtained from Kanto Chemical Co. (Tokyo, Japan).

Fungal Material

Fresh fruiting bodies of *H. fasciculare* were collected at Narusawa village, Yamanashi Prefecture, Japan, in 2018 and identified by one of the author, H. Ko.. The fruiting bodies (aerial part) were cut with a knife and preserved in a refrigerator at -30°C until extraction.

Extraction and Isolation

The fresh fruiting bodies of *H. fasciculare* (9.0 kg) were extracted with EtOH (27 L, 3 times) and then with acetone (15 L, 3 times). The solutions were combined, concentrated under reduced pressure, and partitioned between *n*-hexane and water, ethyl acetate (EtOAc) and water, and the water part concentrated under reduced pressure, and extracted with EtOH, successively. The EtOAc soluble part (35.7 g) was fractionated by silica gel flash column chromatography (50% *n*-hexane/CH_2Cl_2, CH_2Cl_2, 80%, 60%, 50%, 40%, 30%, 20%, 10% CH_2Cl_2/acetone, 70%, 50%, 40%, 30%, 20% CH_2Cl_2/MeOH, MeOH) to obtain 18 fractions (Fractions 1~18), and fraction 12 (1.0 g) was further separated by silica gel flash column chromatography (CH_2Cl_2, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% CH_2Cl_2/MeOH; MeOH; acetone and MeOH; 1.5 L each) to obtain 15 fractions (Fractions 12-1~12-15). Fraction 12-3 (210.3 mg) was further separated by reverse-phase HPLC (ODS-P, 80% MeOH) to afford compound **1** (1.9 mg). Compound **2** (4.0 mg) was purified from fraction 12-2 (90.7 mg) by reverse-phase HPLC (ODS-P, 80% MeOH). Fraction 12-5 (48.6 mg) was separated by reverse-phase HPLC (ODS-P, 80% MeOH) to afford compound **3** (3.8 mg) and compound **4** (3.5 mg).

Compound **1**: Colorless gum; ^{1}H and ^{13}C NMR, see **Table 1**; $[\alpha]_D^{25}$ +30 (*c* 0.20, MeOH); ESIMS *m/z* 744 $[M + Na]^+$; HRESIMS *m/z* 744.4650 $[M + Na]^+$ (calcd for $C_{40}H_{67}NO_{10}Na$, 744. 4657).

Antibacterial Activity

YP medium (yeast extract 5 g/L, peptone 10 g/L, agar 15 g/L) was mixed with 100 μL of suspensions of three plant phytopathogenic bacterial (*Burkholderia glumae* SUPP1744, *Peptobacterium carotovorum* SUPP8, and *Clavibacter michiganensis* SUPP573). 0.1, 0.05, and 0.01 μmol of each compound were dissolved in MeOH, and 40 μL of sample was processed on paper discs. The discs were incubated at 28°C for 3 days and observed.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

H. Ka. conceived the project. H. Ka. and J. Wu designed the chemical experiments. J. Wa., K. T., H. Ko. and J.-H. C. performed the experiments. Y. T. provided the bacteria strains and designed the bioassay. J. Wa., J. Wu and H. Ka. wrote the manuscript. All authors contributed to discussions.

Funding

This work was supported in part by a Grant-in Aid for Scientific Research on Innovative Areas “Frontier Research on Chemical Communications” (No 17H06402).

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Acknowledgment

We thank Mr. Yuji Kamba for providing the picture of *H. fasciculare*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure 1. Structures of **1**−**4**.

Figure 2. $^{1}H-^{1}H$ COSY and HMBC correlations for **1**.

Figure 3. Activity of **1** to **4** against *Burkholderia glumae*, *Pectobacterium carotovorum* and *Clavibacter michiganensis* (positive control, ampicillin).

Table 1. NMR data for **1** in CD_3OD.

Table 2. Inhibitory activity of **1**–**4** for *C. michiganensis* (0.1 μmol/paper disc).

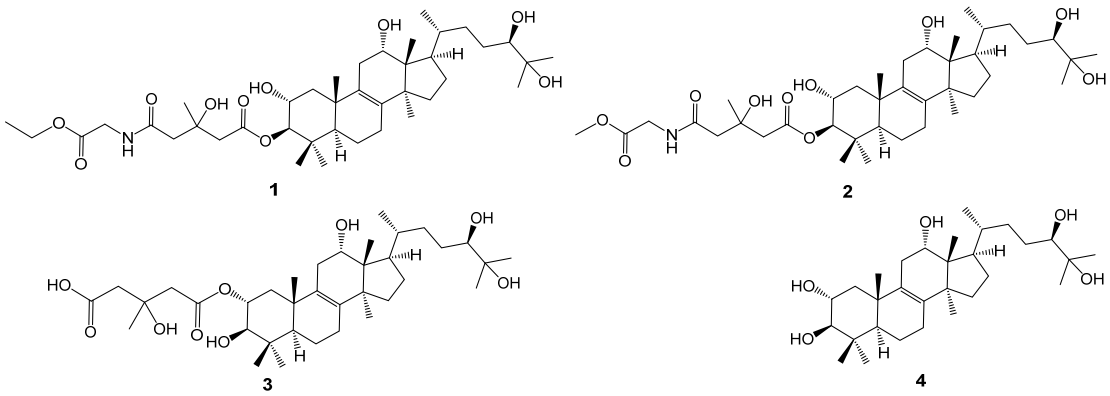

Figure 1. Structures of **1**−**4**.

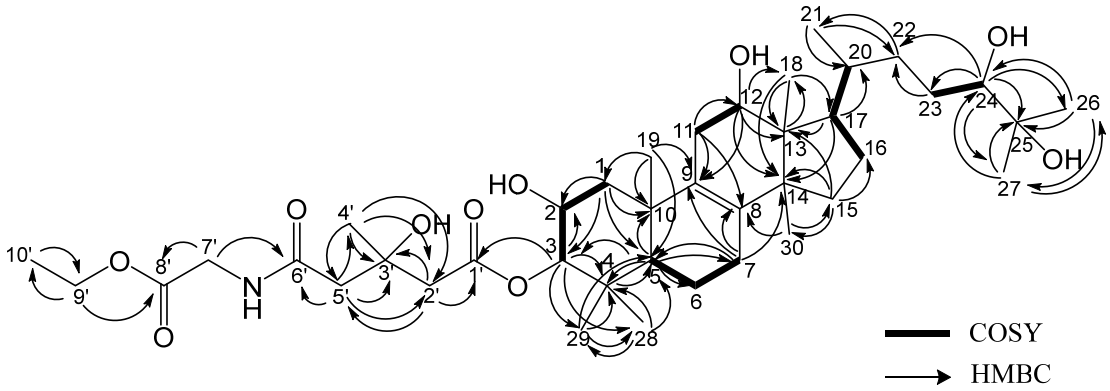

Figure 2. ^{1}H−^{1}H COSY and HMBC correlations for **1**.

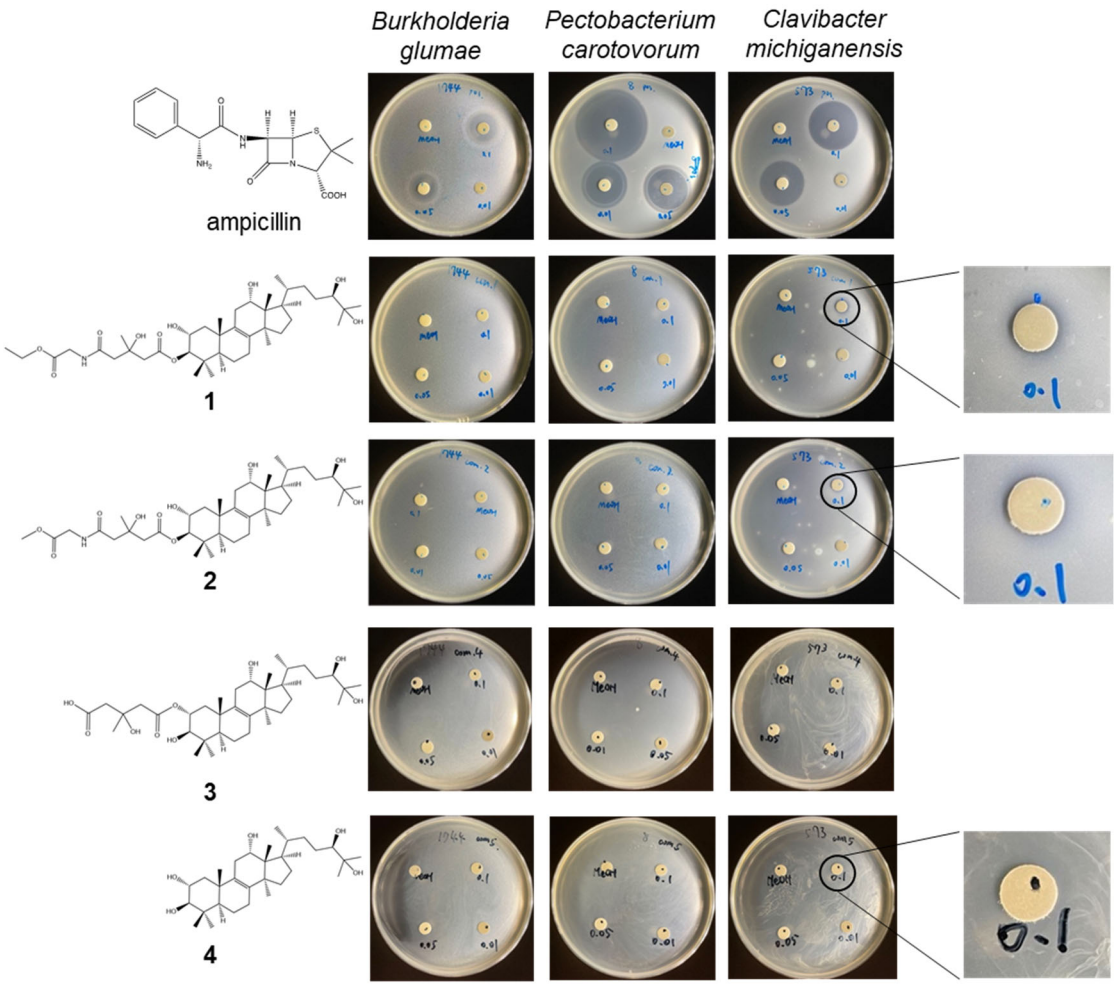

Figure 3. Activity of **1** to **4** against *Burkholderia glumae*, *Pectobacterium carotovorum* and *Clavibacter michiganensis* (positive control, ampicillin).

Table 1. NMR data for **1** in CD_3OD.

Position	δ_C, type	δ_H (type, multiplicity, *J* in Hz)
1	45.0, CH_2	1.36 (m)
		2.09 (m)
2	68.0, CH	3.82 (ddd, 12.0, 10.1, 4.6)
3	85.9, CH	4.57 (d, 10.1)
4	40.1, C	
5	51.8, CH	1.27 (m)
6	19.3, CH_2	1.58 (m)
		1.72 (m)
7	27.3, CH_2	2.10 (m)
8	136.7, C	
9	133.7, C	
10	39.0, C	
11	34.6, CH_2	2.08 (m)
		2.67 (m)
12	73.6, CH	4.00 (d, 7.8)
13	50.6*, C	
14	50.7*, C	
15	33.2, CH_2	1.16 (m)
		1.69 (m)
16	29.0, CH_2	1.38 (m)
		1.53 (m)
17	44.1, CH	2.21 (m)
18	17.0, CH_3	0.65 (s)
19	20.5, CH_3	1.08 (s)
20	37.6, CH	1.44 (m)
21	18.0, CH_3	1.03 (d, 6.5)
22	34.4, CH_2	1.28 (m)
		1.53 (m)
23	29.0, CH_2	1.38 (m)
		1.53 (m)
24	79.8, CH	3.22 (m)
25	73.9, C	
26,27	25.0, CH_3	1.13 (s)
	25.7, CH_3	1.16 (s)
28	29.1**, CH_3	0.91 (s)
29	18.0**, CH_3	0.91 (s)
30	25.3, CH_3	1.11 (s)
1’	173.1, C	
2’	46.9, CH_2	2.73 (s)
3’	71.6, C	
4’	28.0, CH_3	1.41 (s)
5’	47.3, CH_2	2.59 (s)
6’	174.0, C	
7’	42.0, CH_2	3.92 (d, 17.5)
		3.95 (d, 17.5)
8’	171.3, C	
9’	62.3, CH_2	4.18 (q, 7.2)
10’	14.5, CH_3	1.26 (t, 7.2)

Symbols "*" and "**" represent that the values with the same symbol are interchangeable between each other.

Table 2. Inhibitory activity of **1**–**4** for *C. michiganensis* (0.1 μmol/paper disc[a]).

Bacterial	Diameter of inhibition zone (mm)				
	ampicillin	**1**	**2**	**3**	**4**
C. michiganensis	33.0	9.7	10.7	na [b]	10.0
	33.1	10.1	11.8	na	10.1

[a] paper disc (8.0 mm in diameter)
[b] no activity