Fascicularones C and D, Tricyclo[5.4.0.0^{2,5}]undecane Sesquiterpenoids from the Liquid Culture of *Naematoloma fasciculare*

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Two novel sesquiterpenoids of tricyclo[5.4.0.0$^{2.5}$]undecane skeleton, fascicularones C (**5**) and D (**6**) have been isolated from liquid culture of a poisonous mushroom, *Naematoloma fasciculare*. Their structures were elucidated on the basis of spectroscopic methods. Compounds **5** and **6** promoted radicle elongation of lettuce seedling.

Key words: Naematoloma fasciculare, Fascicularones C and D

Introduction

N. fasciculare is a poisonous mushroom widely distributed in northeast part of Japan (Japanese name: Nigakuritake). Phytochemical investigation of this mushroom revealed some biologically active compounds which include new lanostane-type triterpenoids, fasciculols A-F [1 – 3]. In addition, *N. fasciculare*, when grown in liquid culture, produced a complex mixture of sesquiterpene metabolites of a new structural skeleton. Previously, Ito *et al*. and Doi *et al*. have reported the isolation and structure elucidation of some ring fused new sesquiterpenes, naematolin possessing cytotoxicity [4] and naematolins B, C (**1**) and G (**2**) [5 – 7] from the liquid culture filtrates of this fungus. These naematolins C (**1**) and G (**2**), tricyclo[5.4.0.0^{2,5}]undecane sesquiterpenoids, have attracted considerable attention due to their molecular complexity and intriguing biosynthesis. In our continuing search for chemical constituents from the mycelial culture of this fungus, we have isolated two novel sesquiterpenoids named fascicularones A (**3**) and B (**4**) having tricyclic ring skeleton [8]. Further investigation for metabolites of this fungal strain in detail let to the isolation of two fascicularone related compounds, fascicularones C (**5**) and D (**6**). This paper describes the isolation and structure elucidation of **5** and **6**.

Results and Discussion

The fungal strain isolated from cultured tissues of fruiting bodies of mushrooms *N. fasciculare* was fermented at 25 °C for 90 days in a medium containing 4% glucose, 0.1% peptone and 4% malt extract in distilled water. Purification of fascicularones was guided by characteristic coloration by TLC as described previously [8]. The culture filtrate was absorbed onto Amberlite XAD-2. The fraction eluted with MeOH was purified by a combination of Sephadex LH-20 and silica gel column chromatographies to afford fascicularones C (**5**) and D (**6**) (Fig. 1).

The molecular formula of fascicularone C (**5**) was determined to be $C_{15}H_{24}O_4$ by HRFABMS, indicating four degrees of unsaturation. The IR absorption showed that the presence of hydroxyl groups (3400 cm^{-1}) and a carbonyl group (1702 cm^{-1}). The ^{13}C NMR together with the DEPT spectra revealed 15 carbon signals including signals due to four methyls [δ_C 15.6 (C-14), 16.3 (C-15), 25.0 (C-13) and 33.5 (C-12)], two methylenes [δ_C 34.2 (C-10) and 40.2 (C-5)], five methines including two oxygen-bearing methines [δ_C 36.4 (C-9), 37.3 (C-4), 58.5 (C-1), 71.9 (C-6) and 81.5 (C-2)], three quaternary carbons, one of which was linked to an oxygen [δ_C 32.9 (C-11), 59.8 (C-8) and 90.6 (C-3)] and a carbonyl carbon [δ_C 214.1 (C-7)] (Table 1). The ^{1}H NMR and ^{1}H-^{1}H COSY spectra of **5** indicated the presence of three tertiary methyls [δ_H 1.19 (3H, s, H_3-12), 1.21 (3H, s, H_3-13) and 1.40 (3H, s, H_3-15)], a -C*H*$_2$-C*H*-C*H*-C*H* (OH)-linkage [δ_H 1.59 (1H, ddd, J = 11.2, 9.3, 2.0 Hz, H-10), 2.67 (1H, dd, J = 11.2, 7.8 Hz, H-10), 3.99 (1H, m, H-9), 2.53 (1H, m, H-1) and 4.69 (1H, br. s, H-2)], and a C*H*$_3$-C*H*-C*H*$_2$-C*H* (OH)-linkage [δ_H 1.64 (3H, d, J = 6.9 Hz,

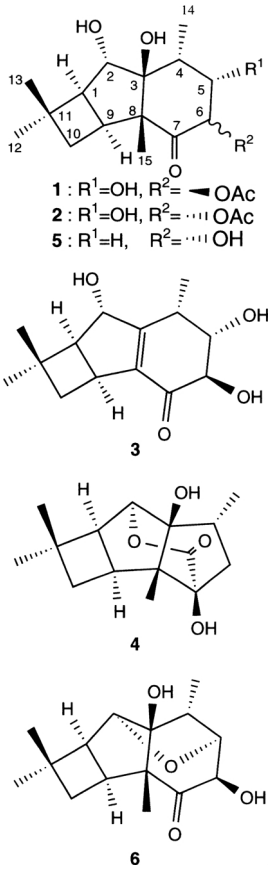

Fig. 1. Structures of naematolins C (**1**), G (**2**), fascicularones A (**3**), B (**4**), C (**5**) and D (**6**).

H-14), 2.50 (1H, m, H-4), 2.33 (1H, ddd, J = 12.2, 7.3, 4.9 Hz, H-5), 2.85 (1H, q, 12.2, H-5), 4.90 (1H, d, J = 12.2, 7.3 Hz, H-6)]. Based on these data, compound **5** was assumed to be a tricyclic sesquiterpenoid. The ^{1}H and ^{13}C-NMR spectra of **5** were similar to those of fascicularone A (**3**) [8]. Resonances in the ^{1}H and ^{13}C NMR spectra of **5** confirmed the presence of a 4-membered ring with *gem*-dimethyl group (C-1, C-9, C-10, C-11, C-12 and C-13) found in **3**. In the HMBC spectrum of **5** (Table 1), the signals H_3-14 correlated with C-3, the signals of H_2-5 with C-3 and C-7, signals H-4 and H-6 with C-8. These data suggested the presence of a cyclohexanone ring made up with C-3, C-4, C-5, C-6, C-7 and C-8. The methyl proton at δ_H 1.40 was correlated with C-3, C-7 and C-9, indicative of methyl at C-8. The H-4 showed HMBC correlations for C-2, and H-9 showed HMBC correlations for C-3 and C-7, thus giving rise to connectivities of C-3 to C-2 and C-8 to C-9. These 2D data let to the

Table 1. ^{1}H and ^{13}C NMR data for fascicularone C (**5**).

No.	δ_C (Mult.)	δ_H (Mult. J Hz)	HMBC (^{1}H to ^{13}C)
1	58.5 d	2.53 (1H, m)	2, 3, 9, 10, 12, 13
2	81.5 d	4.69 (1H, br. s)	1, 3, 8, 9, 11
3	90.6 s		
4	37.3 d	2.50 (1H, m)	2, 3, 5, 6, 8, 14
5	40.2 t	2.33 (1H, ddd,	
		12.2, 7.3, 4.9)	3, 4, 6, 7, 14
		2.85 (1H, q, 12.2)	3, 4, 6, 7, 14
6	71.9 d	4.90 (1H, dd,	
		12.2, 7.3)	4, 5, 7, 8
7	214.1 s		
8	59.8 s		
9	36.4 d	3.99 (1H, m)	1, 2, 3, 7, 8, 11
10	34.2 t	α 1.59 (1H, ddd,	
		11.2, 9.3, 2.0)	1, 8, 9, 11, 12, 13
		β 2.67 (1H, dd,	
		11.2, 7.8)	1, 8, 9, 11, 12, 13
11	32.9 s		
12	33.5 q	1.19 (3H, s)	1, 11, 13
13	25.0 q	1.21 (3H, s)	1, 10, 11, 12
14	15.6 q	1.64 (3H, d, 6.9)	3, 4, 5
15	16.3 q	1.40 (3H,s)	3, 7, 8, 9
2, 6-OH		5.71 (2H, br. s)	
3-OH		5.98 (1H, br. s)	2, 3, 8

Taken in pyridine-d_5 at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR).

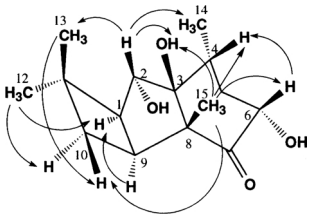

Fig. 2. NOE effects obtained from the NOE difference spectra of fascicularone C (**5**).

complete assignments of ^{1}H and ^{13}C signals of **5** as shown in Table 1. Thus the gross structure of fascicularone C was assigned as **5**. The relative stereochemistry of **5** was established by nuclear Overhauser effect (NOE) difference experiment (Fig. 2) in which NOEs from H_3-15 to H-4, H-6, OH-3 and H-10β, from H-9 to H-1 and from H-2 to 3-OH, H_3-13 and H_3-14, from H_3-13 to H-10β, and from H_3-12 to H-1 and H-10α. These observed NOEs implied that hydrogens at C-2, C-4 and C-6, methyl group at C-8 and hydroxyl group at C-3 had β-configurations and hydrogens at C-1 and C-9 had α-configurations. It is assumed that absolute stereochemistry at C-2 of **5** possesses the same as those of **1** and **3** from a biosynthetic point of view. Consequently, the structure of **5** was determined

Table 2. ^{1}H and ^{13}C NMR data for fascicularone D (**6**).

No.	δ_C (Mult.)	δ_H (Mult. *J* Hz)	HMBC (^{1}H to ^{13}C)
1	54.8 d	2.38 (1H, dd, 7.3, 2.9)	2, 3, 10, 12, 13
2	87.9 d	4.47 (1H, s)	3, 4, 5, 8, 9, 11
3	92.9 s		
4	42.8 d	1.73 (1H, m)	2, 3, 6, 8, 14
5	82.1 d	4.01 (1H, s)	2, 3, 6, 7, 14
6	77.5 d	3.90 (1H, s)	4, 5, 7, 8
7	214.6 s		
8	63.8 s		
9	42.8 d	2.69 (1H, q, 7.3)	1, 2, 3, 7, 10, 11
10	35.1 t	α 1.72 (1H, m)	1, 8, 9, 12, 13
		β 2.06 (1H, dd, 11.7, 7.3)	1, 8, 9, 12, 13
11	32.1 s		
12	31.5 q	1.20 (3H, s)	1, 10, 11, 13
13	25.1 q	1.03 (3H, s)	1, 10, 11, 12
14	12.9 q	1.20 (3H, d, 6.8)	3, 4, 5
15	11.3 q	1.16 (3H, s)	3, 7, 8, 9
3, 6-OH		2.00 (2H, br. s)	

Taken in $CDCl_3$ at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR).

as be (1*S*,9*S*)-2α,3β,6α-trihydroxy-4α,11,11,8β-tetramethyltricyclo[5.4.0.0^{2,5}]undecan-7-one (Fig. 1).

Fascicularone D (**6**) has its molecular formula, $C_{15}H_{22}O_4$, established by HRFABMS, thus requiring five degrees of unsaturation. Its IR spectrum contained absorption bands at 3405 cm^{-1}, 1702 cm^{-1} and 1037 cm^{-1}, characteristic of hydroxyls, a ketone and a C-O-C groups. The ^{13}C NMR spectrum of **6** contained 15 carbons and its DEPT spectrum indicated the presence of four methyls [δ_C 11.3 (C-15), 12.9 (C-14), 25.1 (C-13) and 31.5 (C-12)], a methylenes [δ_C 35.1 (C-10)], six methines including three oxygen-bearing methines [δ_C 42.8 (C-4), 42.8 (C-9), 54.8 (C-1), 77.5 (C-6), 82.1 (C-5) and 87.9 (C-2)], three quaternary carbons, one of which was linked to an oxygen [δ_C 32.1 (C-11), 63.8 (C-8) and 92.9 (C-3)] and a carbonyl carbon [δ_C 214.6 (C-7)] (Table 2). The ^{1}H and ^{13}C NMR spectra of **6** were similar to those of **5**. In the ^{1}H NMR spectrum of **6**, signals at δ_H1.03 (3H, s), 1.20 (3H, s), 1.72 (1H, m), 2.06 (1H, dd, *J* = 11.7, 7.3 Hz), 2.38 (1H, dd, *J* = 7.3, 2.9 Hz) and 2.69 (1H, q, *J* = 7.3 Hz) were assigned to H_3-13, H_3-12, H-10α, H-10β, H-1 and H-9, respectively. These signals supported the presence of a 4-membered ring with *gem*-dimethyl group. A close inspection of the ^{1}H- and ^{13}C NMR of **6** by ^{1}H-^{1}H COSY and HMQC experiments indicated the presence of a C*H*$_3$-C*H*-C*H* (O)-C*H* (OH)- linkage [δ_H1.20 (3H, d, *J* = 6.8, H_3-14), 1.73 (1H, m, H-4), 4.01 (1H, s, H-5) and 3.90 (1H, s, H-6)], one tertiary methyl [δ_H 1.16 (3H, s, H_3-15)] and an oxygenated methine signal [4.47 (1H, s, H-2)]. It

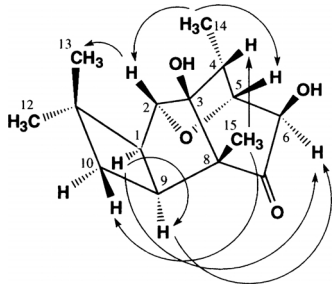

Fig. 3. NOE effects obtained from the NOE difference spectra of fascicularone D (**6**).

can be inferred that **6** contained a tetracyclic sesquiterpene structure considering the degree of unsaturation on 4-membered ring and other substituents. The connection of partial structures and remaining functional groups was determined based on HMBC correlations (Table 2). The correlations between H-1 and C-3, between H-2 and C-4, between H-9 and C-2, and between H-9 and C-3. These correlations indicated that C-2 was linked to C-1 and C-3, and C-3 was also linked to C-4. In addition, connections among C-3, C-9, C-7 and C-15 through C-8 were deduced from HMBC correlations of H-2, H-4, H-10, and H_3-15 to C-8. The existence of an eter bridge between C-2 and C-5 was implied by HMBC correlations of H-2 to C-5, and H-5 to C-2. Thus the gross structure of fascicularone D was elucidated to be **6**. The stereostructure of **6** was ascertained by NOE experiments (Fig. 3). In NOE experiments, compound **6** showed H_3-15 to H-4, but not to H-6, and from H_3-14 to H-2 and H-5, indicating that H-2, H-4 and H-5 all had β-configurations. Further, NOEs from H-1 and H-9 to H-6 implied that these protons had α-configurations. These NOE data also requires a *cis*-juncture between the five-and six-membered rings having β methyl group at C-8 and β hydroxyl group at C-3 with the same stereochemistry as **1**, **2**, **4** and **5**. As is the case for **5**, it is assumed that the absolute configurations of **6** at C-2 was the same as those of **1** and **3** from biogenetic considerations. Based on the following ground, the structure of **6** was determined as be (1*S*,9*S*)-3β,6β-dihydroxy-2α,5α-epoxy-4α,11,11,8β-tetramethyltricyclo[5.4.0.0^{2,5}]undecan-7-one (Fig. 1).

The biological activity of fascicularones A (**3**), B (**4**), C (**5**) and D (**6**) were examined by lettuce seedling. Compounds **3**-**6** accelerated the radicle growth to 148%, 184%, 170% and 188% of control at concentration of 100 ppm, respectively.

Fascicularones A (**3**), C (**5**), D (**6**), naematolins C (**1**) and G (**2**) have a tricyclo[5.4.0.0^{2,5}]undecane skeleton, while fascicularone B (**4**) has a tricyclo [5.3.0.0^{2,5}]decane skeleton. A similar ring system has been found in a compound, hebellophyllene D isolated from *Hebeloma longicaudum* [9]. Interest in the biosynthtic pathway of **1**, **2**, **3**, **4**, **5** and **6** has been focused on the formation of this three ring system. The formation of **1**, **2**, **3**, **5** and **6** would be derived from *cis*-fused caryophyllene sesquiterpene *via* a reduction of the double bond followed by an intramolecular cyclization. In addition, compound **4** would be formed from lactonization reaction on **5**. We are now continuing the search for fascicularone analogs to clarify the biogenetic pathway.

Experimental Section

General experimental procedures

Melting points (mp) data are uncorrected. Optical rotation was measured with a Horiba model SEPA-300 polarimeter, IR spectra were recorded with a JASCO J-20A spectrophotometer, and UV spectra were recorded with a Shimadzu UV mini-1240 instrument. Mass spectra were recorded with a JEOL JMS-700 instrument, and ^{1}H and ^{13}C NMR spectra were obtained with a JEOL EX-400 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was conducted on Sephadex LH-20 (Pharmacia), silica gel 60 (Kanto Chemical Co., Inc.) and Amberlite XAD-2 (ORGANO corporation). TLC was done on a precoated silica gel plate (Merck), and spots were detected by spraying 10% vanillin in H_2SO_4 followed by heating.

Mushroom material

The fruiting bodies of *N. fasciculare* were collected at the foot of Mt. Gassan, Yamagata Prefecture, Japan, in autumn 1997 and identified by one of authors (M. I.). The producing fungal strain was isolated from cultured tissue of the fruiting bodies of *N. fasciculare* [8] and has been deposited in the Faculty of Agriculture, Yamagata University, Yamagata, Japan.

Fermentation

A loopful slant culture of strain of *N. fasciculare* was inoculated into five 1 l flasks containing 300 ml of a medium consisting of 40 g of malt extract, 40 g of glucose, and 1.0 g peptone per 1 liter of water and cultured at 25 °C for 90 days under stationary conditions.

Extraction and isolation

After the incubation period, 1500 ml of culture broth was separated from the mycelia by filtration. The culture filtrate passed through an Amberlite-XAD-2, using H_2O, MeOH and EtOAc as the eluent. The fraction-eluted MeOH (4.68 g) was chromatographed on silica gel column by eluting with $CHCl_3$-MeOH (20:1, v/v) to afford five fractions (Fr.1.1 – 1.5). Fr.1.2 (912 mg) was subjected to column chromatography on silica gel with *n*-hexane-EtOAc (3:1 and 1:1, v/v) and $CHCl_3$ to obtain six fractions (Fr.2.1 – 2.6). Fr.2.1 (69.2 mg) was chromatographed successively on Sephadex LH-20 with MeOH, and then chromatographed on silica gel with a solvent system of *n*-hexane-EtOAc (3:1 and 1:1, v/v) followed by crystallization in *n*-hexane to yield fascicularone D (**6**, 14.5 mg). Fr.2.2 (115.9 mg) was chromatographed on silica gel with a solvent system of $CHCl_3$ to yield crude fascicularone C, which was finally purified by Sephadex LH-20 with MeOH to give fascicularone C (**5**, 10.0 mg).

*Fascicularone C (**5**)*

Colorless needles. – M.p. 149 – 151 °C. – $[\alpha]_D^{20}$ + 117(c 0.47, $CHCl_3$). – IR (KBr): ν = 3400 (OH), 2938, 2859, 1702 (C=O) cm^{-1}. – ^{1}H (400 MHz, C_5D_5N) and ^{13}C {^{1}H} NMR (100 MHz, C_5D_5N) data see Table 1. – MS(EI) *m/z* (%) = 268 (8) [M$^+$], 250 (55), 232 (50), 212 (75), 194 (10), 166 (53), 140 (100), 123 (36), 111 (70), 85 (68). – HRFABMS: m/z = 269.1754 [M+H]$^+$ (calcd. for $C_{15}H_{25}O_4$, 269.1755).

*Fascicularone D (**6**)*

Colorless needles. – M.p. 129 – 131 °C. – $[\alpha]_D^{20}$ – 157(c 0.48, $CHCl_3$). – IR (KBr): ν = 3405 (OH), 2946, 2931, 2851, 1702 (C=O), 1037 (C-O-C) cm^{-1}. – ^{1}H (400 MHz, $CDCl_3$) and ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) data see Table 2. – MS (EI): *m/z* (%) = 266 (59) [M$^+$], 248 (6), 210 (59), 192 (100), 164 (14), 152 (64), 124 (100), 85 (28). – HRMS (FAB): m/z = 267.1600 [M+H$^+$] (calcd. for $C_{15}H_{23}O_4$, 267.1598).

Lettuce seedling assay

This assay was performed as reported [10].

Acknowledgement

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