Sesquiterpenoids from *Fusarium* sp., an Endophytic Fungus in *Agriminia pilosa*

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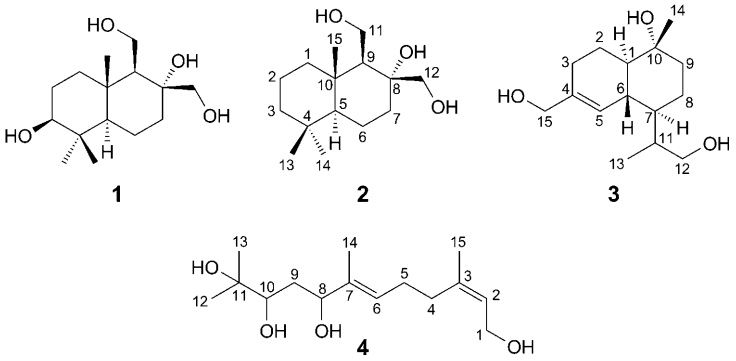
Cultivation of the endophytic fungus isolated from the stems of *Agriminia pilosa* provided four new sesquiterpenoids, namely agripilols A–D (**1**–**4**, resp.). Structures of these isolates were elucidated by NMR and MS data. The isolation of drimane- and cadinane-type sesquiterpenoids from the genus of *Fusarium* is reported for the first time.

Introduction. – The term 'endophyte' refers to a bacterial or a fungal microorganism that colonizes inside the healthy tissues of the host plant, but typically causes no apparent symptoms of disease. The research on endophytes showed that they are potential sources of novel biologically active secondary metabolites for exploitation in medicine, agriculture, and industry [1–4]. Furthermore, results from many researches indicated that endophytic fungi could metabolize the same substance with their own host plants [5–7], which should be considered as a caution that endophytes may be an alternate source of plant secondary metabolites.

Agrimonia pilosa, an erect perennial herb native to Northern Asia and Eastern Europe, has been used in traditional Chinese medicine to treat taeniasis [8]. In the process of our research on the endophytic fungi [9], four new sesquiterpenoids, **1**–**4**, were obtained from the strain *Fusarium* sp. isolated from the stems of *A. pilosa*. Their structures were assigned by MS and NMR analyses, especially 2D-NMR techniques (^{1}H,^{1}H-COSY, HMQC, HMBC, and NOESY).



Results and Discussion. – Agripilol A (**1**) had a molecular formula $C_{15}H_{28}O_4$, established by a HR-ESI-MS (positive-ion mode) *pseudo*-molecular-ion peak at *m*/*z* 295.1898 ($[M+Na]^+$; calc. 295.1885), implying two degrees of unsaturation. The IR spectrum of **1** showed a strong absorption band at 3363 cm^{-1}, typical for a OH group. The ^{1}H-NMR spectrum (*Table*) of **1** revealed the presence of two O-bearing CH_2 groups (δ(H) 3.64 (*s*, CH_2(12)), 3.95 (*dd*, *J* = 11.1, 7.8, 1 H of CH_2(11)), and 3.80 (*dd*, *J* = 11.1, 4.4, 1 H of CH_2(11)), and one O-bearing CH group (δ(H) 3.18 (*dd*, *J* = 8.5, 7.8, H–C(3))), in addition to three tertiary Me groups (δ(H) 0.75, 0.98, and 0.86). The ^{13}C-NMR spectrum of **1** (*Table*) exhibited 15 C-atom signals including those of three quaternary C-atoms, and three CH, six CH_2, and three Me groups. Among them, one CH (δ(C) 79.5) signal, one quaternary C-atom signal (δ(C) 76.9), and two downfield

Table. *^{1}H- and ^{13}C-NMR Data of* **1**–**4** *at 400 and 100 MHz, Respectively.* δ in ppm, *J* in Hz.

Position	**1**[a])		**2**[b])		**3**[a])		**4**[b])	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	1.77 (*dd*, *J* = 13.7, 3.1), 1.24–1.26 (*m*)	39.7	1.08–1.11 (*m*), 1.71–1.73 (*m*)	39.8	1.25–1.27 (*m*)	51.5	4.06 (*d*, *J* = 7.3)	58.9
2	1.57–1.59 (*m*), 1.59–1.62 (*m*)	28.2	1.19–1.21 (*m*), 1.36–1.39 (*m*)	41.6	2.04–2.08 (*m*)	23.8	5.41 (*t*, *J* = 7.6)	124.9
3	3.18 (*dd*, *J* = 8.5, 7.8)	79.5	1.48–1.51 (*m*), 1.57 (*dt*, *J* = 3.5, 13.5)	18.6	1.98–2.01 (*m*), 2.03–2.05 (*m*)	27.8	–	138.1
4	–	40.2	–	33.5	–	140.0	2.15–2.18 (*m*)[c])	31.1
5	0.90–1.00 (*m*)[c])	56.9	1.01–1.03 (*m*)[c])	56.0	5.77–5.80 (*m*)[c])	124.4	2.17–2.19 (*m*)	24.9
6	1.60–1.63 (*m*), 1.34–1.36 (*m*)	20.8	1.27–1.30 (*m*), 1.67–1.69 (*m*)	19.7	1.82–1.84 (*m*)[c])	40.6	5.44–5.46 (*m*)[c])	125.4
7	2.18 (*d*, *J* = 12.5), 1.18–1.20 (*m*)[c])	38.3	2.20–2.23 (*m*)[c]), 1.27–1.29 (*m*)[c])	37.2	1.35–1.38 (*m*)	43.1	–	137.9
8	–	76.9	–	76.0	1.51–1.54 (*m*), 1.20–1.23 (*m*)	23.6	4.23 (*dd*, *J* = 8.5, 4.1)	77.8
9	1.52 (*dd*, *J* = 7.5, 4.5)	62.0	1.72–1.74 (*m*)[c])	59.2	1.75–1.77 (*m*), 1.45–1.47 (*m*)	42.9	1.60–1.64 (*m*)[c])	35.6
10	–	38.7	–	37.5	–	73.2	3.56 (*dd*, *J* = 8.9, 2.5)	78.3
11	3.95 (*dd*, *J* = 11.1, 7.8), 3.80 (*dd*, *J* = 11.1, 4.4)	60.4	3.92 (*dd*, *J* = 13.8, 4.3), 3.96 (*dd*, *J* = 13.9, 11.1)	60.5	2.18–2.20 (*m*)	35.7	–	72.6
12	3.64 (*s*)	65.2	3.69 (*d*, *J* = 10.8), 3.81 (*d*, *J* = 10.8)	63.8	3.41 (*dd*, *J* = 10.4, 7.1), 3.43 (*dd*, *J* = 10.4, 7.4)	67.4	1.12 (*s*)	23.8
13	0.75 (*s*)	16.4	0.79 (*s*)	21.5	0.79 (*d*, *J* = 7.0)	10.9	1.18 (*s*)	26.2
14	0.98 (*s*)	29.0	0.89 (*s*)	33.3	1.08 (*s*)	20.7	1.61 (*s*)	11.7
15	0.86 (*s*)	17.3	0.79 (*s*)	16.5	3.94 (*d*, *J* = 14.5), 3.90 (*d*, *J* = 14.3)	67.7	1.75 (*s*)	23.2

[a]) Recorded in CD_3OD. [b]) Recorded in $CDCl_3$. [c]) Signals that overlapped.

CH_2 signals (δ(C) 65.2, 60.4) were ascribed to O-bearing C-atoms. These data indicated that **1** was a drimane sesquiterpenoid.

Analysis of the ^{1}H- and ^{13}C-NMR, HMQC, and HMBC spectra of **1** enabled us to assign all the H-atoms to corresponding C-atoms. The three partial structures **a** (C(1) to C(3)), **b** (C(5) to C(7)), and **c** (C(9) and C(11)) were established by a combination of 2D-NMR spectra, especially ^{1}H,^{1}H-COSY data (*Fig. 1*). The assemblage of all C-atoms, including quaternary C-atoms and hetero atoms, was mainly accomplished by an HMBC experiment (*Fig. 1*). The connectivity of two partial structures **a** and **b** *via* C(4) was deduced from the HMBC cross-peaks Me(14)/C(4), Me(14)/C(3), and Me(14)/C(5), which also indicated the presence of a OH group at C(3). In the HMBC spectrum, the H-atom signal at δ(H) 0.86 (Me(15)) was correlated with those of C(10) (δ(C) 38.7), C(1) (δ(C) 39.7), and C(9) (δ(C) 62.0) to connect fragments **a** and **c**. The linkage of C(5) and C(10) was established by the HMBCs Me(15)/C(10) and Me(15)/C(5). The H-atom signal of CH_2(12) appeared as *singlet*, indicating the presence of a OH group at C(8), which was confirmed by HMBCs CH_2(11)/C(9) and CH_2(11)/C(8). The connectivity of two subunits **b** and **c** through C(8) was supported by HMBCs CH_2(12)/C(7), CH_2(12)/C(8), and CH_2(12)/C(9). The constitution of **1** was thus outlined.

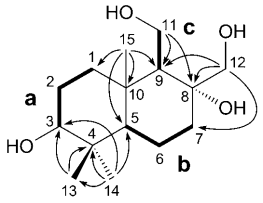


Fig. 1. *Selected ^{1}H,^{1}H-COSY* (—) *and HMBC* (→) *correlations of* **1**

The relative configuration of **1** was deduced from the NOESY correlations as depicted on a three-dimensional structure generated from the molecular modeling (CS Chem 3D Pro Version 8.0) using MM2 force-field calculations for energy minimization (*Fig. 2*). NOE Correlations Me(14)/H–C(3), Me(14)/H–C(5), and H–C(5)/H–C(9) indicated that the H–C(3), H–C(5), H–C(9), and Me(14) were on the same side of the molecular plane, tentatively assumed as α. As a consequence, the configuration of Me(13) and CH_2(11) were β. A strong NOE between Me(13) and Me(15) supported

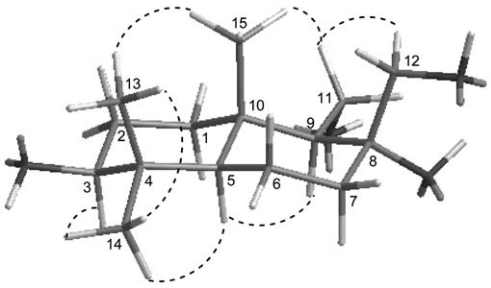


Fig. 2. *Selected NOESY correlations of* **1**

the β-configuration of Me(15) and established the relative configuration of the drimane skeleton with *trans*-fused decalin ring. The NOE correlation $CH_2(11)/CH_2(12)$ indicated that the $CH_2(11)$ and $CH_2(12)$ were β-configured. Complete ^1H and ^{13}C assignments (*Table*) were achieved through a combination of 2D-NMR techniques, including the HMQC, HMBC, and NOESY. Thus, the structure of agripilol A (**1**) was established.

Agripilol B (**2**) had the molecular formula $C_{15}H_{28}O_3$ based on HR-ESI-MS analysis (*m/z* 279.1928 ($[M+Na]^+$; calc. 279.1936)), which was one O-atom less than that of **1**. High similarity was observed between the NMR data of **2** and **1**, implying structural resemblance of the two compounds (*Table*). The major differences were the disappearance of an O-bearing CH signal and the highfield-shifted signals of $CH_2(3)$ in the NMR spectra of **2** compared to those of **1**. The data mentioned above suggested that **2** could be 3-deoxy derivative of **1**, which was confirmed by 2D-NMR ($^1H,^1H$-COSY, HMQC, HMBC, and NOESY) data. Accordingly, the structure of **2** was determined as depicted, *i.e.*, the 8-epimer of a synthetic sesquiterpenoid 8β,11,12-drimanetriol [10].

The molecular formula of agripilol C (**3**) was determined as $C_{15}H_{26}O_3$ by the HR-ESI-MS (positive-ion mode). In the ^1H-NMR spectrum, signals of a tertiary Me group at δ(H) 1.08 and a secondary Me group at δ(H) 0.79 were clearly apparent. A signal at δ(H) 5.77–5.80 and two pairs of signals (δ(H) 3.41, 3.43; and 3.90, 3.94) were easily ascribed to a trisubstituted C=C bond and two O-bearing CH_2 groups, respectively, on the basis of their chemical shifts and coupling patterns. The ^{13}C-NMR spectrum exhibited 15 C-atom resonances, which consisted those of two Me, six CH_2, five CH groups, and two quaternary C-atoms (one O-bearing). On the basis of the above evidence, **3** had the skeleton of a cadinane sesquiterpenoid. High similarity was observed between the NMR data of **3** and those of 15-hydroxy-α-cadinol (HCOL) [11], implying structural resemblance of the two compounds. The major differences were the disappearance of a Me H-atom signal and the downfield-shifted signal of C(12) in the NMR spectra of **3** by compared to those of HCOL, which indicated an additional heteroatom linked to C(12) in **3**. Hydroxy group was preferentially and tentatively considered as the substituent judging from the chemical shift, which was supported by the molecular formula. The interpretation of 2D-NMR spectra further confirmed this conclusion. Thus, the structure of **3** was elucidated as 12,15-dihydroxy-α-cadinol with undetermined configuration at C(11).

Agripilol D (**4**) had the molecular formula $C_{15}H_{28}O_4$ revealed by the HR-ESI-MS spectrum, indicating two degrees of unsaturation. Signals of four Me groups (*singlet*s at δ(H) 1.12, 1.18, 1.61, and 1.75), an O-bearing CH_2 group (δ(H) 4.06 (*d*, $J=7.3$)), two O-bearing CH groups (δ(H) 4.23 (*dd*, $J=8.5, 4.1$); 3.56 (*dd*, $J=8.9, 2.5$)), and two olefinic H-atoms (δ(H) 5.41, 5.44–5.46) were observed in the ^1H-NMR spectrum of **4** (*Table*). Fifteen C-atom signals were detected in the ^{13}C-NMR spectrum of **4**, including those of four Me, four CH_2, four CH groups, and three quaternary C-atoms. From the chemical shifts, it can be deduced that there were two C=C bonds and four O-bearing C-atoms in **4**. These data indicated that agripilol D was a farnesane sesquiterpenoid [12].

The interpretation of 2D-NMR spectra provided three partial structures **a** (C(8) to C(10)), **b** (C(4) to C(6)), and **c** (C(1) and C(2)) (*Fig. 3*). The HMBCs Me(13)/C(11) and Me(13)/C(10) indicated that there were two OH groups at C(10) and C(11),

respectively. The H-atom signal at δ(H) 1.75 (Me(15)) showed cross-peaks with the signals of C(2), C(3) and C(4), which indicated the connectivity of the parts **b**, **c**, and C(15) *via* C(3). The linkage of moieties **a** and **b** was deduced from the HMBCs Me(14)/C(8), Me(14)/C(7), and Me(14)/C(6). The strong NOESY correlations Me(14)/CH_2(5) and CH_2(1)/CH_2(4) indicated the configuration of C(2)=C(3) and C(6)=C(7) bonds were (*Z*) and (*E*), respectively. Thus, the structure of **4** was elucidated as agripilol D with the absolute configurations at C(8) and C(10) undetermined.

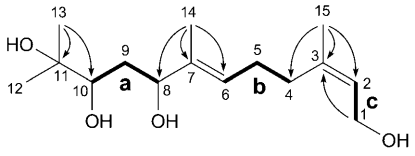


Fig. 3. *Selected* $^1H,^1H$*-COSY* (—) *and HMBC* (→) *correlations of* **4**

In this article, the isolation of sesquiterpenoids of three different types, *i.e.*, drimane (**1** and **2**), cadinane (**3**), and farnesane (**4**), from the strain *Fusarium* sp. is described, and the acyclic sesquiterpenoid (**4**) may be the biosynthetic precursor of **1**–**3**. To the best of our knowledge, many sesquiterpenoids from the genus of *Fusarium* have been reported [13–15], but the isolation of cadinane-type sesquiterpenoids from this genus was reported for the first time. The result showed that the endophytes had different characters as metabolites than other microbes in their living environment, and could be used as potential sources for biologically active secondary metabolites.

Experimental Part

General. All solvents used were of anal. grade (*Hangzhou Gaojin Fine Chemical Plan Chemical Plant*). TLC: pre-coated silica gel GF_{254} plates (*Qingdao Haiyang Chemical Plant*). Column chromatography (CC): silica gel (SiO_2; 230–400 mesh), *MCI CHP20P* gel (75–150 μ; *Mitsubishi Chemical Industries Ltd.*), and *Toyopearl-HW-40C* gel (*Tosoh corporation*). Optical rotations: *Rudolph-Autopol-IV* polarimeter. IR Spectra: *Thermo-Nicolet-6700* spectra-photometer; in cm^{-1}. NMR Spectra: *Bruker AM-400* apparatus; δ in ppm rel. to Me_4Si, *J* in Hz. ESI-MS: *Agilent-6210-Lc/Tof* mass spectrometer; in *m/z*.

Microbial Material and Fermentation. The fungal *Fusarium* sp. was isolated from the healthy stems of *A. pilosa* collected in Hangzhou district, Zhejiang Province, P. R. China. The strain is deposited with the Zhejiang University of Technology (accession No. ZJUT-AP-4303). Plant samples were designed to undergo a process described by *Schulz et al.* as surface sterilization [16]. The surface-sterilized samples were cut into 0.5-cm fragments and placed onto the surface of potato-dextrose agar (PDA) as medium in *Petri* dishes with six fragments in each, which was then incubated at 28° for 4 d. During cultivation, the hyphal-tip method [17] was adopted for the purification of the endophytic fungi. The growing fungi were successively removed into fresh PDA medium and cultivated at 28° for 7 d. The isolated strain *Fusarium* sp. was grown on PDA at 28° for 6 d, before inoculation into 500-ml *Erlenmeyer* flasks each containing 300 ml of potato-dextrose liquid medium for fermentation. The flasks were first inoculated on rotary shakers at 28° for 6 d at 185 r.p.m. and then cultivated for another 20 d at 28° without agitation.

Extraction and Isolation The culture was filtered through cheesecloth. The broth (50 l) was evaporated to dryness under reduced pressure to afford the residue (512 g). The residue was suspended in 3.0 l of H_2O and partitioned successively with hexane, AcOEt, and BuOH. The AcOEt fraction (3.4 g) was subjected to CC (*MCI-CHP20P* gel; MeOH/H_2O, 40 : 60 → 80 : 20) to afford three major fractions *1*–*3*. *Fr. 1* (200 mg) was applied to CC (SiO_2; $CHCl_3$/MeOH 20 : 1 → 15 : 1) to afford **1** (20 mg). *Fr. 2* (0.9 g)

was separated on CC (SiO_2; $CHCl_3$/MeOH 20:1 → 15:1) and then purified by *Toyopearl HW-40C* eluted with MeOH to yield **2** (8 mg) and **3** (7 mg). *Fr. 3* (0.8 g) was subjected to CC (SiO_2; $CHCl_3$/MeOH 30:1 → 20:1) to afford **4** (8 mg).

Agripilol A (= rel-*(2*R*,4a*R*,5*R*,6*S*,8a*S*)-Decahydro-5,6-bis(hydroxymethyl)-1,1,4a-trimethylnaphthalene-2,6-diol*; **1**). Colorless amorphous powder. $[\alpha]_D^{20} = -4.0$ ($c = 0.10$, MeOH). IR (KBr): 3363, 2958, 2869, 1447, 1349, 1043, 975, 715. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS (pos.): 295 ($[M+Na]^+$). HR-ESI-MS (pos.): 295.1898 ($[M+Na]^+$, $C_{15}H_{28}NaO_4^+$; calc. 295.1885).

Agripilol B (= rel-*(1*R*,2*S*,4a*R*,8a*R*)-Decahydro-1,2-bis(hydroxymethyl)-5,5,8a-trimethylnaphthalen-2-ol*; **2**). Colorless amorphous powder. $[\alpha]_D^{20} = -8.0$ ($c = 0.10$, $CHCl_3$). IR (KBr): 3302, 2932, 2867, 1457, 1374, 1048, 1021, 761. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS (pos.): 279 ($[M+Na]^+$). HR-ESI-MS (pos.): 279.1928 ($[M+Na]^+$, $C_{15}H_{28}NaO_3^+$; calc. 279.1936).

Agripilol C (= *(1*R*,4*R*,4a*R*,8a*R*)-1,2,3,4,4a,7,8,8a-Octahydro-6-(hydroxymethyl)-4-[1-hydroxypropan-2-yl]-1-methylnaphthalen-1-ol*; **3**). Colorless amorphous powder. $[\alpha]_D^{20} = +47.6$ ($c = 0.08$, MeOH). IR (KBr): 3342, 2930, 2870, 1661, 1458, 1379, 1298, 1238, 1212, 1028, 931, 832. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS (pos.): 277 ($[M+Na]^+$). HR-ESI-MS (pos.): 277.1774 ($[M+Na]^+$, $C_{15}H_{26}NaO_3^+$; calc. 277.1780).

Agripilol D (= *(2Z,6*E*)-3,7,11-Trimethyldodeca-2,6-diene-1,8,10,11-tetrol*; **4**). Colorless amorphous powder. $[\alpha]_D^{20} = -5.3$ ($c = 0.15$, $CHCl_3$). IR (KBr): 3368, 2966, 2929, 2865, 1663, 1446, 1379, 1306, 1078, 1005, 856. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS (pos.): 295 ($[M+Na]^+$). HR-ESI-MS (pos.): 295.1876 ($[M+Na]^+$, $C_{15}H_{28}NaO_4^+$; calc. 295.1885).

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