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A NEW CHROMONE DERIVATIVE FROM AN ENDOPHYTIC FUNGUS *XYLARIA* sp. ISOLATED FROM *ARDISIA CRENATA*

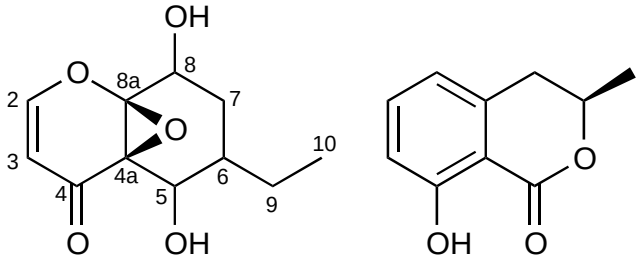
Ken-ichi Nakashima,[1]* Junko Tomida,[2] Masanori Tabata,[1] Takao Hirai,[1] Yuji Morita,[2] Yoshiaki Kawamura,[2] and Makoto Inoue[1]

[1]Laboratory of Medicinal Resources, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464-8650, Japan. [2]Laboratory of Microbiology, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464-8650, Japan. E-mail: nakasima@dpc.agu.ac.jp

Abstract – A new chromone derivative, xylariacetal (**1**), and a known isocoumarin, mellein (**2**), were isolated from *Xylaria* sp. ECN-008, which was derived from the leaves of *Ardisia crenata*. The structure of the new compound was identified by various spectroscopic techniques including 1D- and 2D-NMR. Xylariacetal (**1**) exhibits a characteristic tricyclic structure with an acetal moiety.

Plant endophytes are a group of microorganisms (fungi or bacteria) that are ubiquitous in the tissues and organs of plants, and do not cause diseases or symptoms.[1] Almost all plants in the biosphere have endophytic fungi and bacteria; thus, it is impossible to estimate the biodiversity of endophytes.[2,3] Consequently, it is assumed that the secondary metabolites produced by endophytes have rich chemodiversity. Endophytes produce a number of unique alkaloids, terpenoids, quinones, and polyketides. Some of these compounds show antiarthritic, antimicrobial, anticancer, antidiabetic, insecticidal, and immunosuppressant activities.[4–7] Therefore, we are studying secondary metabolites from endophytes to find new sources of bioactive compounds.

Xylariaceae is a family of ascomycetous fungi that are often found as symbionts in vascular plants.[8,9] *Xylaria* is a major genus of the family and species belonging to this genus are widespread from temperate to tropical areas. A previous review by Song *et al.* described *Xylaria* spp. as producing various bioactive metabolites, including sesquiterpenoids, diterpenoids, diterpene glycosides, triterpene glycosides, steroids, *N*-containing compounds, aromatic compounds, pyrone derivatives, and polyketides.[10] In this work, we determined the structure of a novel chromone derivative, xylariacetal (**1**), isolated as a secondary



1 **2**

Figure 1. Structures of compounds isolated from *Xylaria* sp. ECN-008

metabolite of an endophytic fungal strain *Xylaria* sp. ECN-008 from *Ardisia crenata* Sims (Primulaceae) (Figure 1).

Xylaria sp. ECN-008 was isolated from healthy leaves of *Ardisia crenata*. The leaf surfaces were sterilized by soaking sequentially in 95% EtOH, 0.5% NaClO, and 70% EtOH. The surface-sterilized leaves were cut into 1 cm^2 pieces and put on malt extract agar (MEA) with 0.005% chloramphenicol in a 9 cm petri dish. After a few days, the emergent organisms were isolated in pure culture. The isolated strain was identified as *Xylaria* sp. by sequencing the internal transcribed spacers (ITS) of the ribosomal DNA and 26S rRNA genes.

After being cultured on 30 plates of MEA for a month, the whole mycelia of *Xylaria* sp. ECN-008 were extracted with chloroform to yield the extract (809.6 mg). The extract was subjected to silica gel column chromatography eluted with a chloroform-MeOH step gradient system to yield xylariacetal (**1**) together with a known compound. The known compound was identified as mellein (**2**) by comparison of the spectral data with the literature values.[11]

Xylariacetal (**1**) was obtained as a pale yellow gum exhibiting an UV absorption maximum at 253 nm. The HR-ESI-MS showed an $[M - H]^-$ ion peak at *m/z* 225.0774, indicating that the quasi-molecular formula was $C_{11}H_{13}O_5$ (calcd 225.0763). The ^{1}H-NMR spectrum measured in $CDCl_3$ (Table 1) indicated the presence of a methyl group [δ_H 0.92 (1H, t, J = 7.6 Hz)], two methylene groups [δ_H 1.11 (1H, dt, J = 13.2, 10.0 Hz), 2.15 (1H, ddd, J = 13.2, 6. 8, 2.4 Hz); 1.25 (1H, dquin, J = 13.6, 7.6 Hz), 1.87 (1H, dqd, J = 13.6, 7.6, 3.2 Hz)], and three methine groups [δ_H 4.39 (1H, dd, J = 10.0, 6.8 Hz), 4.11 (1H, d, J = 9.6 Hz), 1.45 (1H, m)], in addition to a set of *cis*-olefin peaks [δ_H 7.18, 5.67 (1H each, d, J = 6.4 Hz)] conjugated with a carbonyl group at δ_C 190.7 (C-4). The DQF-COSY spectrum measured in $CDCl_3$ showed the –CH(O)CH(CH_2CH_3)CH_2CH(O)– moiety. This partial structure was corroborated by HMBC correlations between H-5/C-7, H-5/C-9, H-7/C-9, and H-10/C-6 (Figure 2). The ^{13}C-NMR spectrum also showed two quaternary carbons at δ_C 66.5 (C-4a) and δ_C 89.4 (C-8a). The HMBC correlations between H-2/C-8a, H-3/C-4a, H-5/C-4a, H-7/C-8a, and H-8/C-4a suggested that **1** has a bicyclic

Table 1. ^{1}H and ^{13}C NMR data for **1**[a] (δ in ppm, *J* in Hz)

No.	**1** ($CDCl_3$)		**1** (DMSO-d_6)	
	δ_H	δ_C	δ_H	δ_C
2	7.18 (1H, d, *J* = 6.4)	157.1	7.48 (1H, d, *J* = 6.6)	157.5
3	5.67 (1H, d, *J* = 6.4)	107.2	5.64 (1H, d, *J* = 6.6)	106.8
4		190.7		189.3
4a		66.5		68.3
5	4.11 (1H, d, *J* = 9.6)	69.1	3.93 (1H, dd, *J* = 11.6, 9.6)	66.4
6	1.45 (1H, m)	34.5	1.14 (1H, m)	36.2
7α	2.15 (1H, ddd, *J* = 13.2, 6.8, 2.4)	33.0	1.84 (1H, dd, *J* = 11.2, 5.6)	32.7
7β	1.11 (1H, dt, *J* = 13.2, 10.0)		1.08 (1H, m)	
8	4.39 (1H, dd, *J* = 10.0, 6.8)	65.5	4.11 (1H, dt, *J* = 8.8, 6.8)	64.6
8a		89.4		89.7
9α	1.25 (1H, dquin, *J* = 13.6, 7.6)	23.4	1.14 (1H, m)	23.5
9β	1.87 (1H, dqd, *J* = 13.6, 7.6, 3.2)		1.63 (1H, m)	
10	0.92 (3H, t, *J* = 7.6)	10.5	0.83 (3H, t, *J* = 6.8)	10.8
5-OH			4.95 (1H, d, *J* = 8.0)	
8-OH			5.60 (1H, d, *J* = 6.0)	

[a] ^{1}H- and ^{13}C-NMR spectra were measured at 400 and 100 MHz, respectively.

cyclohexane/pyran ring system bearing an ethyl group at C-6. The chemical shifts of C-4a and C-8a indicated that these quaternary carbons were directly bound to one and two oxygen atoms, respectively, suggesting an acetal or a hemiacetal structure at C-8a. The molecular formula $C_{11}H_{14}O_5$ predicted by HR-ESI-MS indicated the presence of an ether bridge or an epoxy group in addition to two hydroxyl groups.

To detect the proton signals of hydroxyl groups, we measured the ^{1}H-NMR spectrum in DMSO-d_6 (Table 1). The hydroxyl proton signals were two doublets at δ_H 5.60 (1H, d, *J* = 6.0 Hz) and 4.95 (1H, d, *J* = 8.0 Hz), which were correlated with H-8 and H-5, respectively, in the DQF-COSY spectrum (Figure 2). Furthermore, HMBC correlations between 5-OH/C-5, 5-OH/C-6, 8-OH/C-7, 8-OH/C-8, and 8-OH/C-8a

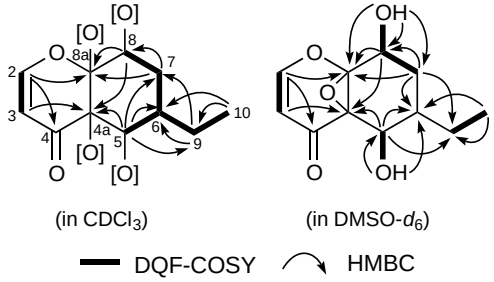


Figure 2. Key HMBC and DQF-COSY correlations of **1** measured in $CDCl_3$ (left) and DMSO-d_6 (right)

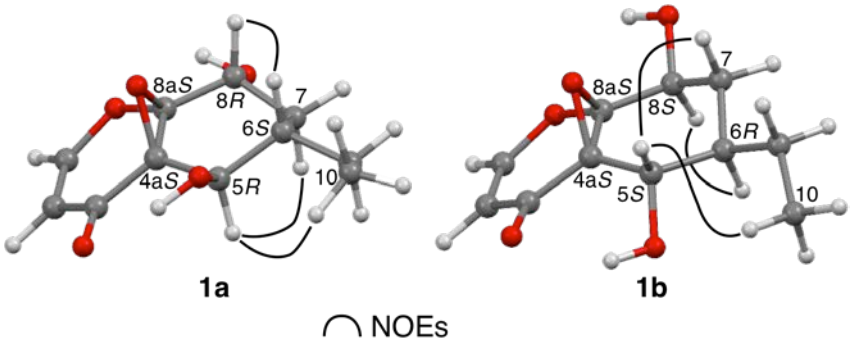


Figure 3. Predicted candidates isomers for **1** and observed key NOEs in the NOESY spectrum

were observed. These results indicated that the hydroxyl groups were on C-5 and C-8, and the epoxy group was attached to C-4a/C-8a.

Next, we measured electronic circular dichroism (ECD) spectrum of **1** to determine the configuration of C-4a and C-8a. **1** exhibited a positive Cotton effect at 325 nm, which was the R-band assigned to be the α,β-unsatu rated epoxyketone s ystem.[12–14] Therefore, the (4a*S*,8a*S*)-configuration was suggested for **1** by Snatzke's inverse-octant rule. The orientation of protons attached at C-5, C-6, and C-8 were determined as 5α, 6β, and 8β, respectively, by the key NOEs between H-5/H-7 and H-6/H-8 in the NOESY spectrum. Additionally, all of the coupling constants between H-5/H-6 (9.6 Hz), H-6/H-7β (10.0 Hz), and H-7β/H-8 (10.0 Hz) indicated that these four hydrogen atoms occupied axial positions in the half-chair conformation. Although the absolute configurations of these positions could not be determined, two possible candidates (**1a** and **1b**) were proposed (Figure 3). We calculated the ECD spectra by using time-dependent density functional theory (TDDFT) calculations to elucidate the absolute structure of **1**. However, no crucial difference between the theoretical spectrum of **1a** and that of **1b** was calculated out.

Xylariacetal (**1**) has a bicyclic cyclohexane/pyran ring system bearing an ethyl group. This characteristic framework can be found in diplosporin and agistatines A–E, which were previously isolated from several fungal strains including *Xylaria* spp.[15–17] However, a compound with a tricyclic cyclohexane/pyran/epoxide ring system has never been detected before.

The cytotoxicity, anti-bacterial properties, and agonistic activities for nuclear receptors were examined for xylariacetal (**1**). However, **1** exhibited no cytotoxicity for HepG2 human hepatocellular carcinoma cells in an MTT assay, and no anti-bacterial effects against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonus aeruginosa*, and *Bacillus subtilis* at a concentration of 100 μM. Furthermore, no agonistic activities for retinoic acid receptor α, retinoid X receptor α, liver X receptors α/β, and peroxisome proliferator-activated receptors γ/δ were detected by luciferase reporter gene assay for each receptor based on the method described in our previous paper.[18]

EXPERIMENTAL

General. NMR spectra were measured on a spectrometer (JNM-AL-400, JEOL) with tetramethylsilane as the internal standard. ESI-MS was performed on a hybrid mass spectrometer (LCMS-IT-TOF, Shimadzu). UV spectra were obtained with a UV spectrometer (U-2900, Hitachi). Optical rotations were recorded on a polarimeter (P-1020, JASCO). IR spectra were recorded on a spectrophotometer (FTIR-8400S, Shimadzu). ECD spectra were obtained using a spectropolarimeter (J-820, JASCO). DNA sequencing was performed with a genetic analyzer (3130, Applied Biosystems).

Isolation of *Xylaria* sp. ECN-008. The leaves of *Ardisia crenata* Sims (Primulaceae) were collected in Gero City, Gifu Prefecture, Japan. The surfaces of the leaves were sterilized by soaking sequentially in 95% EtOH for 30 s, 0.5% NaClO for 2 min, and 70% EtOH for 2 min. The surface-sterilized leaves were cut into 1 cm^2 pieces and put on MEA containing 2% malt extract (Oxoid), 0.1% bacto peptone (BD), 2% D-glucose (Kishida Chemical), and 1.5% agar (Wako Pure Chemical Industries) supplemented with 0.005% chloramphenicol (Wako Pure Chemical Industries) in 9 cm petri dishes. The dishes were incubated at 27 °C in an artificial semidiurnal light/dark environment. Emergent organisms were isolated in pure culture. The isolated strain (ECN-008) was identified as *Xylaria* sp. because sequencing of ITS of the ribosomal DNA and 26S rRNA genes revealed a high homology with sequences from *X. allantoidea* (AY909005) and *X. cubensis* (GU991523) deposited in GenBank (Figure S1).[19] The fungal isolate was deposited at Aichi Gakuin University (Aichi, Japan). The primer used for sequencing was as follows: ITS forward 5'-TCC GTA GGT GAA CCT GCG G-3', ITS reverse 5'-TCC TCC GCT TAT TGA TAT GC-3', 26S rRNA forward 5'-GCA TAT CAA TAA GCG GAG GAA AAG-3', and 26S rRNA reverse 5'-GGT CCG TGT TTC AAG ACG G -3'.[20,21]

Extraction and isolation. *Xylaria* sp. ECN-008 was cultured on 30 plates of MEA (9 cm diameter; each containing 20 mL of MEA). After being cultured for a month, the whole mycelia were extracted with $CHCl_3$ (1.5 L × 2) at room temperature for 24 h and filtered through filter paper. The filtrate was evaporated in vacuo to yield the extract (809.6 mg). The extract was subjected to silica gel (AP-300, Toyota Kako) column chromatography eluted with a $CHCl_3$-MeOH step gradient system (50:1, 40:1, 30:1, 20:1, and 10:1, each 500 mL) and fractioned into 50 mL fractions (frs.). According to their TLC profiles, mellein (**2**; 6.8 mg) and a new compound, xylariacetal (**1**; 38.6 mg), were obtained in frs. 5–7 and frs. 31–34, respectively.

Xylariacetal (1): pale yellow gum; R_f 0.57 (7:1 $CHCl_3$–MeOH); $[\alpha]_D^{25}$ -92.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 253 (3.91) nm; ECD (MeOH) λ (Δε) 252 (–1.44), 296 (–1.57), 325 (1.41); IR (KBr) 3412, 2966, 2934, 2878, 1659, 1607 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HR-ESI-MS (negative ion mode) *m/z* 225.0774 $[M - H]^-$ (calcd $C_{11}H_{13}O_5$ for 225.0763).

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