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

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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

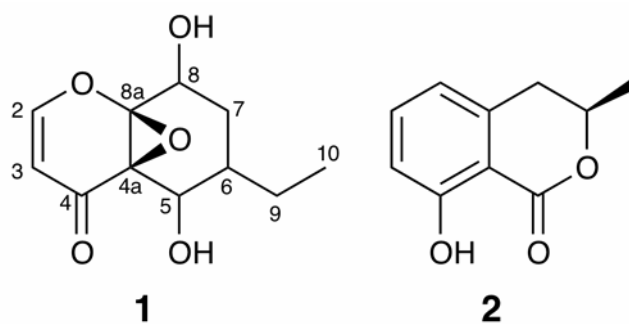


Figure 1. Structures of compounds isolated from *Xylaria* sp. ECN-008

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 cm² 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 C₁₁H₁₃O₅ (calcd 225.0763). The ¹H-NMR spectrum measured in CDCl₃ (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, dqin, $J = 13.6, 7.6$ 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 CDCl₃ showed the $-\text{CH}(\text{O})\text{CH}(\text{CH}_2\text{CH}_3)\text{CH}_2\text{CH}(\text{O})-$ 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 ¹³C-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. ^1H and ^{13}C NMR data for **1**^a (δ in ppm, J in Hz)

No.	1 (CDCl_3)		1 ($\text{DMSO}-d_6$)	
	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
7 α	2.15 (1H, ddd, $J = 13.2, 6.8, 2.4$)	33.0	1.84 (1H, dd, $J = 11.2, 5.6$)	32.7
7 β	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
9 α	1.25 (1H, dquin, $J = 13.6, 7.6$)	23.4	1.14 (1H, m)	23.5
9 β	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 ^1H - 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 $\text{C}_{11}\text{H}_{14}\text{O}_5$ 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 ^1H -NMR spectrum in $\text{DMSO}-d_6$ (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

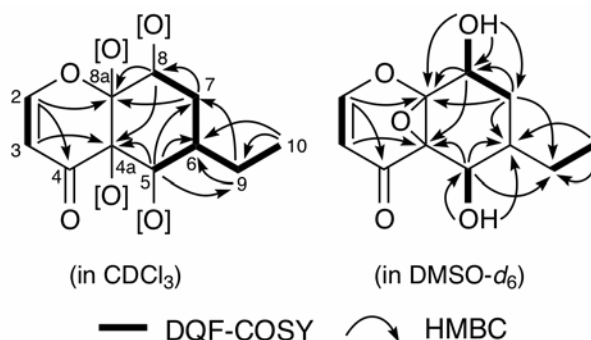


Figure 2. Key HMBC and DQF-COSY correlations of **1** measured in CDCl_3 (left) and $\text{DMSO}-d_6$ (right)

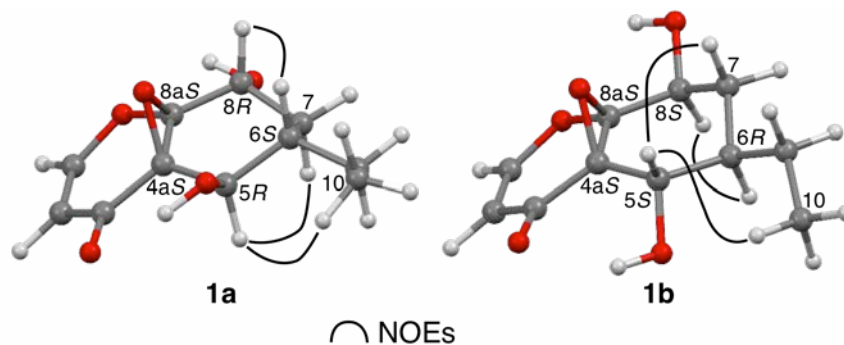


Figure 3. Predicted candidates isomers for **1** and observed key NOEs in the NOESY spectrum

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 -unsaturated epoxyketone system.^{12–14} Therefore, the (4aS,8aS)-configuration was suggested for **1** by Sznatzke'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-7 β (10.0 Hz), and H-7 β /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*, *Pseudomonas 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 cm² 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 CHCl₃ (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 CHCl₃-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 CHCl₃-MeOH); [α]_D²⁵ -92.0 (*c* 0.1, MeOH); UV (MeOH) λ_{\max} (log ϵ) 253 (3.91) nm; ECD (MeOH) λ ($\Delta\epsilon$) 252 (-1.44), 296 (-1.57), 325 (1.41); IR (KBr) 3412, 2966, 2934, 2878, 1659, 1607 cm⁻¹; ¹H and ¹³C NMR see Table 1; HR-ESI-MS (negative ion mode) *m/z* 225.0774 [M - H]⁻ (calcd C₁₁H₁₃O₅ for 225.0763).

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