HETEROCYCLES, Vol. 94, No. 1, 2017, pp. 117 - 121. © The Japan Institute of Heterocyclic Chemistry
Received, 25th October, 2016, Accepted, 7th December, 2016, Published online, 20th December, 2016
DOI: 10.3987/COM-16-13599

A NEW ISOCOUMARIN DERIVATIVE FROM AN ENDOPHYTIC FUNGUS *THIELAVIA* sp. ISOLATED FROM *CRASSULA OVATA*

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Abstract – Thielavic acid (**1**), a new isocoumarin derivative, was isolated from an endophytic fungus *Thielavia* sp. ECN-115, which was obtained from the stems of *Crassula ovata*. The relative structure of **1** was established by spectroscopic analyses including extensive 2D-NMR experiments. The absolute configuration was identified as (4*S*)-thielavic acid by comparing the experimental and calculated electronic circular dichroism spectra.

Plant endophytes are a group of microorganisms that live in the tissues and organs of every plant without provoking diseases or symptoms.[1] Previous studies of the distribution of endophytes have reported the isolation of numerous strains and suggested almost all plants provide habitats for endophytic fungi and bacteria, demonstrating the diversity of endophyte species.[2–4] Endophytes produce diverse compounds with sophisticated structures.[5,6] Some compounds may be valuable for developing drugs with antiarthritic, antimicrobial, anticancer, antidiabetic, insecticidal, and immunosuppressant activities.[7,8] Therefore, we expect endophytic fungi to be a rich natural resource for producing bioactive compounds.

Previous studies of the secondary metabolites of the genus *Thielavia* (Chaetomiaceae) have produced biologically active compounds such as thielavins A–E (prostaglandin synthase, α-glucosidase inhibitors, and glucose-6-phosphatase inhibitors),[9–11] thielocins (phospholipase A_2 inhibitors),[12] and thielavialides A–E.[13] Herein, we report the isolation and absolute structure elucidation of (4*S*)-thielavic acid (**1**) produced by the endophytic fungus *Thielavia* sp.

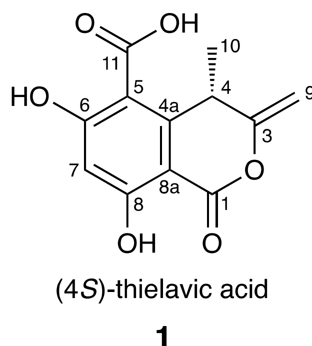


Figure 1. Structure of (4*S*)-thielavic acid (**1**)

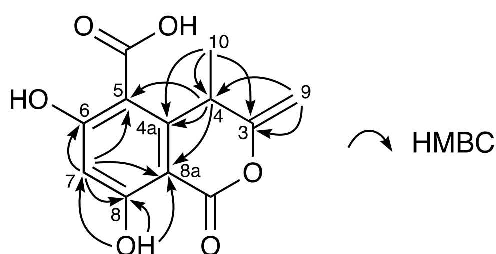


Figure 2. HMBC correlations of **1**

Table 1. ^{1}H and ^{13}C NMR data for **1**[a,b]

No.	**1**	
	δ_H	δ_C
1		167.3 [c]
3		157.4
4	4.95 (1H, q, J = 6.8 Hz)	36.7
4a		152.0
5		104.2
6		170.6
7	6.40 (1H, s)	103.7
8		167.5 [c]
8a		101.3
9	4.79 (2H, br s)	96.9
10	1.51 (3H, d, J = 6.8 Hz)	23.8
11		172.4
8-OH	11.64 (1H, s)	

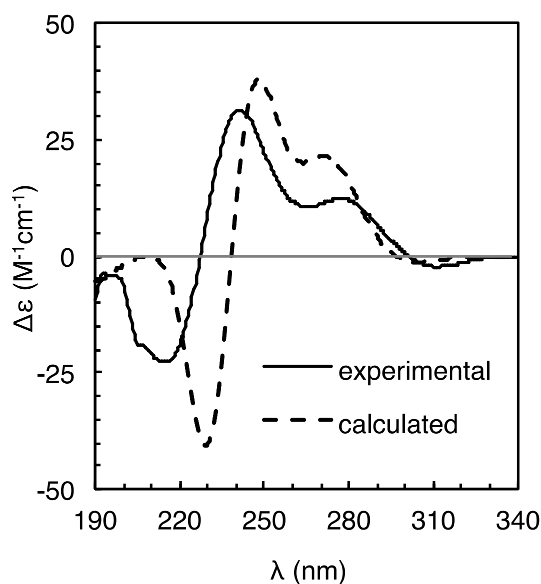
[a] ^{1}H- and ^{13}C-NMR spectra were measured at 400 and 100 MHz, respectively (δ in ppm, J in Hz). [b] Spectra were recorded in acetone-d_6. [c] interchangeable signals.

Thielavia sp. ECN-115 was isolated from stems of *Crassula ovata* (Crassulaceae). The surfaces of the stems were sterilized by soaking sequentially in 95% EtOH, 0.5% NaClO, and 70% EtOH. The surface-sterilized stems were cut into 1 cm 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 *Thielavia* sp. by sequencing 26S rRNA genes. After being cultured on 47 MEA plates for a month, the whole mycelia of *Thielavia* sp. ECN-115 were extracted with CH_3Cl. Thielavic acid (**1**) was recrystallized from CH_2Cl_2 solution of the extract (Figure 1).

Compound **1** was obtained as a colorless powder exhibiting UV absorption maxima at 238, 283, and 315 nm. The HR-ESI-MS data showed an $[M - H]^-$ ion peak at m/z 249.0408, which corresponded to the quasi-molecular formula of $C_{12}H_9O_6$ (calcd 249.0399). The IR spectrum showed two absorption bands associated with C=O bond stretching at 1693 and 1651 cm^{-1}. The ^{1}H-NMR spectrum (Table 1) showed a methyl group [δ_H 1.51 (3H, d, J = 6.8 Hz)] linked to a methine group [δ_H 4.95 (1H, q, J = 6.8 Hz)], a methylidene group [δ_H 4.79 (2H, br s)], an aromatic proton [δ_H 6.40 (1H, s)], and a hydroxy group [δ_H 11.64 (1H, s)]. In addition, the ^{13}C-NMR spectrum contained 12 carbon atom signals assigned to CH_3 (δ_C 23.8), sp^3 CH (δ_C 36.7), sp^2 CH_2 (δ_C 96.9), sp^2 CH (δ_C 103.7), and eight quaternary sp^2 carbons (δ_C 101.3, 104.2, 152.0, 157.4, 167.3, 167.5, 170.6, 172.4). The 1D-NMR data suggested structural similarities with ascochin, a known isocoumarin isolated from an endophytic fungus *Ascochyta* sp.[14] The methylidene group at C-3 and methyl group at C-4 were confirmed by the HMBC correlations between H_2-9/C-3,

H_2-9/C-4, and H_3-10/C-3 (Figure 2). Furthermore, the 6,8-dihydroxy isocoumarin structure with a substituent at C-5 was supported by the following HMBC correlations: H-4/C-4a, H-4/C-5, H-4/C-8a, H-7/C-5, H-7/C-6, H-7/C-8, H-7/C-8a, HO-8/C-7, HO-8/C-8, and HO-8/C-8a. The molecular formula predicted by HR-ESI-MS indicated that there was one more oxygen atom than ascochin. Therefore, the substituent at C-5 was identified as a carboxyl group.

The absolute configuration of **1** was determined by its electronic circular dichroism (ECD) spectrum using time-dependent density functional theory (TDDFT) calculations for the most stable conformation. Because the experimental and calculated ECD spectra agreed well, the absolute configuration of **1** was assigned as (*S*) (Figure 3). Furthermore, the similarity of the experimental ECD spectrum of **1** and that of (4*S*)-ascochin confirmed the absolute structure.[14] As in (4*S*)-ascochin, the positive bands at 244 and 282 nm arise from an aromatic π–π* transition and charge-transfer transitions from alkene π to aromatic π* orbitals, respectively. The weak negative band at 314 nm is assumed to correspond to the carbonyl n–π* transition, although it was not calculated by TDDFT. Thus, the absolute structure of (4*S*)-thielavic acid (**1**) was determined. Further study of the biological activity of **1** is in progress.



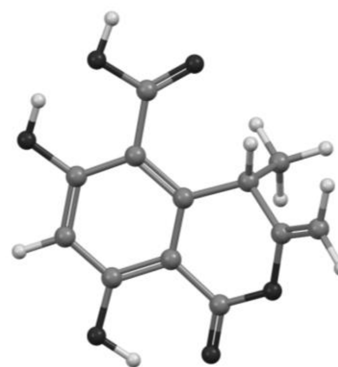


Figure 3. Experimental ECD spectrum of **1** and TDDFT-calculated ECD spectrum for the density functional theory-optimized geometry

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). DNA sequencing was performed with a genetic analyzer (3130, Applied Biosystems). ECD spectra were obtained with a spectropolarimeter (J-820, JASCO). Melting points were measured on a micro melting point apparatus (MP-S3, Yanagimoto).

Isolation of *Thielavia* sp. ECN-115. The stems of *Crassula ovata* were collected in Kasugai City, Aichi Prefecture, Japan. The surfaces of the stems 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 stems were cut into 1 cm 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. The emergent organisms were isolated in pure culture. The isolated strain (ECN-115) was identified as *Thielavia* sp. because sequencing of 26S rRNA genes revealed a high homology with sequences from *Thielavia terrestris* ATCC38088 and *Thielavia terrestris* CBS492.74 deposited in GenBank (Figure S1). The fungal isolate was deposited at Aichi Gakuin University (Aichi, Japan). The primer used for sequencing was as follows: 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'.[15]

Extraction and isolation. *Thielavia* sp. ECN-115 was cultured on 47 MEA plates (9 cm diameter; each containing 20 mL of MEA). After culturing for a month, the whole mycelia and the agar media 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 (112.2 mg). Thielavic acid (**1**; 28.3 mg) was recrystallized from CH_2Cl_2 solution of the extract.

(4*S*)-Thielavic acid (1): colorless powder; mp 222–225 (decomp) (CH_2Cl_2); R_f 0.33 (3:1 $CHCl_3$–MeOH); $[\alpha]_D^{25}$ +259.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.37), 283 (3.86), 315 (3.70) nm; ECD (MeOH) λ (Δε) 217 (–11.12), 244 (15.05), 282 (5.66), 314 (–1.10); IR (KBr) 3225, 3163, 1693, 1651, 1599. 1427, 1346, 1269, 1250, 1229 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HR-ESI-MS (negative ion mode) *m/z* 249.0408 $[M - H]^-$ (calcd $C_{12}H_9O_6$ for 249.0399).

Calculation. The ECD calculations were performed with Gaussian 09 (Gaussian Inc., PA, USA). The conformation was optimized by density functional theory at the B3LYP/6-31G(d) level. TDDFT with the B3LYP/6-31G(d) method was used to calculate the excited energies and rotational strength with 16 states. MeOH was used as the solvent. The rotational strength was converted to Gaussian curves (bandwidth sigma = 1750 cm^{-1}).

ACKNOWLEDGEMENTS

This study was supported by a research grant from the Institute of Pharmaceutical Life Sciences, Aichi Gakuin University. We thank Dr. Naohito Abe and Prof. Masayoshi Oyama (Department Bioactive Molecules, Gifu Pharmaceutical University) for the measurement of the ECD spectrum and HR-ESI-MS of **1**. We also thank Ms. Noriko Ogawa (School of Pharmacy, Aichi-Gakuin University) for her help in calculating the ECD spectrum.

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