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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 A₂ 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.

H₂-9/C-4, and H₃-10/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 (*4S*)-ascochin confirmed the absolute structure.¹⁴ As in (*4S*)-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 (*4S*)-thielavic acid (**1**) was determined. Further study of the biological activity of **1** is in progress.

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).

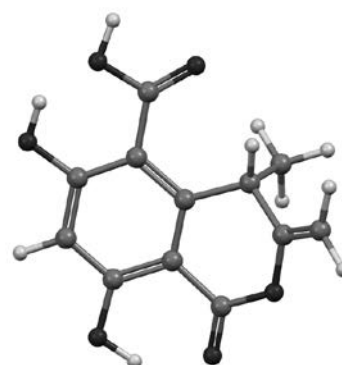
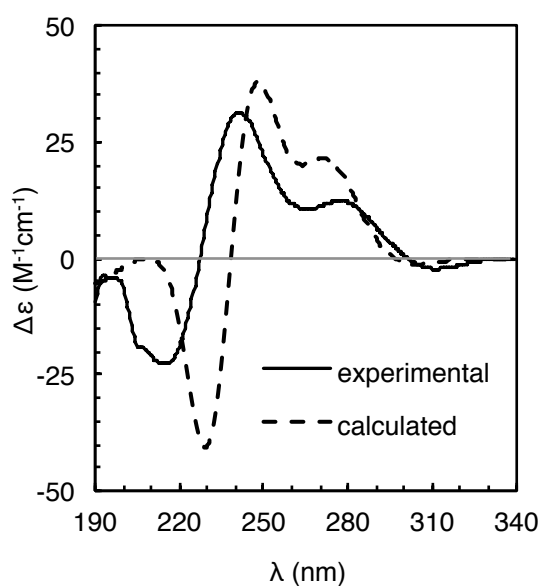


Figure 3. Experimental ECD spectrum of **1** and TDDFT-calculated ECD spectrum for the density functional theory-optimized geometry

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 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 (112.2 mg). Thielavic acid (**1**; 28.3 mg) was recrystallized from CH₂Cl₂ solution of the extract.

(4S)-Thielavic acid (1): colorless powder; mp 222–225 (decomp) (CH₂Cl₂); R_f 0.33 (3:1 CHCl₃–MeOH); [α]_D²⁵ +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 ¹³C NMR see Table 1; HR-ESI-MS (negative ion mode) *m/z* 249.0408 [M – H][–] (calcd C₁₂H₉O₆ 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}).

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