Supporting Information

In Vitro Acanthamoebicidal Activity of Fusaric Acid and Dehydrofusaric Acid from an Endophytic Fungus Fusarium sp. Tlau3

Narumon Boonman¹, Surasak Prachya², Atsadang Boonmee¹, Prasat Kittakoop²,³, Suthep Wiyakrutta¹, Nongluksna Sriubolmas⁴, Saradee Warit⁵, Araya Dharmkrong-at Chusattayanond¹

Affiliation
¹ Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
² Chulabhorn Research Institute, Vibhavadi-Rangsit Road, Laksi, Bangkok 10210, Thailand
³ Chulabhorn Graduate Institute, Chemical Biology Program, Vibhavadi-Rangsit Road, Laksi, Bangkok 10210, Thailand
⁴ School of Pharmacy, Eastern Asia University, Rangsit-Nakornnayok Road, Pathumthani 12110, Thailand
⁵ TB Research Laboratory, National Center for Genetic Engineering and Biotechnology, NSTDA, Pathumthani 10120, Thailand

Correspondence
Assist. Prof. Dr. Araya Dharmkrong-at Chusattayanond, Department of Microbiology, Faculty of Science, Mahidol University, 272 Rama 6 Rd., Bangkok 10400, Thailand.
Phone: +66 2 201 5526 Fax: +66 2 644 5411
E-mail: scacs@mahidol.ac.th, arayachusattayanond@gmail.com
Isolation and identification of the endophytic fungus

The endophytic fungus *Fusarium* sp. Tlau3 was originally isolated from healthy twigs of *Thunbergia laurifolia* Lindl. (Acanthaceae), collected from the forest of Chiang Mai Province, Northern Thailand in July 1999, as described earlier [1]. The plant specimen was identified by Associate Professor Dr. Nijsiri Ruangrungsi, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, by comparison with the authentic sample (voucher specimen no. BK064381) deposited at the Bangkok Herbarium, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The endophytic fungus was identified as a member of the genus *Fusarium* according to its microscopic morphology and nucleotide sequence of ribosomal RNA gene as previously described [1]. Species identification of this fungus was attempted. Macroscopic and microscopic morphologies were examined in details using the method described by Burgess et al., 1994 [2]. The fungus was grown on potato dextrose agar (PDA) at 25°C and 30°C in the dark for 3 days. Colony diameter of *Fusarium* sp. Tlau3 was 2.1 cm at 25°C and 0.8 cm at 30°C. It was grown on PDA and banana leaf agar at 25°C with 12 h photoperiod for 14 days. On PDA, it produced white floccose colony with peach color in the center of the reverse side. On banana leaf, pale orange sporodochia were formed. Macroconidia were long slender (3.0-4.2 × 46.6-66.2 µm), falcate to almost straight, usually 4 to 6 septate, and thin-walled. The apical cells were tapered and hooked. The bases of the basal cells were barely notched. The macroconidia were produced from monophialides on branched conidiophores in the sporodochia and from monophialides formed directly on the hyphae. Microconidia were abundantly produced in false-heads from monophialides and, less frequently, polyphialides. The microconidia were oval (2.2-5.5 × 5.5-16.4 µm), mainly nonseptate and sometime 1 septate. Longer microconidia in spindle shape with 2-3 cells were also produced. Chlamydoconidia could not be found even in 28 days old culture. Phylogenetic analysis was done based on the ITS1-5.8S-ITS2 sequence by comparing with those of *Fusarium* species reference strains registered in the Centraalbureau voor Schimmelcultures (CBS), the American Type Culture Collection (ATCC), and the ARS Culture Collection (also known as the NRRL Collection). By using the CLC Main Workbench 6.6.1 software package, the nucleotide sequences were aligned automatically and adjusted manually. Phylogenetic trees were inferred using the neighbor-joining (NJ) method with 1000 bootstrap
replications for reliability. The Tlau3 fungus was placed in the Giberella fujikuroi species complex clade with 99% bootstrap support, as shown in Fig. 5S.

Based on morphological characteristics and phylogenetic analysis of the ITS1-5.8S-ITS2 sequence, the Tlau3 fungus was tentatively identified as a member of Fusarium fujikuroi species complex.

**Origins and identification of Acanthamoeba isolates**

Four Acanthamoeba isolates were used in this study. Two clinical isolates, AS and AR, obtained from corneal scrapings of Thai keratitis patients were kindly given by Dr. Patoomwanna Supadirekkul (Eye Ear Nose Throat Hospital, Bangkok) and Dr. Kosol Roongruangchai (Mahidol University) since 2000. S3 and S5, the other two isolates were obtained from soil samples collected in Bangkok, Thailand in 2009. Their morphology resembled those of polyphagid (gr II) Acanthamoeba [3]. Trophozoites were axenically grown in peptone-yeast extract-glucose (PYG). Their total cellular DNA was prepared as described earlier [4]. PCR primers CRN5 (5’-CTGGTTGATCCTGCCAGTAG) and 1137 (5’-GTGCCCTTCCGTCGTA) were used to amplify the GTSA.B1 amplimers [5] from the 18S rRNA gene by using GoTag®Green Master Mix (Promega) according to the manufacturer’s instruction. The thermal cycle program was as follows: 7 min at 95°C, followed by 20 cycles of 1 min at 95°C, 1 min at 60°C, and 2 min at 72 °C. This was followed by 25 cycles of 1 min at 95°C and 2 min at 72°C [5]. The amplified DNA was purified and subjected to sequencing reactions by Makrogen, Korea using CRN5, 1137, 892, 373, 570C and 892C as primers [5]. By using NCBI BLAST searching in GenBank AS, AR and S3 were clustered into genotype T4, and S5 was placed in T5. Sequences were aligned by using BioEdit version 7.1.3.0. Phylogenetic relationships of AS, AR, S3 and S5 were inferred by neighbor-joining analysis of the 18S rRNA gene sequence estimated with 1000 bootstrap replications and produced in CLC Main Workbench 6.6.1 (Fig 6S). The AS Acanthamoeba was mostly related to A. castellanii (U07405) at 99% similarity as supported by 100% bootstrap value. AR Acanthamoeba was closely related to A. polyphaga (AF260725) and A. palestinensis (AF260719) with both 99% homology, as supported by 99% bootstrap value. S3 Acanthamoeba was closely related to A. castellanii (U07401) and A. triangularis (AF316547) with both 98%
similarity, as supported by 100% bootstrap value. S5 *Acanthamoeba*, on the other hand, showed 100% homology with *A. lenticulata* (U94739, U94733, U94744, U94741, U94740, U94738, U94737, U94736, and U94735) and 99% homology with U94731, U94732 and U94730. The nucleotide sequences have been submitted to GenBank with the accession numbers JX043489, JX043490, JX104341 and JX043488 for AS, AR, S3 and S5, respectively.

Monoxenic and axenic cultures of these amoebae are maintained at the *Acanthamoeba* laboratory, Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand.

**Scanning electron microscopy**

Scanning electron microscopic study of cFA-treated *Acanthamoeba* was performed as previously described [1]. Trophozoites at $3 \times 10^4$ cells in 50 µL of ASS were treated with $2 \times IC_{50}$ of cFA. Those in ASS and in10 µg/mL PHMB were used as the negative and positive controls, respectively. After centrifugation at $2,620 \times g$ for 5 min, the trophozoites were placed on poly-L-lysine-coated cover slips. Samples were washed 3 times with 0.1 M cacodylate buffer pH 7.4 (sodium cacodylate: 2.054 g, calcium acetate: 0.063 g, distilled water: 100 mL), fixed with 2.5% (w/v) glutaraldehyde in the cacodylate buffer at 4°C for 2 h, and washed 3 times with the buffer. They were post-fixed with 1% (v/v) osmium tetroxide in the buffer at 4°C for 1 h and washed 3 times with the buffer. After dehydration by a graded series of EtOH, the samples were dried in a critical point dryer under CO$_2$ atmosphere. The dried specimens were then coated with platinum-palladium and examined by scanning electron microscope. Experiments and their controls were performed in triplicates.
Spectroscopic data

**Fusaric acid:** UV (MeOH): $\lambda_{\text{max}}$ (log $\varepsilon$) 269 (3.97), 229 (4.22) nm; IR (UATR-solid): $\nu_{\text{max}}$ 2960, 2931, 2863, 1714, 1595, 1382, 1304, 1246, 1189, 1134 cm$^{-1}$; $^1$H NMR (acetone-d$_6$, 600 MHz): $\delta$ 8.65 (1H, s, H-6), 8.16 (1H, d, $J = 7.7$ Hz, H-3), 8.04 (1H, d, $J = 7.7$ Hz, H-4), 2.81 (2H, t, $J = 7.7$ Hz, H-8), 1.71-1.63 (2H, m, H-9), 1.38 (2H, m, H-10), 0.93 (3H, t, $J = 7.3$ Hz, H-11); $^{13}$C NMR (acetone-d$_6$, 150 MHz): $\delta$ 164.8 (C, C-7), 149.6 (CH, C-6), 144.9 (C, C-2), 144.2 (C, C-5), 139.8 (CH, C-4), 124.9 (CH, C-3), 33.5 (CH$_2$, C-9), 32.8 (CH$_2$, C-8), 22.6 (CH$_2$, C-10), 13.7 (CH$_3$, C-11); ESITOF MS: $m/z$ 180.1025 [M + H]$^+$ (calcd. for C$_{10}$H$_{14}$NO$_2$: 180.1025)

**Dehydrofusaric acid:** UV (MeOH): $\lambda_{\text{max}}$ (log $\varepsilon$) 269 (4.19), 228 (4.47) nm; IR (UATR-solid): $\nu_{\text{max}}$ 3081, 2927, 1702, 1638, 1587, 1379, 1273, 1228, 1145, 1028, 997, 919 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 600 MHz): $\delta$ 8.48 (1H, s, H-6), 8.16 (1H, d, $J = 7.8$ Hz, H-3), 7.76 (1H, d, $J = 7.8$ Hz, H-4), 5.84-5.78 (1H, m, H-10), 5.04 (1H, d, $J = 11.2$ Hz, H-11)$_a$, 5.03 (1H, d, $J = 16.0$ Hz, H-11)$_b$, 2.85 (2H, t, $J = 7.4$ Hz, H-8), 2.43 (2H, q, $J = 7.2$ Hz, H-9); $^{13}$C NMR (CDCl$_3$, 150 MHz): $\delta$ 164.3 (C, C-7), 148.3 (CH, C-6), 144.2 (C, C-2), 142.2 (C, C-5), 138.2 (CH, C-4), 136.3 (CH, C-10), 123.5 (CH, C-3), 116.4 (CH$_2$, C-11), 34.6 (CH$_2$, C-9), 32.4 (CH$_2$, C-8); ESITOF MS: $m/z$ 178.0867 [M + H]$^+$ (calcd. for C$_{10}$H$_{12}$NO$_2$: 178.0868)
Fig. 1S $^1$H NMR spectrum (acetone-$d_6$, 600 MHz) of fusaric acid.

Fig. 2S $^{13}$C NMR spectrum (acetone-$d_6$, 150 MHz) of fusaric acid.
Fig. 3S $^1$H NMR spectrum (CDCl$_3$, 600 MHz) of dehydrofusaric acid

Fig. 4S $^{13}$C NMR spectrum (CDCl$_3$, 150 MHz) of dehydrofusaric acid
Fig. 5S Neighbor-joining tree inferred from ITS1-5.8S-ITS2 showing the relationship of the Tlau3 fungus with closely related *Fusarium* species reference strains. The bootstrap values (from 1,000 replicates) of higher than 60% are shown at the nodes. Tlau3 was clustered within the *Giberella fujikuroi* species complex. *Penicillium siamense* and *Paecilomyces victoriae* are used as outgroups.
Fig. 6S Neighbor-joining distance tree based on fragments (~1475 bp) of Acanthamoeba 18S rRNA genes of the T1-T16 genotype representatives. The phylogenetic relationships were estimated with 1000 bootstrap replicates and produced in CLC Main Workbench 6.6.1. Numbers shown above and below the nodes represent bootstrap values. GenBank accession numbers are in parentheses. Balamuthia mandrillaris is used as outgroup.
Fig. 7S Acanthamoebicidal effects of cFA on trophozoites of AR (A), S3 (B), and S5 (C) at 0.14 (□), 0.28 (■), 0.42 (○), 0.56 (▲), 0.70 (◇) and 0.84 (△) µM. Trophozoites in ASS (●) and in 10 µg/mL PHMB (◆) were used as negative and positive controls, respectively. Each value is the mean ± S.D. (n = 3).
Fig. 8S Morphological appearances of AS (A, E, I, M), AR (B, F, J, N), S3 (C, G, K, O) and S5 (D, H, L, P) trophozoites treated with cFA at the 2 × IC₅₀ concentrations, using ASS and 10 µg/mL PHMB as the negative and positive controls. The photographs, taken under an inverted light microscope, show trophozoites in ASS at 30 min (A-D), trophozoites treated with PHMB for 30 min (E-H), trophozoites treated with cFA for 30 min (I-L) and 4 h (M-P). The bar measures 20 µm.
References


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