Endophytic Fungus from *Sinopodophyllum emodi* (Wall.) Ying that Produces Podophyllotoxin

Zizhen Liang¹, Jia Zhang¹, Xuan Zhang¹, Jinjie Li¹, Xiaoqian Zhang¹, and Changqi Zhao¹,2,*

¹Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education, College of Life Science, Beijing Normal University, Beijing 100875, China, and ²Beijing Area Major Laboratory of Protection and Utilization of Traditional Chinese Medicine, Beijing 100875, China

*Author to whom correspondence should be addressed. Email: cqzhao163@163.com

Received 21 January 2015; Revised 13 June 2015

Abstract

The aryltetralin lactone podophyllotoxin, which exhibits pronounced antineoplastic activity, is used as the precursor of the following three clinical anticancer drugs: Etoposide™, Etopophos™ and Teniposide™. The natural occurrence of this arylnaphthalene lignan is scarce and unable to meet the ever-rising demand in the medical industry. Thus, developing alternative sources for the production of podophyllotoxin is extremely urgent. This is the first report of the production of podophyllotoxin from endophytic *Alternaria tenuissima* isolated from *Sinopodophyllum emodi* (Wall.) Ying. The identification of podophyllotoxin was performed using high-performance liquid chromatography and liquid chromatography–mass spectrometry (MS)-MS and confirmed by comparison with authentic standards.

Introduction

The plants of *Sinopodophyllum emodi* (Wall.) Ying, which belong to the family Berberidaceae, are an endangered herbaceous and rhizomatous species. It is a selfing alpine perennial herb that mainly grows in the wild and is native to the lower elevations of the Himalaya-Hengduan Mountain region, including China, north India, Bhutan, Nepal, Pakistan and Afghanistan (1). In China, the populations of *S. emodi* are mainly distributed over the western region of the Qinling Mountains (2) and it was traditionally used as a folk medicine for the treatment of cancer and various verrucoses.

During our ongoing search for bioactive secondary metabolites from *S. emodi* (Wall.) Ying, we have reported podophyllotoxin and a series of its derivatives, including six new compounds (2–4). Podophyllotoxin has also been found in many other plant genera such as *Juniperus* (5–9), *Linum* (10), *Diphylliea* (10), *Teuricum* (11), *Hyptis* (12), *Jeffersonia* (13), *Dysosma* (14), *Nepata* (13) and *Thuja* (15). However, increasing public demand is outpacing the supply and endangering the survival of these plant genera. In addition to the anthropogenic reasons mentioned earlier, these natural sources of podophyllotoxin are subjected to numerous threats such as low population size, narrow distribution ranges and so on. For chemical synthesis, the presence of four chiral positions and the trans-γ-lactonic ring of the podophyllotoxin skeleton make it not economically viable to produce (16–18). Therefore, it is essential to find alternative sources of podophyllotoxin. Microorganisms, especially endophytic fungi, serve as a readily reproducing and inexhaustible source of active secondary metabolites with promising biological activities. Previous works have reported the production of podophyllotoxin from *Podophyllum hexandrum* (19), *Podophyllum peltatum* (20) and *Juniperus recurva* (11).

Herein, we report the isolation and culture of the endophytic fungus *Alternaria tenuissima* from *S. emodi* (Wall.) Ying that grows in the Xinglong Mountains in Lanzhou, Gansu Province, China. The secondary metabolites are evaluated using chemoprofiling of this fungus via chromatographic and spectroscopic methods. Using high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS)-MS, the identification of podophyllotoxin in the fungal biomass was made by comparison with authentic reference standards.
Materials and methods

Collection, identification and authentication of plant material

The fungal strain is an endophyte isolated from fresh roots of *S. emodi* (Wall.) Ying and was deposited in China’s General Microbiological Culture Collection Center (CGMCC no. 5528). The plant materials were collected from natural populations in the Xinglong Mountains in Lanzhou, Gansu Province, China at an elevation of 2300 m, and they were identified and authenticated on the basis of their botanical characteristics. After plant selection, with the help of a sterile scalpel, disease-free parts of the plant, mainly the roots and rhizomes, were cut and placed in sterile plastic bags. These treated plants were stored at 4°C until the procedure for the isolation of endophytic fungi was begun. This plant specimen is presently being preserved in the herbarium of the College of Life Sciences, Beijing Normal University, Beijing, China (no. 09072201).

Authentic podophyllotoxin standard

The podophyllotoxin standard was prepared in our laboratory (21) (Supplementary Figures S3 and S4; Supplementary Table S1).

Isolation of the endophytic fungi

The plants were thoroughly washed in running tap water, followed by washing with double-distilled water. Then, the processes that followed were carried out in a super-clean bench. The roots and rhizomes were surface-sterilized by immersion in 75% ethanol for 1 min, and then they were rinsed three times for 1 min in sterile double-distilled water. Afterwards, they were sterilized by washing in 10% sodium hypochlorite for 6 min and then in 70% ethanol for 1 min. Finally, these surface-sterilized roots and rhizomes were rinsed five times in sterile double-distilled water for 1 min to remove excess surface sterilants.

The excess moisture was blotted in a sterile filter paper. The surface-sterilized plant tissues obtained were evenly distributed in sterilized Petri dishes and stripped of their surface tissue. Then, they were cut into small fragments (0.5 cm × 1 cm), spaced in Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium composed of 200 g of peeled and cut potato L⁻¹, 20 g of glucose L⁻¹ and 15 g of agar L⁻¹, with five fragments in each Petri dish. At the same time, sterilized roots and rhizomes were rolled on the PDA medium and prepared under the same conditions in parallel to isolate the surface-sterilized fungi. Petri dishes were aseptically transferred to slides for microscopy, which monitored the cultures to check the growth of the endophytic fungal colonies from the fragments. Endophytic fungi growing out from the plant tissues over 1–2 weeks were then picked and re-cultured on rich mycological media (PDA), brought into pure culture and incubated at 28°C. A total of 18 putative endophytes were isolated and screened for podophyllotoxin production.

Identification of the endophytic fungi

The endophytic fungi were detected and identified by the morphology of the fungal colony after 1–5 days. Meanwhile, hyphae on the agar plates were aseptically transferred to slides for microscopy, which was used to examine the microscopic features of the fungus. The total genomic fungal DNA was extracted by using a Sunbiotech DNA extraction kit (SG091, Sunbiotech, Beijing, China). The genomic DNA obtained was subjected to polymerase chain reaction (PCR) analysis (ABI GeneAmp 7900, Applied Biosystems, Foster City, CA, USA), and an ITS region was amplified by PCR with the primer pairs ITS1 (TCCGTAATGGTGAACCTGCGG) and ITS4 (TCCTCCGTTATTGATATGC). The PCR mixture consisted of 50 ng of genomic DNA, 1 μL of each primer (10 μM L⁻¹), 1 μL of dNTP for each 2.5 mM L⁻¹, 2 U of Taq polymerase, 1.5 mmol L⁻¹ of Mg²⁺ and 5 μL of 10x PCR buffer. Then, autoclaved double-distilled water was added to 50 μL, and PCR was performed with preheating at 95°C for 3 min, followed by 30 cycles of 30 s at 95°C, 55°C for 30 s, 72°C for 40 s and a final extension at 72°C for 3 min. Then, the PCR products were visualized by electrophoresis and sequenced on an ABI 3730XL DNA Analyzer (Applied Biosystems). The final identification of the endophytic fungus was obtained by matching the sequences against the BLAST database of NCBI.

Preparation of fungal extracts

An axenic culture of the fungus was inoculated in 250 mL conical flasks containing 100 mL of PD liquid medium (200 g of cut potato L⁻¹ and 20 g of glucose L⁻¹). A set of three conical flasks was used, and these flasks were incubated at 28°C with shaking at 120 rpm for 1 week. The cell-free extract was prepared by filtering the incubated culture through muslin cloth under vacuum. The mycelia and broth were treated separately. The mycelial pellet was washed with double-distilled water and resuspended in 100 mL of methanol. The suspension was then sonicated in an ultrasonicator (BILON92-II, Shanghai, China) under chilled conditions (using an ice bath, 4°C), and it was allowed to stand for 2 days. Then, the extract was obtained by filtering that was repeated five times. In the next stage, the combined methanol extracts were evaporated to dryness. The spent broth was extracted five times with isopycnic chloroform and n-butyl alcohol successively, and the organic solvent was removed after each extraction by rotary evaporation in a vacuum at 30°C, yielding the dry organic extract.

Thin layer chromatographic analysis

Thin layer chromatographic (TLC) analyses of the fungal extracts as well as the methanolic solutions of podophyllotoxin, podophyllotoxin glucoside and epipodophyllotoxin standards (>98% purity, NICPBP, China) were carried out on normal-reverse-phase silica gel plates developed in the following solvents: solvent A, chloroform:methanol (99 : 1, v/v); solvent B, chloroform:methanol (8 : 2, v/v); solvent C, chloroform:methanol (65 : 35, v/v); and solvent D, methanol:water (50 : 50, v/v). The spots were detected under ultraviolet (UV) light at 254 nm. The plate was then detected using a chromogenic agent consisting of sulfuric acid:ethanol (15 : 85, v/v), and the colors were developed.

HPLC analysis

The analysis was performed in an Agilent 1200 Series (Agilent Technologies Co. Ltd, Santa Clara, CA, USA) liquid chromatograph using an ODS-C18 reverse-phase column (5 μm, 4.6 × 150 mm) (COSMOSIL 5C18-MS-II, Nacalai Tesque, Kyoto, Japan). The extracts and standards were dissolved in 1.0 mL of methanol (HPLC purity grade) and filtered through a 0.45 μm filter prior to chromatographic separation. A 10 μL aliquot of sample was injected. For the determination of metabolites of the extracts, we used methanol:water (55 : 45, v/v) as the mobile phase at a flow rate of 0.5 mL min⁻¹. This was followed by a 15 min equilibration period using the initial conditions prior to injection of the former sample. The UV signals were monitored at λ = 254 nm.
LC–MS–MS analysis
The separation conditions of HPLC are consistent with those described earlier. Mass spectroscopy was processed on fungal TG9 samples with a positive electrospray ionization mode with a micro-TOF-QII LC–MS–MS (Bruker Daltonics Inc., Billerica, MA, USA). The sample was dissolved in 100% HPLC grade methanol, and it was injected with a spray flow of 2 μL min⁻¹ using the loop injection method. The capillary and end-plate offset voltages were set at 3.5 kV and −500 V, respectively. The nebulizer gas was set at 0.6 bar. The auxiliary nitrogen was adjusted to a constant flow rate of 6.0 L min⁻¹. The dry temperature was set to 180°C. Collision-induced dissociation studies were performed using a collision energy of 20 eV. The ionspray interface and mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution.

Results
Isolation of the endophyte
A total of 18 putative fungal endophytes, which were morphologically different from each other, were isolated from the sterilized plant tissues. After screening these isolated fungi for the production of podophyllotoxin, only one fungus yielded a positive result. This fungus was chosen and coded as TG9.

Identification of the endophyte
Morphological characteristics of the endophyte
TG9 rapidly grew on the PDA medium. After incubation for 24 h, the colony was transparent and smooth. After 48 h, the colony on the plate was white and panniform, and the average colony diameter was 10 mm. The endophytic fungus was examined after 72 h and grew to 22 mm on PDA. Moreover, the center began to turn yellow. The average diameter of a TG9 colony was 79 mm after 168 h, and the colony was orange-yellow and panniform with white edges on the surface of the plate.

The mycelia were dark orange and had septum and branches. The macrospores appearing on the mycelia of the TG9 strain were long and cylindrical shaped. No microspores could be found except for chlamydospores. The colony was flocculent, and the mycelia were fasciculated and combined under the growth state. Based on these typical features, the fungus was identified that it should belong to the Fusarium genus.

Molecular identification of the endophyte
Molecular analysis of endophytic fungus TG9 was conducted, and the ITS region including its 5.8S rDNA, part of its 18S rDNA and its 28S rDNA was successfully amplified by PCR. Searching against the GenBank database, the alignment of the rDNA sequences of strain TG9 revealed 100% homology with the corresponding gene sequences of A. tenuissima.

Identification of podophyllotoxin
Chromatographic analysis
TG9 was cultured in 300 mL of PDA liquid medium at 28 °C and 120 rpm for 1 week (168 h). The production of the fungal culture extract was observed on day 7 (168 h). The yields of chloroform and n-butyl alcohol extracts of spent broth were 50.5 and 348.0 mg, respectively, and the methanol extract of the mycelia was 1139.9 mg. The extracts were examined for the presence of standards by chromatographic analysis. The TLC analysis showed that the chloroform extract of TG9 spent broth exhibited the same Rf value (0.37) as authentic podophyllotoxin. Furthermore, the HPLC analysis demonstrated that the retention time of a single peak (13.80 min) of the chloroform extract is as same as authentic podophyllotoxin (13.80 min) under the same conditions (Supplementary Figure S1).

Spectroscopic analysis
The elucidation of the chloroform extract of TG9 spent broth was performed using HPLC–MS–MS. The retention times of the chloroform extract and authentic podophyllotoxin are nearly the same (13.80 min) in the HPLC analysis (Supplementary Figure S1). Compared with authentic podophyllotoxin, the chloroform extract showed the same molecular ion peak at m/z 437.1 (M + Na⁺). The HPLC–MS–MS data showed a total of seven fragment ions of both authentic podophyllotoxin and the sample. On the basis of the results of the HPLC, HPLC–MS, HPLC–MS–MS and the fragmentation pathways discussed subsequently, the sample at the retention time 13.80 min is podophyllotoxin.

Discussion
As shown in Supplementary Figure S2, a total of seven fragment ions of both authentic podophyllotoxin and the sample were identified from the total ion. All the fragment ions resulted from the major ion at m/z 437.1 (M + Na⁺). Fragment ions at m/z 397.1 and 366.1 were characterized by the integrity of the arylnaphthalene skeleton with the loss of a hydroxyl group and a methoxyl group, respectively. Fragment ions at m/z 313.1 and 282.1 were generated by the loss of furan-2-(5H)-one by the major ion. The fragment ion at m/z 282.1 was produced after further elimination of a methoxyl group. Fragment ions at m/z 247.1 and 229.0 were the result of the loss of 1,2,3-trimethoxybenzene. The fragment ion at m/z 229.0 was generated by the further elimination of a hydroxyl group. The LC–MS–MS data indicated that the metabolite (retention time 13.80 min) was podophyllotoxin. The fragmentation pathway corroborates to the authentic deoxypodophyllotoxin (Supplementary Figure S2) (22, 23). The fragmentation pathway can be explained by Supplementary Figure S3.

The production of podophyllotoxin by A. tenuissima provides evidence to back up the theory that during the long co-evolution of endophytes and their host plants, endophytes have been assumed to have acquired host-derived genes such as transposons (24, 25). The uptake of host genomes by endophytes could enable certain endophytes to biosynthesize some phytochemicals originally associated with the host plant (26). This result also indicated that it would be interesting to further study the podophyllotoxin production and regulation by the cultured endophytes in S. emodi and in axenic cultures. A. tenuissima could be a promising alternative approach for the large-scale production of podophyllotoxin, which is considered to be a lead structure of some antineoplastic drugs, to satisfy clinical needs. However, further strain improvement and the optimization of the fermentation process are required for consistent and dependable podophyllotoxin production.

Conclusion
The aryltetralin lactone podophyllotoxin is used as the precursor of the clinical anticancer drugs Etoposide, Etopophos and Teniposide. Identified by HPLC and LC–MS–MS, endophytic fungi A. tenuissima isolated from S. emodi (Wall.) Ying can produce podophyllotoxin.
This study indicated that *A. tenuissima* may be a promising alternative approach for the large-scale production of podophyllotoxin and supported the theory that during the long co-evolution of endophytes and their host plants, certain endophytes have gained the ability to biosynthesize some phytochemicals originally associated with the host plant.

**Supplementary Material**

Supplementary materials are available at [Journal of Chromatographic Science](http://chromsci.oxfordjournals.org).

**Funding**

This project was supported by the National Science Foundation of China (grant no. 81173503) and Ministry of Science and Technology of China (grant no. 2012BAC01B05-7).

**References**

23. Li, Q., Yan, G., Ge, T.; A fragmentation study of two compounds related to 4-demethylpodophyllotoxin in negative ion electrospray ionization by MSn ion-trap time-of-flight mass spectrometry; *Rapid Communications in Mass Spectrometry*, (2008); 22: 373–378.