The relationship between an endangered North American tree and an endophytic fungus

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Background: The Florida torreya (*Torreya taxifolia*) began a catastrophic decline in the late 1950s and is now the rarest tree in North America for which a full species designation has been established. The trees have common plant disease symptoms, but the reason for the decline has never been identified. *T. taxifolia*'s imminent extinction gains special poignancy through its close relationship to the Pacific yew (*Taxus brevifolia*), which produces the potent anticancer agent, taxol.

Results: An examination of the endophytic fungal communities of wild torreyas consistently found a filamentous fungus, *Pestalotiopsis microspora*, associated with diseased trees and also with most symptomless trees. *P. microspora* can be cultured in the laboratory, and when it is introduced into greenhouse-grown torreyas, it causes disease symptoms similar to those seen in the field. The fungus can then be reisolated from these deliberately infected trees. The phytotoxins pestalopyrone, hydroxypestalopyrone and pestaloside have been isolated and characterized from axenic fungal cultures, and both pestalopyrone and hydroxypestalopyrone can be isolated from artificially infected torreyas. In addition, pestaloside has antifungal activity against other fungal endophytes of *T. taxifolia*.

Conclusions: The filamentous fungus, *P. microspora*, has an endophytic-pathologic relationship with *T. taxifolia*. The fungus resides in the inner bark of symptomless trees, and physiological or environmental factors could trigger its pathological activity. *P. microspora* produces the phytotoxins pestalopyrone, hydroxypestalopyrone, and pestaloside which give rise to the disease. Pestaloside, which also has antifungal activity, could reduce competition from other fungal endophytes within the host.

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Introduction

The Florida torreya (*Torreya taxifolia* Arn. or stinking yew) is the rarest tree in North America for which a full species designation has been established. This yew-like conifer is endemic to 400 km^2 of ravine slopes along a short stretch of the eastern bank of the Apalachicola River in northern Florida and adjacent Georgia [1]. In this small region, it was a common subcanopy tree, but during the late 1950s it began a catastrophic decline, and within a decade, no sexually mature adults remained [2,3]. The decline continues, and the fewer than 1500 remaining trees have been placed on the Federal endangered species list [4]. Why are the torreyas disappearing? The most prevalent symptoms of diseased trees are needle spots, needle death and stem cankers [2,3], but no virulent pathogen has been described. Lack of an associated pathogen has led to speculation that environmental changes resulting from unregulated forestry practices on adjacent lands over the past 80 years rendered the trees vulnerable to attack from native pathogens [3, 5].

The Florida torreya is a member of the plant family Taxaceae and a close relative of the Pacific yew (*Taxus brevifolia*) — the source of the anticancer drug taxol and closely related compounds [6]. The family Taxaceae is one of the smallest and oldest conifer families, and loss of a member presents a greater potential loss of biodiversity than the extinction of a member of a larger family. Thus the torreya's imminent extinction illustrates the possible loss of valuable biosynthetic capabilities before they can be appreciated. The loss of potential pharmaceutical agents through the extinction of plants is a well recognized possibility, but there is an additional, less appreciated, loss. Like most plants, torreya serves as the host for a large number of intimately associated microorganisms such as endophytic fungi, and the host's extinction can lead to the extinction of some of these species as well.

Endophytic fungi are characterized by their ability to invade the intercellular space in the living tissues of plants, and inside virtually every plant there are several species of endophytic fungi [7]. The nature of the association between endophytic fungi and their hosts ranges from symbiotic, to near neutral, to parasitic [7,8]. For example, some grasses are chemically defended against herbivores by indole alkaloids produced by their endophytic fungi [9]. A well documented parasitic relationship is the infection of the leaves of *Hymenaea courbaril*, a tropical resin-producing tree, by the fungus *Pestalotia subcuticularis* [10]. The plant seedling is infected by the fungus, which remains dormant while the plant matures. Following a variable length dormancy period, the fungus produces phytotoxins that ultimately kill the plant. Our understanding of the

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chemistry and biology governing the relationship between endophytic fungi and their hosts is just beginning, and one intriguing possibility is the exchange of genetic information [11]. In this regard, a provocative recent study reports that an endophytic fungus from the Pacific yew produces taxol-like compounds [12].

Our efforts to characterize the endophytic fungi of the Florida torreya and their biosynthetic products have led to the hypothesis that the tree's decline results from a pathogenic relationship with the endophytic fungus *Pestalotiopsis microspora*. Establishing that a pathogen causes a disease typically involves a four-step procedure that was formalized at the end of the last century by the German bacteriologist Robert Koch. The now-familiar Koch's rules [13], phrased for the hypothesis under consideration, are: 1) *P. microspora* must be consistently found in diseased torreyas; 2) *P. microspora* must be grown in pure cultures; 3) when *P. microspora* from a pure culture is introduced into healthy torreyas, it must produce the original disease symptoms; and 4) *P. microspora* must be reisolated from the deliberately infected plants. Today — and especially for the readers of this journal — we would be tempted to add a 'chemical' postulate: compounds isolated from cultured *P. microspora* should reproduce the disease and its symptoms.

Results and discussion

Fulfilling Koch's postulates

The first two steps are to find the pathogen consistently and to develop a pure laboratory culture. Thirty morphologically different endophytic fungi, including *Trichoderma* species, *Cladosporium* species, and *Pestalotia* species, were collected from 30 trees sampled throughout the tree's limited range in northern Florida. One particular fungus was isolated from over 90% of 78 branchlets and needles taken from the 30 trees, and was found on 27 of the 30 trees. The fungus was first isolated from a wild torreya with chlorotic needles (Fig. 1a), but was also usually obtained from the inner bark of a limited number of symptomless trees. The fungus was identified as *Pestalotiopsis microspora* by the characteristic appendages on its conidiophores and the effuse brownish colonies formed by its mycelia (Fig. 2a). The spores are five-celled, with the three center ones being dark and the terminal cells being hyaline. Additional structures are present at the apical ends of the spores, as shown in Figure 2b. *P. microspora* can be cultured in M-1-D medium and maintained on potato dextrose or M-1-D agar. It can be stored for periods exceeding one year in sterile distilled water at 4 °C. The next most commonly found fungus occurred in only 60% of the samples.

To test whether Koch's third postulate applies requires deliberately infecting torreyas with laboratory cultures of *P. microspora*. *T. taxifolia* limbs were inoculated with *P. microspora* by placing a small agar block containing the fungus into a slit on a symptomless torreya limb. The trees were maintained under normal growth conditions in the Montana State University Plant Growth Facility.

Fig. 1. Symptoms of diseased *T. taxifolia* **(a)** A frond on a 1.5 m *Torreya taxifolia* in northern Florida. Many of the needles show chlorosis (yellowing), which is the initial outward symptom of the decline. The initial isolate of the endophytic fungus, *P. microspora*, came from this tree. **(b)** *P. microspora*-induced canker on the limb of a greenhouse grown *T. taxifolia* tree.

Within two weeks, all 20 artificially inoculated trees exhibited canker-like growths with necrotized brownish tissue on their stems along with chlorosis on their terminal leaves (Fig. 1b) — symptoms comparable to those observed in declining trees in the wild. Control experiments using water agar as the inoculum did not cause any symptom formation. The leaf symptoms are vividly

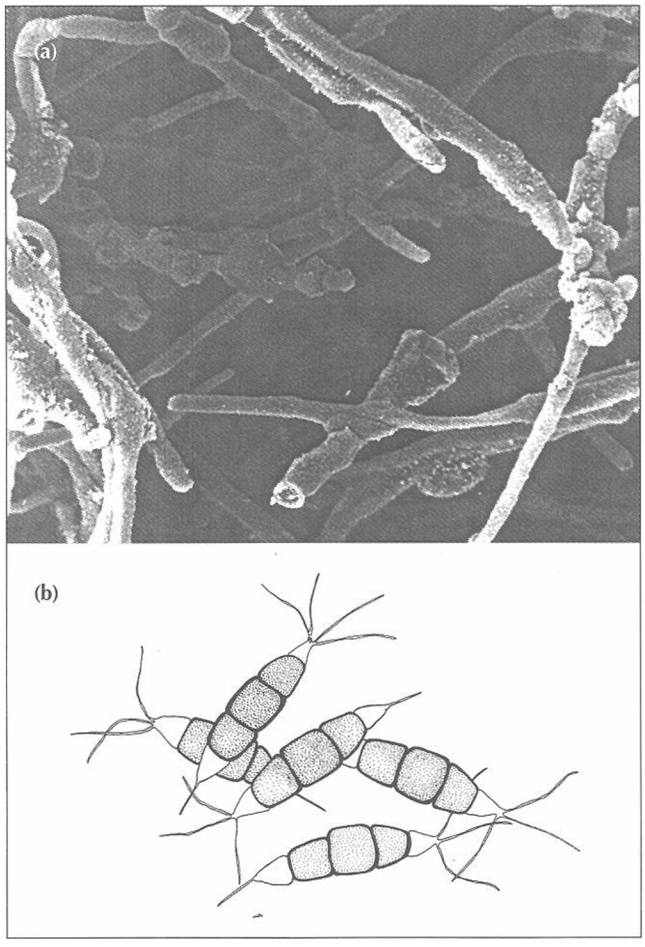

Fig. 2. Identification of *P. microspora*. **(a)** Scanning electron micrograph of the conidiospores of *P. microspora*. **(b)** A schematic drawing of the spores of *P. microspora*.

illustrated by an imaging spectrometer specially designed for use in plant science (Fig. 3) [14].

Finally, the pathogen must be reisolated from the deliberately infected plants. Plant tissues ≈5 cm from the initial point of inoculation were surface-sterilized with 70% ethanol and air-dried. The phloem and cambium were carefully excised and placed on water agar plates for initial mycelial growth, and the isolates were then transferred to nutrient agar plates for physiological and phenotypic differentiation as well as culture purification. A few unidentified fungi were isolated from select artificially inoculated limbs, but *P. microspora* was reisolated in every instance.

The chemical basis for pathogenicity

Since the fungus remained within 4 to 6 cm of the point of inoculation and was never isolated from the leaves in these studies, it seemed plausible that fungal metabolites were responsible for at least some of the leaf disease symptoms. To investigate this possibility, metabolites from fungal cultures grown on a defined medium were examined for phytotoxicity. Phytotoxicity was monitored by a chlorosis/necrosis assay using a leaf puncture test on *T. taxifolia* and *Taxus brevifolia* needles while antimicrobial activity was assayed using *Candida albicans*, *Escherichia coli*, and *Bacillus subtilis*. Only compounds active against *B. subtilis* were found. Using these assays to guide the isolation, three compounds were purified: pestalopyrone, hydroxypestalopyrone, and pestaloside, which were produced at 19, 8 and 9.5 mg g^{-1} extract respectively (Fig. 4). Pestalopyrone, a compound previously identified as a product of *Pestalotiopsis oenotherae* and reported as a weak phytotoxin of evening primrose [15], was identified through NMR spectroscopy and single-crystal X-ray diffraction. Hydroxypestalopyrone was characterized through its close relation to pestalopyrone, spectroscopy data and single-crystal X-ray diffraction (Fig. 4). Such pyrone structures are well known fungal metabolites from a variety of fungal sources. For example, nectriapyrone, which is identical to pestalopyrone except for the fact that it has a methyl substituent on C-2, has been isolated from the terrestrial fungus *Gyrostroma missouriense* and, most recently, from a marine fungus from an Indo-Pacific sponge [16]. The third compound is a lipophenyl β–glucoside, trivially named pestaloside, which was characterized by spectroscopy and chemical analysis.

NMR and mass spectroscopy data indicated a molecular formula of $C_{20}H_{30}O_8$ for pestaloside with six sp^3-hybridized carbons attached to oxygens, a long aliphatic chain, and two rings — one of which was aromatic (Fig. 4). The 9.0 Hz coupling between the only two aromatic protons, H-4 and H-5, established their *ortho* relationship on a tetrasubstituted phenyl ring. The position of the *trans*-heptenyl side chain was identified by the long-range heteronuclear correlations (HMBC) of δ 131.6 (C-7) to δ 6.04 (H-9) and δ 125.9 (C-2) to δ 6.60 (H-8). The chemical shifts of C-3 and C-6 suggested attachments to oxygens, while the observed

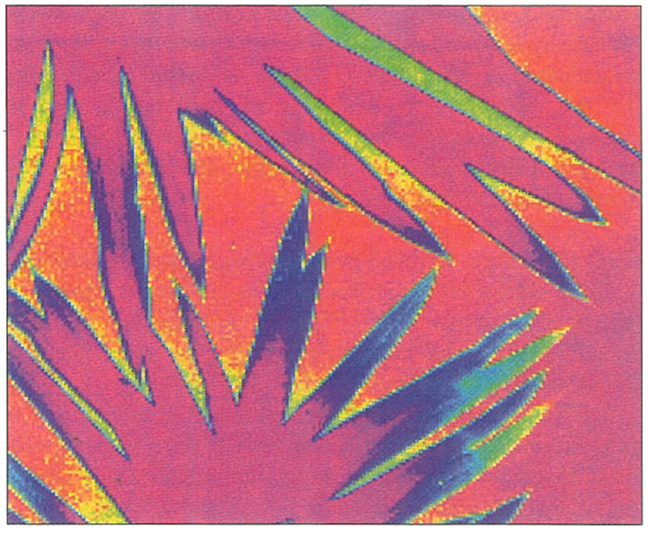

Fig 3. A false color image of the leaves at the stem tip of a fungal-inoculated and control *T. taxifolia*. The inoculation site was 15 cm below the apical meristem. An imaging visible spectrometer was used to give enhanced symptom expression [14]. A distinction between diseased and control tissue is manifested by the greenish-blue coloration at the needle tips on the inoculated stem (bottom). See Materials and methods for details.

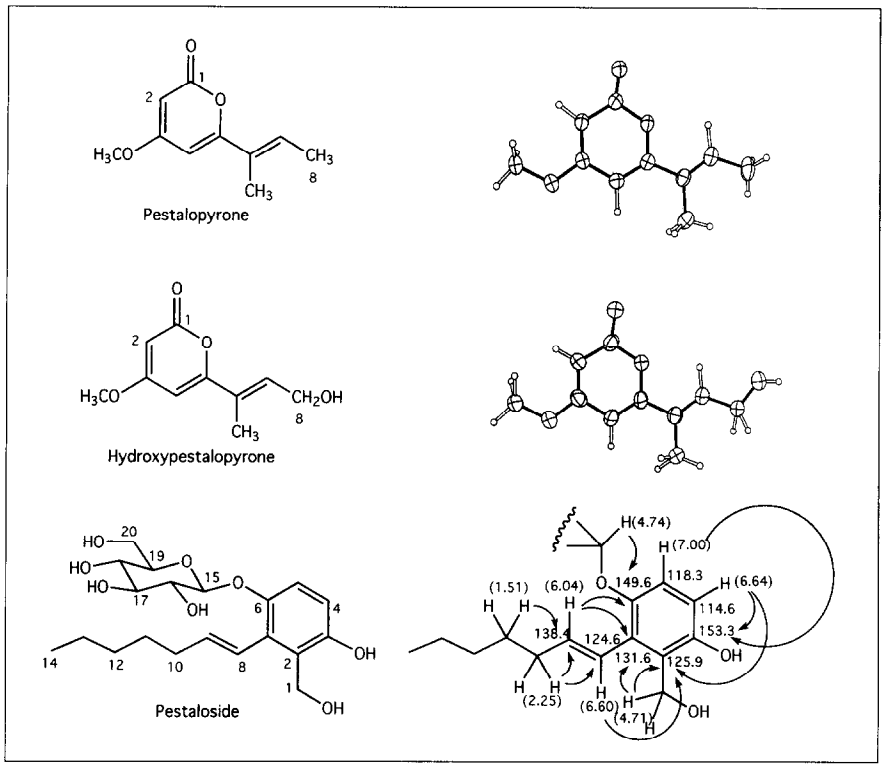

Fig 4. Structures of pestalopyrone, hydroxypestalopyrone and pestaloside. Three-dimensional X-ray derived illustrations of pestalopyrone and hydroxypestalopyrone and important HMBC correlations for pestaloside are on the right.

HMBC correlations of δ 131.6 (C-7) and δ 125.9 (C-2) to δ 4.71 (H_2-1) placed the hydroxymethylene at the C-2 position. Other correlations around the aromatic ring are shown in Figure 4. The remaining signals in the ^{13}C and ^{1}H NMR spectra indicated a sugar fragment, and because the resonance at δ 149.6 (C-6) correlated to the doublet at δ 4.74, the sugar must be attached to C-6. The sugar unit was identified by converting pestaloside to its peracetylated derivative, which clarified the sugar oxymethine resonances, and gas-chromatography mass spectrometry (GC–MS) analysis of the hydrolyzed compound. The β configuration was established by the relatively large *trans*-diaxial coupling of the anomeric proton in both pestaloside and its acetylated derivative. The bioassay data indicated that all three compounds were phytotoxic, and each possessed some antimicrobial activity.

Pestalopyrone, hydroxypestalopyrone, and pestaloside caused chlorosis of torreya needles in four replicates of the leaf puncture test [17]. On *T. taxifolia*, 10 μg of pestalopyrone and hydroxypestalopyrone caused symptoms within two days while on *T. brevifolia*, a much smaller dose, 1 μg, caused symptoms. The difference in sensitivity suggests a modest level of host specificity. Pestaloside, on the other hand, was equally phytotoxic on *T. taxifolia* and *T. brevifolia*.

Pestalopyrone and hydroxypestalopyrone could both be isolated from infected, but not from control, plant tissue at concentrations sufficient to be responsible for symptom expression. Infected and control stem fragments collected three weeks after inoculation were separately ground up and extracted with ethyl acetate. Compounds were isolated by successive preparative thin-layer chromatography, and their identity was confirmed by electrospray ionization mass spectra of both purified compounds.

Since pestaloside was more active in antifungal assays than pestalopyrone or hydroxypestalopyrone (data not shown), we investigated whether it might provide *P. microspora* with a competitive advantage against other endophytic fungi isolated from *T. taxifolia*. In a simple assay (see Materials and methods), pestaloside caused distinct zones of inhibition, especially against a *Cladosporium* species and a sterile hyphomycete isolated from *T. taxifolia*. Pestaloside was also active against *Rhizoctonia solani*, *Geotrichum candidum*, and *Agaricus campestris*, but not against a *Trichoderma* species.

Conclusions

While more than 30 different endophytic fungi were isolated from wild Florida torreyas during this investigation, only *P. microspora* was found to be consistently associated with the tree. This organism, based on the available data, is a plausible causative agent for the Florida torreya's decline. Since hosts and parasites that have been associated for a long time usually co-evolve to a relationship in which the parasite does not kill the host, *P. microspora*'s pathogenicity may indicate a recent association with torreya. One possibility is that it has been introduced via the large scale cultivation of non-native pines on the land surrounding the torreya's habitat. Alternatively it may have long co-existed with torreyas, but became pathogenic as the torreya was stressed by environmental disturbances brought on by intensive forestry practices in the region. Whatever the detailed mechanism, the

involvement of *P. microspora* in the decline of the Florida torreya deserves further consideration. The case for its involvement is increased by the coupling of chemistry with Koch's postulates, and the identification of fungal metabolites that both directly cause disease symptoms and could possibly give *P. microspora* a competitive advantage over other fungi. The urgency of investigating disappearing species for potentially useful compounds is widely accepted; the urgency should expand to include host-specific symbionts and parasites.

Significance

Many useful agents have come from terrestrial plants. For example, our understanding of tubulin and its role in cellular processes has been clarified by the plant derived agents colchicine, vincristine, vinblastine and taxol, and these same agents have been used to treat human diseases ranging from gout to cancer. When a plant species becomes extinct, the possibility of examining the products of its biosynthetic machinery vanish with it; this loss is now generally appreciated. The Florida torreya is rapidly becoming extinct, and as a close relative of the taxol-producing Pacific yew, its extinction could be especially regrettable.

A plant also serves as host to a variety of microorganisms, and to the extent that these microorganisms are host-specific, their biosynthetic capability will disappear as well. These dual losses would also prevent an understanding of the poorly understood relationships between plants and their associated microorganisms, including the degree of host specificity and the range of relationships, from symbiotic to pathogenic.

The present study uses a combination of Koch's postulates and natural-products chemistry to elucidate a pathogenic relationship between the rarest tree in North America and a fungal parasite. Understanding this interaction could eventually lead to treatments that would avoid the tree's extinction in the wild. More generally, the study shows how chemistry and biology can be used to build an understanding of the relationships between hosts and their microorganisms.

Materials and methods

General

^{13}C NMR spectra were recorded on a Varian Unity 400 spectrometer, and all ^{1}H spectra and two-dimensional experiments (correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and HMBC) were recorded on a Varian Unity 500 spectrometer. X-ray data were collected on a Siemens R3m/V diffractometer using CuKα radiation. UV spectra were obtained on a Beckman DU-50 spectrophotometer, and optical rotation was measured on a Perkin–Elmer 241 Polarimeter. Melting points were determined on a Fisher–Johns Melting Point Apparatus.

Fungal isolation and identification

Small branches of *T. taxifolia* were surface-sterilized with 70% ethanol and allowed to dry. A sterile knife was used to slice open the thin outer bark longitudinally and small pieces of the inner bark were placed onto water agar plates (15 g l^{-1}). Microorganisms that had grown radially from the bark pieces were subcultured onto nutrient agar plates for further differentiation. Purity of the strains was attained through successive hyphal-tipping onto nutrient agar plates. The pathogenic fungus was identified as *Pestalotiopsis microspora* on the basis of its colony and spore morphology, which were identical to an existing fungal culture in the Montana State University (MSU) laboratory. The MSU fungus had been identified by Dr. B. Sutton of the Commonwealth Mycological Institute (CMI; Surrey, England). The spores are carrot-shaped with three long, propeller-like structures on the blunt end. Each of these structures is divided into five cells, the three center cells being dark and the two terminal cells being hyaline. The spores of the pathogenic fungus are roughly 20% greater in size than the standard *P. microspora* spores obtained from CMI, indicating that this isolate may be a different strain.

Fungal fermentation

P. microspora was germinated in 500-ml Erlenmeyer flasks containing 50 ml M-1-D seed media containing (g l^{-1}) 0.25 $Ca(NO_3)_2$, 0.08 KNO_3, 0.06 KCl, 0.02 $NaH_2PO_4 \cdot H_2O$, 30.0 sucrose, 1.0 soytone, 0.5 yeast extract; (mg l^{-1}) 2.0 $FeCl_3 \cdot 6H_2O$, 5.0 $MnSO_4$, 2.5 $ZnSO_4 \cdot 7H_2O$, 1.4 H_3BO_3, and 0.7 KI. The culture was placed on a rotary shaker at room temperature for 48 h, after which 25-ml aliquots of the seed culture were transferred into 5-l flasks containing 500 ml of M-1-D fermentation media consisting of (g l^{-1}) 15.0 sucrose, 2.5 malt extract, 0.5 tryptone, 1.0 NaCl, 0.5 $MgSO_4$. Flasks were placed on the rotary shaker at room temperature and harvested after 96 h.

Leaf puncture wound test

T. taxifolia needles were collected from greenhouse-cultivated trees. A 5-μl droplet of sample solution in 5% ethanol was placed on a leaf blade where a puncture wound had been made to enhance the uptake of the compounds to the tissues. The leaves were placed on a moist filter paper in a sealed petri dish and incubated at 25 °C for 48, 96, and 144 h. A 5% ethanol droplet was used as the control. In general 10 μg of each compound dissolved in 5% aqueous ethanol causes an obvious necrotic lesion at the point of application after two days of incubation, and 5 μg causes toxicity after four days. Minimal phytotoxicity is observed at 1 μg after six days.

Isolation of phytotoxins

The 40-l culture of *P. microspora* was filtered through cheesecloth and extracted three times with equal volumes of ethyl acetate. A total of 11.2 g of red crude organic extract was obtained after solvent evaporation *in vacuo*. The extract was first chromatographed on a silica gel column (30 x 1.5 cm), and eluted with 35 ml of each of the following CH_2Cl_2–MeOH solvent compositions: 49:1, 30:1, 33.3:1.7, 32:3, 30:5, 25:10, 20:15, 1:1, and 1:3. Fractions 1–3 and 5–6 caused necrosis of torreya needles in the leaf puncture wound test.

Pestalopyrone

Fraction 1 (2.5 g) was applied to another silica gel column and eluted with varying mixtures of $CHCl_3$–MeOH (75:1 to 10:1). The active fractions (leaf puncture assay) were combined and the active compound purified by two successive fractionations on Sephadex LH-20 (1:1 $CHCl_3$–MeOH) to yield 45 mg of an

off-white powder. The compound crystallized from 1:1 hexane–ethyl acetate as yellow plates, melting point (m.p.) 75–76 °C (The literature value is 101–102 °C; this may be a polymorph). Electrospray ionization mass spectrometry (ESIMS): *m/z* $[M+H]^+$ 181.2; $C_{10}H_{12}O_3$; UV (MeOH) λ_1 238 nm ($\epsilon = 11000$), λ_2 310nm ($\epsilon = 11000$); ^{13}C NMR (100MHz, MeOD) δ 174.1 (C-1, s), 98.8 (C-2, d), 162.6 (C-3, s), 88.5 (C-4, d), 166.9 (C-5, s), 128.3 (C-6, s), 131.0 (C 7, d), 12.0 (C-8, q), 14.3 (C-9, q), 56.9 (C-10, q); ^1H NMR (500MHz, *J* Hz, MeOD) δ 6.12 (H-2, d, 1.0), 5.55 (H-4, d, 1.0), 6.63 (H-7, qq, 7.2, 1.2), 1.85 (H_3-8, d 7.5), 1.87 (H_3-9, d, 1.0), 3.70 (H_3-10, s). A single crystal of pestalopyrone was selected for characterization by single crystal X-ray diffraction. The crystals formed in the triclinic system with a = 9.732 (1), b = 8.090 (2), c = 8.118 (2) Å, α = 86.55 (2)°, β = 66.93 (2)°, and γ = 89.15 (2)° as determined from a least-squares fit of 25 diffractometer-measured 2θ values. A plausible density (1.25 g cm^{-3}) would require two molecules of $C_{10}H_{12}O_3$ per unit cell, and successful refinement verified the choice of space group as centro-symmetric. A total of 1201 independent reflections were collected, and all were judged observed ($F_o \geq 4.0\sigma(F)$). The structure was solved without difficulty using direct methods and refined with anisotropic heavy atoms and riding isotropic hydrogens to a final R = 4.9% using the SHELXTL library of programs. Archival crystallographic data have been deposited with the Cambridge Crystallographic Data Centre.

Hydroxypestalopyrone

Fraction 3 (1.75 g) was subjected to a silica-gel column and eluted with increasing polarity of $CHCl_3$–MeOH (50:1 to 10:1) solvent mixtures. The active fractions (leaf-puncture assay) were combined and the extract chromatographed twice on Sephadex LH-20 to afford 13.5 mg of an off-white powder with a UV spectrum similar to pestalopyrone. The compound crystallized from 1:2 hexane–ethyl acetate as yellow plates, m.p. 165–166 °C. ESIMS: *m/z* $[M+H]^+$ 197.3; $C_{10}H_{12}O_4$; UV (MeOH) λ_1 223 ($\epsilon = 8300$), λ_2 310 ($\epsilon = 2700$); ^1H NMR (500 MHz, *J* Hz, MeOD) δ 6.21 (H-2, d, 2.0), 5.60 (H-4, d, 2.0), 6.58 (H-7, qq, 6.5, 1.5), 4.30 (H_2-8, d, 6.5), 1.88 (H_3-9, d, 1.0), 3.86 (H_3-10, s). A single crystal of hydroxypestalopyrone was selected for characterization by single crystal X-ray diffraction. The crystals formed in the monoclinic system with a = 7.138 (1), b = 9.784 (2), c = 13.982 (2) Å, and β = 100.64 (1)° as determined from a least-squares fit of 25 diffractometer measured 2θ values. A plausible density (1.36 g cm^{-3}) would require four molecules of $C_{10}H_{12}O_4$ per unit cell, or one molecule in the asymmetric unit of space group $P2_1/c$. A total of 961 independent reflections were collected, and 723 (75%) were judged observed ($F_o \geq 4.0\sigma(F)$). The structure was solved uneventfully using direct methods and refined with anisotropic heavy atoms and riding isotropic hydrogens to a final R = 9.0% using the SHELXTL library of programs. Archival crystallographic data have been deposited with the Cambridge Crystallographic Data Centre.

Pestaloside

Fraction 6 was subjected to another silica column and eluted with step gradients of toluene–ethylacetate–methanol (30:5:1 to 1:1:1). The active fractions (leaf-puncture assay) were combined and the extract was further fractionated on a 15 x 2.5 cm silica column (toluene–MeOH, 30:1, 20:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1). The compound was purified from Sephadex LH-20 (1:1 $CHCl_3$–MeOH) as a yellow oil (11.4 mg). $[\alpha]^{25}D = -39.7°$ (MeOH); High resolution fast atom bombardment mass spectroscopy (HRFABMS): *m/z* $[M+H]^+$ 399.1934, observed; calc. for $C_{20}H_{31}O_8$, *m/z* 399.1941; UV (MeOH) λ_1 240nm ($\epsilon = 18000$), λ_2 300nm ($\epsilon = 7400$); ^{13}C NMR (100MHz, MeOD) δ 58.5 (C-1, t), 125.9 (C-2, s), 153.3 (C-3, s), 114.6 (C-4, d), 118.3 (C-5, s), 149.6 (C-6, s), 131.6 (C-7, s), 124.6 (C-8, d), 138.4 (C-9, d), 34.8 (C-10, t), 30.3 (C-11, t), 32.7 (C-12, t), 23.6 (C-13, t), 14.4 (C-14, q), 103.7 (C-15, d), 75.1 (C-16, d), 78.2 (C-17, d), 71.5 (C-18, d), 78.0 (C-19, d), 62.6 (C-20, t); ^1H NMR (500MHz, *J* Hz, MeOD) δ 4.71 (H-1, s), 6.64 (H-4, d, 9.0), 7.00 (H-5, d, 9.0), 6.60 (H-8, dt, 15.0, 1.2), 6.04 (H-9, 15.0, 7.0), 2.25 (H_2-10, qd, 7.5, 1.5), 1.51 (H_2-11, m), 1.38 (H_2-12, m), 1.38 (H_2-13, m), 0.93 (H_3-14, t, 7.2), 4.74 (H-15, d, 6.9), 3.43 (H-16, dd), 3.42 (H-17, t), 3.38 (H-18, t), 3.32 (H-19, ddd), 3.85 (H-20a, dd, 12.0, 2.4), 3.67 (H-20b, dd, 12.0, 5.2).

Acetylation of pestaloside

A 1-mg sample of pestaloside was treated with 1:1 v/v mixture of pyridine and acetic anhydride at room temperature. After 20 h, the solvent and excess acid were evaporated *in vacuo*. The product was washed with acetonitrile and the solvent evaporated to remove trace amounts of acid and pyridine. The reaction afforded a quantitative yield of the hexaacetate of pestaloside.

Carbohydrate analysis of pestaloside

The presence of glucose was confirmed by the University of Georgia's Complex Carbohydrate Research Center for analysis using their standard procedure. A 1-mg sample of pestaloside was treated with 1 M methanolic HCl at 80 °C for 16 h. To check for amino sugars, the sample was N-acetylated using acetic anhydride/pyridine/methanol. The sample was then silylated using Trisil-Z. The resulting derivatized sample was analyzed by both gas chromatography and GC–MS using a DB-1 capillary column. The derivatized sample was purified by passing the sample over glass wool, addition of hexane, and blowing down under nitrogen. Two major peaks were observed in the GC trace, at 21.04 and 21.87 minutes, identified as the α- and β-anomers of glucose. *Myo*-inositol was added as the internal standard (28.85 min).

Artificial infection of T. taxifolia

A 1.5 cm slit was made 10–15 cm from the apical meristem in greenhouse-cultivated trees. *P. microspora* was introduced by placing a 0.5 x 0.5 cm agar block into the slit. The inoculation site was wrapped with a piece of tape, which was eventually removed, and photographed after two weeks. A limb inoculated with water agar block alone was used as the control.

Reisolation of phytotoxins from artificially infected T. taxifolia *limbs*

Infected and control torreya limbs were separately ground up and soaked with ethyl acetate for 8 h. Successive preparative thin-layer chromatography was performed on 0.25-mm silica plates using 8:2 chloroform–methanol, 95:5 ethylacetate–isopropanol, and 6:2 methylene chloride–tetrahydrofuran with R_f values of 0.52, 0.61, and 0.71 for pestalopyrone and 0.09, 0.36, and 0.44 for hydroxypestalopyrone. Compounds were visualized by UV absorbance and reaction with vanillin/sulfuric acid spray reagent. The appropriate molecular ions (M+H and M+Na) of both compounds were observed in ESI–MS.

Reisolation of P. microspora *from artificially infected torreya limbs*

Fungi were isolated from torreya limbs as described above. *P. microspora* was identified from each of the stems after examination of its culture characteristics and its spore formation on γ-irradiated carnation leaves held on water agar.

Antifungal activity of pestaloside

A 5 mg ml^{-1} solution of pestaloside in methanol (10 µl)was placed directly on the center of a potato dextrose agar plate. After evaporation of the solvent, the plate was inoculated with agar plugs of other *T. taxifolia* endophytes (four per plate) placed ≈ 1 cm away from the point of application of pestaloside. All test plates were incubated at room temperature and observed for fungal inhibition after three days.

False color imaging of T. taxifolia

A specially designed imaging fluorometer was used to assess the photosynthetic function of plants [13]. Needles of *T. taxifolia* were kept in the dark for 20 min prior to fluorescence measurements in order to promote maximal light absorption by the chloroplasts. The needles were then irradiated with a 500 W, 120 V projector lamp, and a series of fluorescent images were photographically recorded. The images were exposed for 0.1 s and a fresh image was recorded every 0.1 s. The first ten images were compared and the brightest frame was used to calculate quantum yield, which is proportional to photosynthetic activity. The 150 s image was used as the terminal fluorescence value. In Figure 3, dark red coloration corresponds to highest quantum yield (healthiest tissue); dark blue, to the lowest (least healthy tissue).

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