Glucosylation of the peptide leucinostatin A, produced by an endophytic fungus of European yew, may protect the host from leucinostatin toxicity

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Background: Yew species (*Taxus spp.*) throughout the world are hosts to hundreds, or perhaps thousands, of endophytic organisms. Most commonly, these organisms are fungi, living in a commensal or a symbiotic relationship with their host plant, so the plants exhibit little or no outward evidence that they are supporting these microorganisms. Little is known about any of the biochemical mechanisms that mediate the interactions between the yew host and its associated microbes. We feel that such information may not only contribute to our understanding of endophyte–tree biology, but also may provide novel pharmaceutical leads, because some of the compounds produced by these endophytes have demonstrated pharmacological activities.

Results: *Acremonium sp.* was isolated as an endophytic fungus of the European yew, *Taxus baccata*. Entry of *Acremonium sp.* into the plant may proceed via invasion of natural openings such as stomata.The relationship between *Acremonium sp.* and *T. baccata* may be a symbiotic one, because no symptoms are seen when *Taxus media p.v. Hicksii* is inoculated with this fungus. In culture, the fungus makes leucinostatin A, a peptide with phytotoxic, anticancer and antifungal properties. Although this peptide causes necrotic symptoms in many non-host plants and other cell types, it causes no visible symptoms in the host plant. *T. baccata* and several other plants have a UDP glucose : leucinostatin A glucosyl transferase that catalyzes the production of leucinostatin A β di-O-glucoside from leucinostatin A. This glucoside, also made by the fungus, has a lower bioactivity against plants, fungi and a breast cancer cell line, BT-20, than leucinostatin A.

Conclusions: Leucinostatin A may be one of several potentially toxic peptides produced by *Acremonium sp.* that contribute to the defense of the host, thereby preserving the fungus' own biological niche. The host plant is relatively immune to leucinostatin A because it has an enzyme which transfers two glucosyl residues to leucinostatin A, markedly reducing the peptide's bioactivity. Our results suggest that glucosylation reactions may play a more general role in plant defenses, especially against toxin-mediated disease development.

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Introduction

The European yew (*Taxus baccata*) is found from Scandinavia to NW Africa and from England and Ireland to Central Asia. Limb samples that we have acquired from the European yew throughout this geographical range have yielded a wealth of endophytic fungi. Endophytes are typically recovered from plant samples that have been surface-treated to kill microorganisms living as epiphytes or as contaminants. Ultimately, living tissues are dissected from the plant parts and placed on agar plates to encourage microbial growth. The relationship between these endophytes and their plant hosts may range from near-pathogenic to symbiotic or commensal [1]. Classic examples of symbiosis are certain grass endophytes that effectively defend the grass against herbivores by producing noxious indole alkaloids [2]. Although endophytes have been isolated and taxonomically characterized from only a few plant species, there is so far mostly only speculation as to the exact biochemical, structural or functional relationship between any endophytic microbe and its plant host, however [3].

Of the large number of endophytes that we have isolated from *T. baccata*, one struck us as particularly interesting because it produces leucinostatin A, an extremely bioactive peptide [4–8]. Leucinostatin A has selective toxicity at 1–3 nM against certain cancer cell lines, is broadly antifungal and is phytotoxic [4,7]. The producer of this antimetabolite was identified as an *Acremonium sp.*, which is taxonomically not too unlike the well-known grass endophytes discussed

earlier [2]. Given its toxicity, obvious questions arose as to the potential role and fate of this compound in the endophyte–yew tree relationship and also, structurally, how the fungus endophyte itself may interact with its tree host.

In this report, we unequivocally show, by virtue of inoculation and reisolation experiments (Koch's postulates), that *Acremonium sp.* (isolate Tbp-5) can form an endophytic relationship with *Taxus spp.* [9]. We also demonstrate, by scanning electron microscopy (SEM), how this endophyte may enter the plant, and the structural relationships that it may establish with its host. Furthermore, using ^{14}C-leucinostatin A in aseptic *Taxus* tissues, we show that it is metabolized to a unique product, leucinostatin A β di-O-glucoside, which has a much lower bioactivity than the non-glucosylated form. Acetone powder extracts from various plants were also shown to have UDP glucose: leucinostatin A glucosyl transferase activity. Higher levels of this activity are generally associated with those plants that are relatively resistant to the phytotoxic effects of leucinostatin A, including all yew species tested. The significance of these findings is discussed in relationship to endophytes and plant pathogens in general.

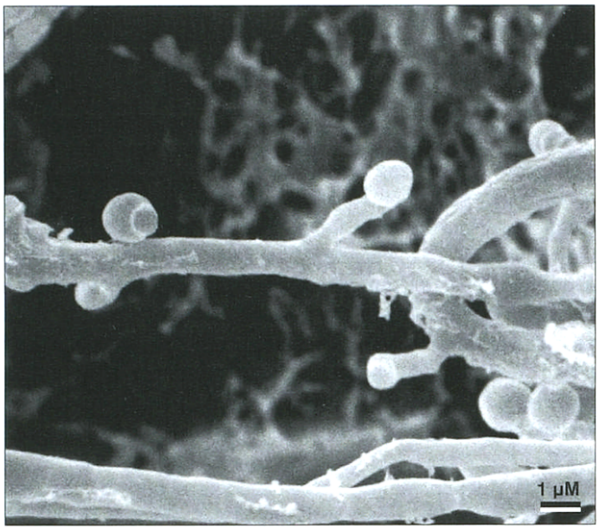
Results and discussion

Acremonium sp. as a yew endophyte

Twig samples were obtained from a mature European yew near Copthorne, England [4]. At least 21 different fungal endophytes were recovered from this tree using the standard techniques for recovering tree endophytes [3,4]. One of the most interesting fungal endophytes was identified as *Acremonium sp.* (Figure 1) [4]. It was especially intriguing because on potato dextrose agar plates it was inhibitory to *Pythium ultimum*, a major plant pathogenic fungus. It was characterized as an *Acremonium* species because it has slender hyphae (1–1.5 μm diameter) with simple upright conidiospores (Figure 1). The conidia are hyaline, oblong, 1–1.5 μm in width and 1.5–2.7 μm in length (Figure 1). W. Gams (CBS, The Netherlands) confirmed the identity of this organism [4].

Although *Acremonium sp.* Tbp-5 was recovered from *T. baccata* in the wild, stronger lines of evidence were needed to establish more firmly that it could form an endophytic relationship with *T. baccata*. To this end, we inoculated greenhouse-grown (1 m high) yew trees, *T. media p.v. Hicksii*. This tree was selected because of its ability to grow under greenhouse conditions and because our seedling trees are relatively free of endophytic fungi [9]. Straight slits were made into small limbs of the tree to form six inoculation sites on each tree. A small piece of agar containing the fungus was placed into the slit, forced beneath the bark on either side of the opening and the wound was wrapped with tape [9]. A comparable set of plants was treated with an agar block alone bearing no fungus. The samples were incubated for three weeks, then limb sections bearing the inoculation sites were removed, surface-treated with ethanol, flamed, and the inner bark dissected with a sterile blade and placed on water agar. Each of the stem pieces treated with the putative endophyte, *Acremonium sp.*, yielded a fungus with identical morphological features to the fungus that was used to inoculate the plants [4] (Figure 1). No microbe of any other type was ever noted in the control stems treated with agar blocks alone. Furthermore, there were no necrotic or abnormal growths seen on the fungal inoculated stems. This result confirms the hypothesis that *Acremonium sp.* Tbp-5 can establish itself in greenhouse-grown yews, and thus fulfills Koch's postulates in a manner comparable to our earlier work on an endophyte of *Torreya taxifolia* [9].

Figure 1

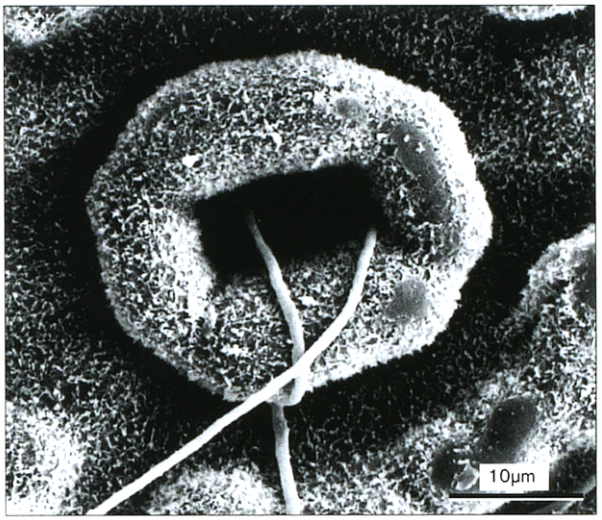


A scanning electron micrograph of *Acremonium sp.* Tbp-5 with its conidia, conidiophores and hyphae. The conditions for SEM are described in the Materials and methods section.

To determine how *Acremonium sp.* might become established in its host, we surface-treated *T. baccata* needles with 70% ethanol and inoculated them with agar blocks (0.5 × 0.5 mm) containing *Acremonium sp.* After 5–7 days, the needles were fixed in buffered gluteraldehyde and examined by SEM. Quite commonly, the fungus was observed entering the leaf directly through the stomatal openings (over the subsidiary cells) on the underside of the needles (Figure 2).

The relationship between *Acremonium sp.* Tbp-5 and the living tissues of the host is difficult to observe directly because the fungus is so diffuse in the plant tissue. In order to solve this problem, we isolated tissues excised from the infested yew stems in the greenhouse and placed them on

Figure 2



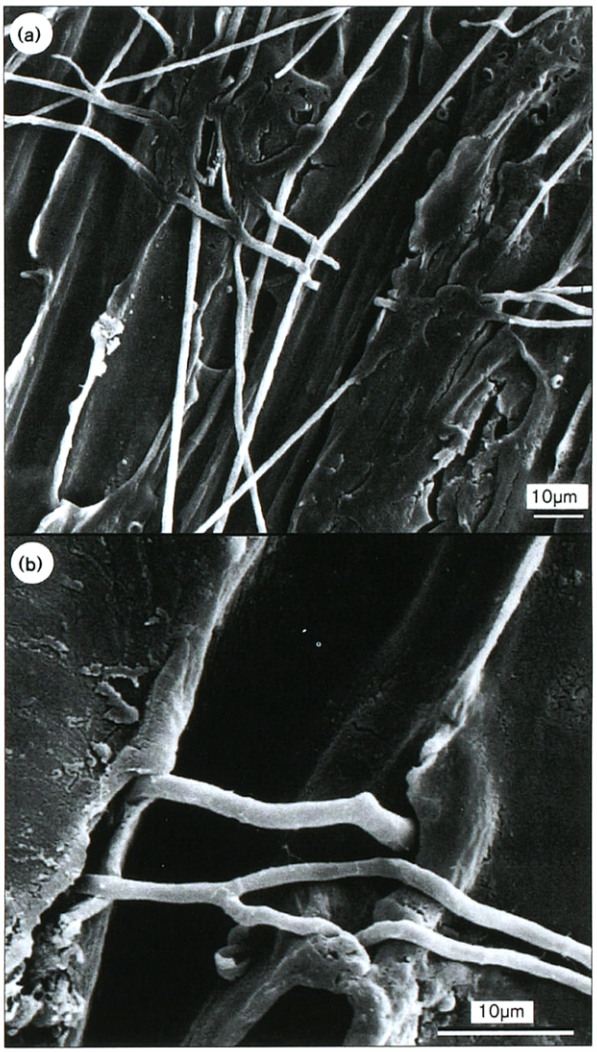
The hyphae of *Acremonium sp.* Tbp-5 entering the stomal area of the undersurface of a *T. baccata* needle. The raised cells surrounding the stomal area over which the hyphae have grown are known as subsidiary cells.

water agar. The fungus that appeared on the tissues after 3–4 days of incubation was examined by SEM. Although most of the fungal hyphae observed are on the wood surface (Figure 3a), some were observed actually penetrating intracellular spaces (Figure 3b), suggesting that this may be the relationship with the host that is actually preferred. An alternative would be tissue invasion via an intracellular mechanism(s) resulting in cellular death. This would cause tissue necrosis, making *Acremonium sp.* Tbp-5 more of a pathogen than a symbiotic endophytic fungus, and this was not observed after stem tissue inoculation.

Leucinostatin A – a metabolic inhibitor

Leucinostatin A is an unusual functionalized commonly known peptide from *Paecilomyces sp.* and *Penicillium sp.* It has been described as having antifungal, anticancer and phytotoxic activities [5–8]. Recently, we have learned that this same compound is produced in culture by *Acremonium sp.* Tbp-5 and is the chief phytotoxic component of *Acremonium* culture fluids [4]. This raises an interesting question about the non-pathogenic relationship of *Acremonium sp.* to its *Taxus sp.* host. It seems an anomaly that the fungus could invade the plant, causing little or no damage, while still producing leucinostatin A, which is a potent phytotoxin. To understand how this could occur, we initially tested leucinostatin A phytotoxicity using a chlorosis/necrosis assay in a stem/peptiole puncture test on a series of *Taxus* species and other plants. After 3 days, some plants showed chlorotic/necrotic discolored areas around the sites of inoculation, whereas others did not (Table 1). Interestingly, each of the *Taxus sp.* tested, with the exception of

Figure 3



The hyphae of *Acremonium sp.* Tbp-5 colonizing the xylem of a young *T. media p.v. media* stem. **(a)** Most hyphae are on the wood surface. **(b)** Some hyphae penetrate the surface.

T. media p.v. Hicksii which showed some slight discoloration, showed no lesion development at the application concentrations of 5 μg/5 μl and 1 μg/5 μl. Similarly, no lesions developed on knapweed petioles, whereas all other plants tested exhibited symptom development (Table 1).

Although some plants are more sensitive to leucinostatin A than others, the unusual insensitivity of yews to leucinostatin A was impressive (Table 1). Conceivably, leucinostatin A was being dispersed or rapidly mobilized away from the site of inoculation, reducing the chances of tissue damage. To test this hypothesis, we prepared ^{14}C leucinostatin A and placed six droplets (10 μl), each containing 20 μg of ^{14}C leucinostatin A (88,600 dpm/μmole), onto

Table 1

Effects of leucinostatin A and leucinostatin A β di-O-glucoside on the stems/petioles of various *Taxus spp.* and other plants.

Plant	Common name	Leucinostatin A		Leucinostatin A β di-O-glucoside
		Lesion diameter (mm) at 5 μg	Lesion diameter (mm) at 1 μg	5 μg
Taxus baccata	European yew	0	0	0
Taxus cuspidata	Japanese yew	0	0	0
Taxus media p.v. Hicksii	'Ornamental yew'	0.2 ± 0	0	0
Centaurea maculosa	Spotted knapweed	0	0	0
Metasequoia glyptostroboides	Dawn redwood	0	0	0
Torreya taxifolia	Florida torreya	1.15 ± 3.5	0.8 ± 0	0
Sequoiodendron giganteum	Sierra sequoia	0.5 ± 0	0.3 ± 0	0
Musa acuminata	Banana	5.3 ± 0.47	5.0 ± 0	0
Sequoia sempervirens	California redwood	1.75 ± 0.2	0.8 ± 0	0

The assays were conducted as described in the Materials and methods section. Each assay was replicated three times and the average lesion size reported along with the population standard deviation of the mean.

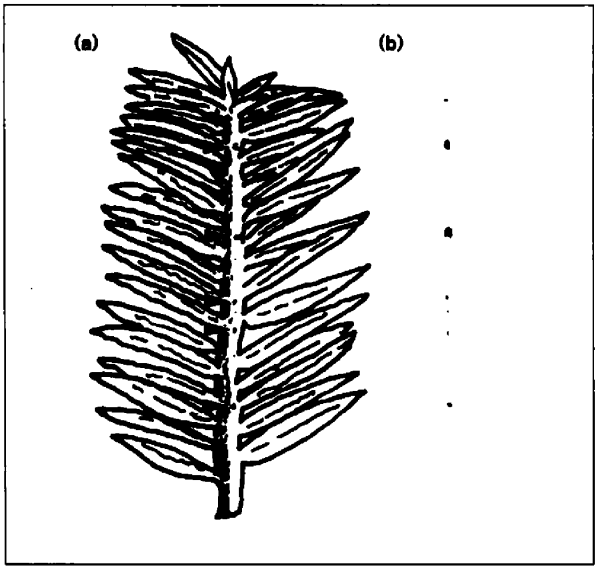
several puncture wounds made in one year old stems of *T. baccata* in a plastic box, with the cut end exposed to free water. After 3 days, only a small amount of necrotic tissue developed around the injection site (< 0.5 mm). The leaf was pressed dry and overlaid with a Kodak X-ray film and exposed for 8 days. The results showed that most of the radioactivity remained at the sites of injection (Figure 4). Thus, although it appeared that the labeling remained at the site of application, leucinostatin A remained relatively innocuous.

Leucinostatin A β di-O-glucoside

Several possibilities exist to explain the unusual insensitivity of *Taxus spp.* and tissues from some other plants to leucinostatin A. Either degradation or derivatization of this compound to a relatively inactive form seemed a likely explanation. Aseptically prepared tissues of *T. media p.v. Hicksii* were therefore incubated with ^{14}C leucinostatin for 3 days in a similar manner to previous studies using ^{14}C taxol precursors and inner bark [10]. After extraction with methanol, thin layer chromatography (TLC) in solvent A (see Materials and methods section) and autoradiography, two major areas of radioactivity appeared on the plate, one with the same R_f as leucinostatin A and the other at R_f 0.24 which is the same value as for an unknown compound that has previously been reported to be produced by *Acremonium sp.* Tbp-5 (MW = 1581) [4]. Residual ^{14}C leucinostatin A in the extract made up over 80% of the recoverable ^{14}C, whereas 5% of the label was in the unknown compound. The possibility that leucinostatin A was being converted by yew tissues to the previously isolated and yet unknown fungal compound was explored further.

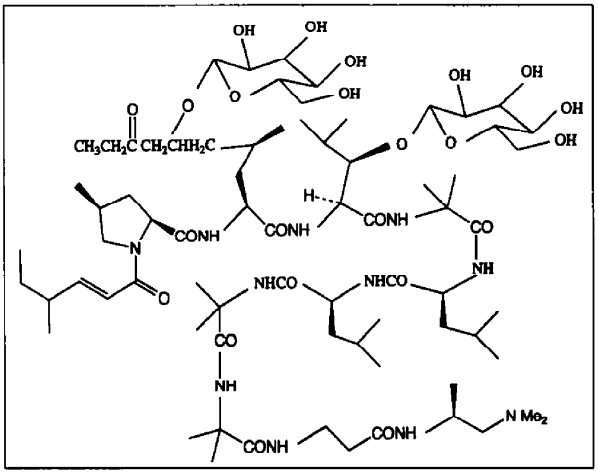
Larger quantities (milligrams) of the unknown fungal compound were isolated by n-butanol extraction of fungal culture fluids followed by TLC in solvents A, B and C (see Materials and methods section). Electrospray ionization mass spectroscopy revealed an $[M+K]^+$ at 1581 and an $[M+Na+K]^{+2}$ at 802 yielding a molecular weight of 1542. This mass is equivalent to two anhydro hexose units linked to leucinostatin A (MW = 1218). Acid hydrolysis in trifluoroacetic acid yielded a compound with the same paper chromatographic mobility as glucose. It also yielded a compound producing an electrospray ionization mass of $[M+Na]^+$ of 1223 or anhydroleucinostatin A (MW = 1200). The β configuration of the glucosyl residues was established by the chemical shift and relatively large coupling constant of the anomeric protons. Furthermore, upon

Figure 4



Injection of leucinostatin A into *T. baccata*. **(a)** A drawing of a one-year old stem of *T. baccata*. The living counterpart had been injected at several locations (arrowheads) with ^{14}C-leucinostatin A, pressed dry and **(b)** an autoradiograph was prepared.

Figure 5



The structure of leucinostatin A β di-O-glucoside.

digestion with a specific β-glucosidase from almonds, glucose and leucinostatin A were detected chromatographically. The results suggest that the fungal product is the β di-O-glucoside of leucinostatin A (Figure 5). The sugar linkages probably exist at the two free hydroxy groups in leucinostatin A, consistent with the ability of β glucosidase to release glucose from this glucoside.

The bioactivity of the glucoside was tested against plants, fungi and a cancer cell line. It showed no activity against any of the plant stems tested (Table 1), but it showed 50% inhibition of the growth of the fungus *Pythium ultimum* after 1 day at 4 μM and after 4 days at 16 μM, whereas leucinostatin A showed 50% inhibition after 1 day at < 1 μM and after 7 days at 4 μM [4]. Leucinostatin A β di-O-glucoside had an LD_{50} of > 25 nM against a breast cancer cell line BT-20, whereas leucinostatin A has an LD_{50} of 2 nM [4]. The noticeable bioactivity of the glucoside against a breast cancer cell line and *P. ultimum* may be related to the ability of each of these biological systems to release leucinostatin A from the glucoside via β-glucosidases present in each system. It would appear that one or both of the free hydroxyl groups on leucinostatin A may be necessary for bioactivity of the molecule to be expressed. This was confirmed by the finding that the acetylated leucinostatin A was inactive in all bioassay systems when at concentrations up to 50 μg/ml in the BT-20 cell line and 10 μg/5 μl in the plant (banana) bioassay system.

The biosynthesis of leucinostatin A β di-O-glucoside

Acetone powders containing a broad mixture of enzymes were prepared from a number of plant sources as well as from *Acremonium sp.* Tbp-5. These were resuspended in Tris buffer and reacted with leucinostatin A plus UDP-[^{3}H] glucose. We reasoned that the most likely plant in which glucosyl residues can be transferred to small peptides is *Centaurea maculosa* (spotted knapweed), which effectively transfers glucose to maculosin, a diketopiperazine phytotoxin [11]. Furthermore, this plant showed no symptoms when treated with leucinostatin A. The results of a controlled enzyme experiment showed that the knapweed extract does indeed have an activity that catalyzes the formation of a compound with the same R_f as the fungal glucoside (Table 2). The acetone powder of *T. baccata* also demonstrated activity, but to a lesser extent (Table 2).

Table 2

Controlled experiments showing UDP glucose : leucinostatin A glucosyl transferase activity in the acetone powders of spotted knapweed and European yew.

Treatment	Leucinostatin A β di-O-glucoside (dpm)
Knapweed enzyme + leu A + UDP-[^{3}H] glucose	2185
Knapweed enzyme + UDP-[^{3}H] glucose	300
Knapweed enzyme (boiled) + leu A + UDP-[^{3}H] glucose	75
Knapweed enzyme + leu A – UDP-[^{3}H] glucose	0
Yew enzyme + leu A + UDP-[^{3}H] glucose	444
Yew enzyme – leu A + UDP-[^{3}H] glucose	110
Yew enzyme (boiled) + leu A + UDP-[^{3}H] glucose	50
Yew enzyme + leu A – UDP-[^{3}H] glucose	0

The enzyme assays were carried out as indicated in the Materials and methods section. All data were recorded as dpm and converted to leucinostatin A β di-O-glucoside per mg protein. Leu A, leucinostatin A.

The product of this reaction was identified as leucinostatin A β di-O-glucoside on the basis that it has an identical R_f to the authentic fungal glucoside in TLC solvent systems A–D. Furthermore, upon either acid hydrolysis or enzyme hydrolysis, the product released [^{3}H] glucose and free leucinostatin A.

We then checked the possible relationship between the bioactivity of the glucoside (Table 1) and the ability of various plant enzyme preparations to convert leucinostatin A to the glucoside. All yew species had a specific enzyme activity of 0.013 picomoles leucinostatin A converted per mg protein, or greater (Table 3). The yews were relatively resistant to the effects of leucinostatin A (Table 1), as were spotted knapweed and dawn redwood, whereas all other plants tested have low transferase activity or none at all, suggesting that their sensitivity to leucinostatin A may be related to their inability to convert leucinostatin A to its glucoside (Tables 1,3). Also, as expected, glucosyl transferase activity was observed in *Acremonum sp.* Tbp-5 (Table 3). There is a direct correlation ($p < 0.05$, median statistical test) between high transferase activity and insensitivity to leucinostatin A (Tables 1,3). This was true for all *Taxus spp.*, dawn redwood and spotted knapweed.

Table 3

Relative UDP glucose:leucinostatin A glucosyl transferase activity in the acetone powder extracts of several yew species and other plants or fungi.

Plant	Relative enzyme activity (picomoles/4.5 h/mg protein)
Taxus baccata	0.013
Taxus media p.v. Hicksii	0.018
Taxus cuspidata	0.027
Centaurea maculosa	0.12
Acremonium sp. Tbp-5	0.025
Metasequoia glyptostroboides	0.038
Sequoia sempervirens	0.006
Sequoiodendron giganteum	0.006
Torreya taxifolia	0.000
Musa acuminata	0.005

Each assay was carried out as described in the Materials and methods section. When the experiments were repeated, within a week of enzyme preparation, the data shown were reproducible to ± 10% of the values shown.

As the glucosyl transferase was not purified from any of these plant preparations, we do not know whether there are several enzymes capable of transferring glucosyl residues to peptides with free –OH groups, or whether one enzyme is present that has little specificity relative to the glucosyl acceptor. For the purposes of discussion, we have therefore tentatively referred to the enzyme as UDP glucose: leucinostatin A glucosyl transferase, which follows the convention of naming such enzymes [12]. We are also aware that some plants, especially those collected from the field, such as *T. cuspidata*, *Metasequoia glyptostroboides*, *Sequoia sempervirens* and *Sequoiodendron giganteum*, may have a contribution to enzyme activity from endophytes. In the case of *T. media p.v. Hicksii* and *T. baccata* and the other greenhouse-grown plants used to assay glucosyl transferase activity, however, there was probably little or no transferase activity from any endophytic organism because few if any organisms could be isolated from these plants, especially *T. media p.v. Hicksii*. Furthermore, even in field-grown plants, if endophytes were present, they would generally contribute less than 0.01% of the total plant mass.

Conclusions

Although *Acremonium sp.* Tbp-5 had been tentatively identified as a *Taxus spp.* endophyte previously, the present work unequivocally shows that this organism, when inoculated into stems of *Taxus media p.v. Hicksii*, can be recovered from the inner tissues of surface-treated tissues several weeks later. The organism could conceivably enter the tree through natural openings such as the numerous stomata with their subsidiary cells on the under surface of the needles (Figure 1). The relationship of the fungus to the host when inside the plant is more difficult to establish because its occurrence there is blanketed by an overwhelming majority of host cells. Nevertheless, the fungus was commonly observed penetrating intracellular spaces on host tissues (phloem and xylem) when inoculated on water agar (Figure 3).

Yews and several other plant species are relatively resistant to the phytotoxic substance leucinostatin A made by *Acremonium sp.* Tbp-5. Although an unequivocal role for leucinostatin A in the endophyte–host relationship has not been established, we can surmise that this compound may contribute to the defense of the host plant by inhibiting, and perhaps by killing, invading pathogens, because the number of fungi affected by leucinostatin A is large [4,7]. It is also conceivable that if the host is inadvertently penetrated (in individual cells) by *Acremonium sp.* Tbp-5, or wounded by other means, the phytotoxin may be rendered relatively innocuous by the presence of UDP glucose: leucinostatin glucosyl transferase that converts it to the β di-O-glucoside (Tables 2,3). Interestingly, the glucoside of leucinostatin A demonstrates reduced bioactivity against plants, the fungus *P. ultimum* and cancer cell line BT-20. Its water solubility is increased, however, which may mean that it could be an improved candidate anticancer drug, given the impressive range of bioactivities of leucinostatin A against certain cancer cell lines such as melanomas and leukemias [4].

The relative activity of the glucosyl transferase as compared with plant tissue sensitivity to the β di-O-glucoside gives a good correlation; high enzyme activity correlates with little or no sensitivity to leucinostatin A (Tables 1,3). This is true for the yews, knapweed and dawn redwood, and might be expected because each of these plant species has relatively few known fungal pathogens [13]. Some plants that are more sensitive to fungal infections, such as banana and the Florida torreya, possess little or no enzyme activity, and are sensitive to leucinostatin A. We are the first to admit that the relationship between toxin insensitivity and high transferase activity in the plants tested may be strictly coincidental, however. There are numerous other factors that may contribute to toxin insensitivity including lack of toxin receptor sites or toxin degradation. Nevertheless, it is interesting to speculate that one or more of the bioactivities associated with the well-known resistance genes in plants may, in fact, be glycosyl transferases.

Although only the leucinostatin A β di-O-glucoside was characterized in this report, other glucosides of the other leucinostatins may also be produced but these were not studied [6]. Furthermore, no evidence could be found of a leucinostatin A monoglucoside, suggesting that a concerted reaction produces the β di-O-glycosylation of leucinostatin A.

Significance

There may be as many as 300,000 different plant species worldwide. Many of these have established a relationship

with one or more endophytic fungi. Although some of these endophytic organisms have been described, the exact biochemical/genetic relationship between any tree endophyte and its host is relatively unknown. This report describes a yew tree endophyte and shows how one of its phytotoxins, leucinostatin A, is converted by the host into a relatively innocuous product — leucinostatin A β-di-O-glucoside. Leucinostatin A may protect the host plant from invading pathogenic fungi, such as *Pythium ultimum*. Similar reactions may also occur in other pathogen–host relationships, resulting in disease resistance. This work sets the stage for other comparable studies to begin.

A cursory examination of the various bioactivities of the glucoside shows that it does not affect any plant tested at concentrations of 5 μg/5 μl. It does, however, show inhibitory activity towards *P. ultimum* and a cancer cell line at higher levels than leucinostatin A. The glucoside also has a higher solubility than leucinostatin A, and, coupled with its higher activity towards certain cancer cell lines than towards normal cell lines, this suggests that the development of leucinostatin A or its derivatives as anticancer agents should be pursued. Thus, our work not only shows how chemistry and biology can be used to understand endophyte–plant relationships, but also sheds new light on how studies on plant–fungal interactions may also serve pharmaceutically. Finally, to us, it seems apparent that the rate of the scientific pursuits on both temperate and tropical rain forest endophytes should be conducted at a rate faster than their destruction is allowed to proceed.

Materials and methods

Fungal isolation, identification and fermentation

The strain of *Acremonium sp.* Tbp-5 used in this study had been previously isolated from a *T. baccata* tree growing in the south of England [4]. The methods used to acquire and identify the fungus are described elsewhere [4]. The methods used to grow the fungus, and to isolate leucinostatin A are also described elsewhere [4].

Fungal inoculation and Koch's postulates

Small slits (0.5 cm) were made into surface-treated (70% ethanol) stems of *Taxus media p.v.Hicksii*. These greenhouse-grown trees were previously shown to be relatively free of endophytic fungi. A small block of agar containing *Acremonium sp.* Tbp-5 or as a control agar supporting no fungal growth at all was placed into each wound [9]. The wound was wrapped once with white laboratory tape. The fungus was re-isolated after three weeks after inoculation (greenhouse conditions). Small stem pieces were place into 70% ethanol and subsequently placed into a flame to remove excess ethanol. Whitish, living phloem/xylem tissues were aseptically excised and placed on water agar. Subsequently, any developing fungal developing were transferred to carnation leaves for growth and identification [14].

Scanning electron microscopy

Fungi and plant tissues were fixed and processed using the methods of Strobel *et al.* [15] by placing them in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2–7.4). The samples were critical-point dried, gold coated with a sputter coater, and observed and photographed with a JEOL 6100 scanning electron microscope.

Radiolabeling techniques

^{14}C leucinostatin A (88,600 dpm/μmole) was prepared by inoculating *Acremonium sp.* Tbp-5 cultures with 10–20 μCi of ^{14}C leucine as previously described [4]. UDP glucose -^{3}H (10.9 Ci/mmole) was obtained from Sigma. All radioactivity was measured by liquid scintillation counting methods and correcting to dpm by quench correction methods. Samples were dissolved in aquasol (New England Nuclear Corp.) prior to counting.

Thin layer chromatography

All TLC was performed on Merck silica gel plates (0.25 mm). The solvent systems used were A) chloroform:methanol:NH_4OH 6:2.5:0.1, B) chloroform:methanol:acetic acid 14:6:1, C) n-butanol:acetic acid:water 4:1:2 , and D) n-butanol:methanol:acetic acid 4:1:1. Most commonly, leucinostatin A and its glucoside can be observed on the plates under shortwave UV light [4]. The leucinostatin A β di-O-glucoside had R_f values of 0.21, 0.24, 0.35, and 0.45 respectively, in the above listed solvent systems.

Preparation and characterization of leucinostatin β di-O-glucoside

Three week old cultures of *Acremonium sp.* Tbp-5 were separated from the mycelium by filtration through four layers of cheesecloth. The fluid was then extracted with two equal volumes of n-butanol. After flash evaporation, the residue was taken up in a small volume of methanol and subjected to preparative TLC in solvents A, B, and C.

The final product gave a single spot in each of the TLC solvent systems A–D. Electrospray ionization mass spectroscopy yielded $(M+K)^+$ at 1581 and $(M+K+Na)^{+2}$ at 802. In the Nuclear magnetic resonance (NMR) produced the typical spectrum of leucinostatin A [4] with the addition of protons classically associated with glucosyl protons at 3.3–3.8 ppm. The anomeric protons appeared at 5.3–5.4 ppm. The glucoside, in methanol, had a single mmolar absorption band ε 217 nm of 4400.

The comparable product (leucinostatin A β di-O-glucoside) prepared from a plant enzyme reaction mixture was characterized by the constant appearance of radioactivity, by rechromatography, in solvent systems A–D. Furthermore, the β di-O-glucoside released its radioactivity after digestion with β-glucosidase with the subsequent appearance of labeling at the same R_f value as glucose in several solvent systems. We noted that without leucinostatin A in the enzyme reaction mixture, little or no labeling appeared at the R_f value of the glucoside (Table 2).

Acid hydrolysis of both the fungal glucoside and the plant enzyme generated product was performed in a trifluroacetic acid: water mixture 1:3 for 12 h at 110°C in a sealed vial. The reaction mixture was blown dry with a stream of N_2 gas. The products were separated by both TLC and paper chromatography. Glucose, as well as anhydro leucinostatin A were recovered as acid hydrolysis products.

Enzyme preparation and assays

Acetone powders of the various plant enzymes tested were obtained by grinding 20 g of freshly prepared cut plant parts in 100 ml of 10 mM Tris buffer pH 7.5 containing 1 g of polyvinylpyrrolidone, and 0.1 g of dithrothreitol. The preparation was kept on ice while grinding at top speed in a Sovall homogenizer for 30 s. The mixture was then filtered through four layers of cheesecloth. The filtrate was precipitated with 1.5 equal volumes of acetone (−20°C). The solution was passed through a single Whatman No. 1 filter paper and the precipitate that was trapped on the paper was rinsed liberally with acetone. The resulting material on the paper was blown dry with N_2 gas, collected and stored at −4°C. The preparation was reconstituted in 1.0 ml of 10 mM Tris buffer, pH 7.4, stirred well and centrifuged at 10,000 x ğ to remove debris. The standard assay contained 0.4 ml of enzyme solution (0.5–1.5 mg protein), UDP glucose-3H-(2μCi), 200 μg of leucinostatin A (dissolved in ethylene glycol monomethyl ether) and incubated at 23°C for 4–5 h. Protein was determined by the method of Lowry using bovine serum as the standard [16]. The products of the reaction

mixture were dissolved in 100 µl of methanol and chromatographed (TLC) in solvent system A.

Purified β glucosidase was obtained from Sigma and all assays used followed their recipes. Typically, 0.4 ml of acetate buffer (10 mM, pH 5.1), 0.4 mg enzyme, 0.4 mg substrate, and incubation times of 12–18 h were used. The release of glucose was followed by chromatography on Whatman No. 1 paper using ethyl acetate–pyridine–H_2O 8:2:1 for 18 h which effectively separates all of the common hexoses. The sugars were detected with an ethanol $AgNO_3$ reagent.

Spectroscopy

NMR spectra were recorded on a Bruker DRX-500 MHZ instrument with samples dissolved in deuterated methanol. Typically, samples were given 64 scans. UV measurements were made in methanol in a Beckman DU-50 spectrophotometer. Electrospray ionization mass spectroscopy was performed on samples dissolved in methanol: water: acetic acid (50:50:1). Samples were injected with a spray flow of 2 µl/min with a spray voltage of 2.2 KV via the loop injection method. Data were gathered on a home built instrument.

Acetylation of leucinostatin A

A sample (0.5 mg) was treated with a 1:1 v/v mixture of pyridine and acetic anhydride at 70°C. After 2 h, the solvents were evaporated and the products washed with methanol to remove excess acid and pyridine. The acetylated derivative was purified in solvent C and shown to be acetylated by electrospray ionization mass spectroscopy.

Bioassays

For the study of plant toxicity, leucinostatin A was first dissolved in ethanol, then the solution made to 5% ethanol by the addition of water. Small puncture wounds were made in plant tissues with a 100 µl Hamilton syringe. Subsequently, the wound was overlaid with a 5 µl droplet of test solution and the plant samples incubated in a plastic box in 100% relative humidity. Controls were the solution without the test compound.

Inhibition of fungal growth ,was measured by placing the samples to be tested in 1.0 ml of potato dextrose agar solution which was, when liquid, transferred to a microtiter plate. A small piece of agar (2 mm × 2 mm), containing *P. ultimum*, was placed at the center of the test well. A control well was placed on each plate containing no test compound. Colony diameters were measured daily for one week.

A BT-20 cancer cell line test was conducted on leucinostatin A and its glucoside according to procedures previously described [4].

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