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THE SEARCH FOR A TAXOL-PRODUCING MICROORGANISM AMONG THE ENDOPHYTIC FUNGI OF THE PACIFIC YEW, *TAXUS BREVIFOLIA*[1]

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ABSTRACT.—Endophytic microbes associated with the Pacific yew tree, *Taxus brevifolia*, were examined as potential sources of the anticancer drug taxol [**1**], a secondary metabolite of the host organism. The first promising organism found was the novel fungus, *Taxomyces andreanae*, which was isolated from the inner bark of a yew tree growing in northwestern Montana. It appears to produce taxol and other taxanes in *de novo* fashion when grown in semi-synthetic liquid media. The presence of **1** in the fungal extract was confirmed by mass spectrometry, comparative chromatographic behavior with "yew" taxol, reactivity with taxol-specific monoclonal antibodies, and 9KB cytotoxicity studies. Both acetate-1-^{14}C and phenylalanine UL-^{14}C served as precursors of taxol-^{14}C in fungal culture labeling studies, confirming the *de novo* synthesis of **1** by the fungus. Immunoassay techniques are currently being used to screen extracts of *Taxomyces andreanae* for new taxanes, and to determine if other endophytic fungi are taxol producers.

In 1971, a novel compound isolated from the bark of the northwest Pacific yew tree, *Taxus brevifolia* Nutt., was described (1). This compound, named taxol [**1**] (also known in the literature as paclitaxel) demonstrated moderate in vivo activity against the P-388, P-1534, and L-1210 murine leukemia, the Walker 256 carcinosarcoma, sarcoma 180, and Lewis lung tumor test systems (2). In the more than twenty years since the initial report of its isolation, structure elucidation, and bioactivity, taxol has garnered support as an anticancer agent, culminating in recent FDA approval of its use against breast and ovarian cancers. There are two main reasons for the attention directed toward this drug. First, it shows promise against refractory breast and ovarian cancers, which are difficult to treat and which are responsible for the deaths of 60,000 women every year (2,3). Second, it exhibits a mode of action unprecedented among cancer chemotherapeutic agents. Taxol targets microtubule formation, but in a unique fashion. Unlike known antimicrotubule agents, which block microtubule production, taxol promotes tubulin polymerization and stabilizes microtubules against depolymerization (4,5). Microtubules are an important subcellular target for chemotherapeutic agents. Antimicrotubule agents, including the vinca (*Catharanthus*) alkaloids, are extremely potent, requiring only a few molecules to disrupt the microtubular structure of cancer cells (2). The discovery of a new compound targeting these structures is of particular importance.

Despite its promise, there is a problem with taxol [**1**]. This highly functionalized diterpene is isolated primarily from the inner bark of the relatively rare and slow-growing Pacific yew tree, *Taxus brevifolia*, and a few related species, in extremely small quantities (<0.02% dry wt) (1). The emergence of **1** as an effective anticancer agent created a difficulty: how to ensure an adequate supply of a natural compound of non-microbial origin. Although the pharmaceutical potential of **1** elevated the status of the

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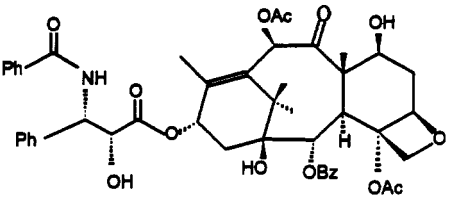

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yew from a nuisance weed to a precious commodity and natural resource, it did not alter the underlying problem—there were simply not enough trees to supply the growing demands for **1** (6). Advanced preclinical and phase I clinical development of **1** required several collections ranging in size from 5,000 to 15,000 pounds of dry bark. A mature Pacific yew (100 years old) yields approximately 10 pounds of dry bark, so each collection required the sacrifice of 500 to 1500 trees (6). As the efficacy of the compound became more apparent, the demand for additional **1** increased (6). In 1987–1988 a 60,000-pound bark collection was undertaken with little controversy. The need for a second 60,000-pound bark collection in 1989, however, sparked concern about the impact such collection sizes may have on the continued existence of the species. Although no accurate inventories of the tree have ever been undertaken, the Fish and Wildlife Service of the U.S. Department of the Interior states that "much of the range of the yew has not been subject to statistical inventories, especially the northern portion (i.e., Alaska and British Columbia). Nonetheless, based on stand information, together with satellite imagery, the U.S. Forest Service estimates that 130 million yew trees occur on 1,778,000 acres of National Forest in the Washington and Oregon Cascades, and Oregon Coast Range" (6).

Even with this estimate, however, it was clear that additional sources of **1** would be necessary. In 1991–1993, a single course of clinical treatment was 125–300 mg of **1**, and typical treatments extended for ten or more courses. Treatment of the 12,000 women who die annually of ovarian cancer alone would consume as much as 36 kg of the drug (6). Isolation methods yielded 1 kg of **1** from 25,000 pounds of dried bark, or the bark of 2,500 yews. Simply treating ovarian cancer over a one-year period would consume 90,000 mature yews (6). The recent approval of **1** for the treatment of breast cancer will triple the projected demand. Should **1** prove as effective against other refractory cancers (head and neck and non-small cell lung cancers) as clinical trials have indicated, it is not unreasonable to assume that the demand for **1** may exceed 300 kg, or 750,000 trees per year (6). This represents an enormous demand on a limited resource. There are simply not enough yews growing in North America to satisfy projected needs of this drug over the next twenty years (6).

Several research groups have addressed the supply problem in a variety of ways. Total syntheses from simple precursors were published virtually simultaneously by Robert Holton (7) and K.C. Nicolaou (8) and their co-workers. Although these syntheses are chemical masterpieces, they will probably not answer the supply question. Recently approved semi-synthetic methods have proven particularly effective, however, and will probably provide much of the pharmaceutical **1** used in the next several years (9,10). Most of these syntheses use baccatin III or 10-deacetylbaccatin III as starting materials, which can be isolated from the needles of the European yew, *Taxus baccata*. Although several strategies have been devised, the most successful methods to date have been designed by Holton (11) and by Georg and Ojima (12). Plant tissue culture also shows promise, and some research groups are reporting yields commensurate with commercialization (13).

Our own attempt at easing the supply dilemma focused on the discovery of a new biological source of the drug: an endophytic microbe colonizing the yew tree. Over the past two years we have isolated more than 300 fungi from the bark and needles of yews grown in Montana, Washington, Idaho, and Oregon. Promising producers of **1** have been studied using a variety of different techniques, including chromatography, mass spectrometry, and antibody-based immunoassays. Immunoassay is proving an effective tool not only in assessing the presence of **1** and taxanes in crude extracts, but also in providing an efficient fractionation guide.

We were painfully aware from the project inception that our chances of success were minimal. In an effort to justify the tremendous time expenditure of this venture, we broadened our research goals. Microbial extracts were evaluated not only for evidence of taxoids but also for other bioactive components. Particular attention was paid to compounds with either antifungal or anticancer potential. This paper, however, will focus primarily on the taxol investigation; auxiliary work will be published at a later date. The significance of finding a microorganism capable of producing **1** should not be understated, since such a discovery might represent a paradigm shift in the search for effective pharmaceutical agents.

From a practical viewpoint, microbial fermentation as a means of producing bioactive substances has several advantages (14): (a) Industrial production of a bioactive substance like **1** requires reproducible, dependable productivity. If a microbe is the source organism, it can be grown in tank fermentors as needed, producing a virtually inexhaustible supply of **1** (14); (b) Microorganisms typically respond favorably to routine culture techniques. Cultivation of macroorganisms (tissue culture) is considerably more challenging, requiring either specialized techniques or months of growth before harvesting is feasible (14); (c) Productivity amplification is relatively easy in microorganisms. In the case of penicillin, improved culture conditions and genetic manipulation of producing strains of *Penicillium* increased drug yield from a few μg/ml to thousands of μg/ml (15,16). With macroorganisms, larger collection sizes are the most reasonable option for improved productivity. In the case of **1**, larger collection sizes will lead to the eradication of the source organism within a few years if all of the demands for it are to be met; (d) Different bioactive compounds can be produced by altering culture conditions. The antibiotic aplasmomycins were produced by *Streptomyces griseus* SS-20 only after NaCl was added to the medium (17). Directed changes in culture conditions can be explored indefinitely as a method of optimizing various biosynthetic pathways, which may lead to even more effective derivatives of taxol (14).

What all of this means is that a **microbial source of taxol could provide an inexhaustible supply of taxol [1] and novel taxanes.**

Although the search for a taxol-producing fungus was prompted by the advantages inherent in a microbial drug source, the real motivation for this search was a discovery made forty years ago by a Japanese plant pathologist. In his study of "foolish rice seedling disease," Yabuta determined that the gibberellins, also highly functionalized diterpenes, were responsible for the disease symptoms induced by the phytopathogenic fungus *Gibberella fujikuroi* (18). It has since been established that the gibberellins are a ubiquitous phytohormone produced by higher plants. The pathways of gibberellin biosynthesis in the fungus and the higher plant are identical up to gibberellic acid-12 (19). This suggests the possibility of intergeneric-genetic exchange between higher plant and fungus. This type of exchange would probably require an intimate association between the cells of the tree and its microbial associates. Therefore, a search for a taxol-producing microorganism should (and did) commence in the tissues of *Taxus* spp., particularly in the portions of the tree from which **1** is isolated (1).

RESULTS AND DISCUSSION

Yew bark, needle, and root samples were collected in several national forests throughout Washington, Oregon, Idaho, and Montana. Samples were taken from both healthy and diseased specimens. Each sample was placed on water agar, and developing microbial colonies were transferred to mycological agar as they appeared. Microbes were established in pure culture using standard methodology. Each microbe was grown in liquid medium (100 ml) following purification. Fungi were grown in mycological broth to which 2% yew needle broth was added. Bacteria were grown in tryptic soy broth with the same amendment. Endophytic microbes often cease production of secondary metabolites when removed from the host organism. Yew needle broth may serve either as a critical precursor reservoir or as a genetic promoter for the biosynthetic mechanism of taxol production. It must be noted, however, that **yew needle broth is added only in the first fermentation.** Subsequent fermentations of promising microbes use strictly defined synthetic or semi-synthetic media, and all yew products are excluded.

All microbial extracts were subjected to first-order examination, which, in the initial phase of this study, consisted of chemical extraction, tlc, and nmr spectroscopy. As the project evolved, however, nmr was dropped from the protocol, because it is not sensitive enough to detect the low taxol titers present in fungal cultures. Monoclonal antibody immunoassay analysis with **1** and taxane-specific antibodies is now included in first-order examination. Following this initial examination, promising microbial extracts were grown in 1-liter cultures **without the addition of yew broth.** These cultures were processed as before, and then subjected to second-order examination, which consisted of a chromatographic step followed by mass spectrometry. Third-order examination involves purification of the potential taxol fraction followed by both intramural and extramural ms analysis, radioisotopic analysis, further monoclonal antibody immunoassay, and 9KB cytotoxicity determinations.

Of the 300 fungi examined, about 10% showed evidence of taxol production based on tlc and monoclonal antibody analysis. Each of these promising organisms is currently under careful scrutiny to verify these initial observations. The first fungus to progress to third-order analysis with consistent evidence of taxol production was isolated from the bark of a yew in an old-growth cedar forest in northern Montana. This previously undescribed fungus, which has been named *Taxomyces andreanae*, has repeatedly demonstrated an ability to produce **1** (20–22). Despite an extensive search, *T. andreanae* has not been found in any other yew examined to date.

The complete third-order analysis of the fungal extract of *T. andreanae* Strobel, Stierle, and Hess combined several different protocols, each of which was critical to confirmation. Fungal taxol, isolated from *T. andreanae*, had R_f values identical to those of authentic **1** in four different tlc solvent systems. Tlc was run on Merck Si gel plates (5×10 cm, 0.25 mm) in the following solvent systems: $CHCl_3$-MeCN, 7:3, $CHCl_3$-MeOH, 7:1; CH_2Cl_2-tetrahydrofuran, 6:2; and EtOAc-*i*-PrOH, 95:5. It showed identical uv characteristics and reacted positively with vanillin/H_2SO_4 spray reagent, yielding a blue spot which turned brown after 12–24 h (23). Fungal **1** had the same hplc R_t as authentic **1** on several different chromatography systems including Si gel chromatography ($CHCl_3$-CH_3CN, 7:3); analytical cyanopropyl bonded-phase hplc (hexane-*i*-PrOH, 3:1); and a Metachem taxsil column (MeOH-H_2O, 66:34). In addition, the uv spectrum of fungal **1** was superimposable on that of authentic **1**, with two maxima at 273 nm and 235 nm (1).

These initial similarities between yew **1** and fungal **1** were corroborated by spectroscopic, immunological, and biological methods. Fungal **1** was examined by several ms techniques. Eims exhibited strong fragment peaks at *m/z* 509 and 569, but

the molecular ion at *m/z* 854 was not apparent (20,21). The eims of authentic **1** exhibited a virtually identical fragmentation pattern. Esms of the fungal **1** fraction yielded primarily peaks at *m/z* 854.3 and 876.6 (20,21). These masses represent the $[M+H]^+$ of **1** and the $[M+Na]^+$ of its sodiated adduct, respectively. Authentic **1** exhibited an identical fabms to fungal **1** following sodiation. In addition, the fabms spectrum of fungal **1** yielded the $[M+H]^+$ ion of **1** (854.3) with peaks characteristic of **1** at *m/z* 509 and 569, when compared to authentic **1** (20,21,24). These data have been corroborated at least five times on different fungal preparations. Evidence was also obtained by lc-ms for the presence of baccatin III in *T. andreanae*: the parent peak at *m/z* 604 is consistent with the $[M+NH_4]^+$ ion of this compound.

Immunological confirmation of the presence of **1** and its analogues could be obtained following the development of monoclonal antibodies specific for **1** and for tetracyclic taxanes. Competitive inhibition enzyme immunoassay (CIEIA) analysis of purified fungal **1** utilizing taxol-specific mAb3C6 and taxane-specific mAb8A10 indicated that **1** isolated from the fungus was identical to **1** isolated from yew bark. CIEIA analysis was then used to verify the presence of **1** in the crude fungal extract and to facilitate bioassay-guided fractionation. Repeated analyses using these antibodies confirmed the presence of taxol in partially and totally purified preparations of fungal taxol. Extracts from twenty different fermentations of *T. andreanae* have been tested by CIEIA with consistent levels of **1** and taxanes in each test. Furthermore, a quantitative comparison between the CIEIA's using monoclonal antibodies specific to **1** with a CIEIA using monoclonal antibodies class-specific to taxanes in general revealed that **1** comprises only 15–20% of the total taxanes present in the semi-purified fungal extract (first tlc step in purification) (20,21).

Mass spectrometry, immunochemistry, and chromatographic methods confirmed that extracts of 21-day cultures of *T. andreanae* contain a compound identical to **1**. To demonstrate that the **1** found in *T. andreanae* is truly produced *de novo* by this fungus, we performed radiolabeling experiments with acetate-1-^{14}C and other radioisotopically labeled precursors. Each precursor was added to a 20-day-old *T. andreanae* culture and then incubated for 4 days at 25°. Phenylalanine-UL-^{14}C was the best precursor for ^{14}C fungal **1**, followed by acetate-1-^{14}C. ^{14}C was also found in baccatin III. Neither benzoate-7-^{14}C, nor leucine-UL-^{14}C yielded any ^{14}C-taxol, although leucine is a very effective taxol precursor in *Taxus brevifolia* (25). Confirmation of the identity of ^{14}C-taxol in the fungal preparations was done by 2D tlc co-chromatography with yew-derived **1** (20,21). The size, shape, and location of the vanillin/H_2SO_4 and uv-absorbing spot on the tlc plate was identical to the exposed single spot on the X-ray film. As a control, EtOH (70%)-killed mycelium was incubated with acetate-1-^{14}C and the culture medium processed in the identical manner (20,21).

Several control tests were performed throughout the course of these studies. These tests eliminated the possibility that the detected **1** was either a carry over from the tree or an accidental contaminant of our fungal cultures. The entire volume of a given culture medium (5 liters) inoculated with agar blocks of fungal mycelium yielded no detectable **1** at time zero. Each batch of cultures was grown with a blank culture (uninoculated, sterile medium). The blanks were autoclaved, "incubated," and processed in exactly the same manner as the inoculated cultures. The blanks were analyzed and tested with the fungal samples. In every case, the blanks had no apparent taxol titer either by immunoassay or by tlc analysis. Likewise, it was not possible to detect **1** in the agar blocks of mycelium (inoculum alone). The addition of 1 mg/liter of chlorocholine chloride (CCC) to the medium completely abolished production of **1**. This compound is also an effective inhibitor of gibberellin production in *G. fujikuroi* (18,19), although it

stimulates petasol (sesquiterpenoid) production in *Drechslera gigantea* (26). We can reasonably conclude that **1** isolated from cultures of *T. andreanae* is actually a product of the metabolism of this organism.

The amounts of taxoids produced by *T. andreanae* are disappointingly low. Estimates made by two different methods, esms and the quantitative monoclonal antibody (CIEIA) technique, indicate that 24–50 ng of **1** are produced per liter. We have observed, however, that many plant-associated fungi require one or more plant metabolites to activate pathways critical to secondary product formation (27). Plant pathogenic fungi often produce mycotoxins at higher yields if the aqueous extract of the host plant is included in the medium (27). This is also the case with the production of **1**. In early controlled experiments, the H_2O-soluble components of yew needles and shoot tips encouraged taxol production by *T. andreanae* (20). Of course, the initial purpose of this investigation was to prove that fungal **1** is produced *de novo* by *T. andreanae*, so plant extracts were not included in any growth media used to verify production of **1** by the fungus. However, such H_2O extracts may indeed augment the production of **1** and may be an important component of yield enhancement.

Using CIEIA to monitor taxol titers, we have attempted to enhance taxol production following several regimens. The complete experimental results will be presented at a later date, but we will outline briefly in this section several of our enhancement experiments. Early experiments showed an increase in taxol titer if yew needle extract were added to the medium. To verify and quantify this effect, we are carefully examining the influence of yew needle extract on taxol production. In repeated experiments, the aqueous extract of yew needles, prepared by steeping 5 g of needles in 1 liter of boiling H_2O for 5 min, added to mycological broth to make a 1% solution, resulted in a hundredfold increase in taxol production. Taxol monoclonal antibody analysis of this 1% yew medium (sterile) showed a taxol titer of between 95 and 200 ng/liter and a taxane titer of 800–1000 ng/liter. *T. andreanae* grown in this medium for 21 days consistently yielded 2,000–3,000 ng/liter taxol and about twice that titer of taxanes, with as much as 80% of the antibody-active material sequestered in the mycelia. Addition of 2% yew broth resulted in even higher titers of taxol/taxanes. The first titers measured for *T. andreanae* in unadulterated mycological broth grown in 21-day still cultures were 24–50 ng/liter taxol. Other amendments added to mycological broth have also resulted in higher taxol/taxane titers. These include different sugars added at different points in the fermentation cycle, and compounds associated with the acetate biosynthetic pathway. We have compared still cultures to shaker cultures. Rapid shaking (>200 rpm) results in poor growth and poor yield, but moderate shaking (100 rpm) resulted in a tenfold increase in mycelial mat. The filtrate of these cultures yielded similar taxol titers to control (still) cultures of *T. andreanae,* but the mycelial mat, after grinding and extracting, yielded several hundred additional ng/liter of **1**. Mycelial extracts of still cultures tended to be quite "wispy" and yield little **1**.

Several other experiments have been run, including a repetition of the original chlorocholine chloride (CCC) experiment. CCC, a known blocking agent of gibberellin biosynthesis in *Gibberella fujikuroi* (18,19) was added to *T. andreanae* in varying amounts. As the CCC content increased, taxol titer decreased, to the point of total suppression. The addition of *N*-(dimethylamino)succinic aicd (Alar), another known plant-growth regulator, increased taxol production at certain concentrations. Other plant growth regulators, particularly those used to increase gibberellin production in *G. fujikuroi*, are currently being studied for their effect on the production of **1** in *T. andreanae*. Such improvements would not be unlike the course taken for virtually all microbes that have been fermented for industrial microbiological purposes (15). Improved culturing

techniques, the addition of "activators," and the application of genetic engineering methods may ultimately permit the commercialization of *T. andreanae* for taxane production.

Several other fungi have shown consistent cross-reactivity with the monoclonal antibodies for **1** and/or taxane. These organisms are currently being subjected to second- and third-order examinations to determine if the observed antibody reactivity is actually attributable to the presence of taxoids. Tables 1 and 2 indicate typical **1** and taxane titers observed for fungi grown in semi-synthetic mycological broth (bacto-soytone and sucrose) devoid of any yew amendments (Table 1) and in mycological broth with 2% yew broth added (Table 2). Titers are determined as taxoid/liter.

Based on immunoassay, tlc and ms analysis, *T. andreanae* also apparently produces baccatin III and several other taxanes. Cephalomannine, however, has never been detected in the fungal extracts. In all yew bark extracts, however, **1** and cephalomannine are isolated together, and are difficult to separate using normal-phase-Si gel hplc. The fungus and the tree may exploit biosynthetic pathways that differ to some degree, which may account for the absence of cephalomannine. An important corollary to this premise is the potential of the fungus to produce unique taxanes. Preliminary ms analyses of compounds that cross-react with the taxane antibody suggest the presence of several known taxanes, including 10-deacetyltaxol and 10-deacetylbaccatin. Several of these compounds, while exhibiting fragmentation patterns suggestive of taxanes, have molecular ions that do not coincide with compounds published in the literature.

These research efforts are significant for both practical and philosophical reasons. First, they could have a profound effect on the supply issues concerning the important anticancer compound taxol. Taxol was originally isolated from the bark of the yew, which represents a finite drug source at best. Although it is currently being produced by semi-synthetic methods using yew needle-derived precursors, production of **1** is still dependent on the yew. A fungus or bacterium capable of producing **1** at a rate of 50 mg/liter would represent an inexhaustible source of the drug. From both an ecological and an economic viewpoint, a microbial source would supplant reliance on the yew. We would no longer be confronted with the choice of saving lives or saving yews. If any of the microbial sources isolated can provide reasonable, reliable quantities of **1**, more drug

TABLE 1. Taxol and Taxane Titers of CH_2Cl_2 Extracts of Endophytic Fungi Grown in Yew-free Media.

Fungus Code No.	Sample Wt[a] (g)	Total Wt[b] (g)	Taxol/ Sample[c] (μg)	Taxol/ Liter[d] (μg/liter)	Taxane/ Sample[c] (μg)	Taxane/ Liter[d] (μg/liter)
CC45BD	0.0045	0.0266	0.0031	0.018	0.4112	2.43
CC50NA1	0.0071	0.0137	0.173	0.669	0.279	1.077
CC53NC	0.0046	0.0254	0.0046	0.025	0.0184	0.102
CC54BE.....	0.0051	0.0655	0.0032	0.041	0.0074	0.095
CC57BC2	0.0057	0.1658	0.0064	0.186	0.0078	0.227
CC64BB	0.0046	0.0699	0.0035	0.053	0.0089	0.135
H10BA2	0.0171	0.0366	0.0135	0.058	0.276	1.189
CC50NA2	0.0046	0.0146	0.0592	0.376	0.282	1.790
CC53NA1	0.0093	0.0193	0.0327	0.135	0.241	1.003
4BA.....	0.0083	0.0198	0.0576	0.274	0.377	1.798
Media.....	0.0085	0.0085	0	0		

[a]Representative sample of CH_2Cl_2 extract analyzed by CIEIA.
[b]Total weight of CH_2Cl_2 extract.
[c]Taxoid titer of sample.
[d]Taxoid titer extrapolated to 1 liter culture.

TABLE 2. Taxol and Taxane Titers of CH_2Cl_2 Extracts of Endophytic Fungi Grown in 0.1 Liter of Media with Yew Broth Added as an Amendment.

Fungus Code No.	Sample Wt[a] (g)	Taxol/ Sample[b] (μg)	Taxol/ Liter[c] (μg/liter)	Taxane/ Sample[b] (μg)	Taxane/ Liter[c] (μg/liter)
CC45BD	0.0027	0.408	4.08	1.67	10.67
CC48BB	0.0045	0.518	5.18	5.63	56.3
CC50NA1	0.0045	0.462	4.62	5.22	52.2
CC50NA2	0.0022	0.880	8.80	8.96	89.6
CC52NC	0.0024	0.758	7.58	7.96	79.6
CC53NA	0.0012	0.622	6.22	7.27	72.7
CC53NC	0.0020	0.614	6.14	5.65	56.5
CC54BA	0.0074 (1/2)	0.304	6.08	4.20	84.0
CC54BE	0.0056 (1/2)	0.174	3.48	1.82	36.4
CC57BC2	0.0065 (1/2)	0.192	3.84	3.59	71.8
CC64BB	0.0060	0.264	2.64	1.87	37.4
blank (1 liter)	0.0057	0.95	0.95	6.61	6.6

[a]Weight of CH_2Cl_2 extract used for CIEIA. Total extract was tested except for fungi designated (1/2). Half of the extract was tested for these organisms.

[b]Taxoid titer of sample.

[c]Taxoid titer extrapolated to 1 liter culture.

would be available for both studies and treatment regimen, at a lower cost to patients, and at no cost to the environment. Taxol is not the perfect drug. Its poor H_2O solubility poses delivery problems that have not been adequately resolved. *T. andreanae* may produce related, more polar compounds with activity approaching that of **1**. These findings open the possibility for an unlimited source of **1** via fermentation technology.

Our results to date have raised some fundamental issues concerning the relationship between endophytes and their hosts. The discovery of two closely associated but taxonomically diverse organisms producing the same complex natural product is the most compelling. Although not the first discovery of this kind (the production of gibberellins by both higher plants and the fungus *Gibberella fujikuroi* predates our discovery by 40 years) this coincident production of **1** suggests that some mechanism for genetic exchange between tree and fungus might exist. If taxol production is indeed coded by a transposable element, then it might be possible to amplify this element and insert it into a fast-growing prokaryote, resulting in improved yields and shorter production time. Microbial production will also facilitate examination of the biosynthetic pathway of **1** and taxoids via feeding studies.

EXPERIMENTAL

FERMENTATION PROCEDURE.—In the initial investigation, *T. andreanae* was established in pure culture via hyphal tip transfer from water agar, on which the bark pieces had been placed, to mycological agar (Difco). The growing mycelium was then serially transferred three to six times, to fresh mycological agar. This eliminated the possibility that fungal hyphae carried a taxol or taxane "contaminant" from the source yew. *T. andreanae* grown on mycological agar was used as an inoculum source for broth cultures. The transfers were made from mycelia 3–7 days after inoculation; older mycelia did not grow as well. *T. andreanae* has been successfully transferred from 2-month-old M-1-D agar cultures, which was used as our maintenance medium. We have since determined that the growth of *T. andreanae* is mitigated by storage on agar plates. It is currently stored in mycological broth or M-1-D broth, which is also our inoculum source.

Because the conidia of *T. andreanae* do not germinate, pieces of agar block (5×5 mm) impregnated with mycelia were added to each autoclaved flask containing mycological (bactosoytone and sucrose) media. Optimum conditions for the production of **1** appear to be 21-day still culture, at 25°, with a surface-volume ratio of 1.3:1 (cm^2/ml).

Twenty-one-day cultures were filtered through cheesecloth. The residue (mycelia) was macerated and extracted thoroughly with CH_2Cl_2-MeOH (1:1). The filtrate was extracted with CH_2Cl_2. The two organic extracts were examined by tlc on Whatman Si gel plates (0.5 mm, 5×10 cm) with yew taxol as the standard, using four different solvent systems.

The plates were visualized both by uv light and by vanillin/H_2SO_4 spray reagent. Extracts with tlc spots reminiscent of **1** were prepared for second-order analysis by flash Si gel chromatography using MeCN as the solvent, followed by hplc in hexane-*i*-PrOH (3:1) (Rainin Dynamax-60A 8 μm cyano, 4.6×250 mm). The appropriate fraction was analyzed by eims and esms. Eims did not give a prominent molecular ion but it did yield the important fragment peaks at *m/z* 509 and 569 mass units; electrospray ms exhibited a prominent sodiated parent ion at *m/z* 876.6.

FUNGAL TAXOL ISOLATION PROTOCOL.—At the end of the incubation period, the culture was filtered through 8 layers of cheesecloth. The filtrate was extracted thoroughly with CH_2Cl_2. The aqueous phase was lyophilized and extracted with CH_2Cl_2-MeOH (1:1). The mycelium was macerated and thoroughly extracted with CH_2Cl_2-MeOH (1:1). The solvent was removed from the organic extracts by rotary evaporation at 30–35°. Tlc of all three organic extracts in four solvent systems indicated that the taxol-like metabolite was concentrated in the CH_2Cl_2 extract of the filtrate.

This organic extract was dissolved in 2 ml of $CHCl_3$ and placed on a 1×5 cm column of Si gel (60–200 mesh) prewashed with $CHCl_3$, and eluted with 20 ml of CH_3CN. The resulting fraction was dried and the residual oil was chromatographed on a Merck prep. tlc Si gel plate (0.5 mm Si gel) and developed in $CHCl_3$-CH_3CN (7:3). The region at the R_f of taxol-baccatin III at ca. 0.17–0.30 was removed from the plate by scraping, and extracted with CH_3CN.

The CH_3CN-soluble extract was subjected to hplc (Si gel 1.5×25 cm column) using $CHCl_3$-CH_3CN (7:3) in an isocratic mode. The peak eluting with the same R_f as **1** was collected, dried, and subjected to the final prep. tlc on a pre-washed Merck Si gel plate (0.25 mm) in EtOAc-*i*-PrOH (95:5). The area with the identical R_f to **1** was eluted with CH_3CN and dried. Various modifications of this extraction and purification method were also successful in yielding fungal **1**.

DEVELOPMENT OF MONOCLONAL ANTIBODIES SPECIFIC TO TAXOL AND ITS CONGENERS.—A hydridoma cell line derived from a mouse immunized with keyhole limpet hemocyanin-7-succinyltaxol conjugate produces a high affinity mAb to taxol and its C-7 derivatives (20). This mAb 3C6, is twentyfold less reactive with cephalomannine and is virtually unreactive with baccatin III. MAb 8A10 was derived from a mouse immunized with 7-succinylbaccatin III: it cross-reacts with taxol, cephalomannine, baccatin III, and 10-deacetylbaccatin III. It did not react, however, with an analogue lacking the C-20 oxetane ring, 20-acetoxy-4-deacetyl-5-*epi*-20,*O*-secotaxol. Thus mAb 8A10 appears to bind a determinant common to the intact tetracyclic diterpenoid ring structure common to many natural taxanes.

CIEIA ANALYSIS OF FUNGAL EXTRACTS.—The assay is performed in 10% MeOH to facilitate dissolution and processing. The CIEIA is conducted in 96-well microtiter plates coated with 100 μl of a bovine serum albumin (BSA) conjugate of the hapten, either 7-succinyltaxol for mAb 3C6 or 7-succinylbaccatin III for mAb 8A10. Additional BSA (200 μl) is then added to prevent non-specific antibody binding to the solid phase. To generate standard curves, analytical standards are serially diluted between 0.5 and 300 nM in phosphate buffered saline containing 0.25% BSA, 0.05% Tween-20 and 20% MeOH: 50 μl are combined with an equal volume of optimally diluted antibody in phosphate-buffered saline containing 0.25% BSA and 0.05% Tween-20. Dried extracts of fungal cultures are suspended in 0.2 ml MeOH, and diluted (1:4) with phosphate-buffered saline containing 0.25% BSA and 0.05% Tween-20. The suspended extract is serially diluted in phosphate-buffered saline containing 0.25% BSA, 0.05% Tween-20, and 20% MeOH, and combined with antibody as described for analytical standards. Analyte present in standard or test wells competitively inhibits antibody binding to the solid-phase BSA-hapten conjugate. The bound antibody is detected indirectly using an anti-mouse immunoglobulin enzyme-conjugate and appropriate chromogenic substrate, to generate a colorimetric assay endpoint which is inversely proportional to the analyte concentration.

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