Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*

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*Pestalotiopsis microspora* was isolated from the inner bark of a small limb of Himalayan yew, *Taxus wallachiana*, and was shown to produce taxol in mycelial culture. Taxol was identified by spectroscopic and chromatographic comparisons with authentic taxol. Optimal taxol production occurred after 2-3 weeks in still culture at 23 °C. [¹⁴C]Acetate and [¹⁴C]phenylalanine served as precursors for fungal [¹⁴C]taxol. These observations on *P. microspora* are discussed in relation to the biological importance of taxol production by fungi in general.

**Keywords**: taxol, anticancer drugs, endophytes, Himalayan yew

**INTRODUCTION**

Taxol is a highly functionalized diterpene that is found in extremely small quantities in all *Taxus* species (Wani *et al.*, 1971; Georg *et al.*, 1994). It was originally isolated and characterized from the inner bark of Pacific yew, *Taxus brevifolia* (Wani *et al.*, 1971). The US National Cancer Institute, in collaboration with Bristol Myers Squibb Co. and other workers, have demonstrated the efficacy of taxol against certain human cancers (Georg *et al.*, 1994). Taxol is now marketed as an anticancer drug. Its mode of action is unique in that it prevents the depolymerization of tubulin during the processes of cell division (Schiff *et al.*, 1979; Schiff & Horowitz, 1980).

A full course of treatment for a patient may require 2 g of taxol, administered several times over many months. In the past, this amount of taxol would require the felling of 12 or more large Pacific yews, and cost over US$100,000 for the drug alone (Hartzell, 1991). Currently, taxol is being produced by the chemical conversion of baccatin III to taxol by synthetic methods (Georg *et al.*, 1994). However, baccatin III is also obtained from *Taxus* spp., although also from the renewable portions of the tree, small stems and leaves. Although the total synthesis of taxol has been realized (Nicolaou *et al.*, 1994), this multi-step process seems cost-prohibitive. Ultimately, in order to lower the price of taxol and make it more available, a fermentation process involving a micro-organism would be the most desirable means of supply. The discovery of *Taxomyces andreanae* was the first demonstration that any organism other than *Taxus* spp. could produce taxol (Stierle *et al.*, 1994). However, the yields of taxol and taxanes have been low.

Our search for other yew-associated microbes has taken us to the foothills of the Himalayas, where the Himalayan yew, *Taxus wallachiana*, grows on steep, moist mountain slopes at altitudes of about 1500–3000 m. Areas were found that had not been logged or massively disturbed by agricultural practices with the rationale that yews growing in these areas would have a maximum advantage to form host–microbe relationships that might involve taxol biosynthesis.

This report shows that one isolate of an endophytic fungus, *Pestalotiopsis microspora*, obtained from the inner bark of *T. wallachiana*, produces taxol in culture. Furthermore, the taxol isolated from this source is biologically active against certain cancer cell lines, is spectroscopically identical to authentic taxol, and accumulates in cultures at the level of micrograms per litre. These results indicate that *P. microspora* is an excellent candidate for consideration in fermentation technology.

**METHODS**

**Endophytic fungi of T. wallachiana.** The fungus which was the subject of this report was one of many endophytic fungi isolated...
from small branches of *T. wallachiana* obtained in the foothills of the Himalayas. These organisms were isolated by treating the yew stems with 70% (v/v) ethanol, allowing the alcohol to evaporate and removing the outer bark with a sterilized sharp blade. Small pieces of the inner bark were placed on the surface of water agar in Petri plates. After several days, fungi were observed growing from the stem fragments in the plates. Individual hyphal tips of the various fungi were removed from the agar plates and placed on either potato dextrose agar (PDA) or MID agar medium (Pinkerton & Strobel, 1976), and incubated at room temperature for at least 10 d. Each fungal culture was checked for purity and transferred to another agar plate by the hyphal tip method. In the case of the *T. wallachiana* collection, we were able to acquire at least 33 different endophytic fungi. Endophytic fungi frequently do not readily produce spores on either PDA or MID agar media, or on autoclaved stems/leaves of the host plant. However, fruiting structures accompanied by spores are commonly produced on gamma-irradiated carnation leaves placed on the surface of a water agar Petri plate after 1–2 weeks of incubation at 23 °C (Nelson et al., 1983). Such too was the case of the fungus used in this study. Numerous dark fruiting structures were produced on carnation leaves, which proved useful for identification purposes.

**Fungal growth and preservation.** The organism used in this study was grown in 2 litre Erlenmeyer flasks containing 500 ml MID medium supplemented with 1 g soytone 1L (Pinkerton & Strobel, 1976). Three agar plugs, containing mycelium 0.5 x 0.5 cm, were used as inoculum. The fungus was grown at 25 °C under still conditions for various times as indicated. It could easily be held in a viable condition after storage as agar plugs in distilled H2O at 4 °C or as spores and mycelium in 15% (v/v) glycerol at -70 °C.

**Taxol isolation procedures.** After an appropriate incubation time, the entire culture (1 litre) was passed through four layers of cheesecloth. In order to reduce the amount of fatty acids that may contaminate taxol, we added 0.25 g NaCO3 to the culture fluid with shaking. The culture fluid was extracted with two equal volumes of methylene chloride and the organic phase was taken to dryness under reduced pressure at 35 °C. The residue was dissolved in 1 ml methylene chloride and placed on a 1 x 30 cm column of silica gel (Baker 40g). Elution of the column was performed in a step-wise manner starting with 70 ml 100% methylene chloride followed by mixtures of methylene chloride/ethyl acetate at 20:1, 10:1, 6:1, 3:1 and 1:1 (v/v). A compound having the same chromatographic mobility as authentic taxol was found from the 6:1 to 1:1 fractions. These fractions were combined and evaporated to dryness, and subjected to preparative thin-layer chromatography (TLC) on a Merck 1 mm (20 x 20 cm) silica gel plate developed in solvent A (chloroform/methanol, 7:1, v/v). After separation in solvent A, TLC was successively done in each of the following solvents on Merck 0.25 mm silica gel plates (20 x 20 cm): B (chloroform/acetonitrile, 7:3, v/v), and C (ethyl acetate/2-propanol, 95:5, v/v). After each chromatographic step, the area of the plate containing putative taxol was carefully removed by scraping off the silica at the appropriate Rf and exhaustively eluting it with acetonitrile. After drying under a stream of dry N2, the residue was then ready for rechromatography, or in the case of the final separation step in solvent C, for spectroscopic analyses.

**TLC.** All comparative TLC analyses were carried out on Merck 0.25 mm silica gel plates developed in solvents A, B and C indicated above, and additionally in solvents D (methylene chloride/tetrahydrofuran, 6:2, v/v) and E (methylene chloride/methanol/dimethylformamide, 90:9:1, by vol.). Taxol was detected with the 1% (w/v) vanillin/sulfuric acid reagent after gentle heating (Cardellina, 1991). It appeared as a bluish spot fading to dark grey after 24 h.

**Spectroscopic analyses and taxol quantification.** After purification, taxol was estimated by its UV absorption, dissolved in 100% methanol, at 273 nm (Wani et al., 1971). Its millimolar absorption coefficient, ε, at this wavelength is 1.7.

Nuclear magnetic resonance spectroscopy (NMR) was done on taxol preparations in a Brucker 500 MHz instrument with the sample dissolved in 100% deuterated methanol. The sample was subjected to 2048 scans with a sweep width of 6024 and 8K real points.

Mass spectroscopy was done on taxol samples using both the electrospray and fast atom bombardment (FAB) techniques. For electrospray, the sample was dissolved in methanol/water/acetic acid (50:50:1, by vol.). It was injected with a spray flow of 2 μl min⁻¹ and a spray voltage of 2.2 kV by the loop injection method. For FAB, the sample was placed in a solution of glycerol to 'magic bullet' solution 1:1 (v/v). The 'magic bullet' is a 5:3 mixture of dithiothreitol and dihioerythritol. The accelerating voltage was 5 kV. A FAB xenon atom gun was used at 8 keV adjusted to 1.0 mA. FAB spectra were acquired on a VG 70E-HF double-focusing mass spectrometer operating at a resolution of 2000.

**Scanning electron microscopy.** Spores of the fungus obtained by growing it on irradiated carnation leaves were fixed and processed using the methods of Upadhyay et al. (1991), except that they were placed in 2% (v/v) 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.

**Taxol production as a function of culture time.** The fungus was grown in MID medium as described above for various times and the mycelium was washed, dried at 80 °C, and weighed. Taxol was isolated from the fluid of each culture and was quantified spectrophotometrically. The experiment was repeated four times and the data presented as the mean value for each incubation time, with the population standard deviation.

**Taxol precursors and experiments with 14C.** Various 14C-labelled compounds were tested as potential precursors to fungal taxol. Each of these compounds was separately added to 10-d-old cultures (95±2) of the fungus, followed by incubation for 7 d at 24 °C. The cultures remained free of contaminating microbes during the experiment. The culture fluid was harvested as described above, and specific radioactivity of taxol was measured. In this manner, the ratio of the specific radioactivity of the taxol (product) to the specific radioactivity of the compound administered (precursor) could be determined. The closer this ratio is to 1.0, the more likely the compound is a precursor to taxol (Strobel et al., 1992).

All radioactivity measurements were made with compounds dissolved in 10 ml methanol and placed into 10 ml Aquasol (New England Nuclear). Counting was done on a Packard Tri Carb liquid scintillation counter and all counts corrected to d.p.m. by the quench correction method.

**Biological evaluations.** Fungal taxol was evaluated in the standard 26 cancer cell line test that is conducted routinely at Wyeth-Ayerst Research (R. Kramer, unpublished methods). Fungal taxol was also evaluated for its ability, when compared to authentic taxol, to inhibit cell division (SchiI & Horowitz, 1980). This was done by the standard method of exposing CCRF-CEM cells (5 x 10⁴ ml⁻¹) to fungal taxol for 24 h. The cells were fixed in 70% ethanol at -20 °C overnight;
rehydrated in PBS and stained with 50 µg propidium iodide ml⁻¹ in the presence of 200 kU ribonuclease for 1 h at 22 °C. Results were acquired using a Becton Dickinson FACsort flow cytometer. The instrument was calibrated with Calibrite Beads (CB) and fluorescence emission was collected through a 585/42 band pass filter. Events (10000) were acquired for each sample and a doublet discrimination program was used to eliminate data produced by cells present in doubles or larger clumps. All analyses were done using the Lysis II software (Becton Dickinson).

**Materials.** All solvents used for chromatography were HPLC grade. Solvents used for extraction were ACS grade. Radioisotopes were obtained from New England Nuclear Corp. Taxol was a generous gift of the National Cancer Institute.

**RESULTS AND DISCUSSION**

**Identity of the fungus**

The fungus used in this study was identified as *Pestalotiopsis microspora* (Speg.) Bat. & Peres. It typically possesses a brown to hyaline branched septate mycelium with conidiomata that are acervular and are brownish with a thin wall. The conidia are fusiform with four septa, with both the basal and terminal cells being hyaline and the median cells brown. The characteristic terminal cell appendages are not branched and may number three or four (Fig. 1). The conidia are typically 24–26 µm × 5.5–6.0 µm, making them, on average, somewhat larger than many of the other representatives of this species (Raj, 1993). The identity of the fungus was determined by Dr B. C. Sutton of the International Mycological Institute, Egham, UK. The fungus was deposited at Montana State University Mycological Collection, no. 1040.

**Fig. 1.** Conidiospores of *P. microspora*. (a) Light micrograph, (b) scanning electron micrograph. Bars, 10 µm. (c) An artist’s drawing.

**Fig. 2.** NMR spectra of taxol. (a) Authentic taxol (see Methods for details). (b) Fungal taxol (X denotes a contaminant peak).
Taxol from *P. microspora*

A compound having chromatographic properties comparable to taxol in solvent systems A and E, and giving a colour reaction with the vanillin/sulfuric acid reagent, was consistently isolated from *P. microspora*. It was never seen either in inoculated, extracted and processed culture media at zero time, or in culture media alone that had been extracted and examined. This eliminated the possibility that any taxol had been carried from the original tree source of the fungus via the mycelium (inoculum plugs). The compound isolated from the fungus yielded a UV absorption spectrum that was identical to authentic taxol (Wani et al., 1971).

The fungal compound produced an NMR spectrum identical to that of authentic taxol (Fig. 2). However, the signal at 1.54 p.p.m. in the fungal taxol sample is a contaminant peak primarily contributed by palmitic acid. This was ascertained by derivatizing a taxol preparation with a methylating reagent (Meth-Prep; Alltech), and subjecting the preparation to standard gas-liquid chromatography techniques. The identity of the palmitic acid was further confirmed by mass spectroscopy. This fatty acid can be removed or reduced in quantity from fungal taxol preparations by adding Na$_2$(CO$_3$) to the crude culture extract before the methylene chloride extraction step. Effectively, the Na$_2$(CO$_3$) saponifies the fatty acid and this permits its removal at the silica gel chromatography step.

Further convincing spectroscopic evidence for the identity of taxol was obtained by electrospray mass spectroscopy. Characteristically, authentic taxol yielded both an $(M+H)^+$ peak at 854 and an $(M+Na)^+$ peak at 876. By comparison, fungal taxol also yielded a peak $(M+H)^+$ at 854 and an $(M+Na)^+$ peak at 876 (Fig. 3). FAB mass spectroscopy of fungal taxol yielded $(M+H)^+$ at 854 with characteristic fragment peaks at 509 and 569 mass units. Authentic taxol produced an identical spectrum (McClure et al., 1992).

Other activities of fungal taxol

Taxol from *P. microspora* reacted positively with the specific monoclonal antibodies prepared to authentic taxol by P. Grothaus and G. Bignami at the Hawaii Biotechnology Group Inc., Aiea, Hawaii, following their earlier work on high-titre polyclonal antibodies to taxol (Grothaus et al., 1993). All of the appropriate controls, including other authentic plant taxanes, were run in this test. The results were comparable to the monoclonal antibody reaction produced with taxol from *T. andreanae* (Stierle et al., 1993).

In the 26 cancer cell line test, fungal taxol showed a pattern of activity comparable to that produced by standard authentic taxanes in these cell lines (R. Kramer, unpublished). In addition, the ability of taxol obtained from *P. microspora* to induce mitotic arrest (G2/M) in human CCRF-CEM leukaemia cells was comparable to authentic taxol (Fig. 4). Both authentic taxol and that from *P. microspora* produced the maximum mitotic arrest at a concentration of 37 ng ml$^{-1}$ (only the *P. microspora* results are shown), consistent with a tubulin-stabilizing mode of action (Schiff & Horowitz, 1980).
Taxol production by *P. microspora*

Taxol production was followed as a function of time in still culture. The taxol peaked after 2–3 weeks of incubation and rapidly declined after 5 weeks even though the mycelium continued to grow (Fig. 5). The mean maximum yield of taxol per flask was 55 ± 10 μg (Fig. 5).

Taxol accumulated in the mycelium consistently at the level of 20–30% of the amount excreted into the culture medium. This was determined by thoroughly rinsing the mycelial mat with distilled H₂O and then lyophilizing the mat, extracting it exhaustively with methylene chloride and separating and quantifying the taxol as described in Methods. The taxol acquired in this manner gave UV and mass spectra identical to those found for taxol from the culture medium. The total amount of taxol produced per litre was about 60–70 μg. This is three orders of magnitude more than is produced by *Taxomyces andreanae* and about one order of magnitude less than that found in the inner bark of *Taxus brevifolia* (on a comparative dry weight basis of fungal mycelium and bark). Commonly, taxol represents 0.01–0.02% of the weight of dry bark, and the taxol content of 1 litre of *P. microspora* culture is about 0.001% of the total dry weight of the culture contents. It is to be noted that our measure of taxol production is via the absorption coefficient of taxol. Trace amounts of compounds other than taxol may perturb the absorbance reading, giving weight estimates for taxol greater than are actually present. Nevertheless, we feel reasonably confident that our estimates are close to reality given the fact that the fungal taxol and authentic taxol gave identical UV spectra.

[14C]Taxol from cultures of *P. microspora*

Fungal cultures were individually administered either 50 or 100 μCi (1.85 or 3.70 MBq) of various 14C-labelled precursors (sodium [1-14C]acetate, [U-14C]phenylalanine, [2-14C]mevalonolactone or [U-14C]glucose) and incubated for 7 d at 23°C. The specific radioactivity of the taxol isolated from the culture broth was determined. In order to make proper comparisons, all radioactivity incorporated into taxol was calculated on the basis of 100 μCi of the precursor administered to 10 g (dry weight) of the fungus culture. The compounds best incorporated, based on the relatively high ratio of the specific activity of the taxol isolated to the specific radioactivity of the radioactive precursor, were sodium acetate and phenylalanine. The other compounds tested were relatively ineffective as precursors. Glucose, may be ineffective given the large amount of unlabelled glucose in the medium, whereas mevalonolactone may not be an effective precursor because of membrane passage difficulties.

The radiochemical purity of taxol derived from both [1-14C]acetate and [U-14C]phenylalanine was checked by two-dimensional co-chromatography (with authentic taxol) in solvents D and E. Subsequently, the plate was overlaid with a Fuji RX new medical X-ray film and exposed for three months. The single spot that appeared on the X-ray film was identical in size, shape and location to the bluish spot (taxol) that appeared on the TLC plate after spraying with vanillin/sulfuric acid (Strobel et al., 1993). This clearly indicated that the radioactivity being determined was largely in taxol and not radiochemical impurities.

We further checked the radiochemical purity of [14C]taxol derived from fungal cultures by its co-crystallization with authentic taxol. The rationale is that if the radioactivity in the fungal taxol preparation resides exclusively with taxol, then the radioactive fungal taxol will co-crystallize with authentic taxol to a constant specific radioactivity (d.p.m. mg⁻¹)(Strobel et al., 1994). We did four steps of recrystallization using 1 mg authentic taxol and radioactive fungal taxol in the presence of methanol/water (1:1, v/v) at 0°C (Strobel et al., 1994). After the first crystallization, the taxol preparation yielded a constant specific radioactivity in subsequent crystallizations, which is a further demonstration that the radioactivity in the preparation was due to taxol.

**Ecological considerations**

The discovery that fungi other than *T. andreanae* make taxol increasingly adds to the possibility that horizontal gene transfer may have occurred between *Taxus* spp. and its corresponding endophytic organisms (Steirle et al., 1993). The unequivocal demonstration that taxol is produced by *P. microspora* supports the idea that certain endophytic microbes of *Taxus* species may make and tolerate taxol in order to better compete and survive in association with these trees (Young et al., 1992). Since *Taxus* spp. grow in places that are generally damp and shaded (Hartzell, 1991), certain plant-pathogenic fungi (water moulds) also prefer this niche. It is uncommon to find root rot in *Taxus* species caused by water moulds (G. Strobel, unpublished). Taxol is toxic to the water moulds by the same mode of action as that on human cells (Young et al., 1992).
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