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Taxol and Taxane Production by *Taxomyces andreanae*, an Endophytic Fungus of Pacific Yew

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Taxomyces andreanae, a fungal endophyte, was isolated from the phloem (inner bark) of the Pacific yew, *Taxus brevifolia*. The fungus is hyphomyceteous and, when grown in a semi-synthetic liquid medium, produced taxol and related compounds. Taxol was identified by mass spectrometry, chromatography, and reactivity with monoclonal antibodies specific for taxol. Both [1-^{14}C]acetic acid and L-[U-^{14}C]phenylalanine served as precursors of [^{14}C]taxol in fungal cultures. No taxol was detected in zero-time cultures or in the small agar plugs used to inoculate the culture flasks.

Taxol, a highly derivatized diterpenoid (*1*), has shown promise as an anti-tumor agent in breast and ovarian cancers (Fig. 1) (*2*, *3*). The primary source of taxol has been the harvested and dried inner bark (phloem-cambial tissues) of *Taxus brevifolia* (Pacific yew) (*4*, *5*). Pacific yew is a slow-growing tree found in moist soils near streams and lakes in certain regions of the Pacific Northwest (*6*). Only 0.01 to 0.03% of the dry phloem weight is taxol, yet as much as 2 g of purified taxol is required for a full regimen of anti-tumor treatment (*5*). The supply issue is further complicated by the scarcity of the yew tree. Although all 11 species of *Taxus* make taxol, natural stands of these trees are often small and located in remote areas.

Here we report the production of taxol by *Taxomyces andreanae*, an endophytic fungus associated with *Taxus brevifolia* (*7*). We confirmed the presence of taxol and related taxanes in 3-week-old cultures of the fungus by mass spectrometry, immunochemistry, chromatographic methods, and radiochemical techniques.

The search for yew-associated microbes that produce taxol is justified by previous examples of plant-associated microbes producing "plant" compounds, such as gibberellins (*8*). The pathways of gibberellin biosynthesis in the fungus and the higher plant are identical up to GA_{12} (*9*). We examined microorganisms isolated from more than 25 *T. brevifolia* trees from 20 locations. Of the 200 microbes screened to date, only *T. andreanae* has demonstrated the ability to produce taxol. This fungus was isolated from the surface-disinfected (80% ethanol) inner bark of one tree in an old-growth cedar forest in northern Montana.

Taxomyces andreanae was cultured by transferring hyphal tips from water agar, on which bark pieces had been cultured, onto a modified-mycological agar (*10*). The mycelium was then successively transferred to eliminate traces of taxol or other taxanes carried over from the original tree source. Transfers of small agar plugs to broth cultures were made from mycelia that had grown 3 to 7 days. After this period, mycelia appeared to go into a quiescent state. *Taxomyces andreanae* was stored in water at 4°C and grown on a semi-defined culture medium.

The conidia of *T. andreanae* do not germinate, therefore we transferred pieces (0.5 by 0.5 cm) of agar block containing the mycelial mats to sterilized S-7 medium (*10*). Optimum conditions were as a still culture, at 25°C, with a surface-to-volume ratio of 1.3 (cm^2:ml).

After 21 days of incubation, the culture was filtered through cheesecloth. The residue was ground in a Sorvall Omnimixer and filtered again. The combined fluids were extracted with an equal volume of dichloromethane or chloroform-methanol (10:1 v/v). Solvent was removed from the organic phase by rotary evaporation at 30°C, yielding the organic extract. Thin-layer chromatography (TLC) of the organic extract demonstrated the presence of a compound that mimicked taxol in three solvent systems (*11*).

The organic extract was dissolved in 2 ml of chloroform and placed on a silica gel column. The column was rinsed with chloroform and eluted with 20 ml of acetoni-

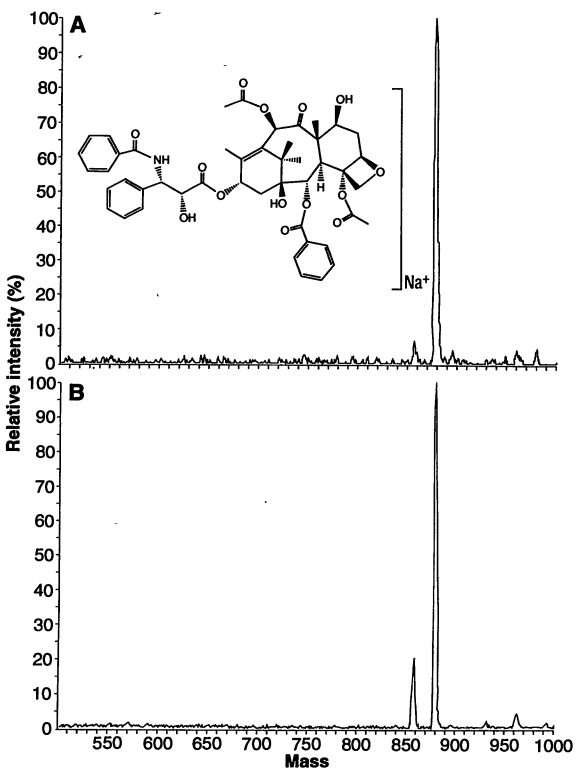

Fig. 1. Electrospray mass spectrum of fungal taxol and structure of yew taxol (**A**). The solvent was methanol–H_2O–acetic acid 50:50:1 v/v/v with a flow of 2 μl/min under a voltage of 2.2 kV. The sample was loop injected. Electrospray mass spectrum of sodiated yew taxol (**B**).

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trile. The eluant was dried, chromatographed on a preparative TLC plate (0.5-mm silica gel), and developed in chloroform-acetonitrile 7:3 v/v. The region of chromatographic retention corresponding to taxol (R_F of 0.17 to 0.30) was eluted with acetonitrile. The resulting residue was subjected to high-performance liquid chromatography (HPLC) with the use of chloroform-acetonitrile 7:3 v/v in an isocratic mode. The peak eluting with a retention time of 5.0 min was collected, dried, and analyzed by preparative TLC on a Merck silica gel plate (0.25 mm) in ethyl acetate-isopropanol 95:5 v/v. The area with the identical R_F to taxol was eluted with acetonitrile.

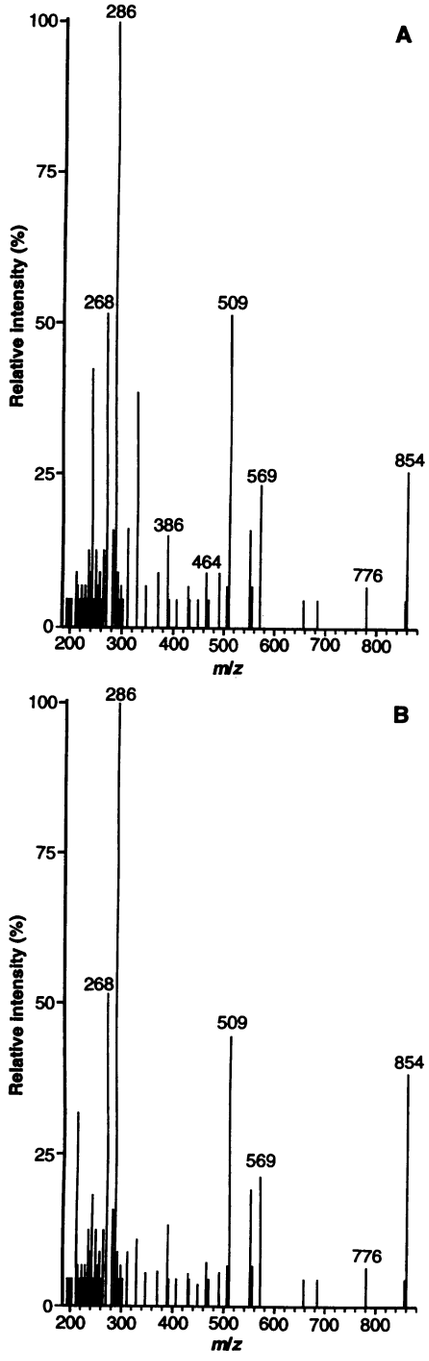

Fig. 2. Fast atom bombardment mass spectra of fungal taxol (**A**) and yew taxol (**B**). The matrix was glycerol-dithiothreitol-dithioerythritol 8:5:3 v/v/v. The accelerating voltage was 5000 V. A Xenon atom gun at 8 keV at 1 mA was used.

The electron impact mass spectrum (EIMS) of fungal taxol was virtually identical to published spectra (*12*), with fragment peaks with a mass-to-charge (*m/z*) ratio at 509 and 569, and an undetectable molecular ion. Electrospray mass spectrometry of fungal taxol yielded peaks primarily at *m/z* 854 and 876 with no evidence of other contaminating taxanes. These masses represent the $(M + H)^+$ of taxol and the $(M + Na)^+$ of its sodiated adduct, respectively (Fig. 1). Authentic taxol yielded essentially the same spectrum as fungal taxol following sodiation (Fig. 1). The fast atom bombardment mass spectrum of fungal taxol yielded the $(M + H)^+$ of taxol (854) with fragment peaks characteristic of authentic taxol at 509 and 569 mass units. (Fig. 2) (*12*). The presence of baccatin III in the fungal extract was also indicated. Liquid chromatography–mass spectrometry depicted a compound with a molecular ion at *m/z* 604, which is consistent with $(M + NH_4)^+$ of baccatin III.

Taxol and baccatin III isolated from *T. andreanae* had the same R_F values as yew taxol and baccatin III in five different TLC systems (*11*). These compounds reacted positively with the vanillin-sulfuric acid spray reagent, yielding a blue spot that turned brown after standing for 12 to 24 hours at 25°C (*13*). Fungal taxol had the same retention time as yew taxol in HPLC (silica gel, chloroform-acetonitrile 7:3 v/v; retention time was 5.0 min). The ultraviolet (UV) spectrum of fungal taxol was identical to that of yew taxol, with maxima at 273 nm and 235 nm (*1*).

Indirect competitive inhibition enzyme immunoassay (CIEIA) with the use of monoclonal antibody (mAb) 3C6, specific to taxol, confirmed the presence of taxol in crude and purified preparations of fungal extract. With the use of mAb 3C6 and mAb 8A10, which reacts with taxanes in general, we determined that taxol comprised 15 to 20% of the total taxanes present in the organic extract of *T. andreanae* (*14*).

Biological activity of fungal taxol was evaluated in the 9KB (epidermal carcinoma) cytotoxicity test (*15*). The 50% effective dose (ED_{50}) for fungal taxol was $7.8 \pm 1.5 \times 10^{-3}$ mg/ml and authentic taxol was $4.3 \pm 3.2 \times 10^{-3}$ mg/ml. These results suggest that the stereochemical configuration of fungal taxol is similar enough to yew taxol to have the same immunological and cytotoxic activities (*16*).

Radiolabeled precursors were used to demonstrate that taxol is produced by *T. andreanae* (6). Precursors were added to cultures of *T. andreanae* 20 days after inoculation. The cultures were incubated for 4 days at 25°C (Table 1). Of the precursors tested, L-[U-^{14}C]phenylalanine resulted in the most labeled taxol. The benzoyl portion of yew taxol is derived from phenylalanine (*6, 17*). L-[U-^{14}C]leucine did not label fungal taxol, although leucine is a precursor of taxol in *Taxus brevifolia* (6). The identity of [^{14}C]taxol in the fungal preparations was confirmed by two-dimensional TLC co-chromatography with yew taxol in solvents c and d (*11*) (Fig. 3). The size, shape, and location of the vanillin-sulfuric acid and UV-absorbing spot on the TLC plate was identical to the single radiolabeled spot.

Culture medium and agar, alone or inoculated with fungal mycelium, yielded no

Table 1. Incorporation of ^{14}C into taxol and baccatin III by *Taxomyces andreanae* from possible precursors. All data are normalized on the basis of 100 μCi administered per 2 g of dry weight of fungal mycelium.

Precursor	Taxol (dpm)	Baccatin III (dpm)
[1-^{14}C]Na acetate	261 ± 32	128 ± 20
[1-^{14}C]Na acetate (mycelium killed with 70% ethanol)	0	0
L-[U-^{14}C] Phenylalanine	1241 ± 40	268 ± 25
[7-^{14}C] Na benzoate	0	0
L-[U-^{14}C]Leucine	0	0

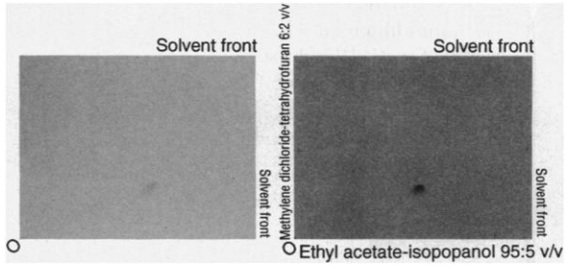

Fig. 3. Two-dimensional TLC analysis of fungal taxol cultured with 100 μCi of [1-^{14}C]Na acetate, with yew taxol as an internal standard. The plate was exposed to Kodak X-OMAT x-ray film, which was subsequently developed (left), and sprayed with vanillin-sulfuric acid (*13*) to detect taxol (right). O represents the origin.

detectable taxol at time zero. Chlorocholine chloride (at 1 mg/ml) in the medium (*10*) abolished taxol production. This compound is also an effective inhibitor of gibberellin production in G. *fujikuroi* (*9*), although it stimulates petasol (sesquiterpenoid) production in *Drechslera gigantea* (*18*). We conclude that the taxol isolated from cultures of *T. andreanae* was a product of the metabolism of this organism.

The amounts of taxol and taxanes produced by *T. andreanae* are low, as reflected by the limited incorporation of ^{14}C-labeled precursors into taxol (Table 1). Quantitation by electrospray mass spectrometry and CIEIA indicated that 24 to 50 ng of taxol were produced per liter. However, many plant-associated microbes require one or more plant metabolites to activate the synthesis of secondary natural products (*19*). Improved culturing techniques and the application of genetic engineering may improve taxol production by *T. andreanae* (*20*).

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10. S-7 medium consists of 1 g of glucose, 3 g of fructose, 6 g of sucrose, 1 g of Na^+-acetate, 1 g of soytone, 1 mg of thiamine, 1 mg of biotin, 1 mg of pyridoxal, 1 mg of Ca^{2+}-pantothenate, 3.6 of mg $MgSO_4$, 6.5 mg of $CaNO_3$, 1 mg of $Cu(NO_3)_2$, 2.5 mg of $ZnSO_4$, 5 mg of $MnCl_2$, 2 mg of $FeCl_3$, 5 mg of phenylalanine, 100 mg of Na^+-benzoate, and 1 ml of 1M KH_2PO_4 buffer (pH 6.8) per liter. Sugar ratio is identical to that occurring in the inner bark of Pacific yew. Modified mycological agar consists of 10 g of bacto-soytone, 40 g of glucose, 15 g of bacto-agar, 1 g of Na^+-acetate, and 50 mg of sodium benzoate per liter.
11. Thin-layer chromatography solvent systems, v/v: a, chloroform-acetonitrile 7:3; b, chloroform-methanol 7:1; c, ethylacetate-isopropanol 95:5; d, dichloromethane-tetrahydrofuran 6:2; and e, hexane-acetone 1:1.
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15. Cytotoxic properties of fungal taxol were tested as described [T. Mossman, *J. Immunol. Methods* **65**, 55 (1983)]. KB cells (epidermoid carcinoma) were incubated in 96-well plates at 37°C for 24 hours. Test samples of taxol were dissolved in dimethyl sulfoxide (DMSO) and diluted with medium. The samples were added to the plates in triplicate for each concentration, and incubation was continued at 37°C for 7 days. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (10 μl of MTT in 20 ml of phosphate-buffered saline) was added, and the plates incubated at 37°C for 6 hours. Medium was then removed, 100 μl of DMSO was added, and plates were shaken gently for 10 min to dissolve the formazan crystals. Absorbance at 535 nm was measured and ED_{50} was calculated as the dilution of sample with an effect one-half that of the control (no agent added).
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Frequency-Dependent Natural Selection in the Handedness of Scale-Eating Cichlid Fish

Michio Hori

Frequency-dependent natural selection has been cited as a mechanism for maintaining polymorphisms in biological populations, although the process has not been documented conclusively in field study. Here, it is demonstrated that the direction of mouth-opening (either left-handed or right-handed) in scale-eating cichlid fish of Lake Tanganyika is determined on the basis of simple genetics and that the abundance of individuals with left- or right-handedness depends on frequency-dependent natural selection. Attacking from behind, right-handed individuals snatched scales from the prey's left flank and left-handed ones from the right flank. Within a given population, the frequency of the two phenotypes oscillated around unity. This phenomenon was effected through frequency-dependent selection exerted by the prey's alertness. Thus, individuals of the rare phenotype had more success as predators than those of the more common phenotype.

Mechanisms that maintain balanced polymorphisms in nature have attracted much attention (*1*). Frequency-dependent selection has been suggested as one such mechanism (*2*), and evidence has been accumulated to support it (*3–5*). However, almost all the recent work done on this topic is concentrated around the sex ratio (*4*) and alternative mating phenotypes (*5*) in which the agents of selection are conspecifics, and little work has involved prey-predator interaction. Although some data suggest that selection by enemy or prey maintains polymorphism (*6*), this process has not been demonstrated conclusively under natural conditions. I report here a simple example of frequency-dependent selection effected through the differential guarding response of prey toward the two phenotypes of a predator.

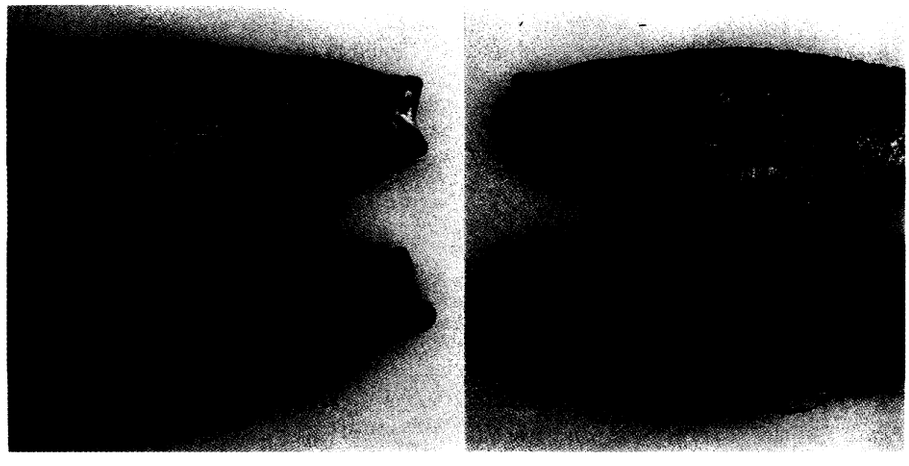

Fig. 1. The handedness of mouth opening of a Lake Tanganyikan scale-eating cichlid, *P. microlepis*. A right-handed (upper) and a left-handed (lower) individual are shown from both sides. [Photo provided by H. Yamasaki]