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ORIGINAL PAPER

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Development of a process for the production of the anticancer lead compound pleurotin by fermentation of *Hohenbuehelia atrocaerulea*

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Abstract Pleurotin is a naphthoquinone antibiotic originally isolated from *Pleurotus griseus*. Two pleurotin producing strains of *Hohenbuehelia atrocaerulea* have been identified, which, on solid substrate fermentation for 2 months yield 1–2 mg/l of the antibiotic. Described here is the lengthy developmental process which resulted in a production protocol being developed which reliably yields pleurotin from liquid fermentation at >300 mg/l. Critical to obtaining this increase in titer was inclusion in the media of an aqueous extract of alder wood.

Keywords Pleurotin · *Hohenbuehelia atrocaerulea*

Introduction

Pleurotin (Fig. 1) (2aα,4a*β*,5*β*,6*β*,8aα,12b*β*,12c*β*,12d*β*)-(−)-2a,3,4,4a,5,6,7,8a,12b,12c-Decahydro-6-methyl-2H-5,12*d*-ethanofuro[4′,3′,2′:4,10] anthra[9,1-bc]oxepin-2,9,12-trione, CAS 1404-23-5, NSC 401005, $C_{21}H_{22}O_5$, MW 354.40, a naphthoquinone antibiotic, was discovered by Robbins in 1947 as the substance produced by *Pleurotus griseus* which is toxic to gram positive bacteria [6, 7]. The same compound, but named geogenine, was subsequently reisolated from *Geopetalum geogerium*, and subsequently found from *Hohenbuehelia* spp. and its anamorph *Nematoctonus* spp. [1, 3, 5, 9].

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In the National Cancer Institute (NCI) anticancer 60 cell line testing panel pleurotin has an LC_{50} of 42 mM without selectivity toward a tissue type, thus failing to meet selection criteria [8]. Renewed interest in pleurotin as an anticancer lead compound was stimulated by the discovery that it inhibits the thioredoxin–thioreductase system [10]. Lack of a supply constrained work on pleurotin in anticancer research, so to provide this compound, the Developmental Therapeutics Program (DTP) of the NCI requested an exploration of methods through which gram quantities of pleurotin could be produced by fermentation.

Materials and methods

Strains

Hohenbeuhelia atrocaerulea (ATCC 60515) was purchased from the American Type Culture Collection. *Hohenbuehelia petalodes* (Q68C3354), *Pleurotus elongatipes* (Q68C4113), *Pleurotus rafienbunyi* (Q68C4215), and *Hohenbuehelia atrocaerulea–var. grisea* (0G0S3036) were selected from the DTP Natural Products Repository Collection at Frederick, MD. All fungal strains were maintained on potato dextrose agar. *Caenorhabditis elegans* (N2 wild-type strain) was purchased from Carolina Biological Supply.

Culture media

Robbins medium: (Czapek-Dox broth 35 g/l, dextrose 40 g/l, corn steep liquor 5 g/l) [6].

Potato dextrose broth (PDB)**:** (Difco Potato Dextrose Broth 24 g/l, $MgSO_4$ 1 g/l, $CaCO_3$ 1 g/l).

Malt extract medium: (dextrose 20 g/l, malt extract 2 g/l, yeast extract 2 g/l, peptone 2 g/l, KH_2PO_4 2 g/l, $MgSO_4$ $7H_2O$ 2 g/l, thiamine 0.05 g/l).

Sing medium: (dextrose 18 g/l, malt extract 3.5 g/l, KH_2PO_4 0.5 g/l, asparagine 0.5 g/l, casein hydrolysate 0.5 g/l, ammonium tartrate 0.35 g/l, $MgSO_4$ 0.25 g/l) [5].

Yeast-malt broth: (dextrose 10 g/l, Bacto Peptone 5 g/l, yeast extract 3 g/l, malt extract 3 g/l).

Soy glucose starch medium (SGSM): (soy peptone 10 g/l, glucose 40 g/l, soluble starch 10 g/l, Tween 80 1 g/l, $CaCO_3$ 0.5 g/l, adjust to pH 6.5 with HCl).

Glucose sucrose fructose medium (GSF): (glucose 10 g/l, sucrose 40 g/l, fructose 15 g/l, casamino acids 2 g/l, asparagine 2 g/l, yeast extract 1 g/l, Na_2HPO_4 0.5 g/l, $MgSO_4$ $7H_2O$ 1 g/l, $CaCO_3$ 1 g/l, plus trace elements).

Czapek-Dox medium: (Bacto Saccharose 30 g/l, $NaNO_3$, K_2HPO_4 1.0 g/l, $MgSO_4$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4$ 0.01 g/l).

Fermentation equipment

Shake flask fermentations were carried out in New Brunswick Scientific Incubator Shakers, model series 25, at 200 rpm. Stirred tank fermentations were performed using a 14 l New Brunswick Scientific BioFlow 110 benchtop fermentor with temperature control and pH and dissolved oxygen monitoring.

Extraction and purification

Whole fermentation broths were processed with an Omni–Macro Homogenizer #17505 with generator probe G55-195 (Omni International, 6530 Commerce Ct., Gainesville, VA, 22065), then extracted by partitioning twice against an equal volume of water-saturated ethyl acetate (EtOAc). After a thorough mechanical mixing, the thick emulsion required time for the layers to separate, which was sometimes accomplished by centrifugation. The EtOAc extract was concentrated by rotary evaporation and residual water removed through high vacuum drying, leaving a waxy material. For real-time analysis of whole broth, a 1.0 ml aliquot was mixed with 1.0 ml of acetonitrile (AcCN) and sonicated for 30 s, then passed through a 0.2 μm syringe filter. Analysis of EtOAc extracts and column fractions was done by dissolving 1.0 mg of high-vacuum dried extract in 1.0 ml of methanol (MeOH), then passing through a 0.2 μm syringe filter. Injection volume was typically 20 μl MeOH or AcCN/water.

HPLC analysis was performed using a Waters 600E pump, Waters 996 Photodiode Array Detector, and Waters 717plus autosampler controlled with Waters Millennium 32 software version 4.0. The column was an HP Hypersil ODS, 5 μm, 4.0×250 mm, PN 79926OD-584 eluted with AcCN/20 mM ammonium acetate (55:45) pH 4.0, isocratically at a flow rate of 1.0 ml/min. Standard curves for the quantitation of pleurotin in broth were constructed from data collected by making six serial dilution injections of authentic pleurotin while measuring UV absorbance at 248 nm. Typically, the run time was 10 min per assay. All solvents and chemicals used were analytical reagent or HPLC UV transparent grade.

Results

Since a viable culture of the original pleurotin producing organism, *P. griseus* [7], could not be located, and following a review of the literature, four fungal cultures thought to have the potential for pleurotin biosynthesis were selected from the DTP Natural Products Repository for exploration: *Hohenbuehelia petalodes* (Q68C3354), *Pleurotus elongatipes* (Q68C4113), *Pleurotus rafienbunyi* (Q68C4215), and *H. atrocaerulea–var. grisea* (0G0S3036). The first fermentations were done with PDB, stationary, and SGSM and GSF media, at the 125 ml scale shaking in Erlenmeyer flasks. Because the literature suggested that pleurotin might be unstable to light and alkaline pH, and temperature sensitive, fermentations were carried out in the dark, with temperature control and pH monitoring of broth. After 8+ weeks, an organic solvent extract was made from each whole ferment and examined by reverse phase HPLC with diode array detection and comparison of retention time with an authentic standard for the presence of pleurotin. Only from the extract of the PDB culture of *H. atrocaerulea* was a trace amount, ~1–2 mg/l, of

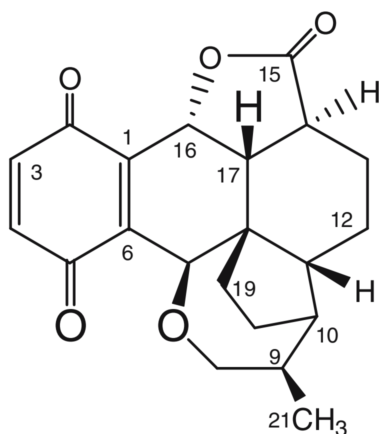

Fig. 1 Pleurotin

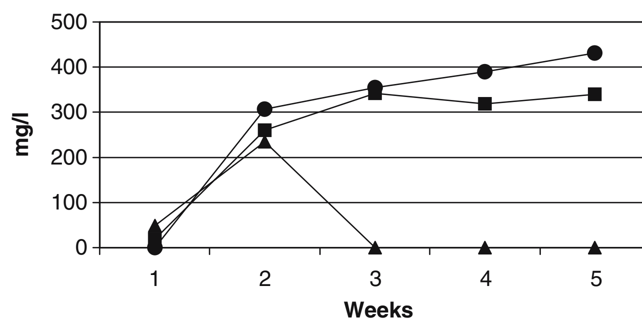

Fig. 2 Media comparison profile: (*filled circle*) Robbins, (*filled square*) PDB, (*filled triangle*) malt

pleurotin detected. This culture, originally isolated from a dead elm from Argyle State Park, Illinois, had been obtained from the Center for Forest Mycology Research, United States Department of Agriculture, 1 Gifford Pinchot Dr., Madison, WI.

A second strain of *H. atrocaerulea*, ATCC 60515, listed as having been isolated from a dead elm in Canada, was purchased from the American Type Culture Collection. Upon re-fermentations of these two *H. atrocaerulea* strains under identical conditions for 8 weeks, equally low levels of pleurotin were found to have been produced by both.

With two pleurotin-producing cultures identified, the task became development of fermentation conditions which increased the titer of the desired compound, with a secondary goal of devising a process which would allow for eventual scale-up fermentation. Since Hohenbuehelia is a wood rotting fungus, it was considered to be a reasonable experiment to include wood fiber in media development trials. Therefore, stationary fermentations for 8+ weeks in 1 l Erlenmeyer flasks were carried out using 500 ml of various liquid media plus 150 g of Silvacel,[1] a shredded wood product made of aspen and used as a filtration aid in the food processing industry, or without this added wood fiber. From flasks lacking the wood fiber, no pleurotin was detected, but when wood fiber was present during fermentation, pleurotin was found consistently at titers of 40–60 mg/l (analyses not shown). A more extensive media replacement study was conducted with Silvacel wood fiber present during stationary fermentation, resulting in no discernable improvement of pleurotin titer (data not shown). Not until fermentation was performed in shake flasks, in the dark, at 25 °C and agitated at 250 rpm with wood fiber present, was a significant improvement in pleurotin titer achieved, reaching as much as 400 mg/l when *H. atrocaerulea* ATCC 60515 was fermented on Robbins medium [6] with Silvacel wood fiber present (Fig. 2). When Silvacel was incorporated into PDB or malt extract media the titer of pleurotin was consistently lower.

These studies clearly indicated that some compound present in wood fiber, or perhaps the physical texture of the fiber itself, was required for the observed significant increase in the production of pleurotin by *H. atrocaerulea* ATCC 60515. But Silvacel had been discontinued as a commercial product, so lacking a reliable, long-term supply of a required media component, the next study to be undertaken was to identify an alternate wood fiber which supported high-level pleurotin production. Wood shavings and saw dusts of beech, birch, poplar, elm, etc. of various sizes and textures were prepared plus several commercially available wood fiber products were tried as replacements for Silvacel, all of which, included in Robbins medium at ~100 g/l liquid, were fermented with *H. atrocaerulea* ATCC 60515. This strain was selected for development because of its slightly more robust growth and slightly more consistent production of pleurotin. Representative results from analysis of an organic solvent extract made from whole broth after 4 weeks fermentation are presented in Fig. 3. Next to Silvacel, the highest pleurotin production was found when the aspen/birch wood fiber, MAT wood #116[1] was present during fermentation. From a single flask to which had been added MAT#116, distilled water, and the seed inoculum of *H. atrocaerulea* ATCC 60515, and following stationary fermentation for ~6 weeks, no pleurotin was detected.

Anticipating that there would be difficulties with incorporation of this fibrous MAT #116 into a fermentation method in stirred tanks, and wishing to avoid those problems, studies were initiated to determine whether an extract of wood fiber, when incorporated into the fermentation media, would support production of pleurotin. MAT #116 was extracted by percolation with a variety of organic solvents (i.e., hexane, dichloromethane, MeOH) and water, the solvent removed by rotary evaporation, and the extractables included in Robbins media during fermentation. Little or no production of pleurotin was detected when the organic solvent extracts were included, but pleurotin was found when the water extractables were added to Robbins media (data not shown). Therefore, MAT #116 wood fiber was extracted by percolation variously with hot tap water, or cold water, and a second pass of cold water, with a portion of each extract frozen and lyophilized. Material from each processing regimen was incorporated into Robbins media (Table 1). The highest titer of pleurotin was found in the cold water extract, less from a second pass of cold water or from hot water extracts and much lower yields from both freeze dried water extracts. While the exact chemical or nutrient composition of this water extract is not known, this data strongly suggested some water-soluble compound present in the wood fiber which was lost due to heating, freezing, or in the freeze drying process, was important for maximum pleurotin production. Whether this compound(s) served as a nutrient or an elicitor for pleurotin production remains unknown.

H. atrocaerulea was the first nematophagous fungus described [2], and is known to produce sticky bulbous hyphae that trap and subsequently digest nematodes. Rotten wood, on which *H. atrocaerulea* typically grows, is a nutrient-poor, nitrogen-depleted substrate, and it has been speculated that the trapping and digestion of nematodes is an adaptation to obtain needed nutrients. Preliminary trials were pursued to investigate whether the presence of nematodes during *H. atrocaerulea* fermentation increased the titer of pleurotin, and/or whether pleurotin had an effect upon nematodes as an attractant or toxin. However, on solid substrate fermentation of *H. atrocaerulea*, no alteration of pleurotin

[1]In the manufacturing of both Silvacel and Mat Wood #116 there is a sterilization step. The woods used to produce the data described in Fig. 3, and additional fermentations not described in Fig. 3, came from a number of sources, including branches of trees, green sawn wood, and kiln-dried lumber

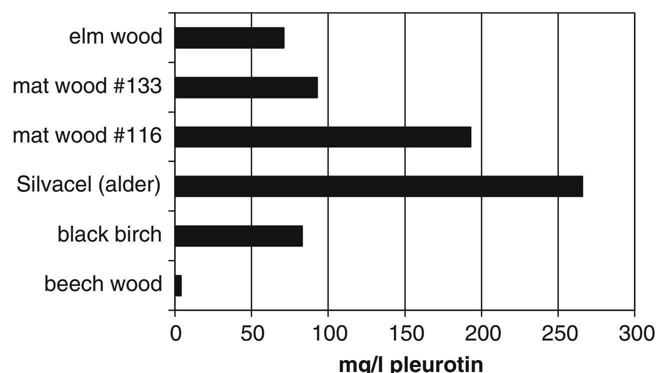

Fig. 3 Wood fiber study

Table 1 Effect of extraction temperature and lyophilization on pleurotin titer

	Room temperature water extraction (mg/l)	Hot water extraction (mg/l)
Freeze-dried	129	174
Not freeze-dried	310	258

titer was detected when the nematode *Caenorhabditis elegans* was present, nor, aside from becoming entrapped in fungal hyphae, was any effect observed. When nematodes were grown on agar in Petri dishes (with *E. coli* present as their food), and a series of paper discs onto which varying concentrations (50–200 μg) of pleurotin had been dried, were laid upon the surface of the agar, (i.e., disc diffusion assay), the nematodes were observed to move about very close to the discs without any apparent effect. By contrast, a circular 'clear zone' of dead *C. elegans* was observed if a paper disc containing Cavicide disinfectant was placed onto the agar.

Based on these findings, the following standard protocol for extraction, media preparation and fermentation was adopted: MAT Wood #116 was packed into a 10 cm diameter borosilicate glass percolator (Kontes Glass 584800-4000), covered with cold tap water and allowed to soak at room temperature for 16+ h. The percolator was drained (one pass only) and this dilute, yellow, aqueous extract used in place of tap water when Robbins fermentation medium was prepared, i.e., "modified Robbins medium". This modified Robbins medium was autoclaved as usual prior to inoculation of a seed culture of *H. atrocaerulea* ATCC 60515. Additional media component replacement studies utilizing wood fiber aqueous extract in place of water have been done in an attempt to further increase the pleurotin titer and decrease fermentation time, but Robbins medium modified as described here proved to be the medium of choice, providing the highest pleurotin yield (Fig. 4).

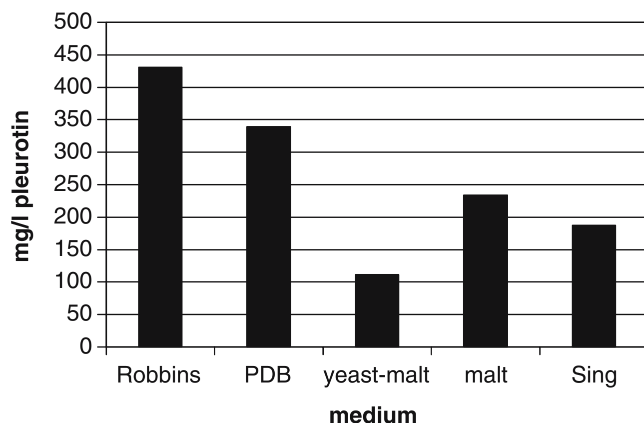

Fig. 4 Maximum pleurotin titers; 6 week media study

Production of multi-gram quantities of pleurotin was now accomplished by moderate up-scaling of fermentation conditions into multiple 1 l shake flasks by the following method: A seed stock of *H. atrocaerulea* ATCC 60615 was prepared in 500 ml baffled flasks containing 200 ml PDB, growing at room temperature in darkened shakers at 200 rpm for 2 weeks. Multiple 1 l baffled flasks containing 250 ml of modified Robbins medium, were inoculated with 50 ml of seed culture. These flasks were shaken in the dark at 215 rpm at room temperature for 6+ weeks. The broth maintained a dark brown color and thin consistency throughout fermentation. Tan pellets and pieces of cellular mat become visible at 3 weeks and appeared microscopically as entangled bundles of hyphae. An aliquot was taken weekly for titer determination. Pleurotin was not detected at 1 or 2 weeks. At 3, 4, 5, and 6 weeks pleurotin titer increases, typically reaching ~300 mg/l at 5 weeks (Fig. 5). Longer fermentation under these conditions did not result in an increase in titer of pleurotin. At termination, the pool of whole broth formed by combining multiple flasks was high shear homogenized, followed by liquid/liquid partitioning twice against equal volumes of water-saturated EtOAc to give an organic solvent extract which was rotary evaporated to dryness. When analyzed by C-18 reverse phase HPLC, this extract was found to contain ~20% by weight pleurotin. This crude extract was flash chromatographed over Davison grade 22 silica gel, 40–63 μm, by step gradient elution from 100% hexane to hexane/EtOAc (7:3). Pleurotin eluted at hexane/EtOAc (7:3). After concentration with a rotary evaporator and on standing overnight in the refrigerator, from some fractions impure pleurotin crystallized as dark yellow needles. Additional fractions containing pleurotin were identified by HPLC/diode array analysis and a pool was formed of the less pure fractions and the mother liquors from those fractions where crystallization had occurred. This pool was preparatively chromatographed over a Rainin C-18 HPLC column. From the enriched fractions, dried, then resolubilized in dichloromethane/MeOH, pleurotin crystallized as yellow needles. Multiple recrystallizations of the impure pleurotin from these same solvents gave slightly yellowish needles of pleurotin of >95% purity by HPLC/diode array analysis. Identity was established by comparison of ^{1}H and ^{13}C NMR signals with published values [4], and by MS to establish molecular weight. In excess of

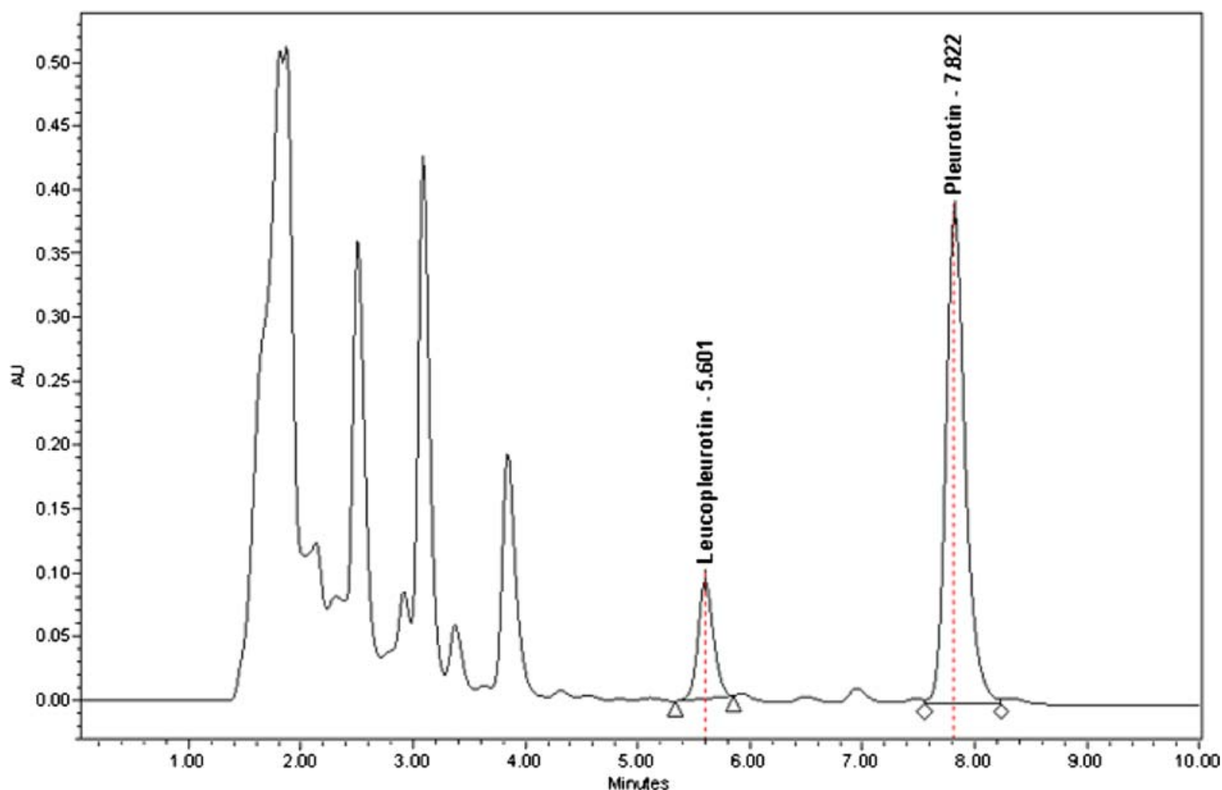

Fig. 5 C18 HPLC analysis with diode array detection, EtOAc extract of *H. atrocaerulea* from BioFlow fermentation at 5 weeks

10 g of crystalline pleurotin produced as described here was delivered to the DTP to meet the needs in developmental anticancer research.

While multiple fermentations in 1 l shake flasks for production of pleurotin was in progress, adaptation of shake flask methods to a 14 l New Brunswick BioFlow 110 benchtop fermentor were pursued. *H. atrocaerulea* ATCC 60515 seed culture prepared as above, inoculated into 12 l of modified Robbins production medium contained in the BioFlow chamber, with the tank operating at 24 °C and 325 rpm with a flat blade turbine impeller, in the dark, resulted in a detectable amount of pleurotin being produced after 2 weeks. The titer of the desired compound continued to increase, reaching ~300 mg/l in 4–5 weeks, followed by a marked decrease. A major new component which had not been seen during the analysis of shake flask fermentation extracts, and whose increase seemed to inversely correlate with pleurotin titer, was detected by HPLC/diode array analysis. Having a UV spectrum similar to pleurotin, molecular weight 2 amu greater than pleurotin, and retention time on the C-18 analytical chromatographic media 2 min less than pleurotin, it seemed probable that this component was leucopleurotin, a compound previously reported to be produced by *Nematoctonus* sp., the anamorph of *Hohenbuehelia*, which has likewise been cited as a pleurotin producer [9]. It was discovered that, if at the termination of fermentation the whole broth was allowed to sit without agitation for 48 h at ambient temperature in the BioFlow tank, this presumed leucopleurotin peak was nearly completely converted to pleurotin. High sheer homogenization of the whole broth, extraction with EtOAc and purification of pleurotin could then be carried out as already described.

Discussion

Hohenbuehelia, a genus in the family Pleurotaceae [11], is comprised of slow growing, wood rotting fungi, and contains approximately 100 species of worldwide distribution. *H. atrocaerulea* has been found in Eastern North America and Europe. Since dead wood is a nitrogen-poor substrate, it would be tempting to speculate that some biologically active, diffusible compound being produced by the fungus has a role in nematophagy, such as attracting or immobilizing the nematode prey as a way of 'capturing' nitrogenous nutrients, but our preliminary observations suggest that pleurotin is not this compound. Other nematophagous fungi include *Nematoctonus*, an imperfect basidiomycete closely related to *Hohenbuehelia* which is likewise known to produce pleurotin and homologous compounds, *Arthrobotrys, Pleurotus* and *Hyphoderma.*

It is clear that neither growth of *H. atrocaerulea*, nor production of pleurotin is dependent upon the presence of wood fiber, yet some component of wood is needed to achieve a titer significantly enhanced over baseline pro-

duction. The fascinating puzzle of what water-soluble component(s) extracted from certain wood fibers is responsible for the observed marked increase in titer of pleurotin, or how this component acts, remains to be solved by others.

Because of the slow growth of *H. atrocaerulea*, followed by extraction and analysis, each successive trial in this developmental process required 10–12 weeks, until a determination could be made whether the change in fermentation conditions had resulted in an improved titer of pleurotin. The results reported here are but a portion of a lengthy fermentation development process which took place over 5+ years, but was successfully completed with the delivery of 10 g of pleurotin to the DTP, NIH, making it possible for research on pleurotin as an anticancer lead compound to be pursued. Should those studies prove favorable, with the methods reported here, the up-scaling of fermentation of *H. atrocaerulea* to produce much greater amounts of this anticancer lead substance is now possible.

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