Development of a Process for the Production of the Anticancer Lead Compound Pleurotin by Fermentation of *Hohenbuehelia atrocaerulea*

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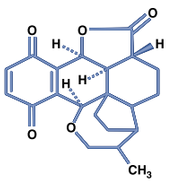
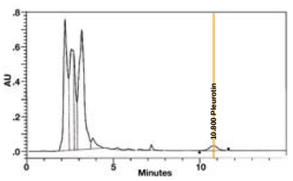
Introduction

Pleurotin,(2aα,4aβ,5β,6β,8aα,12bβ,12cβ,12dβ)-(-)-2a,3,4,4a,5,6,7,8a,12b,12c-Decahydro-6-methyl-2H-5,12d-ethanofuro[4',3',2':4,10]anthra[9,1-bc]oxepin-2,9,12-trione, CA 1404-23-5, NSC 401005, $C_{21}H_{22}O_5$, MW 354.40, a naphthoquinone antibiotic, was discovered by Robbins as the substance produced by the fungus *Pleurotus griseus* which is toxic to gram positive bacteria (1, 2, 3). Renewed interest in pleurotin has been stimulated by the discovery that it exhibits anticancer activity through inhibition of the thioredoxin-thioreductase system (4). To provide sufficient pleurotin for anticancer research, the Developmental Therapeutics Program (DTP) of the National Cancer Institute at Frederick (NCI) requested an exploration of methods through which gram quantities of pleurotin could be produced by fermentation.

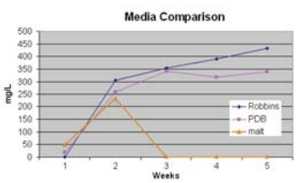


Experimental

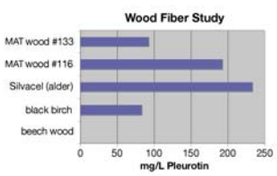
A. **Initial studies were aimed at finding a producing organism. A viable culture from the original producing organism, *P. griseus*, could not be located, so four cultures thought to have the potential of pleurotin biosynthesis were selected for examination: *Hohenbuehelia petalodes (Q68C3354C), Pleurotus elongatipes (Q68C4113C), Pleurotus rafienbunyi (Q68C4215O),* and *Hohenbuehelia atrocaerulea*–var. *grisea (0G0S3036J).* After 8+ weeks fermentation on potato dextrose broth (PDB), stationary, and Soy peptone, Glucose, Soluble starch (SGSM), and Glucose, Sucrose, Fructose (GSF), shaking in Erlenmeyer flasks, an organic solvent extract was made from each and examined by C-18 reverse phase HPLC with diode array detection for the presence of pleurotin. Only from the extract of the PDB culture of *Hohenbuehelia atrocaerulea* was a trace amount of pleurotin detected.**



B. **Having found a producing organism, the task became development of fermentation conditions which increased the titer of the desired compound. A second strain of *Hohenbuehelia atrocaerulea* was purchased, ATCC 60515, and further of media studies were carried out. This genus is a slow-growing, nematophagous, wood-rotting fungus (5, 6), so this next set of trials involved fermentation of these two strains in various media, both in the absence and in the presence of wood fiber. After 8+ weeks of stationary fermentation, extracts were made and analyzed by HPLC. Without wood fiber, no pleurotin was detected, but when wood fiber was present, pleurotin was found consistently at titers of 40–60 mg/L. In shake flasks with wood fiber present, after 8+ weeks, the titer of pleurotin reached ~400 mg/L when *H. atrocaerulea*, ATCC 60515 was fermented on modified Robbins' media.**



C. **Additional fermentations were carried out to determine whether the type of wood fiber affected pleurotin yield. Several commercial wood products were tried as well as wood shavings we made in the lab. Though fermentation in the presence of Silvacel resulted in the highest titer of pleurotin, it is a product now discontinued by the Weyerhaeuser Co., so could not be considered for long-term use. An aspen/birch wood fiber, MAT wood #116, gave the best titer among other readily available wood products.**

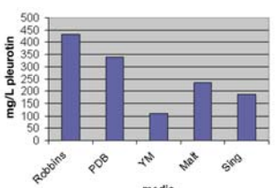


D. **The use of wood fiber in fermentations carried out in stirred tanks is problematic. Therefore, studies were initiated to determine whether an extract of wood fiber, when incorporated into the fermentation media, would support production of pleurotin. MAT #116 was extracted by percolation with organic solvents (i.e., hexane, dichloromethane, methanol) and water, the solvent removed, and the extracts included in Robbins' media during fermentation. Little or no production of pleurotin was detected after 8+ weeks fermentation when the organic solvent extracts were present, but pleurotin was detected from the water extract.**
Therefore, MAT #116 wood fiber was extracted by percolation with hot water or room temperature water, with a portion of each frozen and lyophilized, then each was incorporated into Robbins' media. Following 8+ weeks fermentation, the following results were obtained:

	Room Temperature Water Extraction	Hot Water Extraction
Freeze-dried	129 mg/L	174 mg/L
Not freeze-dried	310 mg/L	258 mg/L

E. **Based on these results, the following standard protocol was adopted: MAT #116 wood fiber was packed into a borosilicate glass percolator, covered with tap water, and allowed to soak 16+ hours. The extract is drained (one pass only) and the thin, yellow aqueous extract used in place of tap water when Robbins' fermentation media is prepared. This modified Robbins' media is autoclaved, as usual, prior to inoculation of a seed culture of *H. atrocaerulea.***

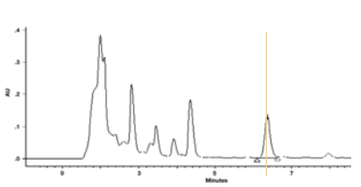
F. **Additional media substitution studies utilizing aqueous extract of MAT #116 wood fiber in place of water have been done to try to increase the pleurotin titer and decrease fermentation time, but Robbins' media is still the best choice, providing the highest yield.**



Production Mode

The Developmental Therapeutics Program requested 10 gm of pleurotin to be produced by fermentation of *H. atrocaerulea* by the following protocol:

1. **A two-week seed culture growth period for *H. atrocaerulea* ATCC 60515 in 500 ml–baffled flasks containing 200 ml potato dextrose broth at room temperature in dark shakers at 200 rpm.**
2. **Transfer 50 mls of seed culture into 1L. baffled flasks containing 250 ml of modified Robbins' medium, made with MAT #116 water extract in place of tap water. Shake in the dark at 215 rpm at room temperature. After two weeks, the titer of pleurotin is found to steadily increase, typically reaching ~300 mg/L at 5 weeks.**
3. **Whole fermentation broth is processed with a high shear homogenizer, then extracted by partitioning twice against an equal volume of water-saturated ethyl acetate. The combined EtOAc extracts are rotary evaporated to dryness, giving a waxy material which, when analyzed by C-18 reverse phase HPLC, is found to contain ~20% pleurotin. Flash chromatography over silica with hexane/ethyl acetate yields pleurotin-enriched fractions from which impure yellow crystals will grow. Further purification by reverse phase HPLC gives a colorless, crystalline product at >98% purity.**



HPLC Analysis

Analysis was performed using a Waters 600E pump, Waters 996 Photodiode Array Detector, and Waters 717plus autosampler controlled with Waters Millennium 32 software (v 4.0). The column was an HP Hypersil ODS, 5 μm, 4.0x250 mm, PN 79926OD-584 eluted with acetonitrile/20 mM ammonium acetate (55:45) pH 4.0 isocratically with a flow rate of 1.0 ml/min. For real-time analysis of whole broth, a 1.0 ml aliquot was mixed with 1.0 ml of acetonitrile and sonicated for 30 sec, then filtered through a 0.2 μm syringe filter into a vial. Analysis of ethyl acetate extracts and column fractions was done by dissolving 1.0 mg of high-vacuum dried extract in 1.0 ml of MeOH, then filtering through a 0.2 μm syringe filter into a vial. Injection volume was typically 20 μl extract dissolved in methanol or acetonitrile/water. Detection/quantitation was based on absorption at 248 nm. Typical run time was 10 min.

Conclusions

After a development period extending over six years, a method has been formulated by which pleurotin can be produced in gram quantity by fermentation of *Hohenbuehelia atrocaerulea ATCC 60515* on modified Robbins' medium. Further methods developmental work will be directed toward adaptation of this method into 12–liter stirred, instrumented, benchtop fermenters, with larger up-scaled fermentation in mind.

Acknowledgements

Sharon Wiles, Fungal Metabolites Lab, SAIC Frederick, Inc., for her excellent technical work.

References:

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Plus much useful information on nematophagus fungi on Dr. Barron's web site at:
http://www.uoguelph.ca/~gbarron/MISC2003/illustra.htm

Footnotes:

a) *Hohenbuehelia atrocaerulea* 0G0S3036J, obtained from Center for Forest Mycology Research, USDA, 1 Gifford Pinchot Dr., Madison, WI, was isolated from a dead elm from Arglye State Park, Illinois.

b) *Hohenbuehelia atrocaerulea* ATCC60515 was isolated by G. Thorn from a dead elm from Ontario.

c) SILVACEL, a shredded alder wood, was formerly produced by Weyerhaeuser Co. It was supplied gratis by McCutcheon Apple Products, 13 South Wisner St., Frederick, MD.

d) MAT #116 and #133 were supplied gratis by MAT, Inc., 12402 Highway 2, Floodwood, MN.

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