

# Development of a process for the production of the anticancer Lead compound Wortmannin by fermentation of *Penicillium duclauxii*, T.G. McCloud<sup>1</sup>, A. Burnette<sup>1</sup>, R. Collins<sup>2</sup>, S. Graf<sup>2</sup>, J. Zhu<sup>3</sup>, S. Reeb<sup>3</sup>, R. Testerman<sup>3</sup>, S.-D. Wang<sup>3</sup>, and D. J. Newman<sup>4</sup> <sup>1</sup>Chemical Synthesis and Analysis Laboratory, <sup>2</sup>Fungal Production Laboratory, <sup>3</sup>Biopharmaceutical Development Program, SAIC-Frederick Cancer Research and Development Facility, and <sup>4</sup>Developmental Therapeutics Program, National Cancer Institute, Frederick, Maryland 21702-1201, USA

**ABSTRACT:** Wortmannin was discovered as an antifungal antibiotic produced by *Penicillium wortmanii* in 1957, and subsequently in several other genera. In addition to antifungal activity, this mycotoxin has been reported to possess hemorrhagic activity in domestic animals and to inhibit myosin light chain kinase. More recently, interest in the use of wortmannin as an anti-cancer chemotherapeutic agent has grown after it was determined to be an inhibitor of phosphatidylinositol-3-kinase, an enzyme important in the intracellular signaling pathways that mediate cell growth. Because of these findings, wortmannin was selected by the Developmental Therapeutics Program (DTP), of the National Cancer Institute (NCI), for production to provide a sufficient quantity for research and development. The development of fermentation conditions for the production of wortmannin in a liquid culture of *Penicillium duclauxii* are described. An analytical HPLC method was developed which allowed rapid quantitation of the compound during fermentation. Extraction optimization and up-scale purification studies were performed which resulted in an isolated yield >1gm/liter at the 12 liter fermentation scale.

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**INITIAL STUDIES: FERMENTATION:** *Talaromyces wortmanii*, ATCC 60775, *Fusarium oxysporum*, URI-414, and *Penicillium duclauxii*, NRRL 21122, all reported to be producers of wortmannin, were grown on 250 mls of five different media: potato dextrose (PDB), soy glucose starch (SGSM), glucose sucrose fructose (GSF), cotton flour (COTT), and molasses (MOL), in shake flasks for 7 days at 25 degrees and 200 rpm. The mat plus broth was homogenized, extraction performed with ethyl acetate and the organic solvent solubles examined by HPLC.

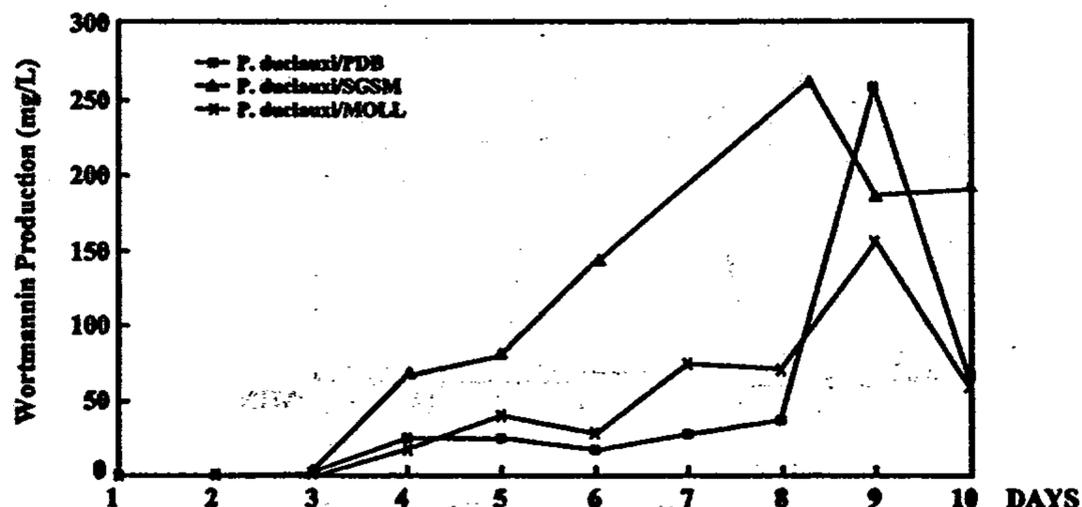
Wortmannin was detected in only three extracts: from the *Penicillium* in PDB and SGSM giving about equal amounts of the desired compound, and MOL somewhat less. PDB was selected for further development. Subsequent shake flask fermentations were performed with PDB at half, or double strength, and with different buffers, and multiple 4L shake flasks each containing 1L of optimized PDB were grown, with yields as great as 200mg/L being obtained.

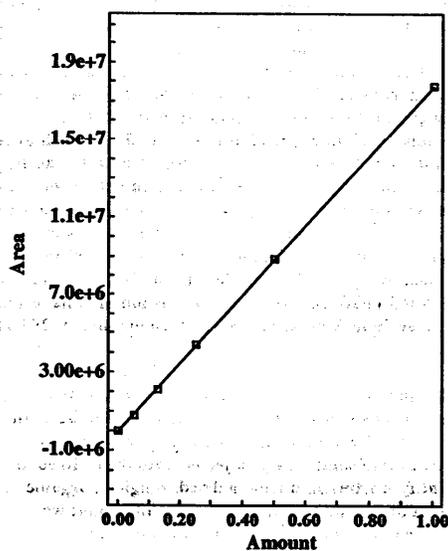
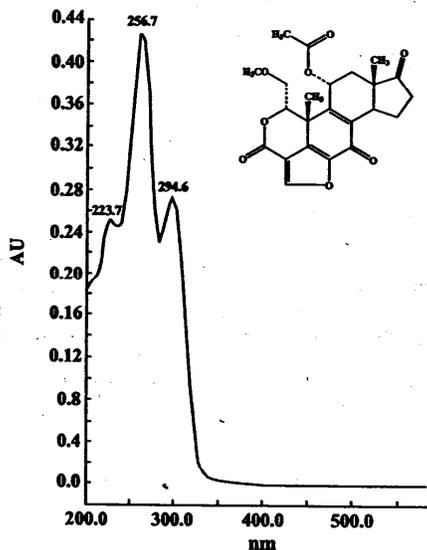
**CHROMATOGRAPHIC METHODS: HPLC METHODS:** Analytical reverse phase HPLC methods were developed which allowed rapid and accurate quantitation of wortmannin in whole broth, or in extracts.

The HPLC system consisted of a Waters 717 plus Autosampler, Waters 600E pump and System Controller, Waters 996 photodiode array detector, and data collection and analysis by Waters Millennium 2010 Chromatography Management Software. A Rainin Dynamax phase-bonded silica C18 column, 4.6 x 250mm, 8 $\mu$ m with matching guard column (cat# 83-201-C; G) was employed. Flow rate was 1.0 ml/min and the eluate was monitored at 255.8 nm. Wortmannin had a retention time of 10.5 min. with isocratic elution by 50% acetonitrile, 50% water, and 1% acetic acid. Initial standard curves were constructed with a wortmannin standard obtained from Sigma, (cat# W 1628). Following the initial purification trials, all standard curves were produced using wortmannin of a higher level of purity, purified by the NPSG, and identity confirmed by mass spec and NMR analysis. Wortmannin was found to have a linear response from 0.001 to 1.0 mg/ml with maxima at 255.8 and 296.0 nm.

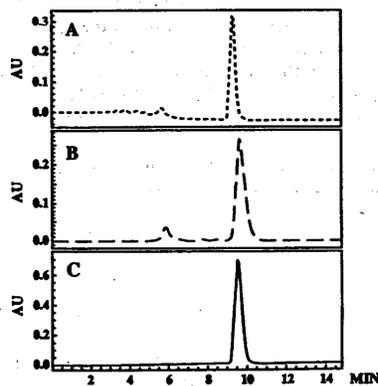
To prepare a whole broth sample for analysis, an aliquot was vigorously mixed with an equal volume of acetonitrile. Following filtration through a 0.2 $\mu$ m pore-size nylon syringe filter, 10 $\mu$ l of solution was injected onto the C-18 column. To quantify wortmannin from a dried, weighed, organic solvent extract or chromatographic fraction, an aliquot was dissolved in acetonitrile at ~1mg/ml and injected.

WORTMANNIN TIME COURSE AND MEDIA STUDY



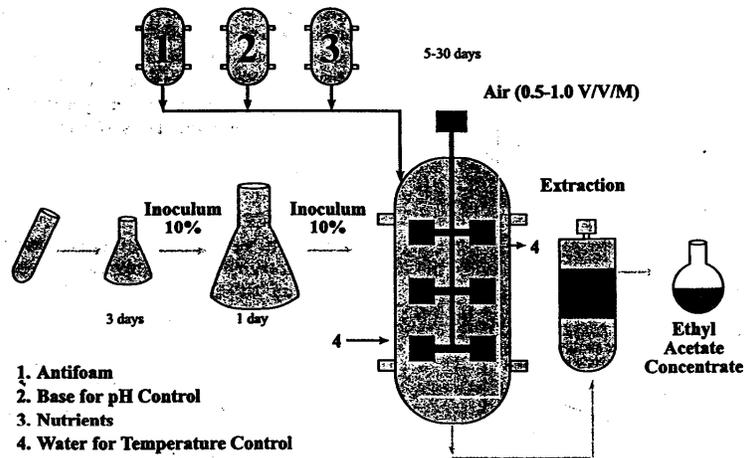


**EXTRACTION:** Despite repeated fermentations under identical conditions, it was noted that the color of the broth varied from cream colored to deep orange, and though generally exceedingly viscous at the completion of the fermentation cycle, that too was variable. Highly viscous broths were not easily filterable nor could they be centrifuged to remove the fungal mat, and were problematic to extract. Freeze drying of whole mat plus broth followed by trituration of the dry solids by several methods, or treatment of the viscous broth with glycosidases or urea, or high-shear homogenization were not successful in producing a consistency that was more amenable to solvent extraction. The amount of wortmannin extracted into several halogenated and non-halogenated organic solvents and mixtures was quantified, with the highest yields obtained simply by vigorous mechanical agitation and partitioning of the homogenized mat plus broth against ethyl acetate. For example: 3L of viscous broth including the fungal mat, and 0.5 L of ethyl acetate were placed into a 5 L explosion-proof stainless steel Waring blender can and homogenized at high speed for 5 minutes. Several batches processed in this way were added to a 20 L borosilicate glass vessel along with an equal volume of ethyl acetate, which was vigorously mechanically stirred using a pneumatic motor for at least 15 minutes, then the layers were allowed to separate. The ethyl acetate was removed and the whole broth was re-extracted 2 or 3 more times. The combined ethyl acetate extractables were rotary evaporated to dryness. HPLC analysis of the extracted broth showed a residual content of wortmannin of ~10mg/L, while the weight of ethyl acetate extractables was ~30gm/L, and by HPLC analysis contained ~35 % wortmannin. With this data and experience, and a need to produce wortmannin at a larger scale, the developmental process was transferred to a 20L fully instrumented, stirred fermentor.



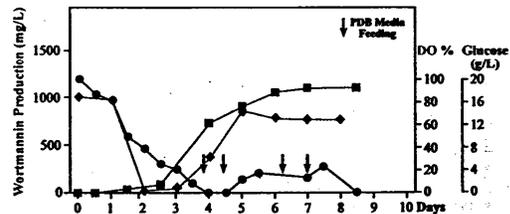
- A Ethyl acetate extract, 39% wortmannin
- B Enriched by silica flash chromatography, 53% wortmannin
- C Final product >99% wortmannin

### Wortmannin Fermentation Flow Chart

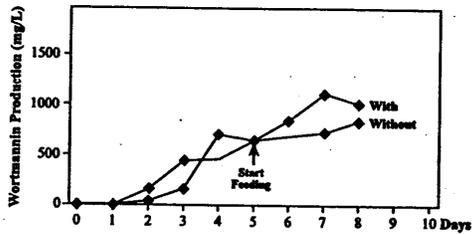


**INSTRUMENTED FERMENTATION :** Method development was continued in a 20 L, fully instrumented BioFlow IV (NBS Co. New Brunswick, New Jersey) fermentor. Experiments to determine the effect of dissolved oxygen, agitation rate, temperature, and pH were performed. Though not critical, pH control between 3 & 4 by automatic addition of base gave an improved yield of the desired product. Addition of an antifoam did not adversely affect wortmannin production or extraction and purification of the product. Maintenance of dissolved oxygen at 60% by aeration at 0.5 to 1.0 V/V/M increased wortmannin titre. Then the effect of nutrient level with time on the production of wortmannin was investigated. Glucose concentration was monitored and observed to decrease to undetectable in 4 days of fermentation, at which time the titer of wortmannin reached ~800mg/L. It was found that if glucose was supplemented after 4 days, production of wortmannin continued, reaching >1,000mg/L at 7 days. Next the effect of a 90% harvest and re-feed with media was investigated. It was found that partial harvest at 6 days and refeeding with PDB gave continued production of wortmannin, which reached a titer of ~500mg/L at 12 days total elapsed fermentation time. A second 90% volume harvest and refeed resulted in a wortmannin titer of <500mg/L at 20 days, and a third 90% harvest and refeed resulted in no further wortmannin production by 25 days. More than 80 grams of crystalline wortmannin at >99% purity have been produced by these methods.

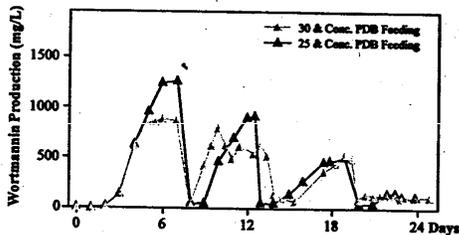
Wortmannin Fermentation Profile



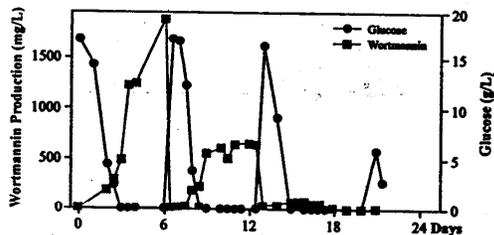
Effect of Feeding on Wortmannin Production



Effect of Temperature on Repeat Fed-Batch Production



Fermentation Profile with Repeat Fed-Batch Process



**CONCLUSIONS:** Fermentation conditions have been developed which, in a stirred, fully instrumented fermentor, result in the production of the anticancer lead compound wortmannin at a titer of >1,000 mg/L. Maintenance of glucose level at 0.5-4 gm/L was important in maximizing wortmannin production. Partial harvest and refeed with media allows wortmannin production to be extended to 20 days, significantly increasing the yield of product over single batch fermentation. A further upscaling in fermentation to produce kilogram quantities of wortmannin is achievable.

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