PNEUMOCANDINS FROM *Zalerion arboricola*

I. DISCOVERY AND ISOLATION

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HPLC bioautography of the directed biosynthesis of *Zalerion arboricola* led to the discovery of pneumocandin B_0 (L-688,786), a new antifungal and anti-*Pneumocystis carinii* lipopeptide. Isolation techniques were developed to separate this component from pneumocandin A_0 (L-671,329) in fermentations of a mutant of *Zalerion arboricola*. A number of related compounds were also isolated, which differ from pneumocandins A_0 and B_0 in the hydroxylation patterns on the ornithine, homotyrosine, and proline.

Aculeacin[1,2] and echinocandin B[3,4] are representatives of a large class of naturally-occuring antifungal lipopeptides which are characterized by potent fungicidal activity against *Candida* sp. A semi-synthetic derivative of echinocandin B, cilofungin, has been shown to protect animals with systemic fungal infections.[5] Another representative, pneumocandin A_0 (L-671,329),[6~8] is active against *Pneumocystis carinii*[6~9] an important opportunistic organism which causes pneumonia in AIDS patients. The common mechanism of action against these two pathogens is presumed to be inhibition of 1,3-β-glucan synthesis.[9] This paper describes the discovery, production, and isolation of pneumocandin B_0 and related lipopeptides, which have biological activity against both *Candida* sp. and *P. carinii*.

Materials and Methods

Identification of the Producing Strain

The strain of *Zalerion arboricola* Buczacki (ATCC 20868) was recovered from filtrates of water and sediments from a farm pond in the Valle del Rio Lozoya, near Madrid, Spain. This unusual isolate was sent to the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, where Dr. G. S. DEHOOG made the original identification. The characteristics of this strain coincide well with the original descriptions of BUCZACKI[10] and with the comparative treatment of the original strain by GOOS.[11] In the description of the organism presented in the "Results and Discussion" section, capitalized color names are from RIDGWAY.[12]

Analytical and Preparative HPLC Systems

HPLC analysis of pneumocandin B_0 was performed on a system consisting of a Spectra-Physics 8700 pump, a Spectra-Physics 8780 autoinjector, a DuPont Zorbax ODS 4.6 mm i.d. × 25 cm column, an LKB 2151 variable wavelength UV detector equipped with a 10 mm pathlength cell and a Spectra-Physics 4200 integrator. Column temperature was maintained constant at 40°C *via* jacketing and a constant temperature bath. The isocratic solvent system was CH_3CN-H_2O (45:55), the flow rate was 1.0 ml/minute, and the UV absorbance of the effluent was monitored at 210 nm. Under these conditions the retention times of

pneumocandin B_0 and pneumocandin A_0 were 13.2 and 14.5 minutes, respectively.

HPLC analysis of mixtures of pneumocandins B_0 and C_0 was performed on a system identical to the one described above except that the Spectra-Physics 4200 integrator was replaced with a Spectra FOCUS Forward Optical Scanning Detector and Data System, and the ODS column was replaced with a DuPont Zorbax 4.6 mm i.d. × 25 cm silica column. HPLC conditions consisted of an isocratic solvent system of CH_2Cl_2 - MeOH - H_2O (80 : 20 : 2), a flow rate of 1.0 ml/minute, and UV detection at 276 nm. Samples for analysis were reconstituted in mobile phase. The retention times of pneumocandin B_0 and pneumocandin C_0 were 11.45 and 14.05 minutes, respectively.

HPLC bioautography was performed on a system identical to the one described for reverse phase analytical HPLC, except for a Waters U6K manual injector (instead of the autoinjector) and the addition of a Gilson Model 203 Microfraction collector, equipped with a rack for 96-well plates.

Semi-preparative HPLC of pneumocandin C_0, pneumocandins A_1, A_3, A_4 and pneumocandins A_2 and B_2 was performed on a system consisting of two Rainin Rabbit HPX solvent delivery systems equipped with 25 ml/minute pump heads, sample injection *via* a Rainin Rabbit HPX solvent delivery system equipped with a 5 ml/minute pump head or a Waters U6K injector, a Gilson model 116 UV detector equipped with a 0.05 mm pathlength cell, a Pharmacia FRAC-300 fraction collector and a Spectra-Physics 4270 integrator. The pumps and injectors were controlled by an IBM AT computer equipped with Gilson 712 software running in a Microsoft Windows environment. Reverse phase HPLC columns were maintained at a constant temperature of 40°C *via* jacketing and a constant temperature water bath.

Another semi-preparative HPLC system, utilized for the pneumocandin B_0 base conversion product, consisted of two LDC Constametric III solvent pumps controlled by an LDC Gradient Master, a Knauer variable wavelength UV detector equipped with a 0.4 mm pathlength cell, a Valco EC6W injector valve with 5 ml injection loop, a Spectra-Physics 4200 integrator, and a Buchler LC200 linear fraction collector.

Preparative HPLC of pneumocandins A_2 and B_2 was performed on a Separations Technology LAB 800A Preparative HPLC, with an LDC/Milton Roy Gradient Master, and a Knauer Variable Wavelength UV detector equipped with a variable pathlength UV cell. An Amicon 10 cm × 50 cm column with Matrex 20 μm, 100 Å C18 packing was used for the separation.

Preparative HPLC of pneumoncandins B_0 and C_0 was performed on a large-scale HPLC system which consisted of a Peak Performer Solvent Delivery/Detection System (Dorr-Oliver, Milford, CT), equipped with an LC 150EV (Prochrom, Brownsburg, IN) axial compression column charged with 5.8 liters of Matrex irregular silica gel (20 μm particle size, 60 Å pore size, Amicon, Danvers, MA).

Antifungal Assays used to Monitor Isolation

Seeded agar plates were prepared as follows. Potato-Dextrose broth (50 ml, Difco) was inoculated with a lyophilized pellet of *Candida albicans* (MY1028), and the culture was incubated, with agitation at 220 rpm, for 24 hours at 28°C. This culture was used as a 1% inoculum to seed Potato-Dextrose agar (PDA, Difco) used for the plates. Assay samples (20 μl) were applied to 9.5 mm filter disks and air dried at room temperature before being placed on the seeded assay plates. After incubation at 28°C for 24 hours, zones of inhibition were measured from the extreme edges of clearing against the background lawn.

Fermentation of *Zalerion arboricola* (ATCC 20868) for the Production of Pneumocandins A_1, A_3, and A_4

Seed flasks were prepared containing 54 ml of KF medium[6] of the following composition: Corn steep liquor, 0.5%; tomato paste, 4%; oat flour, 1%; glucose, 1%; $FeSO_4 \cdot 7H_2O$, 10 mg/liter; $CaCl_2 \cdot 2H_2O$, 1 mg/liter, H_3BO_3, 0.56 mg/liter; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.19 mg/liter; $ZnSO_4 \cdot 7H_2O$, 2 mg/liter. Following inoculation these seed cultures were incubated from 3 to 6 days at 25°C with agitation at 220 rpm and a 5 cm throw on a rotary shaker. Sixteen ml of the resulting culture growth was used to inoculate 2-liter flasks of solid production medium[6] consisting of 120 g millet and 120 ml of nutrient solution of the following composition in distilled water: Yeast extract, 3.33%; sodium tartrate, 0.67%; ferrous sulfate, 0.07%; monosodium glutamate 0.67%; and corn oil 0.67 ml/100 ml. The flasks were autoclaved for 20 minutes, an additional 80 ml of distilled water added, and autoclaved for an additional 20 minutes. The inoculated flasks were incubated under static conditions at 25°C and 50% relative humidity for 14 days.

Isolation of Pneumocandins A_1, A_3 and A_4

Nineteen 2-liter flasks of solid fermentation medium were extracted with 1 liter of MeOH per flask. The contents of the flasks were combined, stirred, and filtered. The resulting cake was then reextracted with 6 liters of MeOH ($\times 2$) and filtered. The 3 filtrates were combined and concentrated *in vacuo* to 3 liters. This concentrate was diluted with 1 liter of H_2O and extracted with 3 liters of EtOAc ($\times 2$). The two 3-liter EtOAc extracts were combined, dried over Na_2SO_4, and concentrated under reduced pressure to 100 ml.

This 100-ml concentrate was mixed with 100 ml dry silica gel (Kieselgel 60, 70~230 mesh) and dried by removing the solvent on a rotary evaporator. The dry, coated silica gel was then applied to a 500-ml silica gel column which was slurry-packed with EtOAc and eluted with EtOAc followed by EtOAc - MeOH (9 : 1).

The EtOAc - MeOH (9 : 1) rich cut from the silica gel chromatography was concentrated to an oil, dissolved in 200 ml of CH_2Cl_2 - hexane - MeOH (10 : 10 : 1) and combined with 40 ml of Sephadex LH-20 (prepared by steeping overnight in MeOH and washing with 200 ml of CH_2Cl_2 - hexane - MeOH (10 : 10 : 1), $\times 2$). After soaking for a few minutes the supernatant was removed *via* filtration, the LH-20 washed with 200 ml of CH_2Cl_2 - hexane - MeOH (10 : 10 : 1), and filtered. These filtrates were discarded. The active constituents were recovered by extracting the LH-20 with 200 ml of MeOH ($\times 2$).

The MeOH washes were combined, concentrated to an oil, reconstituted in 5 ml of EtOAc - MeOH (75 : 25), and chromatographed on a 200-ml silica gel (Kieselgel 60, 230~400 mesh) column using EtOAc - MeOH (75 : 25), to yield 435 mg of pneumocandin A_0 and 123 mg of a mixture of biologically active components, which did not contain pneumocandin A_0. This 123-mg sample was dried onto 2 ml of silica gel and chromatographed on a 70-ml silica gel (Kieselgel 60, 230~400 mesh) column using an EtOAc - MeOH step gradient. The antifungal components eluted with EtOAc - MeOH (50 : 50) to yield 54 mg of material.

This mixture was fractionated, by semi-preparative HPLC on the Rainin/Gilson system, on a 21.2 mm i.d. $\times$ 25 cm) DuPont Zorbax ODS column using a CH_3CN - H_2O (50 : 50) isocratic solvent system and a flow rate of 20 ml/minute. A 30-ml void volume was discarded and 20-ml fractions were collected. Fractions were evaluated and recombined based on UV monitoring of the column effluent at 210 nm, analytical HPLC of the fractions, and antifungal activity of the fractions. Upon concentration, fractions 11~14 yielded 5 mg of pneumocandin A_1, fractions 18~22 yielded 5 mg of pneumocandin A_3 and fractions 26~32 yielded 4 mg of pneumocandin A_4.

Fermentation of *Zalerion arboricola* (ATCC 20958)[13] for the Production of Pneumocandins A_2 and B_2

Erlenmeyer flasks (250 ml) were prepared containing 54 ml of KF medium. The flasks were inoculated from an agar slant of *Z. arboricola* (ATCC 20958), and incubated at 25°C for four days at 220 rpm. A 20-ml sample from the 250-ml flasks was used to inoculate each of four 2-liter flasks containing 500 ml of KF medium, which were then incubated at 25°C for three days at 220 rpm. The flasks contents were then pooled for use as inoculum for a 300-liter seed fermenter containing 180 liters of KF medium and 0.2% polypropylene glycol P-2000 to reduce foaming. The seed fermenter was operated for four days at a temperature of 25°C, an air flow of 90 liters/minute, a pressure of 0.7 kg/cm^2 gauge and an agitator speed of 200 rpm.

Four 800-liter production fermenters were used, each containing 475 liters of Medium G[14] of the following composition: D-Mannitol, 4%; NZ-Amine type E, 3.3%; Fidco 8005 Yeast Extract, 1%; $(NH_4)_2SO_4$, 0.5%; KH_2PO_4, 0.9%; polypropylene glycol P-2000, 0.2%. Each production fermenter was inoculated with 25 liters of broth from the seed fermenter, and operated at a temperature of 25°C, an air flow of 250 liters/minute, a pressure of 0.7 kg/cm^2 gauge, and an agitator speed of 150 rpm. The pH was allowed to decrease from an initial value of 6.0 down to 5.5, and then maintained at 5.5 ± 0.5 using NaOH and H_2SO_4. After four days the broth was harvested for product isolation.

Isolation of Pneumocandins A_2 and B_2

Two thousand liters of MeOH was added to an equal volume of whole broth and the mixture was agitated overnight. The extract was centrifuged and the supernatant was adsorbed onto a 70-liter Sepabead SP-207 (Mitsubishi Kasei Co.) column in an upflow direction (fluidized bed). The column was washed with 570 liters of MeOH - H_2O (65 : 35) and eluted with MeOH. The rich cuts were combined to yield

230 liters, adjusted to a solvent composition of MeOH - H_2O (50 : 50) based on Karl Fischer analysis, and adsorbed onto a 100-liter Diaion HP-20 (Mitsubishi Kasei Co.) column in a downflow direction. The column was washed with 760 liters MeOH - H_2O (65 : 35) and eluted with MeOH. Rich cuts were combined to yield a 114 liters composite.

Four liters of this HP-20 rich cut was adsorbed onto a 400 ml HP-20 column, washed with 6 liters MeOH - H_2O (65 : 35), and eluted with 1.5 liters MeOH. The rich cuts were combined, concentrated *in vacuo*, and reconstituted in 25 ml of MeOH - H_2O (70 : 30). This preparation was fractionated on the Separations Technology Preparative HPLC system using a flow rate of 200 ml/minute and a gradient from MeOH - H_2O (70 : 30) to MeOH - H_2O (85 : 10) over a period of three hours. Fractions containing pneumocandins A_2 or B_2 were combined and concentrated to yield 4.9 g and 150 mg, respectively. Additional purification could be obtained *via* semi-preparative HPLC chromatography on the Rainin/Gilson system utilizing a Zorbax ODS 21.2 mm i.d. × 25 cm column and MeOH - H_2O (68 : 32) as the mobile phase.

Directed Biosynthesis

Vegetative growth of the culture was prepared by aseptically inoculating a lyophilized pellet of *Z. arboricola* (ATCC 20868) into a 250-ml 3-baffled Erlenmeyer flask containing 20 ml of seed medium. The seed medium consisted of Cerelose, 2.0%; Pharmamedia, 0.25%; 85% lactate, 0.2%; KH_2PO_4, 1.5%; and trace elements, 1.0%, in distilled water at pH 7.0. The trace element mixture contained per liter: $FeSO_4 \cdot 7H_2O$, 1 g; $MnSO_4 \cdot 4H_2O$, 1 g; $CuCl_2 \cdot 2H_2O$, 25 mg; $CaCl_2$, 100 mg; H_3BO_3, 56 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 19 mg; and $ZnSO_4 \cdot 7H_2O$, 200 mg.

Seed flasks were incubated for 5 days at 25°C, with agitation at 220 rpm and a 5 cm throw on a rotary shaker. This seed was used to inoculate (5%) screening medium containing amino acid analogs or to prepare frozen vials (2.5 ml) for subsequent seed preparations. Analog screening was done in 7.5 ml of the same medium contained in 25 × 125 mm tubes. The analog was added 5 days after inoculation to a final concentration of 5 ~ 8 mg/ml. The compounds tested for the ability to modify the pneumocandin molecule

Table 1. Compounds tested for directed biosynthesis of pneumocandin.

Proline analogs	Miscellaneous analogs
D-Proline	L-Canalinepicrate
cis-4-Hydroxy-L-proline	D,L-*p*-Fluorophenylalanine
3,4-Dehydro-L-proline	D,L-*allo*-3-Hydroxyglutamine
5-Oxoproline	*β*-Chloro-L- alanine
L-Proline	D,L-*p*-Aminophenylalanine
α-Methylproline	D,L-Fluorotyrosine
L-4-Hydroxyproline	D,L-5-Fluorotryptophan
trans-3-Methylproline	Pyridazine-3-carboxamide
N-[4,5-Dihydro-2-thiazoyl]proline	D,L-Homoserine
5-*p*-Chlorophenylproline	α-Ammonium-5-Chlorobenzoate
4-Hydroxy-L-proline	5-Hydroxy-D,L-tryptophan
L-Thiazolidine-4-carboxylate	D,L-2-Amino-4-phosphonobutyrate
L-2-Pyrrolidine-5-carboxylate	α-Phenylpyroglutamate
2-Methyl-5-pyrrolidinecarboxylate	L-Pipecolic acid
D,L-5,5-Dimethyl-4-thiazolidinecarboxylic acid	2,6-Piperdinedicarboxylate
Leucine analogs	D-Homophenylalanine
D,L-Methylallylglycine	L-Homotyrosine
D,L-*β*-Hydroxynorvaline	L-Ornithine
β-Hydroxy-D,L-leucine	L-Glutamine
Trifluorononorvaline	5-Hydroxy-L-pipecolic acid
3-Hydroxyleucine	L-Tyrosine
β-Methylnorleucine	L-Azetidine-2-carboxylic acid
α-Methylallylglycine	Cyclopent-2-en-1-yl-glycine
Threonine analogs	L-α-Ammoniumbutyrate
D-Threonine	2-Ammonium-3-methyl-4-hexynoate
O-Methylthreonine	
Ethylthreonine	
4,4-Difluorothreonine	

are tabulated in Table 1. The screening tubes were incubated for a total of 14 days with agitation (220 rpm) at 27°C.

The incubation was terminated by the extraction of the whole broth with an equal volume of methyl ethyl ketone (MEK). The MEK was removed under vacuum, and the residue was dissolved in a known volume of MeOH, dried with Na_2SO_4, and examined by HPLC bioautography.

HPLC bioautography was performed on a DuPont Zorbax ODS 4.6 mm i.d. × 25 cm column, maintained at 40°C, using a CH_3CN-H_2O (50:50) isocratic solvent system, a flow rate of 1.0 ml/minute, and UV detection of the effluent monitored at 210 nm. Under these conditions the retention time of pneumocandin A_0 was 8.5 minutes. A secondary solvent system, consisting of CH_3CN-H_2O (40:60), was utilized for bioautographs of a selected number of directed biosyntheses. The retention time of pneumocandin A_0, in this system, was 39 minutes.

The HPLC effluent was collected in 96-well microtiter dishes (Costar, 200 μl/well after a 1.5 ml void volume). Following removal of the solvent by evaporation, the wells were filled with indicator culture prepared as follows. Potato-Dextrose broth (50 ml, Difco) was inoculated with a lyophilized pellet of *Candida albicans* (MY1028) and the culture was incubated for 24 hours at 28°C, with agitation at 220 rpm. This culture was used as a 1% inoculum to seed Potato-Dextrose agar (PDA, Difco). This seeded agar (0.05 ml) was used to fill the microtiter wells. After incubation at 28°C for 24 hours, antifungal agents were detected as clear wells in the microtiter plates.

Fermentation of *Zalerion arboricola* (ATCC 20957)[13] for the Production of Pneumocandins B_0 and C_0

Two hundred fifty-ml Erlenmeyer flasks were prepared containing 54 ml of P34-2 medium of the following composition: Corn steep liquor, 0.5%; D-mannitol, 2.5%; glucose monohydrate, 1.0%; Pharmamedia, 0.9%; KH_2PO_4, 0.9%; $FeSO_4 \cdot 7H_2O$, 0.001%; $MnSO_4 \cdot H_2O$, 0.001%; $CuCl_2 \cdot 2H_2O$, 0.000025%; $CaCl_2 \cdot 2H_2O$, 0.001%; H_3BO_3, 0.000056%; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.000019%; $ZnSO_4 \cdot 7H_2O$, 0.002%. The medium was inoculated with mycelium from a frozen vial of *Z. arboricola* (ATCC 20957), and incubated at 25°C for four days at 220 rpm (New Brunswick Scientific, 5-cm throw). A 20-ml sample was used to inoculate each of four 2-liter flasks containing 500 ml of P34-2 medium per flask, which were incubated at 25°C for four days at 220 rpm (New Brunswick Scientific, 5-cm throw). The flask contents were then pooled for use as inoculum for a 300-liter seed fermenter containing 180 liters of P34-2 medium and 2 ml/liter polypropylene glycol P-2000 to reduce foaming. This seed fermenter was operated for six days at a temperature of 25°C, an air flow of 90 liters/minute, a pressure of 0.7 kg/cm^2 gauge, and an agitator speed of 200 rpm. A 25-liter sample was used to inoculate an 800-liter seed fermenter containing 475 liters of P34-2 medium and 2 ml/liter P-2000. This final seed fermenter was operated for four days at a temperature of 25°C, an air flow of 250 liters/minute, a pressure of 0.7 kg/cm^2 gauge, and an agitator speed of 150 rpm.

One 19,000-liter and five 800-liter production fermenters were used, containing medium H[14] of the following composition: D-Mannitol, 10%; NZ-Amine type E, 3.3%; Fidco 8005 yeast extract, 1%; $(NH_4)_2SO_4$, 0.5%; KH_2PO_4, 0.9%; P-2000, 2 ml/liter. The production fermenters were inoculated from the final seed fermenter, using an inoculum volume equal to 3% of the production batch volume. The fermenters were operated at a pressure of 0.7 kg/cm^2 gauge, and at various temperatures between 25°C and 28°C (no effect of temperature variations in this range was observed). The air flow rates and agitator speeds were 6,300 liters/minute and 80 rpm for the 19,000-liter fermenter, and 250 liters/minute and 150 rpm for the 800-liter fermenters. The pH was allowed to decrease from an initial value of 6.0 to 5.5, and then maintained at 5.5±0.2 using NaOH and H_2SO_4. After twelve days the broth was harvested for product isolation.

Isolation of Pneumocandin B_0

The fermentation broths from the 19,000-liter and the five 800-liter production fermenters were combined upon harvest to yield 10,750 liters of whole broth containing 5.4 kg of pneumocandin B_0. The whole broth was extracted with MeOH using a 1.5:1 whole broth-MeOH ratio. The larger suspended solids were separated *via* a high-speed solid bowl centrifugal decanter (Westfalia model CA-226) and reextracted with MeOH using a 1:2 solids-MeOH ratio. The solids were again separated *via* centrifugation and then extracted a third time with 80% aqueous methanol using a 1:2 solids-MeOH ratio. After the

third extraction, less than 5% of the pneumocandin B_0 remained in the solids which were discarded.

Each centrifugate, which still contained some fine particulates, was adjusted with water to less than 50% MeOH, measured *via* Karl Fischer analysis, and then passed through a fluidized bed of the SP-207 in an upflow direction to adsorb the pneumocandin B_0 and other non-polar compounds. The resin was washed with MeOH - H_2O (65 : 35) and the pneumocandin B_0, along with the other compounds, was eluted using 100% MeOH.

The combined SP-207 rich cuts were diluted with water to less than 50% MeOH (Karl Fischer analysis) and passed downflow through a bed of HP-20 to again adsorb the pneumocandin B_0 and other non-polar impurities. The resin was washed with MeOH - H_2O (50 : 50) and eluted using 100% MeOH. An additional SP-207 adsorption/elution step was performed to further concentrate the material and reduce the H_2O content.

Pneumocandin B_0, and closely related compounds, were separated from the more non-polar compounds (primarily the antifoam P-2000 used in the fermentation) *via* precipitation with isopropyl acetate as described below. SP-207 MeOH fractions, containing $\leq 5\%$ water content, were vacuum concentrated to ~ 100 g/liter pneumocandin B_0 (final Karl Fischer analysis $\sim 15\%$). Pneumocandin B_0 was precipitated by slowly adding the concentrate to isopropyl acetate at 25°C. The precipitated solids were separated by both filtration and centrifugation. After vacuum drying there was 4.8 kg total solids, which contained 3.23 kg pneumocandin B_0 (purity 67%). Yield loss in the mother liquors was 1%, but losses caused by physical handling problems during the filtration step resulted in an 84% material balance for the step.

The silica gel chromatography step was performed in 4 runs using 500 liters of silica gel per run. Total solids for the 4 runs were 1 kg (0.67 kg pneumocandin B_0), 1.4 kg (0.94 kg pneumocandin B_0), 1.3 kg (0.94 kg pneumocandin B_0), and 1.1 kg (0.80 kg pneumocandin B_0), respectively. As a result of the limited product solubility in the 85 : 10 : 5 EtOAc - MeOH - 5% aq AcOH mobile phase used to elute the column, column feed was carefully prepared to avoid gelling in the column or before charging onto the column. Pneumocandin A_0 (L-671,329) impurity levels in the feeds were $\sim 3\%$.

Each silica gel rich cut was vacuum concentrated to remove EtOAc and then the concentrate composition was adjusted to 50% MeOH by the addition of MeOH. When necessary, additional 50% MeOH was added until all solids were completely in solution. Feed was charged to an HP-20 column, washed with 1 bed volume of 50% MeOH, and the product was eluted with 100% methanol. Early fractions, containing significant amounts of water and pneumocandin B_0, were recycled back to the HP-20 feed of the next run.

Rich cuts were vacuum concentrated and pneumocandin B_0 precipitated by addition of acetonitrile to the concentrated solution over ~ 1 hour. After 3 hours of aging the precipitate was easily filtered on a 45.7-cm filter pot and then vacuum dried. Silica gel rich cuts from run 1 were separately processed to pure precipitate while runs $2 \sim 4$ were combined after the HP-20 column and precipitated in two equal parts.

Isolation of Pneumocandin C_0

A portion of the material that had been processed through the SP-207, HP-20, and a second SP-207 columns (as described in the "Isolation of Pneumocandin B_0" section) was used for the isolation of pneumocandin C_0. The pneumocandins were precipitated from a 500 ml MeOH sample containing ~ 70 g of pneumocandins and ~ 50 g of P-2000, by the dropwise addition of 4.5 liters of isopropyl acetate. The precipitate, which contained the pneumocandins, was filtered and vacuum dried. The P-2000 remained in the filtrate. The precipitate was dissolved in 550 ml of EtOAc - MeOH - 5% aq AcOH (2 : 2 : 1). Portions of this sample were diluted to a solvent composition of EtOAc - MeOH - 5% aq AcOH (76 : 16 : 8) prior to silica gel HPLC chromatography.

A 700-ml aliquot of this preparation was chromatographed on the Peak Performer preparative HPLC system utilizing EtOAc - MeOH - 5% aq AcOH (85 : 10 : 5) as the mobile phase, a flow rate of 605 ml/minute, and collecting 1.75 liters fractions.

Fractions 16 and 17 were concentrated *in vacuo* to yield 1.76 g and 1.66 g of total solids, respectively. A 716-mg portion of fraction 16 was reconstituted in 14 ml of CH_2Cl_2 - MeOH - H_2O (80 : 20 : 2) and filtered through a 0.5 μm syringe filter. Seven 2 ml injections of this preparation were chromatographed on the Rainin/Gilson semi-preparative HPLC system using a Whatman Partisil 10, 21.2 mm i.d. $\times$ 50 cm silica gel column, using CH_2Cl_2 - MeOH - H_2O (80 : 20 : 2) as the mobile phase, a flow rate of 10 ml/minute, and

monitoring the effluent by UV at 276 nm. In addition, a 55-mg and a 112-mg portion of fraction 17 were prepared and chromatographed in a similar fashion.

The rich cuts, based on the UV monitoring and analytical HPLC of the fractions from these preparative HPLC chromatographies, were combined, concentrated, and reconstituted in 100 ml of MeOH - H_2O (1 : 1), and adsorbed onto a 20-ml HP-20 column. The column was then washed with MeOH - H_2O (1 : 1) and eluted with MeOH to yield 91 mg of pneumocandin C_0.

Base Conversion Product of Pneumocandin B_0

Approximately 35 mg of pneumocandin B_0 in 80 ml of CH_3CN - H_2O (45 : 55) was stirred with 8 ml 1 M Na_2CO_3, pH 10.5 (adjusted with concentrated H_3PO_4) for 50 minutes and then adjusted to pH 6 with 1 M H_3PO_4. The reaction mixture was lyophilized and the residue was taken up in 8 ml of H_2O - CH_3CN - MeOH (4 : 1 : 1) yielding, after centrifugation, an upper phase (7 ml) and a lower phase (1.5 ml). The product was located in the upper phase, which was concentrated *in vacuo* and chromatographed on the LDC semi-preparative HPLC system using a Rainin Dynamax 8 μm C18, 21.4 mm i.d. × 25 cm column, equipped with a 50 mm guard column. The mobile phase consisted of CH_3CN - H_2O (45 : 55), the flow rate was 10 ml/minute, and 10 ml fractions were collected. Column temperature was maintained constant at 50°C *via* jacketing and a constant temperature water bath. Fractions 38 ~ 46 were combined based on UV detection of the column effluent at 210 nm and analytical HPLC of the fractions, filtered, lyophilized, and taken up in 2 ml of MeOH. A 1-ml portion of this MeOH solution, containing 18 mg of purified base conversion product, was utilized for structural elucidation.

Crystallization of Pneumocandin B_0

A solution of pneumocandin B_0 was prepared in *n*-propanol, at a concentration of 50 mg/ml, by heating in a 60°C water bath. After filtration through glass wool the solution was subdivided, various amounts of H_2O were added, and the samples were sealed and allowed to stand at room temperature. Crystals were observed in the tubes containing 2 ~ 8% H_2O. Optimum solvent compositions for crystallization were in the range of 3 ~ 5% H_2O in *n*-propanol. Good quality crystals could be obtained in the concentration range 20 to 200 mg/ml.

Results and Discussion

Nomenclature and Structure of the Pneumocandins

The structures and suggested nomenclature for the pneumocandins are shown in Fig. 1. The accompanying papers describe the production of *Z. arboricola* mutants,[13] structural elucidation,[15] biological activity,[16] and the biosynthesis[17] of the pneumocandins discussed in this paper.

Description of the Producing Strain

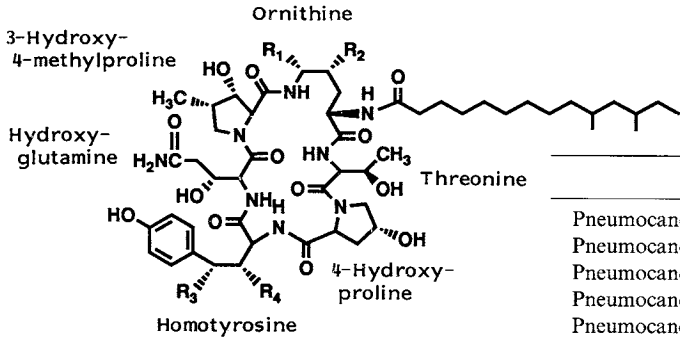
The diagnostic microscopic features of the wild-type strain of *Z. arboricola* (ATCC 20868) are illustrated in Fig. 2. Radial growth of this strain is slow on most mycological agar media. Morphological features on Potato-Dextrose agar (Difco) at 20°C, for a 12 hours photoperiod, are described below.

Colonies attained a diameter of 8 ~ 12 mm in one week. Mature colonies (3 ~ 4 weeks) were effuse, with submerged and aerial hyphae, surface hairy, lanose, or funiculose, dull to moderately shiny, forming raised, densely compact colonies, with a substromatic texture due to dense conidia formation. Colony colors were pale olive-brown, olive, olive-brown, finally olive-black, Isabella Color, Sayal Brown, Tawny-olive, Saccardo's Umber, Sepia, Brownish Olive, Raw Umber, Dark Olive, Olivaceous Black, similar in reverse. Odor, exudates, and soluble pigments were absent.

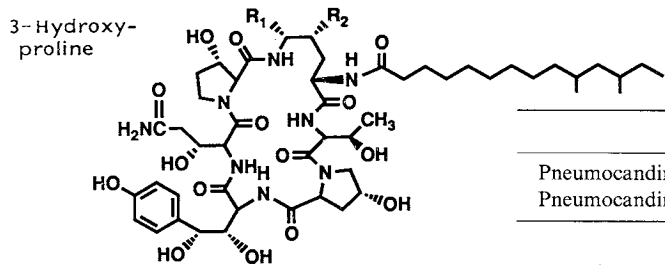
Hyphae were pale yellow-brown to olive-brown in 3% KOH, septate, branched, often with irregular lateral or terminal lobes, 1 ~ 3 μm wide, thin- to slightly thick-walled, with walls smooth to slightly incrusted or verrucose. Aerial hyphae often adhered together in erect fascicles. Setae and hyphopodia were absent.

Fig. 1. Structure and nomenclature of the pneumocandins.

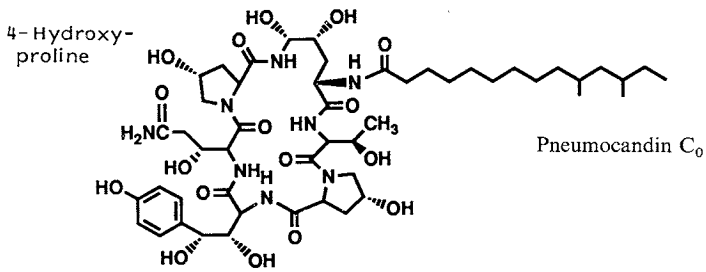
Pneumocandins are divided into three groups according to the identity of the proline residue occupying the position of 3-hydroxy-4-methylproline in pneumocandin A_0. Subscripts refer to the number of hydroxyl functions absent.



	R_1	R_2	R_3	R_4
Pneumocandin A_0	OH	OH	OH	OH
Pneumocandin A_1	OH	OH	OH	H
Pneumocandin A_2	H	H	OH	OH
Pneumocandin A_3	OH	H	H	H
Pneumocandin A_4	H	H	H	H



	R_1	R_2
Pneumocandin B_0	OH	OH
Pneumocandin B_2	H	H



Conidiogenous cells were monoblastic, scattered to dense, integrated, terminal and intercalary, arising directly from undifferentiated hyphae, at right, to slightly acute angles. Conidia originated as irregular chains, filaments, or coils, later developing as compact, irregular masses of 6~25 cells. Individual conidial cells were 3~6 μm in diameter, globose, subglobose, or slightly irregular to lobed, smooth to finely verruculose, yellow-brown to olive brown.

Zalerion arboricola has previously been known only from very specific habitats, *i.e.*, stem wounds of *Picea abies* Karst. and stem cankers of *Larix decidua* Mill. caused by *Trichoscyphella wilkommii* (Hart.) Nannf. in Great Britain.[10,18] The presence of propagules of this organism in pond water in Spain remains unexplained. Possibly propagules were introduced incidentally by wind or rain dispersal from trees in the vicinity. During an investigation of the microflora associated with natural healing of stem cankers of *L. decidua*, BUCZACKI observed that the causal organism, *T. wilkommii*, was displaced from mature natural

Fig. 2. Conidia and conidiogenous cells of *Zalerion arboricola* (ATCC 20868).

Standard bar = 10 μm.

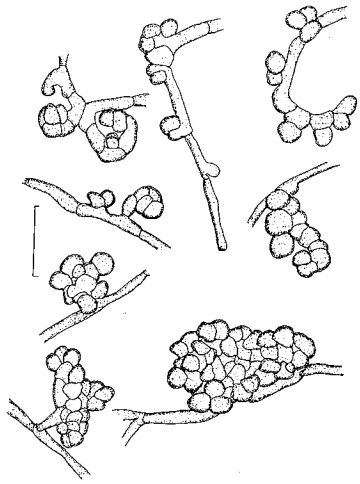
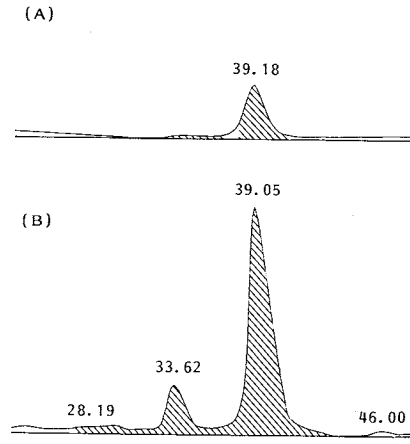


Fig. 3. HPLC bioautograph (CH_3CN - H_2O, 60 : 40) of a control fermentation and a 3,4-dehydro-L-proline fed directed biosynthesis of *Zalerion arboricola*.

Stippling indicates areas of antifungal activity. A; Control, B; 3,4-dehydroproline.



cankers by *Z. arboricola*.[18] When *T. wilkommii* and *Z. arboricola* were co-inoculated into wood blocks in the laboratory, *Z. arboricola* replaced the pathogen, and *Z. arboricola* caused hyphal death of *T. wilkommii* in simultaneous agar culture. When living stems of *L. decidua* were co-inoculated with the two fungi, the rate of extension of stem cankers was decreased compared to controls inoculated only with the causal agent, *T. wilkommii*. In the same study, *Cryptosporiopsis abietina* Petrak was also associated with displacement of *T. wilkommii* from natural cankers and was shown to have similar effects on the *Larix* canker pathogen. Fungi of the genus *Cryptosporiopsis* also produce antifungal compounds of the echinocandin class, including pneumocandin A_0 (L-671,329).[19~21] Therefore, it is possible that these compounds may be responsible for the antagonistic effects of these fungi against *T. wilkommii* and confer these bark-inhabiting fungi with a selective advantage in natural communities.

Pneumocandins A_1, A_3 and A_4

These three compounds were produced by the wild-type producer of pneumocandin A_0. These compounds are related to pneumocandin A_0, in that they all contain the 3-hydroxy-4-methylproline, but differ in their hydroxylation patterns on the dihydroxy-homotyrosine and the dihydroxy-orthinine moieties.[15] Very small quantities of these compounds were isolated from a relatively large fermentation.

Pneumocandins A_2 and B_2

A mutant strain of *Z. arboricola*,[13] that did not produce significant quantities of pneumocandin B_0, produced large quantities of a compound that was thought, initially, to be pneumocandin A_3 by analytical HPLC. Subsequent isolation and structure determination indicated that it was pneumocandin A_2, which is *bis*-reduced (compared to pneumocandin A_0) on the ornithine. The *bis*-reduced version of pneumocandin B_0, pneumocandin B_2,[15] was also found as a minor constituent of the fermentation of this mutant.

Directed Biosynthesis

The wild-type *Z. arboricola* was grown in the presence of a variety of unusual amino acids in the

hope of producing compounds related to pneumocandin A_0, in which the normal amino acids were replaced by the unusual amino acids. An HPLC bioautography approach was utilized, using antifungal activity as the detection method, since the pneumocandins lack a strong, characteristic UV chromophore. Pneumocandin B_0 production was observed in fermentations grown in the presence of L-proline, L-azetidine-2-carboxylic acid, 3,4-dehydro-L-proline, D,L-*p*-fluorophenylalanine, α-methylallylglycine, L-α-ammoniumbutyrate, 5-hydroxy-D,L-tryptophan, D,L-methylallylglycine and under control conditions. By far the highest titers of pneumocandin B_0, as well as pneumocandin A_0, were detected in the directed biosynthesis using 3,4-dehydro-L-proline, as seen in the HPLC bioautographs in Fig. 3. No other pneumocandin-type antifungal agents were detected in any of the other directed biosynthesis experiments.

Production of Pneumocandin B_0

Although the directed biosynthesis method was useful for the original detection of pneumocandin B_0, the only structural information that could be obtained from samples generated by this method was MS data indicating that pneumocandin B_0 was simply a des-methyl analog of pneumocandin A_0. Pneumocandin B_0 was detected as a very minor constituent of the fermentations of the wild-type *Z. arboricola* strain and a sufficient quantity was isolated from a 70-liter liquid fermentation for structural elucidation and initial biological evaluation.[15,16] Neither of these methods, however, was appropriate for producing the quantities of compound required for further chemical and biological investigations. As shown in Fig. 1, pneumocandin B_0 contains a residue of 3-hydroxyproline at the position occupied by 3-hydroxy-4-methylproline in pneumocandin A_0.

The isolation of a mutant strain of *Z. arboricola* (ATCC 20957) was the first step in developing a practical means of producing pneumocandin B_0.[13] The pneumocandin B_0/pneumocandin A_0 ratio in fermentations of this mutant was approximately 3 : 1 compared to 1 : 7 for fermentations of the wild-type strain. Development of a practical isolation procedure was the next step.

The isolation scheme can be conceptually divided into three steps or sequence of steps, *i.e.*, 1) extraction and preparation for chromatography, 2) silica gel chromatography, and 3) workup of the fractions from the chromatography. A summary of the results of the isolation is tabulated in Table 2.

The first sequence consisted of extraction with methanol/water and sequential adsorption/elution from SP-207 (fluidized bed), HP-20, and SP-207 (all polystyrene/divinyl benzene resins useful for reversed phase-type solid phase extractions) followed by precipitation with isopropyl acetate. This series of steps increased the purity of pneumocandin B_0 from 0.65% in the broth extract to 67%, or about 100-fold, with

Table 2. Pilot plant yield summary.

Isolation step	Pneumocandin B_0 total kg	Purity (%)	% Yield step	% Yield overall
Fermentation	5.40			
Methanol extraction	5.06	0.65	94	94
Sepabead SP-207 FBA	4.25	23	84	79
Diaion HP-20	4.17	31	98	77
Sepabead SP-207	3.86	47	93	72
Precipitation	3.23	67	84	60
Silica gel	2.47		76	46
Diaion HP-20	2.44		99	45
Precipitation	2.38	100*	98	44

* 100% by definition.

Table 3. Yield and material balance for four silica gel columns.

Run Number	Pneumocandin B_0 yield in g	Pneumocandin B_0 % yield	Material balance %	Pneumocandin A_0 %
1	520	78	92	0.2
2	728	77	88	NA
3	622	71	85	NA
4	580	78	87	1.3
Total	2450			0.4

an overall yield of 60%.

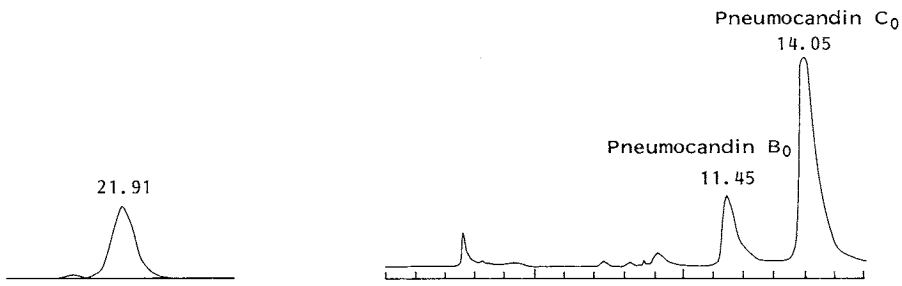
Filtrates from the three methanol/water extractions of the whole broth and collected mycelial cake, contained 47%, 39%, and 8% yields of pneumocandin B_0 for a total of 94% yield. Average purity of the combined rich cut fractions from the SP-207 fluidized bed adsorption step was 23%, a 35-fold purification. The HP-20 and second SP-207 steps were necessary to minimize the amount of water (since water causes severe foaming upon subsequent concentration) and to reduce the volume of the rich cut to facilitate its concentration, in preparation for the precipitation step. The precipitation of pneumocandin B_0 from the concentrate significantly improves the purity, primarily by eliminating the P-2000 antifoam, which remains in the supernatant. The main purpose for this set of steps was to separate the pneumocandins from non-pneumocandin impurities.

The second step was the silica gel chromatography. The silica gel chromatography solvent system was derived from the organic phase of a two phase countercurrent chromatography system involving CH_2Cl_2 - MeOH - H_2O, which showed a significant improvement in the separation of the pneumocandins on TLC, compared to conventional CH_2Cl_2 - MeOH solvent systems. Stepwise modification of the solvent system, dictated by chromatographic and non-chromatographic considerations, led to the reported EtOAc - MeOH - 5% aq AcOH (85 : 10 : 5) solvent system. A key difficulty in utilizing this type of chromatography was preparing the feed for the column. Feed reconstituted in mobile phase resulted in gels which could not be applied to the column in a homogenous fashion. On the other hand, utilizing a solvent composition for the feed that was significantly different (higher in MeOH - H_2O) from the mobile phase composition compromised an already difficult separation. In early laboratory scale work, the problem was solved by drying the feed onto silica gel and applying the coated silica gel to the head of the column. However, this was not practical for larger scale work. The eventual solution was to dissolve the feed in the MeOH - 5% aq AcOH portion of the mobile phase, add the EtOAc portion, and immediately charge the feed onto the column before gelling could occur.

The results of the four silica gel column runs are summarized in Table 3. The purpose of this silica gel step was to remove the quantities of related pneumocandins from pneumocandin B_0 preparations, especially pneumocandin A_0, which was accomplished quite effectively.

The third sequence of steps was a workup of the silica gel rich cuts. As a result of the aqueous/methanol portion of the silica gel solvent system, significant amounts of dissolved silica gel were present in the rich cut fractions containing pneumocandin B_0. These fractions were concentrated to remove EtOAc and diluted with H_2O. The pneumocandin B_0 was adsorbed and eluted from HP-20, while the silica gel was unretained. The HP-20 rich cuts were vacuum concentrated, precipitated, and filtered to yield purified pneumocandin B_0 in 44% overall yield from broth and 100% purity (defining this final preparation as the standard).

Fig. 4. Analytical HPLC of a mixture of pneumocandin B_0 and pneumocandin C_0.



A C-18 column (CH_3CN-H_2O, 43:57) (left panel) and a silica column (CH_2Cl_2-MeOH-H_2O, 80:20:2) (right panel).

Isolation of Pneumocandin C_0

On reverse phase HPLC, the difference in retention time for pneumocandin B_0 and its isomer, pneumocandin C_0, was negligible. Mixtures of the two compounds resulted in a barely noticeable broadening of the HPLC peak (Fig. 4). Column chromatography on silica gel partially separated the two compounds which allowed initial characterization of pneumocandin C_0 by MS and ^1H NMR as an isomer of pneumocandin B_0. A preparative, silica-based HPLC system was developed and utilized to provide pure material for structural elucidation, which indicated that the hydroxyl group found at the 3-position of the proline in pneumocandin B_0 was at the 4-position in pneumocandin C_0[15] (Fig. 1). Based on the preparative silica gel separation of these two components, an analytical silica-based HPLC system was developed that easily distinguished the two as seen in Fig. 4. While the resolution was greatly improved compared to the reverse phase system, the solvent system used for the silica gel HPLC system necessitated the use of the extremely weak 276 nm chromophore from the homotyrosine residue for UV detection. As a result, this analytical system was considerably less sensitive than the reverse phase system.

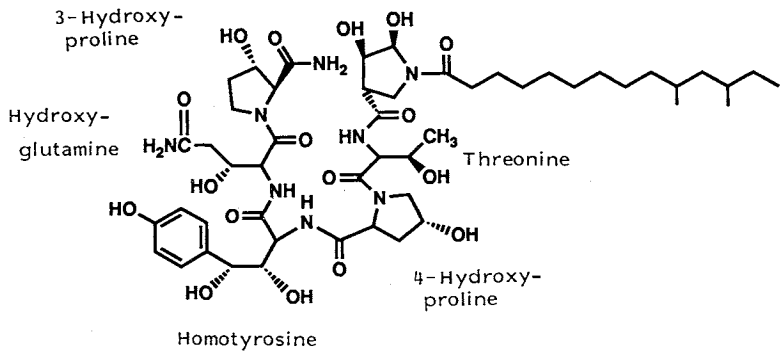
The large-scale preparative silica gel column chromatography described in the "Isolation of Pneumocandin B_0" section was useful for producing pneumocandin B_0, containing a minimum amount of pneumocandin C_0, once an analytical HPLC system was available to detect the pneumocandin C_0.

The Base Conversion Product of Pneumocandin B_0

Irreversible conversion of pneumocandin A_0 to a chromatographically similar compound, in the presence of base, had been previously noted, although the conversion product was never structurally characterized. The base conversion product of pneumocandin B_0, on the other hand, was so chromatographically similar to pneumocandin B_0 that is was originally not detected by analytical reverse phase HPLC. Eventually an analytical HPLC system that could reproducibly detect and quantitate both components was developed. The structure of the base conversion product of pneumocandin B_0 is shown in Fig. 5.[15]

The preparative separation of these two components was extremely difficult and impractical and, therefore, steps were taken to prevent the formation of the base conversion product during the isolation. Since pH stability studies indicated that base conversion product forms slowly even near neutral pH, but not under acidic conditions, acetic acid was added to the aqueous portion of the silica gel chromatography solvent system in this isolation and to all steps in subsequent isolations.

Fig. 5. Structure of the base conversion product of pneumocandin B_0.



Crystallization

Attempts to crystallize pneumocandin B_0 by vapor diffusion, in a large variety of solvents and conditions, were unsuccessful leading to gels and, in a few cases, precipitates. Slow evaporation of solvents also led to gels.

Pneumocandin B_0 was dissolved, at a variety of concentrations, in hot (60°C) isopropanol, hot *n*-propanol, or hot *n*-butanol. These samples were filtered hot and allowed to cool to room temperature in a sealed container. Gel formation was observed for isopropanol and *n*-propanol and a clear solution was observed for *n*-butanol. However, when these gels were redissolved by heating and resealed, the compound remained in solution upon cooling to room temperature. The sample containing 50 mg/ml in *n*-propanol was apparently not well sealed and after 3 weeks a significant volume reduction was noted along with small, but very birefringent, spike-like crystals.

One possible explanation for the gel formation in the sealed container and crystal formation in the open container was that in the open container a significant amount of water may have condensed into the *n*-propanol and changed its overall composition. Another possibility was that the slow evaporation of *n*-propanol resulted in a supersaturated solution, causing pneumocandin B_0 to crystallize.

A number of experiments with varying amounts of water/*n*-propanol in sealed containers were performed to determine if the solvent composition was responsible for the observed crystallization and, if so, to develop optimum conditions for crystallization. Based on these experiments it was apparent that the presence of H_2O was essential to the crystallization process. No crystallization was observed in other alcohol/H_2O mixtures, indicating that *n*-propanol was also an essential ingredient in the crystallization of pneumocandin B_0.

These crystals can be characterized as hexagonal columns, which begin decomposing at >200°C, and splinter badly upon drying. The Karl Fischer analysis of dried crystals indicated a water content of 9.9%. This is equivalent to 6 moles of H_2O per mole pneumocandin B_0, suggesting that the crystals obtained by this method were hydrates.

In conclusion, the discovery of pneumocandin B_0 and isolation of sufficient quantities for initial biological evaluation was difficult, but the development of practical methods of producing this compound was the truly challenging aspect of this project. The isolation of a compositional mutant provided a means of producing significant quantities of pneumocandin B_0, while a silica gel chromatography step allowed the isolation of high-purity material, free of related pneumocandins such as A_0 and C_0. Crystallization,

on the other hand, may or may not be useful for the preparation of pneumocandin B_0, but was scientifically important because it is a prerequisite for stereochemical studies of this molecule,[15] which may lead to a better understanding of its biology and chemistry.

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