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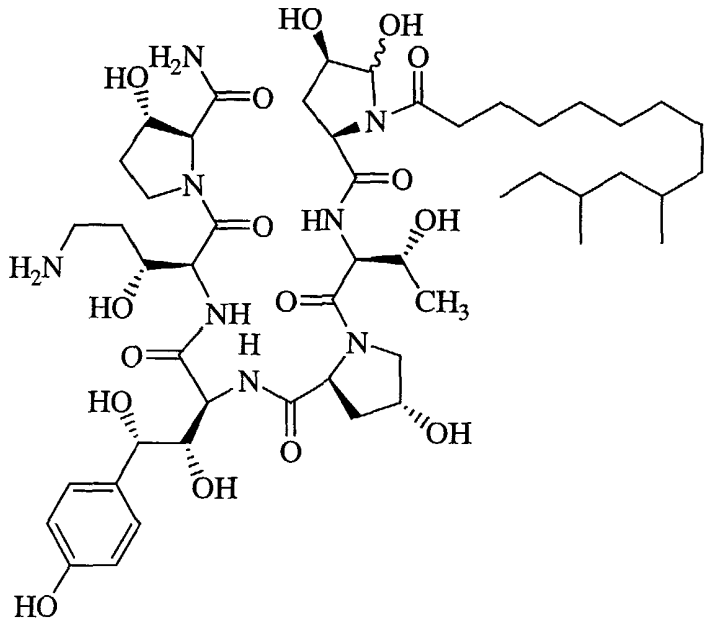
(54) Title: ACTIVE METABOLITE OF ANTIFUNGAL COMPOUND



(I)

(57) Abstract: This invention relates to a method for treating a fungal infection comprising administering to a mammalian subject in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt, Other aspects of the invention include a method of treating a fungal infection using a combination of the compound of Formula I and a second antifungal agent and pharmaceutical compositions of said combinations.

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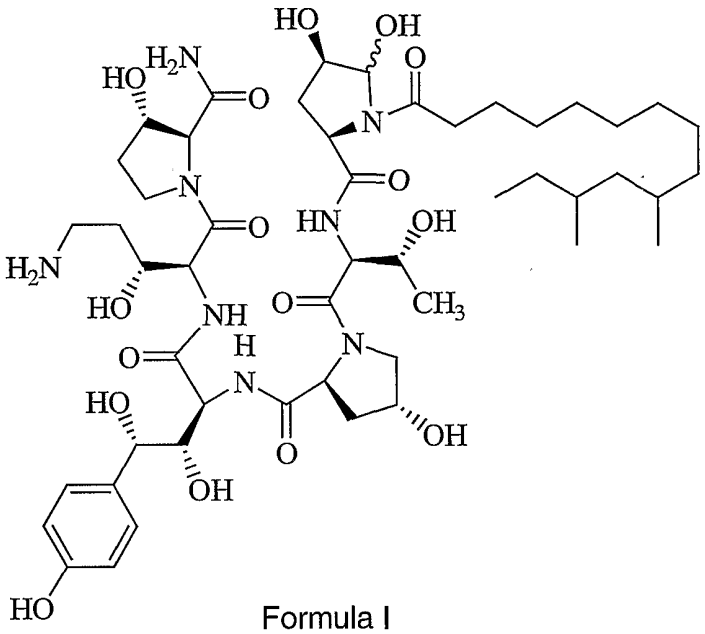
TITLE OF THE INVENTION

ACTIVE METABOLITE OF ANTIFUNGAL COMPOUND

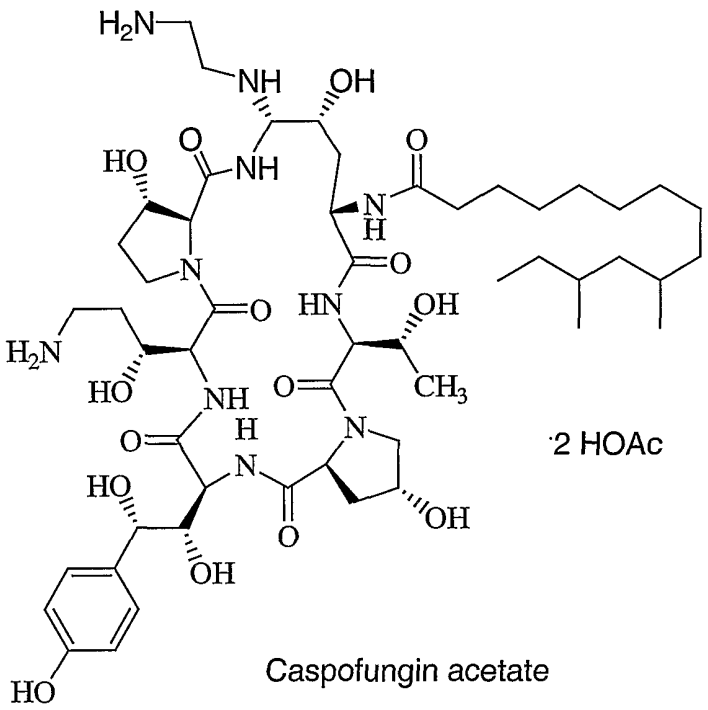
BACKGROUND OF THE INVENTION

This invention relates to the identification of a metabolite compound of Formula I



Formula I

having antifungal activity. This metabolite has been found to be the major metabolite of CANCIDAS™ (caspofungin acetate) produced in human plasma. Caspofungin acetate is a semisynthetic antifungal agent developed for the parenteral treatment of *Candida*, *Aspergillus*, *Pneumocystis carinii* and other mycotic infections. Its activity is mediated by inhibition of the synthesis of β-(1,3)-glucan, an integral component in the cell wall of the target organisms. Caspofungin is a macrocyclic peptide with a molecular weight of 1094 disclosed in US Patent No. 5,378,804. This compound possesses a variety of potentially reactive functionalities that include an aminal moiety. The metabolite is formed by the hydrolysis of the aminal in the ornithine residue, ring opening and recyclization to form two isomeric 5-membered cyclic hemiaminal products. These two isomers are not easily separable by high performance liquid chromatography and thus are isolated together as a single product and collectively termed "the metabolite".



Caspofungin acetate

In prior work, it had been determined that a related ring-opened analog of pneumocandin B_0 was found to possess very poor β-(1,3)-glucan synthesis activity. This hexapeptide metabolite of Formula I, however, has been found to have surprising activity against *Candida* isolates.

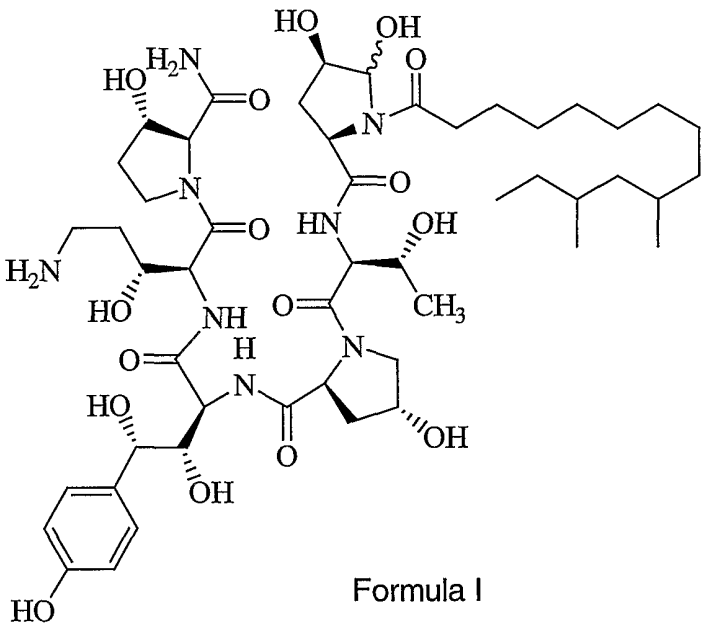
A ring-opened analog of pneumocandin B_0 was disclosed by Merck Research Laboratories. See J. M. Balkovec et al., "Synthesis, Stability and Biological Evaluation of Water Soluble Prodrugs of a New Echinocandin Lipopeptide. Discovery of a Potential Clinical Agent for the Treatment of Systemic Candidiasis and *Pneumocystis carinii* Pneumonia (PCP)" *J. Med. Chem.* **1992**, *35*, 194-8. The synthesis of compound 2 an intermediate in the synthesis of the compound of Formula I was first described by F.A. Bouffard et al., "Synthesis and Antifungal Activity of Novel Cationic Pneumocandin B_0 Derivatives" *J. Med. Chem.* **1994**, *37*, 222-5. The preparation of the compound of Formula I begins with pneumocandin B_0 that can be isolated by following the procedures disclosed by Merck Research Laboratories. See R.E. Schwartz, et al., Pneumocandins from *Zalerion arboricola* I. Discovery and isolation. *J. Antibiot.* (1992) **45**:1853-1866, and P. S. Masurekar et al., Pneumocandins from *Zalerion arboricola* II. Modification of product spectrum by mutation and medium manipulation. *J. Antibiot.* (1992) **45**:1867-1874, and US Patent

Nos. 5,194,377 and 5,202,309. US Patent Nos. 5,854,212 and 5,939,384 also disclose a method for the preparation of Pneumocandin B_0 See Example 1.

Recent disclosures discuss the formation of a metobolite/degradation byproduct of caspofungin. See XU, X., DELUNA, F., YUANG, A., CYLC, D., DAVIS, M., SAHLY, Y. and LIN, J.H. Slow hepatic uptake and elimination play an important role in the disposition of L-743,872, a potent antifungal agent, in rats. For presentation at: American Association of Pharmaceutical Scientists 10th Annual Meeting (AAPS), Seattle, Washington, 10/26/1996 - 10/31/1996; KAUFMAN, M.J. and NERURKAR, M. Degradation of the macrocyclic antifungal agent L-743,872: Reaction products and kinetics, and stabilization strategies. For presentation at: American Chemical Society 31st Middle Atlantic Regional Meeting, Pleasantville, New York, 05/27/1997 - 05/30/1997; KAUFMAN, M.J. and NERURKAR, M. Degradation of the macrocyclic antifungal agent L-743,872: Reaction products and kinetics, and stabilization strategies. For presentation at: American Chemical Society 31st Middle Atlantic Regional Meeting, Pleasantville, New York, 05/27/1997 - 05/30/1997; BALANI, S.K., XU, X., ARISON, B.H., SILVA, M.V., GRIES, A., DELUNA, F.A., CUI, D., KARI, P.H., LY, T., HOP, C.E.C.A., SINGH, R., WALLACE, M.A., DEAN, D.C., LIN, J.H., PEARSON, P.G. and BAILLIE, T.A. Metabolites of caspofungin acetate, a potent antifungal agent, in human plasma and urine. For submission to: Drug Metabolism and Disposition; and MCQUADE, M.S., FORSYTH, R.J., ZIMMERMAN, J. and ROBERTS, J.C. Stability of reconstituted Cancidas™ (caspofungin acetate) in commonly used i.v. solutions and flexible polyvinyl chloride containers. For submission to: American Journal of Health-System Pharmacy.

SUMMARY OF THE INVENTION

A method for treating a fungal infection comprising administering to a mammalian subject in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt,

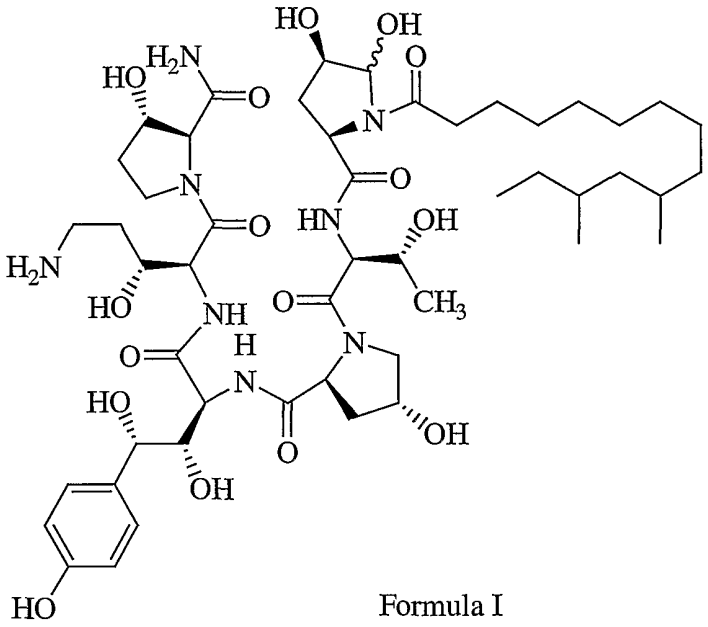


Formula I

Additional aspects of the invention include: 1) a method for controlling mycotic infections comprising administering to an immune-comprised patient in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt; 2) a method for controlling *Pneumocystis* pneumonia comprising administering to an immune-comprised patient in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt; 3) a pharmaceutical composition comprising a compound of Formula I or its pharmaceutically acceptable salt and a second antifungal agent selected from the group consisting of: azoles or polyenes, purine or pyrimidine nucleotide inhibitors, chitin synthesis inhibitors, elongation factor inhibitors, mannan-binding antifungal agents, bactericidal/permeability-inducing (BPI) protein products, and complex carbohydrate antifungal agents; and 4) a method of treating a fungal infection comprising administering to a mammalian subject in need of such treatment an effective amount of a combination of a compound of Formula I or a pharmaceutically acceptable salt thereof and a second antifungal agent selected from the group consisting of: azoles or polyenes, purine or pyrimidine nucleotide inhibitors, chitin synthesis inhibitors, elongation factor inhibitors, mannan-binding antifungal agents, bactericidal/permeability-inducing (BPI) protein products, and complex carbohydrate antifungal agents.

DETAILED DESCRIPTION OF THE INVENTION

A method for treating fungal infections comprising administering to a mammalian subject in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt,



Formula I

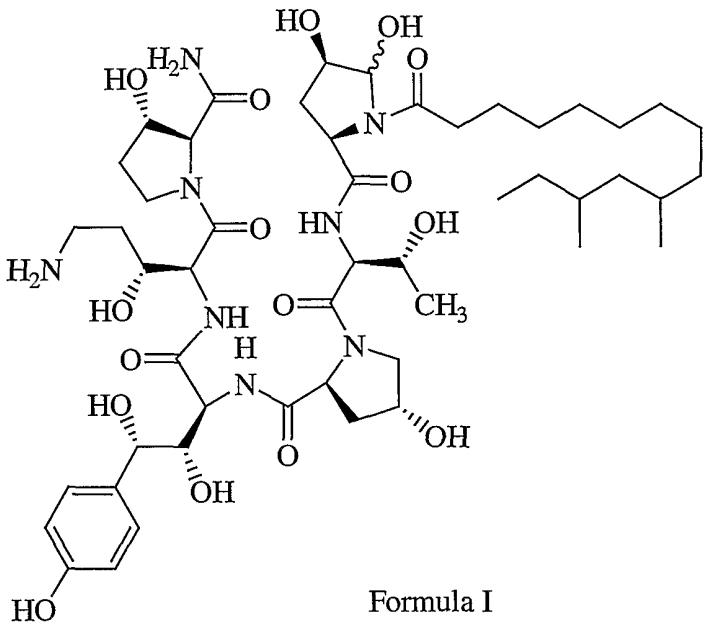
The method as recited above, wherein the compound of Formula I is administered as a pharmaceutical composition of said compound with a pharmaceutically acceptable carrier.

The method as recited above, wherein the pharmaceutical composition is administered parenterally or orally.

The method as recited above, wherein the pharmaceutical composition is administered parenterally.

The method as recited above, wherein the pharmaceutical composition is administered by intravenous infusion.

A method for controlling mycotic infections comprising administering to a mammalian subject in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt,



Formula I

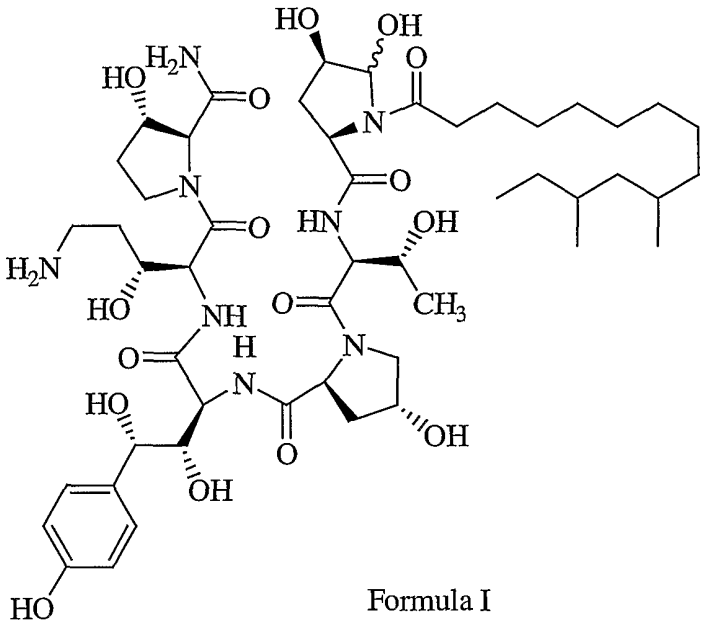
The method as recited above, wherein the compound of Formula I is administered as a pharmaceutical composition of said compound with a pharmaceutically acceptable carrier.

The method as recited above, wherein the pharmaceutical composition is administered parenterally.

The method as recited above, wherein the pharmaceutical composition is administered by intravenous injection.

The method as recited above, wherein the pharmaceutical composition is administered by intravenous infusion.

A method for controlling *Pneumocystis* pneumonia comprising administering to an immune-comprised patient in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt,



Formula I

The method as recited above, wherein the compound of Formula I is administered as a pharmaceutical composition of said compound with a pharmaceutically acceptable carrier.

The method as recited above, wherein the pharmaceutical composition is administered parenterally.

The method as recited above, wherein the pharmaceutical composition is administered by intravenous injection.

The method as recited above, wherein the pharmaceutical composition is administered by intravenous infusion.

The present invention also relates to a pharmaceutical composition comprising a combination of the compound of Formula I and a second antifungal agent, as well as an antifungal combination therapy comprising the administration of the antifungal compound of Formula I and a second antifungal agent.

It is understood that this combination therapy would involve the sequential, simultaneous or concomitant administration of these two agents. More particularly, the invention relates to antifungal combination therapy comprising the use of a second antifungal agent such as: azoles or polyenes, purine or pyrimidine nucleotide inhibitors, chitin synthesis inhibitors, elongation factor inhibitors, mannan-binding antifungal agents, bactericidal/permeability-inducing (BPI) protein products, and complex carbohydrate antifungal agents.

Representative examples of such second antifungal agents are: azoles, such as fluconazole, voriconazole, itraconazole, ketoconazole, miconazole, ravuconazole, posaconazole; polyenes such as amphotericin B, nystatin or liposomal and lipid forms thereof such as ABELCET, AMBISOME and AMPHOCIL; purine or pyrimidine nucleotide inhibitors such as flucytosine; or polyoxins such as nikkomycins, in particular nikkomycin Z or other chitin synthesis inhibitors, elongation factor inhibitors such as sordarin and analogs thereof, mannan-binding antifungal agents such as the pradamicins, bactericidal/permeability-inducing (BPI) protein products such as XMP.97 or XMP.127 or complex carbohydrate antifungal agents such as CAN-296.

In particular, this combination therapy has been shown to be useful against such opportunistic pathogens as *Cryptococcus* spp., *Candida* spp., *Aspergillus* spp., *Histoplasma* spp., *Coccidioides* spp., *Paracoccidioides* spp. *Blastomyces* spp., *Fusarium* spp., *Sporothrix* spp., *Trichosporon* spp., *Rhizopus* spp., *Pseudallescheria* spp., dermatophytes, *Paeciliomyces* spp., *Alternaria* spp., *Curvularia* spp., *Exophiala* spp., *Wangiella* spp., *Penicillium* spp., *Saccharomyces* spp., *Dematiaceous* fungi and *Pneumocystis carinii.*

CANCIDAS™ (Caspofungin acetate) is disclosed in U.S. Patent No. 5,378,804 and its preparation is described in that patent, as well as U.S. Patent No. 5,552,521.

The azole, polyene or other antifungal agent may be administered orally or parenterally. The compound of Formula I is preferably administered parenterally, but is not limited to that route and may also be administered by other routes such as oral, intramuscular or subcutaneous.

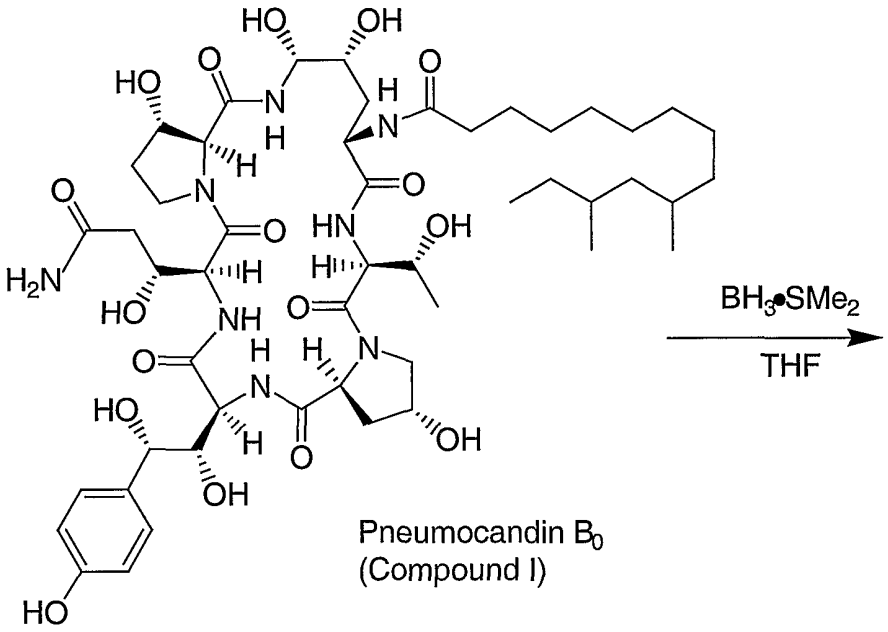
Combination therapy results in enhanced effects using sub-inhibitory concentrations of all agents. These effects may be demonstrated *in vitro* and *in vivo* using clinical and environmental strains of *C. neoformans, C. albicans* and *A. fumigatus.*

The compound of Formula I is formed from caspofungin by hydrolysis of the aminal group, followed by ring opening and recyclization onto N2 of the ornithine group.

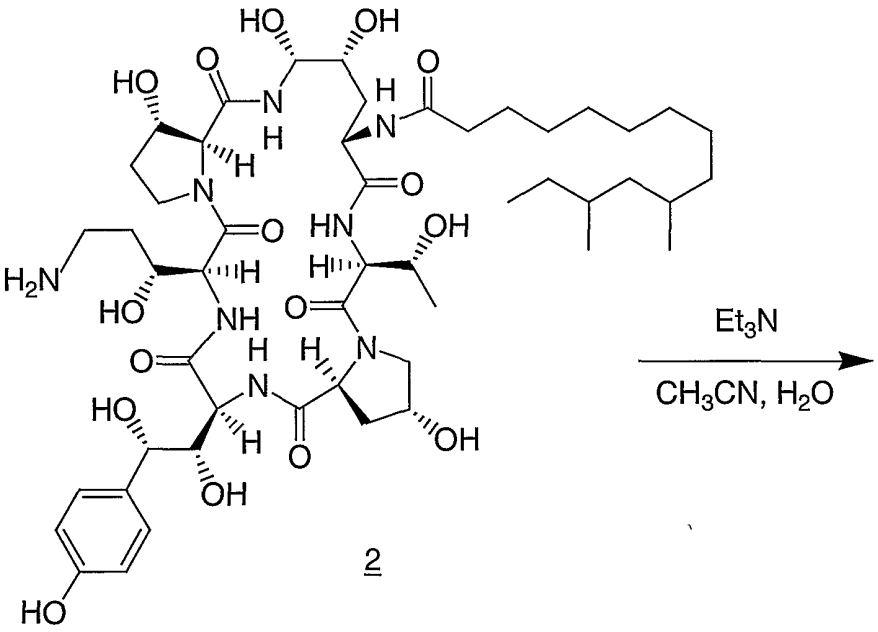
The compound of Formula I may be prepared from pneumocandin B_0 (Compound I) following the procedures outlined in SCHEME 1. Briefly, the 3-hydroxyglutamine residue of pneumocandin B_0 may be reduced to give Compound 2. Borane dimethylsulfide complex is a preferred reducing agent. Compound 2 may be

treated with a base such as triethylamine, to effect ring opening and subsequent recyclization to give the compound of Formula I. The compound of Formula I is formed as a mixture of two isomers at the newly formed hemiaminal group. The isomers are not easily separable by standard chromatographic techniques and thus may be used as a mixture.

SCHEME 1



Pneumocandin B_0
(Compound I)



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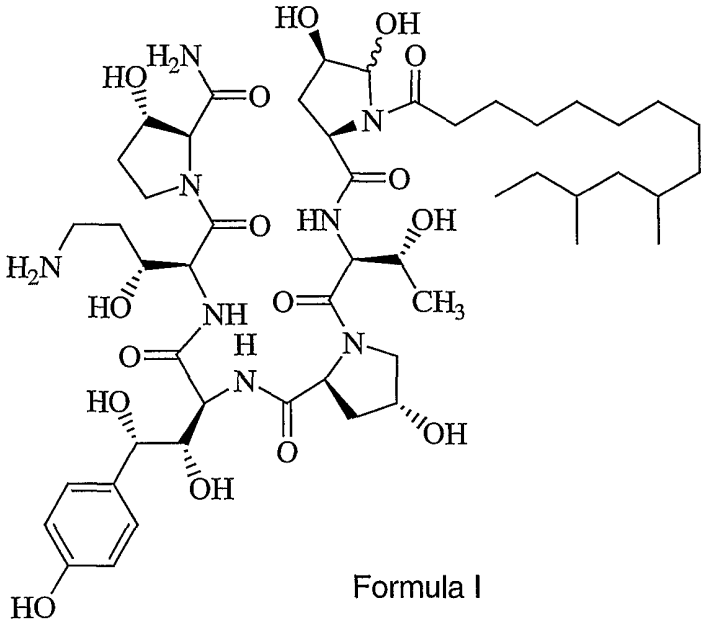
SCHEME 1 (continued)



Formula I

The invention also embraces acid addition salts. The compound of Formula I in the normal course of isolation is obtained as an acid addition salt. The salt thus obtained may be dissolved in water and passed through an anion exchange column bearing the desired anion. The eluate containing the desired salt may be concentrated to recover the salt as a solid product.

The compound of Formula I was tested for yeast susceptibility following protocol M27-A. This protocol is the standard by which all hospital clinical laboratories test their yeast isolates for susceptibility to antifungal agents. See J.N. Galgiani et al. *Reference Method for Broth Dilution Antifungal Susceptibility Testing: Approved Standard M27-A (Reference F-7)*, National Committee for Clinical Laboratory Standards1997; 17(9):1-29. A description of this method follows: The inoculum was standardized with a spectrophotometer (optical density, 550 nm) and was diluted to a final concentration of 0.5 x 10^3 to 2.5 x 10^3 in RPMI 1640 medium with L-glutamine, without sodium bicarbonate, buffered with 0.165 M MOPS (morpholinepropanesulfonic acid) (BioWhittaker, Walkersville, MD).

The compound of Formula I along with appropriate control standards were prepared as concentrated stock solutions in sterile deionized water and diluted in RPMI 1640 medium, and tested at concentrations ranging from 128 μg/mL down to

0.06 μg/mL in two-fold serial dilutions. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of compound which completely inhibited visible growth after incubation at 35°C for 24 or 48 hr.

The same method as described above was used for *Aspergillus* except that the reference standard is M38-P [National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. Proposed standard M38-P. Wayne, PA: National Committee for Clinical Laboratory Standards; 1998.] and the inoculum final concentration was 1 to 5 x 10^4 conidia/ml and the MIC is defined as the lowest concentration of compound which produces prominent reduction in growth as compared to the growth control (approximately 50% of control). The results are shown in Table A:

Table A. Minimum Inhibitory Concentrations of Compound I Against *Candida* and *Aspergillus* species

		MIC (μg/mL)	
Organism	**Isolate**	**24 h**	**48 h**
C. albicans	MY 1055	1-2	2
C. albicans	ATCC 24433	1-2	2
C. albicans	ATCC 90028	1	2
C. tropicalis	ATCC 750	1	1
C. krusei	ATCC 6258	16	16-32
A. fumigatus	MF 5668	64	>64
A. fumigatus	MF 5669	64	>64

The compound of Formula I also shows *in vivo* effectiveness against fungi which may be demonstrated using the following *in vivo* assay.

Growth from an overnight SDA culture of *Candida albicans* MY 1055 was suspended in sterile saline and the cell concentration determined by hemacytometer count and the cell suspension adjusted to 3.75 x 10^5 cells/ml. Then 0.2 milliliter of this suspension was administered I.V. in the tail vein of mice so that the final inoculum was 7.5 x 10^4 cells/mouse.

The assay then was carried out by administering aqueous solutions of compound of Formula I at various concentrations intraperitoneally (I.P.), thrice daily

(t.i.d.) for one day to 18 to 20 gram female DBA/2 mice, which previously had been infected with *Candida albicans* in the manner described above. Deionized water was administered I.P. to *C. albicans* challenged mice as controls. After 24 hours, the mice were sacrificed by carbon dioxide gas, paired kidneys were removed aseptically and placed in sterile polyethylene bags containing 5 milliliters of sterile saline. The kidneys were homogenized in the bags, serially diluted in sterile saline and aliquots spread on the surface of SDA plates. The plates were incubated at 35°C for 48 hours and yeast colonies were enumerated to determine the number of colony forming units (CFU) per gram of kidneys. The results are shown in Table B. Compound of Formula I reduced the tissue burden in the kidneys of infected animals at doses of 0.31, 1.25, 5 and 10 mg/kg/day.

Table B. In Vivo Anti-*Candida* Activity of Compound of Formula I

Compound	Dose mg/kg/day	Mortality	Log CFU/gm Kidney	Reduction from sham
Formula I	10	0%	5.19	-0.41
Formula I	5	0%	5.26	-0.34
Formula I	2.5	0%	5.48	-0.12
Formula I	1.25	0%	5.24	-0.36
Formula I	0.63	0%	5.51)	-0.09
Formula I	0.31	0%	5.23	-0.37
Deionized water	0	0%	5.60	---

The compound of Formula I is also useful for inhibiting or alleviating *Pneumocystis carinii* infections in immune-compromised patients. The efficacy of the compounds of the present invention for therapeutic or anti-infection purposes may be demonstrated in studies on immunosuppressed rats.

In a representative study, Sprague-Dawley rats (weighing approximately 250 grams) are immunosuppressed with dexamethasone in the drinking water (2.0 mg/L) and maintained on a low protein diet for seven weeks to induce the development of *Pneumocystis* pneumonia from a latent infection. Before drug treatment, two rats are sacrificed to confirm the presence of *Pneumocystis carinii* pneumonia (PCP). Five rats (weighing approximately 150 grams) are injected twice

daily for four days subcutaneously (sc) with compound of Formula I in 0.25 ml of vehicle (distilled water). A vehicle control is also carried out. All animals continue to receive dexamethasone in the drinking water and a low protein diet during the treatment period. At the completion of the treatment, all animals are sacrificed, the lungs are removed and processed, and the extent of disease determined by microscopic analysis of stained slides.

The outstanding properties are most effectively utilized when the compound is formulated into novel pharmaceutical compositions with a pharmaceutically acceptable carrier according to the conventional pharmaceutical compounding techniques.

The novel compositions contain at least a therapeutic antifungal or antipneumocystis amount of the active compound. Generally, the composition contains at least 1% by weight of the compound of Formula I. Concentrate compositions suitable for dilutions prior to use may contain 90% or more by weight. The compositions include compositions suitable for oral, topical, parenteral (including intraperitoneal, subcutaneous, intramuscular, and intravenous), nasal, and suppository administration, or insufflation. The compositions may be prepacked by intimately mixing the compound of Formula I with the components suitable for the medium desired.

Compositions formulated for oral administration may be a liquid composition or a solid composition. For liquid preparation, the therapeutic agent may be formulated with liquid carriers such as water, glycols, oils, alcohols, and the like, and for solid preparations such as capsules and tablets, with solid carriers such as starches, sugars, kaolin, ethyl cellulose, calcium and sodium carbonate, calcium phosphate, kaolin, talc, lactose, generally with lubricant such as calcium stearate, together with binders disintegrating agents and the like. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage form. It is especially advantageous to formulate the compositions in unit dosage form (as hereinafter defined) for ease of administration and uniformity of dosage. Compositions in unit dosage form constitute an aspect of the present invention.

Compositions may be formulated for injection and may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles such as 0.85 percent sodium chloride or 5 percent dextrose in water and may contain formulating agents such as suspending, stabilizing and/or dispersing agents. Buffering agents as well as additives such as saline or glucose may be added to make the solutions

isotonic. The compound may also be solubilized in alcohol/propylene glycol or polyethylene glycol for drip intravenous administration. These compositions also may be presented in unit dosage form in ampoules or in multidose containers, preferable with added preservative. Alternatively, the active ingredients may be in powder form for reconstituting with a suitable vehicle prior to administration.

The term "unit dosage form" as used in the specification and claims refers to physically discrete units, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the pharmaceutical carrier. Examples of such unit dosage forms are tablets, capsules, pills, powder packets, wafers, measured units in ampoules or in multidose containers and the like. A unit dosage of the present invention will generally contain from 100 to 200 milligrams of one of the compounds.

When the compound is for antifungal use any method of administration may be employed. For treating mycotic infections, oral or intravenous administration is usually employed.

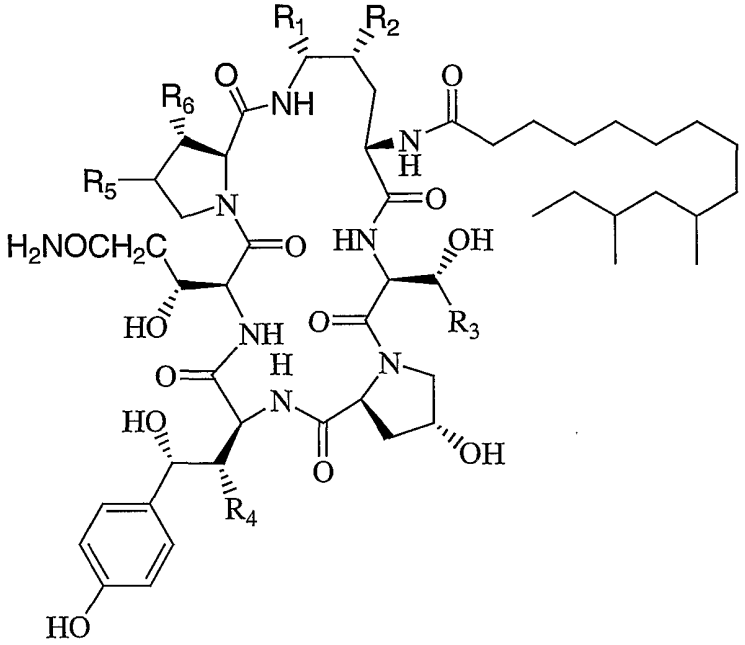
When the compound is to be employed for control of *Pneumocystis* infections it is desirable to directly treat lung and bronchi. For this reason inhalation methods are preferred. For administration by inhalation, the compounds of the present inventions are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or nebulizers. The preferred delivery system for inhalation is a metered dose inhalation (MDI) aerosol, which may be formulated as a suspension or solution of compound of Formula I in suitable propellants, such as fluorocarbons or hydrocarbons.

Although the compounds of the present invention may be employed as tablets, capsules, topical compositions, insufflation powders, suppositories and the like, the solubility of the compounds of the present invention in water and aqueous media render them adaptable for use in injectable formulations and also in liquid compositions suitable for aerosol sprays.

The following examples illustrate the invention but are not to be construed as limiting.

EXAMPLE 1

Preparation of Pneumocandin B_0



Compound	R_1	R_2	R_3	R_4	R_5	R_6
I	OH	OH	CH_3	OH	H	OH
II	OH	H	CH_3	OH	H	OH
III	OH	=O	CH_3	OH	H	OH
IV	OH	OH	H	OH	H	OH
V	OH	OH	CH_3	H	H	OH
VI	OH	OH	CH_3	OH	H	H
VII	H	H	CH_3	OH	OH	OH
VIII	H	OH	CH_3	OH	H	OH
IX	H	H	CH_3	OH	H	OH
X	OH	OH	CH_3	OH	OH	H
XI	OH	OH	CH_3	OH	OH	OH
XII	OH	OH	CH_3	OH	CH_3	OH

Part A. Pneumocandin B_0 (also referred to as compound I) is disclosed in U.S. Patent No. 5,202,309 which issued April 13, 1993 and is produced by cultivating the fungus *Glarea lozoyensis* (formerly identified as *Zalerion arboricola*) under aerobic

conditions. A process for the production of Pneumocandin B_0 is disclosed in U.S. Patent 5,194,377 which issued March 16, 1993. Pneumocandin B_0 is produced by cultivating *Glarea lozoyensis*, ATCC No. 20868, deposited under the Budapest Treaty in the Culture Collection of American Type Culture Collection at 12301 Parklawn Drive, Rockville, Md. 20852. Pneumocandin B_0 can also be prepared following the procedures described in US Patent No. 5,939,384. Alternatively, Pneumocandin B_0 can be isolated following the procedure described below.

Part B. Alternate Synthesis for Pneumocandin B_0

Culture

The fungus *Glarea lozoyensis* (ATCC 74030) is used to produce Compound I and the structurally related analogues. This improved production strain was derived ultimately from the wild-type organism, ATCC 20868, (isolated from a sample of fresh water) by sequential steps of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. The culture was maintained as aliquots of a mycelial suspension in 5% (v/v) glycerol stored at -70°C.

Shake-Flask Scale Fermentations-Control Process

A 250 ml Erlenmeyer flask containing 50 ml of LYCP-5 medium was inoculated aseptically with 1 ml of a thawed culture stock. This first stage seed culture was incubated at 25°C with 220 rpm agitation for 3-5 days. A 1 ml aliquot of the first stage seed was transferred to a second 250 ml Erlenmeyer flask containing 50 ml of LYCP-5 medium. This second stage seed culture was incubated as above for 3 days.

Table 1: LYCP-5 seed medium

Component	Concentration (per liter)
KH_2PO_4	9 g
Lactic acid (80-90%)	2 mL
Glucose	25 g
Yeast Extract	5 g
Pharmamedia	10 g
$FeSO_4 \cdot 7H_2O$	10 mg
$MnSO_4 \cdot H_2O$	10 mg
$CuCl_2 \cdot 2H_2O$	0.25 mg
$CaCl_2 \cdot 2H_2O$	0.1 mg
H_3BO_3	0.56 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.19 mg
$ZnSO_4 \cdot 7H_2O$	2 mg

For each variable tested (i.e., treatment group), several 250 ml Erlenmeyer flasks each containing 25 ml FGY medium (Table 2) or a variation thereof (described below) were inoculated at 5% (v/v) with second stage seed. The flasks were incubated at 25°C with 220 rpm agitation for 14 days. The pH for each treatment group was adjusted as required by removing one flask from the group, adding acid or base to return the pH to 5.0-5.5, and then adding this same volume of sterile titrant to the remaining flasks in the group. Where required, a volume of a sterile fructose solution was added during the fermentation to maintain the residual concentration within a specific range.

Table 2: FGY production medium

Component	Concentration (per liter)
Monosodium glutamate	10 g
Fructose	125 g
Yeast Extract	8.3 g
KH_2PO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	0.4 g
Proline	15 g
MES Buffer	15 g
$FeSO_4 \cdot 7H_20$	8.3 mg
$MnSO_4 \cdot 7H_20$	8.3 mg
$CuCl_2 \cdot 7H_20$	0.21 mg
$CaCl_2 \cdot 7H_20$	0.83 mg
H_3BO_3	0.47 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.16 mg
$ZnSO_4 \cdot 7H_2O$	1.7 mg

Analysis of the pneumocandins produced was carried out by extracting the whole broth with organic solvent followed by chromatographic analysis using standard reverse phase and normal phase procedures. The titer of Pneumocandin B_0 is expressed as arbitrary "units". The levels of the structural analogues is expressed as a ratio percent of the amount of Pneumocandin B_0 produced.

Amino Acid Supplementation

On or about day 6 (i.e., mid-cycle) of the fermentation, sterile solutions of L-proline, *trans*-3-hydroxy-L-proline, *trans*-4-hydroxy-L-proline, threonine, serine, arginine, ornithine or glutamine were added to the fermentation to give appropriate final concentrations. Pneumocandin extraction and analysis was carried out after 14 days of fermentation.

Increasing the proline concentration in the base medium (0-15 gm/l) resulted in a dose-dependent reduction in the levels of Compounds X and XI while the level of Compound VI increased as a function of proline concentration (Table 3). A

15 gm/l addition of proline to each of these treatments on or around day 6 resulted in comparable titers for each treatment but was unable to off-set the effects of the initial level of proline in the medium.

Table 3: Effect of varying the initial proline concentration in the base medium

Compound	0 gm/L Proline	5 gm/L Proline	10 gm/L Proline	15 gm/L Proline
I	100 units	112	105	106
X	7.0 %	5.8	5.1	4.4
VI	2.1 %	2.5	2.6	2.7
XI	0.9 %	0.5	0.4	0.3
IX	2.6 %	3.6	3.4	3.7

The mid-cycle addition of hydroxyprolines impacted the fermentation as well (Table 4). A 5 gm/L addition of *trans*-3-hydroxy-L-proline resulted in a 50% improvement in titer with the levels of Compounds X, VI and XI reduced dramatically. Conversely, a 5 gm/L addition of *trans*-4-hydroxy-L-proline resulted in a doubling of the level of Compound X with minimum impact on the other analogues or the titer of Pneumocandin B_0.

Table 4: Effect of 15 gm/L *trans*-3 and *trans*-4-hydroxyproline added on or about day 6

Compound	Control Process	*trans*-3-hydroxyproline	*trans*-4-hydroxyproline
I	100 units	140	110
X	4.8 %	0.1	9.4
VI	2.7 %	0.7	2.4
XI	0.5 %	0.2	0.7
IX	2.7 %	4.3	2.5

Amino acids such as glutamine, arginine, and ornithine which can be metabolized to Δ^1-pyrroline-5-carboxylate (P5C) also appear to have an impact on the analogues which are defined by the specific amino acid incorporated at the position "occupied" by 3-hydroxyproline in pneumocandin B_0 (Table 5).

Table 5: Effect of proline "related" amino acids added (5 gm/L) on or about day 6.

Compound	Control Process	Arginine	Ornithine	Glutamine
I	100 units	105	97	107
X	6.5 %	9.8	6.6	8.1
VI	2.9 %	2.2	1.8	4.0
XI	0.9 %	1.2	0.9	0.5

Supplementation of the medium with 5 gm/L threonine or serine resulted in a complete elimination or large increase in the level of Compound IV respectively (Table 6). In both cases, the titer of Compound I was reduced by 30%. Additional work has shown that 1 gm/L threonine is sufficient to maintain Compound IV at acceptable levels while having no impact on the titer of Compound I.

Table 6: Effect of adding 5 gm/L serine or threonine on or about day 6

Compound	Control Process	Threonine	Serine
I	100 units	70	70
IV	2.2 %	0	17.4

Effect of Trace Elements

Several trace elements were examined for their impact on the titer of Compound I and the spectrum of structural analogues produced. When added at concentrations equal to the ferrous salt, zinc, cobalt, and nickel salts had the most

pronounced effects (Table 7). Zinc reduced the titer of Compound I by 50% and doubled the level of Compound VI. Cobalt affected a 25% reduction in the titer of Compound I while increasing the levels of Compounds VI, VIII, IX and V. The addition of nickel had no impact on the titer of Compound I but increased the level of Compound V.

Table 7: Effects of trace elements

Compound	Control Process	Zinc	Cobalt	Nickel
I	100 Units	50	75	100
II	4.8 %	3.9	7.2	4.4
VIII	0.5 %	0.8	2.5	0.7
IV	2.8 %	1.9	6.0	3.1
V	1.6 %	2.8	14.1	7.5
VI	2.2 %	4.3	1.8	1.7

Osmolarity

In this fermentation, osmolarity can be controlled by maintaining the residual fructose concentration at high (>75 gm/L) or low (<30 gm/L). The initial fructose concentration in the control process is 125 gm/L and is kept high by making two 50 gm/L additions during the 14 cycle. Alternatively, the initial fructose concentration can be lowered to 40 gm/L and several 25 gm/L additions made during the course of the fermentation to maintain a low residual sugar level. When the "low" fructose process is run, there is a increase in the titer of Compound I along with an increase in the level of Compound X (Table 8). This increase in the level of Compound X can be offset by adding an inorganic salt such as sodium chloride or sodium sulfate. The addition of inorganic reduces the effects of running at a reduced concentration of fructose. These results suggest that osmolarity plays a role in pneumocandin synthesis.

Table 8: Effect of osmolarity

Compound	Control Process	Low Fructose, No Salt	Low Fructose, 150 mM NaCl	Low Fructose, 116 mM Na_2SO_4
I	100 units	134	111	110
X	4.3 %	8.2	5.5	6.1

In summary, hydroxylation patterns of amino acids of Pneumocandin B_0 are sensitive to zinc, cobalt and nickel. Additionally, amino acid additions to the production medium have a direct effect on the pneumocandins produced by the fermentation. Supplementation of the production medium with proline, *trans*-3-hydroxyproline and *trans*-4-hydroxyproline effects the incorporation of *trans*-3 or *trans*-4-hydroxyproline residues in Pneumocandin B_0. The addition of threonine to the fermentation controls the level of the serine analogue, Compound IV.

Thus, the impact of amino acids and trace elements on the fermentation provides insights into factors affecting the biosynthesis of Pneumocandin B_0 (Compound I) and has provided for an improved fermentation process by decreasing the levels of structural analogue and increasing the titer of Pneumocandin B_0.

EXAMPLE 2

Preparation of the Compound of Formula I

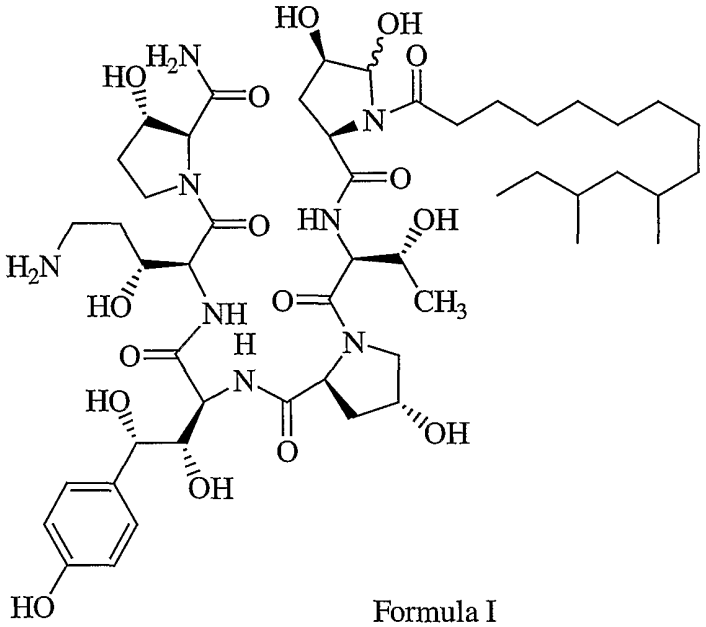
Part A. Pneumocandin B_0 (15.9 g, 89% area % pure, 3.4 wt % water, 0.0128 mol) was added to dry THF (0.64 L) and the suspension was dried to <10 mol% water by refluxing through a bed of 3 Å molecular sieves. Additional dry THF was added to reconstitute the mixture to the original volume and the suspension was cooled to <4° C. with an ice/water/methanol bath. Neat $BH_3{\cdot}SMe_2$ (10.91 g, 0.144 mol) was added over ten minutes and the reaction mixture was monitored by HPLC until the ratio of starting material to product was 1:1 indicating the end of the reaction (3.5 h). At 4 hours, the mixture was cooled to –12° C. and slowly quenched with 2N HCl (0.036 L. This solution was diluted to 1.14 L with water. The assay yield of Compound II was 6.60 g (47%). The quenched solution was diluted to 4 L with water and loaded onto a

medium-pressure column of LiChroprep RP-C18 adsorbent (158 g). After loading, the column was washed with 1.2 L of water and the amine was eluted with 1.9 L of 1:4 v/v acetonitrile/water, and then 0.38 L of 1:3 v/v acetonitrile/water. The rich cuts (>80 area %) were combined and diluted with water to a 1:7.3 v/v acetonitrile/water (1.70 L total). This mixture was loaded to the same column described above, and the column was washed with 0.57 L of water. The desired compound was eluted with 0.57 L methanol. The rich cut fractions (>85 area %) were combined and concentrated by rotary evaporation and static high vacuum to give 6.81 g (87 wt % pure, 6.8 wt % water) containing 5.92 g of Compound 2 hydrochloride salt for an isolated yield of 43%. Partial ^{1}H NMR (400 MHz, CD_3OD): δ 7.12 (d, 2H), 6.75 (d, 2H), 5.18 (d, 1H), 4.97 (d, 1H), 1.19 (d, 3H), 0.89 (t, 3H), 0.86 (d, 6H). Mass spectrum (FAB) m/z $(M+Li)^+$: 1058.

Part B. Compound 2 (0.220 g, 0.202 mmol), as recited in Scheme 1, was dissolved in 1:1 v/v acetonitrile/water (8 mL). Triethylamine (0.060 mL, 0.43 mmol) was added and the clouded mixture was stirred at room temperature overnight. In the morning, an additional 0.060 mL of triethylamine was added, and the mixture was stirred an additional 2 hours. HPLC analysis (45:55 v/v acetonitrile/water/0.1% TFA, Zorbax C18, 1.5 mL/min) showed a preponderance of the desired product (RRT 5.68 min) over the starting material (RRT 4.83 min). The reaction mixture was acidified with 0.5 mL of glacial acetic acid and concentrated in vacuo. Purification by preparative HPLC (42:58 v/v acetonitrile/water/0.1% TFA, Waters DELTAPAK C-18 19X300 mm, 12 mL/min) gave the compound of Formula I as a mixture of two isomers at the hemiaminal center (98% pure by analytical HPLC). Partial ^{1}H NMR (400 MHz, CD_3OD): δ 7.20 and 7.18 (d, 2H), 6.74 (d, 2H), 5.32 and 5.27 (d, 1H). Mass spectrum (ESI) m/z $(M+H)^+$: 1051.7.

WHAT IS CLAIMED IS:

1. A method for treating a fungal infection comprising administering to a mammalian subject in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt,



Formula I

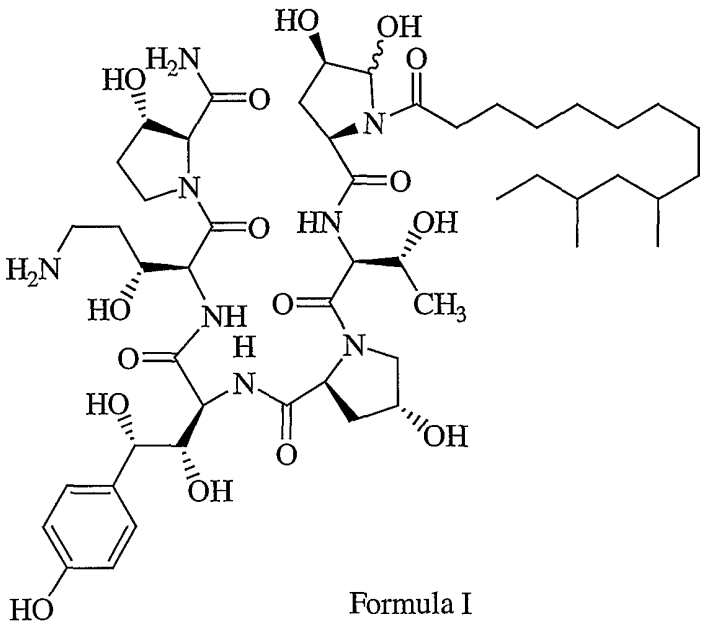
2. The method as recited in Claim 1, wherein the compound of Formula I is administered as a pharmaceutical composition of said compound with a pharmaceutically acceptable carrier.

3. The method as recited in Claim 2, wherein the pharmaceutical composition is administered parenterally or orally.

4. The method as recited in Claim 3, wherein the pharmaceutical composition is administered parenterally.

5. The method as recited in Claim 4, wherein the pharmaceutical composition is administered by intravenous infusion.

6. A method for controlling mycotic infections comprising administering to a mammalian subject in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt,



Formula I

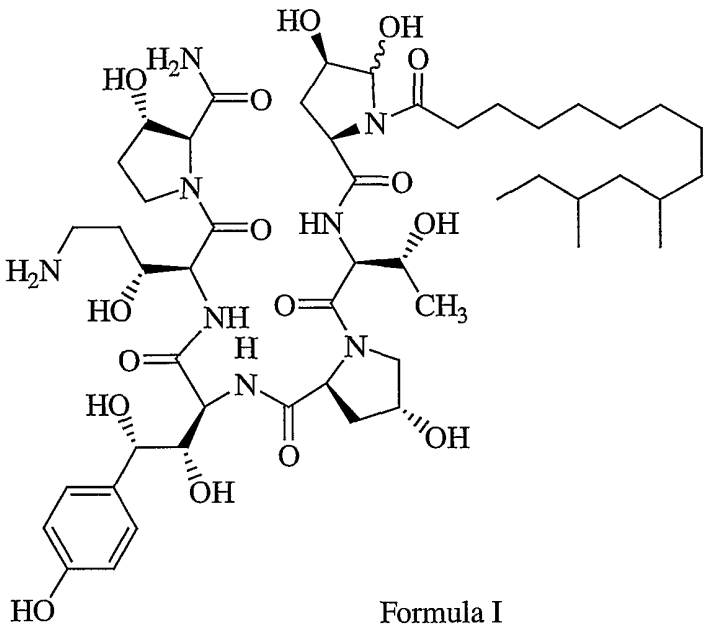
7. The method as recited in Claim 1, wherein the compound of Formula I is administered as a pharmaceutical composition of said compound with a pharmaceutically acceptable carrier.

8. The method as recited in Claim 7, wherein the pharmaceutical composition is administered parenterally or orally.

9. The method as recited in Claim 8, wherein the pharmaceutical composition is administered by parenterally.

10. The method as recited in Claim 9, wherein the pharmaceutical composition is administered by intravenous infusion.

11. A method for controlling pneumocystis pneumonia comprising administering to an immune-comprised patient in need of such treatment, an effective amount of a compound of Formula I or its pharmaceutically acceptable salt,



Formula I

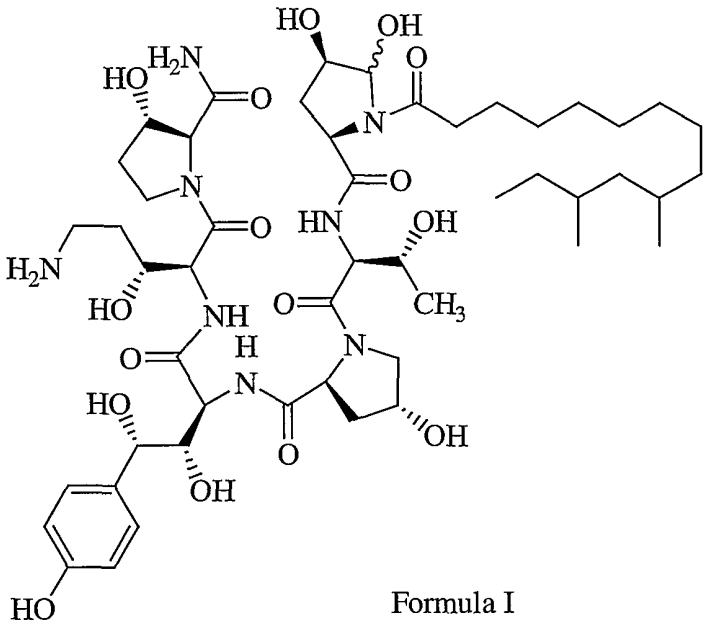
12. The method as recited in Claim 11, wherein the compound of Formula I is administered as a pharmaceutical composition of said compound with a pharmaceutically acceptable carrier.

13. The method as recited in Claim 1, wherein the pharmaceutical composition is administered parenterally or orally.

14. The method as recited in Claim 1, wherein the pharmaceutical composition is administered by parenterally.

15. The method as recited in Claim 1, wherein the pharmaceutical composition is administered by intravenous infusion.

16. A pharmaceutical composition comprising a compound of Formula I or its pharmaceutically acceptable salt



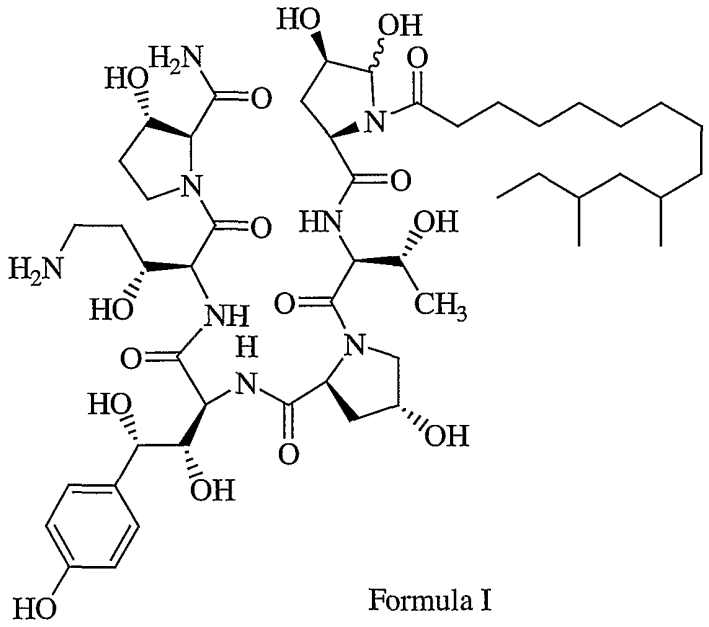
Formula I

and a second antifungal agent selected from the group consisting of: azoles or polyenes, purine or pyrimidine nucleotide inhibitors, chitin inhibitors, elongation factor inhibitors, mannan-binding antifungal agents, bactericidal/permeability-inducing (BPI) protein products, and complex carbohydrate antifungal agents.

17. The pharmaceutical composition as recited in Claim 16, wherein the second antifungal agent is the azole: fluconazole, voriconazole, itraconazole, ketoconazole, miconazole, ravuconazole, or posaconazole.

18. The pharmaceutical composition as recited in Claim 16, wherein the second antifungal agent is the polyene: amphotericin B, nystatin or liposomal, ABELCET, AMBISOME or AMPHOCIL.

19. A method of treating a fungal infection comprising administering to a mammalian subject in need of such treatment an effective amount of a combination of a compound of Formula I or a pharmaceutically acceptable salt thereof,



Formula I

and a second antifungal agent selected from the group consisting of: azoles or polyenes, purine or pyrimidine nucleotide inhibitors, chitin synthesis inhibitors, elongation factor inhibitors, mannan-binding antifungal agents, bactericidal/permeability-inducing (BPI) protein products, and complex carbohydrate antifungal agents.

20. The method of treatment as recited in Claim 19, wherein the administration of said combination is sequential, simultaneous or concomitant.

21. The method of treatment as recited in Claim 20, wherein the second antifungal agent is the azole: fluconazole, voriconazole, itraconazole, ketoconazole, miconazole, ravuconazole, or posaconazole.

22. The method of treatment as recited in Claim 20, wherein the second antifungal agent is the polyene: amphotericin B, nystatin or liposomal, ABELCET, AMBISOME or AMPHOCIL.