June 25, 1957

E. L. HAZEN ET AL

2,797,183

NYSTATIN AND METHOD OF PRODUCING IT

Filed Jan. 7, 1952

2 Sheets-Sheet 1

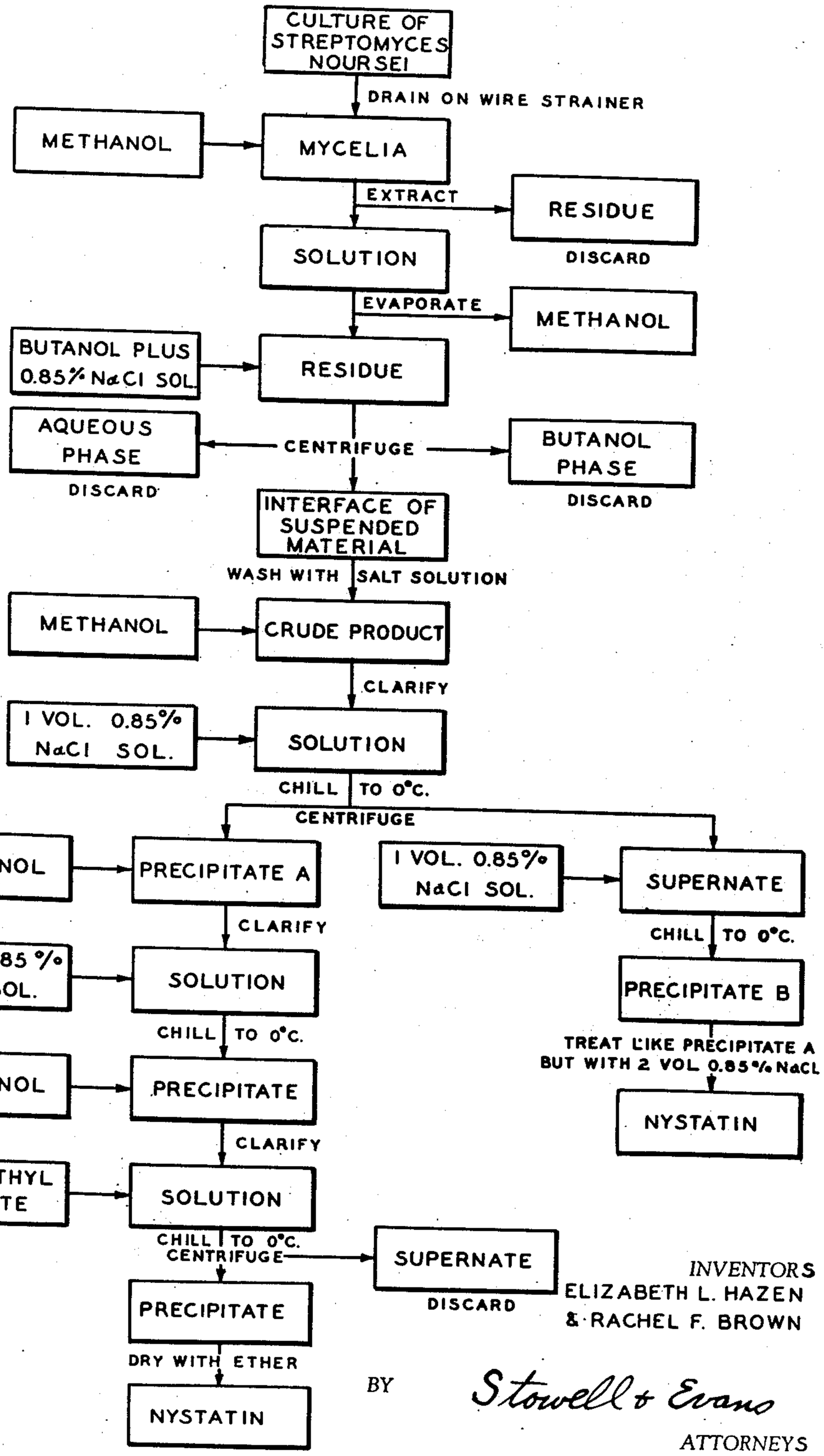

Fig. 1

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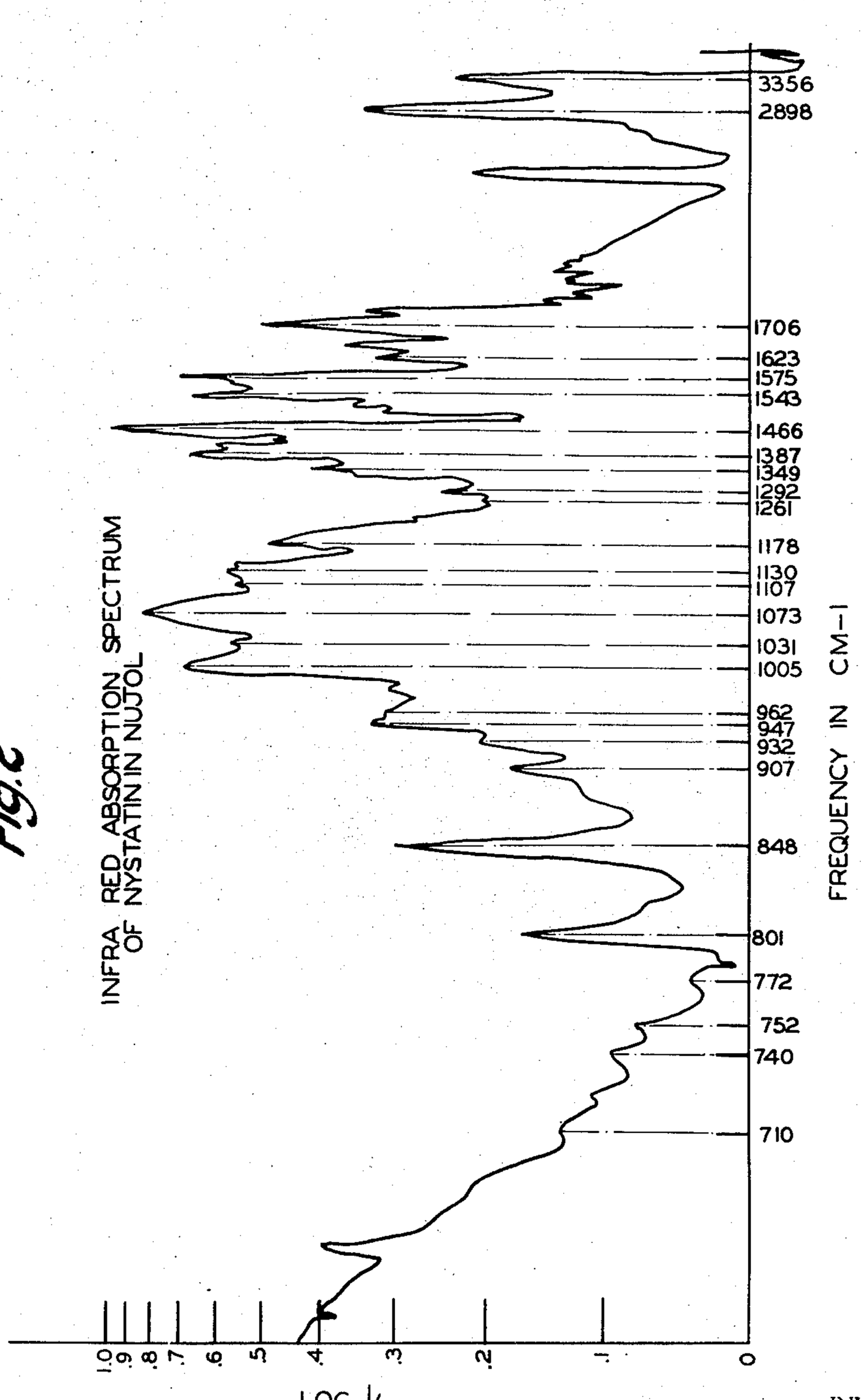
Fig. 2



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United States Patent Office

2,797,183

Patented June 25, 1957

2,797,183

NYSTATIN AND METHOD OF PRODUCING IT

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Application January 7, 1952, Serial No. 265,293

8 Claims. (Cl. 167—65)

This invention relates generally to antibiotics and their production, and more particularly to a new and useful antibiotic and methods of producing it.

Most antibiotics known at the present time are characterized by high antibacterial activity with little or no antifungal activity. In an investigation of newly isolated actinomycetes for antagonists to pathogenic fungi an organism has been found which when cultivated under particular controlled conditions yields an apparently-intracellular antifungal antibiotic, which is both fungistatic and fungicidal and apparently lacks antibacterial activity.

The organism belongs to a species under the genus Streptomyces, the particular strain having been isolated from a farm soil in Fauquier County, Virginia. The species has been designated as *"Streptomyces noursei."* A pure culture of the organism has been filed with the "Collection of Type Cultures" of the Division of Laboratories and Research, New York State Department of Health, Albany, New York, under the number 48240.

Cultures of the organism have also been filed with the American Type Culture Collection, wherein they have been assigned the identifying designation: ATCC 11455.

The antibiotic has been designated as "nystatin" (formerly designated "fungicidin"). It is a quite stable, relatively water-insoluble compound which is relatively nontoxic in antibiotically-effective doses and active in vivo and in vitro against a wide variety of saprohytic and pathogenic fungi.

Nystatin is obtainable by the following general procedure: the organism *Streptomyces noursei* is grown in contact with (i. e., in or on) a sterile liquid nutrient medium containing a source of assimilable carbon and nitrogen, and the nystatin formed is recovered, preferably from the mycelium (i. e. organism growth). In the preferred method, the mycelium is first separated from the culture liquid; the separated mycelium is extracted with an organic solvent for nystatin, especially a lower aliphatic alcohol; and the solution of nystatin thus obtained is treated to further purify and concentrate the nystatin therein. Alternatively, the mycelium may be extracted while in the culture liquid, in which case the organic solvent for nystatin employed must be substantially water-insoluble.

In the accompanying drawings

Fig. 1 is a flow sheet of a typical isolation and recovery procedure as described in Example 1; and

Fig. 2 is an infra-red absorption spectrum of the new antibiotic.

The organism *Streptomyces noursei* is characterized by straight, curved and wavy mycelium, and by round and oval spores produced in chains from curved and spiral-forming hyphae (loose, closed spirals). When grown on Sabouraud's glucose agar at 28±1° C. for seven days, the colony is heaped and folded; the aerial mycelium is at first white, then later (at sporulation) gray; and on the under surface, the color is at first light tan, and after two weeks it is usually dark brown and the medium

is darkened throughout. There is no hemolysis on blood agar medium, but the medium is darkened.

The organism *Streptomyces noursei* is also characterized by the following:

Odor: strong earthy.

Gelatin (12%): rapid liquefaction.

Sabouraud's glucose agar:

(At 35–6° C.)—good growth, heaped and folded; white aerial mycelium, infrequently becoming rusty pink; in about 50% of the cultures, a diffusible pigment of pomegranate purple color (Ridgeway's color standards, Plate 12) is produced.

(At 28±1° C.)—good growth, heaped and folded; white aerial mycelium becoming gray; on reverse of growth, pigment is brown and medium is often darkened throughout. On one occasion in two years a diffusible pigment, pomegranate purple, was produced in one culture.

(At 40° C.)—slight growth, glabrous.

Szapek's agar:

(At 35–6° C.)—no growth.

(At 28±1° C.)—growth very scanty; no aerial mycelium; colony small, colorless, flat and deeply embedded.

Asparagin-glucose-agar (synthetic):

(At 35–6° C.)—growth cerebriform in center, with finely-wrinkled periphery; aerial mycelium shell-pink and scant; under surface of growth dark gray; small amount of diffusible, shell-pink pigment produced.

(At 28±1° C.)—good growth; gray and white knob-like projections on a background of more flattened and wrinkled tan growth; on under surface pigment is dark gray.

Ammonium chloride-glucose-agar (synthetic):

(At 35–6° C.)—growth cream-colored; slightly heaped and folded in center, and flattened at periphery; no pigment on under surface; no diffusible pigment.

(At 28±1° C.)—growth slightly heavier than at 35–6° C., but otherwise the same.

Hydrolyzed casein-glucose-agar:

(At 35–6° C.)—growth heaped and folded in center, flattened at periphery; aerial mycelium has tannish tint; no diffusible pigment.

(At 28±1° C.)—good growth; dark-gray aerial mycelium; under surface dark gray; no diffusible pigment.

Cellulose agar (both at 28±1° C. and 35–6° C.): growth very poor.

Starch agar (both at 28±1° C. and 35–6° C.): good growth of discrete colonies with white aerial mycelium in center and periphery colorless and embedded; starch hydrolyzed.

Blood agar (both at 28±1° C. and 35–6° C.): good growth; colonies convex, lobate, with central perforation; heavy, white, chalky aerial mycelium; no hemolysis, but blood is darkened; occasionally glabrous colony found, which may show pink pigment; on transfer to Sabouraud's dextrose, such colony may or may not develop white aerial mycelium.

Infusion agar (both at 28±1° C. and 35–6° C.): good growth of dry, rough, discrete, buff-colored, convex colonies with depressed centers, some showing chalky aerial mycelium; no diffusible pigment.

Milk (both at 28±1° C. and 35–6° C.): coagulation, followed by peptonization.

Potato plug:

(At 35–6° C.)—good growth, heaped and folded, chiefly glabrous; reddish-purple pigment, potato slightly pink in color.

(At 28±1° C.)—growth heaped and folded, with white, chalky, aerial mycelium; no pigment.

Carrot plug:

(At 35–6° C.)—growth heaped and folded, chiefly glabrous; small amount of dirty-white mycelium; no pigment.

(At 28±1° C.)—growth heaped and folded, with white, chalky aerial mycelium.

Nitrites: trace produced from nitrates.

Infusion broth (with 1% glucose, at 28±1° C.): white growth on surface; flocculent sediment; clear broth.

Honey broth:

(At 35–6° C.)—small amount of white growth on surface; later heavy white collar, flocculent sediment.

(At 28±1° C.)—heavy white collar; flocculent sediment; clear broth.

Glucose-tryptone-agar:

(At 35–6° C.)—growth dry, slightly heaped and folded in center and more flattened on periphery; aerial mycelium cream-colored; under surface pinkish; small amount of light-pink diffusible pigment.

(At 28±1° C.)—growth heavier, more heaped and folded than at 35–6° C.; aerial mycelium powdery and gray; under surface tannish-brown; small amount of tannish-green diffusible pigment.

Glucose-tryptone broth plus 0.1% agar:

(At 28±1° C.)—surface of medium covered with heavy growth, aerial mycelium heaped, white turning to gray; soluble pigment, pomegranate purple, rarely produced; H_2S produced; slight amount of acid produced.

(At 35–6° C.)—type of growth same as at 28° C., but less abundant; soluble pigment, pomegranate purple, frequently produced.

The organism *Streptomyces noursei* may be grown to produce nystatin either under stationary or surface (aerobic) conditions or under submerged aerobic conditions, the latter being effected by means of agitation and aeration, as well known in the art. The time required for maximum yield of systatin varies with the method of cultivation. In the case of surface culture, elaboration of nystatin is usually complete in about 5–7 days; and in the case of submerged culture, in about 3–4 days. The optimal temperature for production of nystatin is around 28° C., the yield of nystatin dropping off at higher and lower temperatures of cultivation; and the starting pH may range between about 5.5 and about 7.5, but is preferably about 7.0–7.2.

Various liquid nutrient media containing sources of assimilable carbon and nitrogen may be employed. The assimilable carbon source may be an assimilable polyhydric alcohol (e. g., glycerol) or (preferably) an assimilable saccharide (mono-, di-, or poly-; e. g., glucose). The assimilable source of nitrogen is preferably an assimilable proteinaceous material, especially a protein degradation or hydrolysis product (e. g., tryptone). Other utilizable saccharides include maltose and lactose; and other utilizable proteinaceous materials include yeast extract, malt extract, casein digest, soybean digest, and corn steep liquor. Preferably, also, the medium includes added essential salts, where necessary, inter alia, potassium, sodium, magnesium and iron salts.

The concentration and purification of nystatin may be accomplished in a number of ways, representative methods being described in detail hereinafter.

The new antibiotic shows no pronounced basic or acidic properties. No known salts have been isolated. The antibiotic decomposes when heated in aqueous solutions of acids or alkalis. In methanol solution it withstands a temperature of 55° C. for 1 hour at pH 5 to 8. It contains the elements C, H, and N. Chemical analysis shows carbon 58.13%; hydrogen 8.77% and nitrogen 1.76%. All of the nitrogen appears to be in the amine form. The substance shows no optical rotation. In 0.025% solution in methanol the ultraviolet absorption curve gave

maximum absorption at 2350 A., 2914 A., 3043 A., and 3193 A. at E=298, 536, 754, and 596 respectively.

The infrared spectrum was obtained on a sample of highly purified nystatin suspended in hydrocarbon oil using a Perkin Elmer model 12B spectrometer. The bands obtained are listed below in terms of their frequencies (cm.$^{-1}$).

(cm.$^{-1}$)	(cm.$^{-1}$)
3,356	1,073
2,898	1,031
1,706	1,005
1,623	962
1,575	947
1,543	932
1,466	907
1,387	848
1,349	801
1,292	772
1,267	752
1,178	740
1,130	710
1,107	

The antibiotic is almost insoluble in water. It is soluble in methanol to the extent of 0.25 mg./ml., in methanol N/50 hydrochloric acid to the extent of 5 mg./ml., in 70% methanol to the extent of 0.5 mg./ml., in 70% ethanol to the extent of 2 mg./ml., and in 60% acetone to the extent of 0.25 mg./ml. In N hydrochloric acid it dissolves to the extent of 0.45 mg./ml., and in N sodium hydroxide to the extent of 5 mg./ml. The solubility decreases with the purity of the product. It starts to decompose about 155° and does not melt when heated up to 295°.

The new antibiotic shows reducing properties in that Benedict's solution is reduced; bromine water, and iodine-potassium iodide solution are decolorized by it. The following colors appear with concentrated acids: red violet with sulfuric; pink with phosphoric; yellow with hydrochloric. It gives no biuret or ninhydrin reaction. It produces no color with ferric chloride.

IN VITRO ACTIVITY OF NYSTATIN

Nature of tests.—(All tests performed with a stock suspension of partially-purified nystatin containing 5 mg./ml., obtained by dissolving the nystatin in cold N/50 hydrochloric acid and adjusting immediately to pH 7.0–7.2 with cold 0.1% sodium carbonate solution, the stock suspension being kept in a refrigerator and used for only 72 hours.)

(1) Determination of the sensitivity of *Cryptococcus neoformans, Candida albicans,* and other fungi to nystatin: Serial 2-fold dilutions are made in 4.5 ml. glucose-tryptone broth and heated for ten minutes at 70° (for sterilization). To two series of tubes is added, respectively, 0.5 ml. of a saline suspension of *C. neoformans* and *C. albicans* in concentrations of 1,000,000 and 300,000 cells/ml. Readings of visible growth are made after 5, 24, 72 and 96 hours' incubation at 28°, and sensitivity recorded as the least amount of nystatin inhibiting growth, as evidenced by absence of gross turbidity after 96 hours.

(2) Determination of the antimicrobial activity of nystatin: The agar dilution method of Reilly, Schatz and Waksman (J. Bact., 1945, v. 49, 585–94) is used, except that infusion agar is employed for the bacterial cultures and the temperature of incubation is 35–6°.

(3) Further determination of the effect of nystatin on the growth of *C. neoformans*: Two sets of serial 2-fold dilutions in glucose-tryptone broth are inoculated, respectively, with 10 million and 1 million microorganism/ml. After incubation at 3, 4, 5, 24 and 48 hours, aliquot portions are removed and plated on glucose-tryptone agar; and colony counts are made after 48 hours incubation.

(4) Determination of the effect of defibrinated horse blood and serum: 0.1 ml. of nystatin suspension diluted serially in sterile saline so as to contain from 100–3.13

micrograms is added to a series of tubes containing 0.25 mil. whole blood or serum, and to each dilution is added 0.05 ml. of a 48-hour glucose-tryptone broth culture of *C. neoformans* containing 26 million microorganisms/ml. The tubes are then incubated for 48 hours with frequent shaking, after which the contents are streaked on plates of Sabouraud's glucose agar which are then incubated for 48 hours.

Results of tests.—Some results of these tests are given in Tables I and II, hereinafter. They indicate that nystatin is strongly fungistatic against a wide variety of saprophytic and pathogenic forms, amounts of the order of 1.56–6.25 micrograms/ml. being effective against many fungi.

TABLE I

[Fungistatic action of nystatin.]

Test species	Sensitivity: micrograms of nystatin per ml. at 28±1° C.	
	72 hrs.	96 hrs.
C. neoformans	1.56	1.56
C. albicans	3.13	3.13

TABLE II

[Antifungal spectrum of nystatin.]

Fungi	Strain No.	Least amount inhibiting growth, micrograms/ml.
Cryptococcus castellanii	45232	3.13
Cryptococcus glutinis	4676	1.56
Candida guilliermondi	45211	3.13
Candida krusei	45214	6.25
Candida stellatoidea	45213	3.13
Saccharomyces cerevisiae	45217	3.13
Sporobolomyces salmonicolor	4550	3.13
Schizosaccharomyces octosporus	45231	1.56
Endomycopsis fibuliger	45230	3.13
Geotrichum lactis	47462	6.25
Aspergillus fumigatus	50248	6.25
Penicillium notatum	43281	3.13
Penicillium sp	50526	13
Penicillium claviforme	49470	3.13
Rhizopus nigricans	50524	3.13
Fusarium sp	50527	3.13
Alternaria sp	50523	1.56
Cephalosporium sp	45226	25
Phoma sp	50522	6.25
Ceratostomella ulmi (plant pathogen)	50525	6.25
Hormodendrum sp	4893	3.13
Histoplasma capsulatum (yeastlike)	4894	1.56
Blastomyces dermatitidis (yeastlike)	45223	1.56
Paracoccidioides brasiliensis (yeastlike)	45224	1.56
Coccidioides immitis (spherules)	50521	6.25
Cryptococcus neoformans	45215	1.56
Candida albicans	4657	3.13
Trichophyton mentagrophytes	45141	6.25
Trichophyton rubrum	4516	6.25
Trichophyton rosaceum	4974	3.13
Epidermophyton floccosum	44253	1.56
Microsporum audouini	4896	3.13
Microsporum canis	4817	13
Sporotrichum schenckii (mycelial) (yeastlike)	50251	13
Monosporium apiospermum	45221	100
Allescheria boydii	48102	>100
Phialophora verrucosa	45229	13

Inhibition of growth of the following bacteria is not obtained even with 100 micrograms/ml. of the partially purified nystatin: *Staphylococcus aureus* (No. 45142), *Streptococcus hemolyticus* (No. 44131), *Bacillus subtilis* (No. 45137), *Bacillus cereus* var. *mycoides* (No. 48294), *Salmonella typhosa* (No. 38351), *Shigella paradysenteriae* (No. 45300), *Bacterium mucosum capsulatum* (No. 4767), and *Bacillus circulans* (No. 48205). Growth of a strain of *Mycobacterium tuberculosis* (No. 50539) freshly isolated from a human lung is not inhibited in Dubos medium containing 100 micrograms nystatin/ml.

Nystatin is strongly fungicidal (as well as fungistatic). In the presence of one million microorganisms (*C. neoformans*), 6.25 microgram/ml. mystatin brings about a progressive decline in the population and no viable cells are detected at 24 hours; and with 12.5 micrograms/ml., no viable cells are detected at 3 hours. With a tenfold increase in microorganisms, and 12.5 micrograms/ml. nystatin, no viable cells are detected at 4 hours.

Incubation of nystatin in the presence of defibrinated horse blood or serum gives no indication of diminishing or otherwise modifying its activity.

IN VIVO ACTIVITY OF NYSTATIN

Nature of tests.—All tests were performed with stock suspension described hereinbefore, diluted with sterile physiologic salt solution, and in white mice (Albany strain) of 20–25 g. which were infected by intraperitoneal and intravenous injections of saline suspensions of *C. neoformans* or intraperitoneal injections of *Histoplasma capsulatum* in large doses.

Results of tests.—(I. *Toxicity*).—The approximate LD50 of the partially-purified nystatin administered intraperitoneally is between 20 and 26 mg./kg.; injected subcutaneously, however, 2 g./kg. does not kill, but there is induration and necrosis at the injection site. Mice receiving 473 mg./kg. show no evidence of necrosis. Mice receiving a total of 735 mg./kg. over a period of four weeks show no ill effects, and compare favorably with an untreated control group in appearance and weight (the nystatin being administered subcutaneously or intraperitoneally in doses of 200–400 micrograms, five days each in the first and second weeks, four days the third week, and three days the fourth week).

(II. *Therapeutic activity*).—It was definitely established that mice injected with large amounts of *C. neoformans* or *H. capsulatum* and given repeated parenteral does of nystatin have a milder form of infection, and that life is prolonged beyond that of the controls.

The following examples are illustrative of the invention (all temperatures being in centigrade, all solutions not otherwise identified being aqueous, and all strain numbers given being those of the aforementioned collection of type cultures):

Example 1

(*a*) A glucose-tryptone medium is prepared with distilled water, the medium containing 1% glucose (of high purity, free of other sugars, starch, proteins, alcohol and heavy metals), 0.5% tryptone, 0.1% agar, 0.2% K_2HPO_4, 0.2% NaCl, and 0.001% $FeSO_4$, and being adjusted to pH 7.0–7.2 by addition of normal hydrochloric acid solution; and 400 ml. aliquots of the medium are placed in 3-liter Erlenmeyer or Fernbach flasks. The flasks are then plugged with cotton, sterilized by autoclaving at 15 lbs. pressure (121°) for 20 minutes, cooled, inoculated with a *Streptomyces noursei* inoculum (7–10 day old pellicle produced on the same medium), and incubated at 28±1° for five to seven days.

(*b*) The pellicle is harvested from 5- to 7-day-old cultures, drained on wire strainers, and macerated in a Waring blendor for 1 minute with methanol, 1 ml./g. The material is centrifuged at 10° C. and the extract removed. Two more extractions are made by stirring the residue for 1 hour with methanol in the same proportion. The extracts are chilled at 0±2° C. for at least 24 hours. They are then clarified by centrifugation at below 10° C. and the solvent removed in vacuo at below 45° C. Acetone may be substituted for methanol using pure solvent for the first extraction and 50% for subsequent extractions. The residue is suspended in 50 ml. each of butanol and 0.85% sodium chloride solution per 1–2 liters of extract and chilled at 0±2° C. for at least 24 hours. The suspension is separated by centrifugation at below 10° C. and filtration into 3 parts. The bottom aqueous phase and the top butanol phase are discarded. The insoluble material at the interface contains the major part of the nystatin. It is washed on the filter with 0.85% saline saturated with butanol to remove inert pigments, and next with 0.85% saline to remove butanol and more pigments. The washed residue is dissolved in a minimum of methanol using heat (temperature up to 55° C.) if

desired. The extract, clarified by centrifugation at below 10° C. and by filtration if necessary, is treated with an equal volume of 0.85% sodium chloride solution. After 2 hours at 0±2° C. the precipitate (A) is collected by centrifugation at below 10° C. and the supernate treated further with 0.85% sodium chloride solution up to a total of 2 volumes. After standing at least 24 hours at 0±2° C. the precipitate (B) is collected by centrifugation at below 10° C. Each of the two saline precipitates, designated A and B, respectively, is separately dissolved in methanol (with warming up to 55° C. if desirable) and reprecipitated with one and two volumes of 0.85% sodium chloride solution, respectively. After chilling to 0±2° C. the precipitates are collected by centrifugation at below 10° C. Each of the two saline precipitates is separately dissolved in methanol (with warming up to 55° C. if desirable), and reprecipitated with 4 volumes of ethyl acetate. After standing overnight at 0±2° C. the precipitates are collected and dried with ether. The dry products represent purified antibiotic. Both products are yellow; that obtained by one volume saline precipitation is more highly colored. The antibiotic may be recovered in one step by a single precipitation with two–three volumes of saline.

Example 2

(*a*) A medium is prepared, inoculated and incubated as described in Example 1 (*a*).

(*b*) The surface growth in each flask is then harvested, heated at 70–72° for ten minutes, and pressed between filter papers to remove adhering medium, the pressed mycelium containing about 70–83% moisture (as determined by loss on heating at about 105° for five hours). Methanol is then added to the pressed mycelium, in the proportion of 1.5–3 ml./g., the mixture is stirred in a Waring blender for a half minute at room temperature, centrifuged cold (below about 10°), and the supernatant decanted. The residue is extracted with the same amount of methanol for 2–3 hours at room temperature; and the residue obtained on centrifuging this mixture and decanting the supernatant is again extracted in the same manner. The pooled methanol extracts (supernatants) are chilled to 0–5°, centrifuged, and the resulting methanol solution of nystatin is recovered by decantation.

(*c*) Ethyl acetate is added to the methanol solution of nystatin, first in a proportion of 0.3–2.0 times the volume of the methanol to precipitate inert material (removed by filtration or centrifugation), then to a total of 4–5 times the volume of the methanol, and the resulting precipitate is recovered. The precipitate is then washed with 0.85% NaCl solution, dissolved in methanol, and the ethyl-acetate precipitation repeated. The precipitate thus obtained is again dissolved in methanol, and the solution mixed with 3–4 volumes ethyl ether; and the resulting precipitate is separated, washed with ether, dried and ground to a powder.

The product thus obtained in a yield of about 30–50 mg/liter. broth (a partially-purified nystatin) is a fine yellow powder that can be stored in the cold for several months without loss of activity. It is slightly soluble in methanol, 65–70% ethanol, wet butanol, 70% acetone, propylene glycol, ethylene glycol monomethyl ether, and water at pH 2–3 or 8–9; less soluble in 95% ethanol, butanol, and acetone; and insoluble in ether, chloroform, ethyl acetate, amyl acetate, benzene, petroleum ether (low boiling), and water (around neutrality). It contains about 1.5% Kjeldahl nitrogen (at that stage of purification), evidences no sulfur or halogen content, shows strong reducing properties, fails to react in tests for protein, and gives no color with ferric chloride; and its molecule is relatively small, being ultrafiltrable through fine gradocol membranes (thin membranes of collodion or similar substances graded as to porosity). Aqueous solutions of the product prepared at pH 2 and pH 9 are very unstable. The product may be used as a therapeutic agent without further purification, or may be further treated to concentrate and/or purify the nystatin therein.

Example 3

(*a*) A medium is prepared, inoculated, and incubated as described in Example 1(*a*).

(*b*) The whole culture is then mechanically stirred for 2–3 hours with ¼–1 volume of n-butanol at room temperature, and the n-butanol phase separated and centrifuged cold (if necessary). The aqueous phase is mechanically stirred for 2–3 hours with ⅛–½ volume n-butanol, the n-butanol phase separated, and the remaining aqueous phase again extracted in the same manner with n-butanol. The pooled n-butanol extracts are chilled to 0–5°, centrifuged, and the supernate decanted. The n-butanol is then removed from the supernate in vacuo at below 45°, the residue extracted with methanol, and the methanol extract is further treated as described for the methanol solution of nystatin in section *c* of Example 2. The partially-purified nystatin thus obtained is qualitatively the same as that obtained in Example 2.

Example 4

The medium employed in Example 1 is replaced by a glycerol-yeast extract broth, the fermentation, extraction and purification being the same as in Example 1. This medium is prepared with distilled water, and contains 2.5% glycerol, 0.3% yeast extract, 0.1% agar, 0.2% NaCl, 0.1% K_2HPO_4, 0.02% $MgSO_4$, and 0.001% $FeSO_4$; and it is adjusted to pH 7.0–7.2 by addition of normal sodium hydroxide solution, and sterilized by autoclaving at 15 lbs. pressure (121°) for 20 minutes. The nystatin preparation thus obtained is qualitatively the same as that obtained in Example 1 and described hereinbefore, but is obtained in lower yield.

Example 5

(*a*) A glucose-tryptone medium is prepared as described in Example 1(*a*) (except that the agar is omitted). 100 ml. aliquots of the medium are placed in 300 ml. Erlenmeyer flasks, and the flasks are plugged with cotton, sterilized by autoclaving at 15 lbs. pressure (121°) for 20 minutes. The flasks are then cooled, inoculated with a saline suspension of a 7–9 day old culture of *Streptomyces noursei* on Sabouraud's dextrose medium, and incubated at 28±1° for 3–4 days on a shaking machine which gives the flasks a rotary and tilting motion simulating that given by hand shaking.

(*b*) The whole culture is then treated as described in Example 1, the partially-purified nystatin thus obtained being qualitatively the same as that obtained in Example 1.

The following additional media, inter alia, may be employed for the submerged-culture production of nystatin as described in Example 5:

A. Medium described in Example 5 with agar omitted.

B. Medium described in Example 1 with agar omitted and with crude dextrose in place of the highly-pure dextrose.

C. Medium described in Example 1 with agar omitted and with crude maltose in place of the dextrose.

D. Medium described in Example 1 with agar omitted and with malt extract in place of the dextrose.

E. Medium described in Example 1 with agar omitted and with proteose peptone in place of the tryptone.

F. Distilled water containing following ingredients in percentages indicated:

Casein digest	1.7
Soybean digest	.3
NaCl	.5
K_2HPO_4	.25
Dextrose	.25

G. Distilled water containing following ingredients in percentages indicated:

Dextrose	2.0
KCl	.4
$CaCO_3$	.8
$(NH_4)_2SO_4$	.5
Dried brewer's yeast	.25
KH_2PO_4	.02

The following other variations may be made in the procedure described in Example 2 without qualitatively affecting the production and recovery of nystatin:

1. Heating of the surface growth after harvesting may be omitted.

2. Amyl acetate may be used in place of ethyl acetate.

3. Extraction may be effected by grinding the surface growth with the methanol, or percolating methanol through the surface growth.

4. n-butanol, ethanol, or a mixture of equal parts of methanol and ethanol may be substituted for the methanol.

The production of nystatin may, of course, be effected on a larger scale, utilizing where applicable the well-known techniques and apparatus employed in the commercial, large-scale production of other antibiotics, both by stationary or submerged culture.

The invention may be variously otherwise embodied within the scope of the appended claims.

This application is a continuation-in-part of our application Serial No. 208,978 filed February 1, 1951, now abandoned.

We claim:

1. The method of producing a fungistatic substance from the organism *Streptomyces noursei* grown in contact with a sterile liquid nutrient medium containing sources of assimilable carbon and nitrogen, which comprises separating the mycelium of the organism from the culture medium, extracting the separated mycelium with a solvent of the group consisting of lower alcohols and lower alkyl ketones, and recovering from the extract the fungistatic substance extracted thereby from the mycelium.

2. The method as defined in claim 1 wherein the fungistatic substance is recovered from the extract by evaporating the solvent.

3. The method as defined in claim 1 wherein the fungistatic substance is precipitated from the extract by the addition of an aqueous solution of sodium chloride.

4. The method as defined in claim 1 wherein the fungistatic substance is precipitated from the extract by the addition of a lower alkyl acetate.

5. The mehod as defined in claim 1 wherein the fungistatic substance recovered from the extract is purified by repeated solution in a lower alcohol and precipitation from the solution with an aqueous solution of sodium chloride.

6. The method as defined in claim 1 wherein the fungistatic substance recovered from the extract is purified by suspending it in a mixture of butanol and an aqueous solution of sodium chloride, and thereafter separating the insoluble material including the fungistatic substance at the interface between the butanol phase and the aqueous phase of the mixture.

7. The method as defined in claim 1 wherein the mycelium is extracted with methanol, the methanol extract is evaporated leaving a solid residue, the solid residue is treated with a mixture of butanol and an aqueous solution of sodium chloride, the solid material is separated from the interface between the butanol and the aqueous phases of the mixture and is thereafter subjected to repeated solution in methanol and precipitation with an aqueous solution of sodium chloride.

8. An antifungal substance obtained from the mycelium of a culture of *Streptomyces noursei,* which is relatively insoluble in water, sparingly soluble in the lower alcohols and lower alkyl ketones, contains carbon, hydrogen and nitrogen, gives no color with ferric chloride, fails to react in tests for protein, is active in vivo and in vitro against many saprophytic and pathogenic fungi and exhibits characteristic absorption bands in the infrared region of the spectrum when suspended in solid form in a hydrocarbon oil at the following frequencies expressed in reciprocal centimeters: 3356, 2898, 1706, 1623, 1575, 1543, 1466, 1387, 1349, 1292, 1267, 1178, 1130, 1107, 1073, 1031, 1005, 962, 947, 932, 907, 848, 801, 772, 752, 740, and 710.

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