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PATENT SPECIFICATION

DRAWINGS ATTACHED

L083.662

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COMPLETE SPECIFICATION

A Novel Antibiotic, methods for its production and
Therapeutic Compositions containing it

We, SCHERICO LTD., of Winkelriedstrasse 56, Lucerne, Switzerland, a body corporated constituted under the laws of Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for the production of a novel antibiotic possessing useful activity as a therapeutic agent, which is especially effective in combatting infections engendered by gram-positive organisms, to the novel antibiotic itself and to therapeutic compositions in which it is present. More particularly, this invention relates to a process for the fermentation production of a novel antibiotic in which there is utilized a hitherto undescribed variant of the micro-organism *Micromonospora halophytica* of the order *Actinomycetales*, to the novel antibiotic itself and to therapeutic compositions in which it is present.

When considered from one of its aspects, this invention provides a process for the production of a novel antibiotic, herein for convenience referred to as antibiotic No. 467 or simply No. 467, which comprises incubating the aforementioned variant of *Micromonospora halophytica* or an antibiotic No. 467-producing mutant thereof in contact with an aqueous nutrient medium containing assimilable carbon and nitrogen until a product having substantial antibiotic activity is produced, and thereafter isolating the antibiotic No. 467 from the product.

Information relating to this process, with specific reference to some of the more important features associated therewith, is given in the appropriately identified passages which follow.

of this invention is a variant of *Micromonospora halophytica* which has been designated *M. halophytica* var. *nigra*, and it will be referred to as such herein. A strain of this micro-organism found particularly convenient for use in the process was isolated from mud at the bottom of a salt pool in Syracuse, New York. A culture of the living organism from this source has been deposited, and made a part of the stock culture collection of the United States Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois, from which it is available under the NRRL number assigned thereto, namely NRRL 3097.

M. halophytica var. *nigra* is characterized by a long branched mycelium of approximately 0.5 microns in diameter and abundant sporulation. The spores are ellipsoidal to spherical and average 1.2 microns in the large diameter. In older cultures, they appear smooth walled and dark colored. The spores appear to be borne randomly along the mycelial length on short or long sporophores; the spores are occasionally sessile. The vegetative mycelium does not normally break up into polymorphic elements but appears to retain its integrity. The mycelium is non-acid and gram-positive.

To isolate the micro-organism, a portion of a dried mud sample which, when the particular strain of *Micromonospora halophytica* var. *nigra* (NRRL 3097) mentioned hereinbefore is employed in the process, is obtained from the bottom of a salt pool in Syracuse, New York, is shaken in sterile distilled water and, after being suitably diluted, the suspension is plated on screen agar medium containing 0.5% tryptose, 2.0% soluble starch, 0.3% calcium propionate and 2.0% agar, all in distilled water.

The cultures so obtained may then be tested for antibiotic activity by first growing in the following medium for up to 60 days at 26°

THE MICRO-ORGANISM

The micro-organism utilized in the process

[Price 4s. 6d.]

C: 0.3% beef extract, 0.5% tryptose, 0.1% dextrose, 2.4% soluble starch, 0.5% yeast extract, 1.5% agar, all in tap water. The whole aqueous agar is then extracted with butanol and the butanol-water extract is concentrated. Sufficient antibiotic is extracted by the butanol-water mixture to provide a concentrate which, by disc test, inhibits the growth of *Staphylococcus aureus* and *Bacillus subtilis*.

Antibiotic activity against the same organisms is observed after growth in submerged fermentation for 96 hours in an aqueous medium such as one containing 1% NZ-Amine Type A (a peptone resulting from the enzymatic digestion of casein which serves as a source of nitrogen and is available from Sheffield Chemical Co., Norwich, New York) and 5% soluble starch (sold under the Registered Trade Mark Difco—Difco Laboratories, Inc., Detroit, Michigan).

Colony observations, set forth in Table I which follows, were made on the aforementioned microorganisms after 14 days incubation at 24–26° C. in the designated media. In describing the color formations the following system and references are employed: The color designation consists of two designates. The first is a color name taken from the "Descriptive Color Name Dictionary", by Taylor, Knoche and Granville, published by the Container Corporation of America, 1950 (U.S.A.), with a color chip number corresponding to the color name; said chip number taken from the "Color Harmony Manual", 4th Edition, 1958, published by the Container Corporation of America, U.S.A. The second designate consists of a color name and number referring to the synonym or near synonym found in the National Bureau of Standards (U.S.A.) Circular 553, November 1, 1955.

TABLE I
Colony Observations on Various Media

Medium	Observations
Bennett's Agar + 0.1% CaCO ₃	Growth: good; colony plicate. Color: luggage tan-g4NE, strong brown-55.
Emerson's Agar + 0.1% CaCO ₃	Growth: good; colony crenate-convolute Color: sepia brown g3PN, dark yellowish brown-78.
Tomato Paste/Oatmeal-Agar + 0.1% CaCO ₃	Growth: good; colony plicate. Color: terracotta g5PE, strong brown-55.
Glucose/Asparagine-Agar + 0.1% CaCO ₃	Growth: fair; colony flat. Color: orange g4LA, strong orange-50.
Glucose/Yeast Extract-Agar (0.5% yeast, 1.0% dextrose) + 0.1% CaCO ₃	Growth: good; colony plicate. Color: terracotta g5PE, strong brown-55.

Further culture characteristics of *M. halophytica* var. *nigra*, as observed on common laboratory media are as follows: *Potato slice*—growth: sparse, represented by several large macro-colonies which develop from an inoculum, otherwise showing no growth. *Carrot slice*—growth: poor. *Czapek's agar*—growth: fair, plicate; color: chocolate brown g4PN, dark brown—59. *Tyrosine agar*—growth: fair; no color reaction. *Peptone/iron agar*—growth: fair; no color reaction.

On an agar medium composed of 1 percent NZ-Amine Type A plus 1 percent glucose and 0.1 percent CaCO₃, growth is fair, weakly plicate; colony color is terracotta g5PE, strong

brown—55. When the nitrogen source is 0.5 percent Difco yeast extract, growth is good, plicate; and color is also terracotta g5PE, strong brown—55.

An acceptable growth of *M. halophytica* var. *Nigra* is ensured when the pH of the medium is between 6.5 and 8 but the best growth is generally achieved within the pH range of 6.8–7.8. The colonies on many natural agar media are initially orange, but as sporulation progresses turn olive brown and eventually black. The copious black sporulation layer typical of this culture on many media easily distinguishes it from the light brown to brown sporulation layer present in

M. halophytica var. *halophytica*. Also *M. halophytica* var. *nigra* fails to produce the reddish-brown diffusible pigment produced on certain media by *M. halophytica* var. *halophytica*. The typical black sporulation layer produced by *M. halophytica* var. *nigra* is commonly found on plates containing the following carbohydrate in the carbohydrate utilization study: D-fructose, D-galactose, D-lactose, raffinose and D-trehalose.

While the strain of *M. halophytica* var. *nigra* identified as NRRL 3097 constitutes, primarily because of its ready availability, a particularly convenient starting material for the process, it will be appreciated that other antibiotic No. 467-producing strains of the variant will provide acceptable alternatives. Further, mutants of *M. halophytica* var. *nigra*, obtained by subjecting the micro-organism, say of the strain NRRL 3097, to the action of a mutating agent such as high frequency radiation [including X-ray, and ultra-violet radiation], actinophages and/or nitrogen mustards, may also be suitable as starting materials providing, when incubated under appropriate conditions, a product having substantial antibiotic activity and from which the antibiotic No. 467 can be isolated.

FORMATION AND ISOLATION OF THE ANTIBIOTIC

The micro-organism described heretofore, by the controlled fermentation described herein, produces a product with substantial antibiotic activity. As most of this activity is generally associated with the broth rather than the mycelium, it is generally preferred to utilize the former as the source of antibiotic, following its separation from the mycelium. By paper chromatographic studies in several systems, the isolated antibiotic appears to be a single substance. However, in certain systems, some of which will be described hereinafter, resolution into as many as four fractions has occurred.

In the process for producing antibiotic No. 467, *M. halophytica* var. *nigra* may be grown at a suitable temperature of from 25 to 40° C., under submerged aerobic conditions in an aqueous nutrient medium containing an assimilable carbon and nitrogen source. Suitable nitrogen sources include both organic and inorganic nitrogen, preferably the former, such as soybean meal and peptones, e.g. the commercial preparation NZ-Amine Type A. Suitable carbon sources include carbohydrates such as starch, dextrin and sugars. The fermentation is preferably carried out at a pH of from about 6.5 to 8.0, with a pH in the range from 6.8 to 7.8 frequently being the most advantageous, for a period of about 96 to 120 hours, although shorter or longer periods may be employed. At the end of the fermentation period, peak antibiotic production has usually been attained and the

product, which comprises a mycelium and a broth, has overall a substantial antibiotic activity, most of which generally resides in the broth. The mycelium is removed, typically by filtration, and the antibiotic No. 467 isolated from the broth conveniently by solvent extraction.

In a typical application of the solvent extraction technique, the crude broth, with its pH adjusted to a value of between 2 and 9.5, is extracted once or twice with a water-immiscible organic solvent, such, for example, as butyl alcohol, ethyl acetate, methylene chloride, chloroform, carbon tetrachloride, benzene or ether. The antibiotic which is recovered from the extract by evaporation of the solvent to a residue, may be purified, for instance, by washing with a suitable organic solvent, and/or by recrystallization from such a solvent.

The Examples which follow are given by way of illustrating certain features of the process of this invention, especially the fermentation of *M. halophytica* var. *nigra*, the separation of the broth of the resulting product from the mycelium thereof, the extraction of the antibiotic from the broth, and the isolation of antibiotic No. 467, and they are not to be regarded as limiting the invention. The assay values quoted were determined by standard disc assay methods using *Staphylococcus aureus* as the reference organism.

EXAMPLE 1

Shake Flask Fermentation of *M. Halophytica* var. *nigra* NRRL 3097

A. Germination Stage:

Add a lyophilized culture of *M. halophytica* var. *nigra* to a 300 ml. shake flask containing 100 ml. of the following sterile medium:

Beef extract	3 g.	105
Tryptose	5 g.	
Dextrose	1 g.	
Soluble starch	24 g.	
Yeast extract	5 g.	
Water	to 1 liter	110

Incubate on a rotary shaker [280 rotations per minute (RPM), 5 cm. stroke] for 5 days at 37° C.

B. Fermentation Stage:

Transfer a 25 ml. inoculum from the above germination stage to each of four 2-liter flasks, each containing 500 ml. of the following medium:

Soluble starch	50 g.	120
NZ-Amine Type A	10 g.	
Soft water to	1 liter	
pH after sterilization	7.45	

Incubate the flasks and their contents for

96 to 120 hours at 28° C. on a rotary shaker.

- Sometimes it is advantageous to employ a second germination stage whereby a 25 ml. inoculum from the first germination stage is aseptically transferred to 500 ml. of the germination medium and shaken as above for 72 hours.

- C. Alternate Fermentation Stage Procedure:
There is utilized 500 ml. of a growth medium comprising 30 g. soybean meal, 40 g. dextrose, 5 g. calcium carbonate and 1000 ml. tap water. To each of four 2-liter flasks containing the foregoing is added a 25 ml. inoculum from the germination stage, and the flasks are then incubated at 28° C. for five days on a rotary shaker.

D. Extraction of No. 467

- Filter the 40 liters of incubated product obtained from the pooling of 20 runs of fermentation of *M. halophytica* var. *nigra* according to part B or C of this Example. Wash the filter cake with water and combine the filtrates, obtaining about 40 liters. Extract the harvest broth twice with equal volumes of ethyl acetate (40 liters each) and concentrate to a residue. Take up the dark oil in 100 ml. of ethyl acetate and add it slowly to 1 liter of ethyl acetate/petroleum ether (1:1) while stirring. Filter, then concentrate the filtrate to a residue, 5.2 g. of a dark brown oil. Add to the oil 150 ml. of benzene/chloroform (1:1) and filter again. Add the filtrate slowly, with stirring, to 1.5 liters of hexane. Filter the precipitate, wash with hexane and dry in vacuo, obtaining 1.12 g. of No. 467 as an amorphous powder which exhibits (on agar plates) antibiotic activity against *Staphylococcus aureus* (ATCC 209P) down to 0.1 µ g./ml., *Streptococcus faecalis* 1 µ g./ml., and *B. subtilis* 10 µ g./ml.

EXAMPLE 2

Tank Fermentation of *M. halophytica* var. *nigra* NRRL 3097

- A. Germination Stage:

Add a lyophilized culture of *M. halophytica*

var. *nigra* to a 300 ml. shake flask containing 100 ml. of the sterile medium set forth in Example 1 (germination stage).

Incubate on a rotary shaker (280 RPM, 5 cm. stroke) for five days at 37° C.

B. Inoculum Preparation Stage:

Transfer a 25 ml. inoculum from the germination stage to each of four 2-liter flasks, each containing 500 ml. of the sterile medium utilized for germination. Incubate the flasks and contents for 5 days at 28° C. on a rotary shaker (280 RPM, 5 cm. stroke). Pool the contents of the flasks.

Add a 25 ml. inoculum (taken from the pool) to each of ten 2-liter flasks, each containing 500 ml. of the aforescribed sterile medium. Incubate the flasks and their contents for 3 to 5 days at 28° C. on a rotary shaker (280 RPM, 5 cm. stroke). Pool and aseptically transfer the broth into a sterile inoculum flask having a side arm (total volume—about 5 liters).

C. Fermentation Stage:

Aseptically transfer the 5 liters of inoculum obtained from part B of this Example to a 130-liter fermenter containing about 90 liters of the following sterile medium:

Soluble starch	4500 g.	
NZ-Amine Type A	900 g.	
Antifoamer	100 ml.	
Soft water	to 90 liters	

Aerobically ferment for 55 to 65 hours [until the packed cell volume (PCV) is about 4.5—5.0% as determined by centrifuging a 10 ml. sample at 2800 RPM for 5 minutes] under the following conditions:

Temperature: 26° C.

Sterile air input: about 75 to 80 liters/minute

Pressure: about 0.5 to 0.6 atm. above normal atmospheric pressure

Agitation: 160 to 165 RPM

The following data is typical of such fermentation run:

Hours	Temp.	Air input in litres per minute	Pressure in atm. above normal	Rpm	pH	PCV in %	Disc Assay with <i>Staph. aureus</i> as the reference organism
0	26	76	0.49	160	6.80	—	—
8	26	76	0.55	160	6.95	1.0	—
16	26	76	0.53	160	6.85	1.0	—
23	26	76	0.52	160	7.10	1.5	14 mm zone
31	26	76	0.53	160	7.35	1.5	—
38	26	79	0.49	165	7.60	2.5	—
46	26	79	0.54	165	7.75	2.5	21 mm zone
54	26	79	0.52	165	7.90	4.5	—
65	26	79	0.51	165	7.80	5.0	25 mm zone

D. Extraction of No. 467

Separate the mycelium from the broth by filtration, utilizing a filter aid. Extract 90 litres of the harvest broth with two volumes of ethyl acetate after first adjusting the pH to 7.0. Concentrate the extract to 3 litres in vacuo. Remove the water layer which separates on standing, and concentrate the ethyl acetate solution further to approximately 200 ml. Chill the concentrate overnight and filter. Add the filtrate to 10 volumes of petroleum ether/ethyl ether (1:1) and filter. Wash the precipitate with several portions of solvent mixture and add the washes (petroleum ether/ethyl ether) to the filtrate. Concentrate the filtrate plus washings containing the antibiotics in vacuo to a residue consisting of a dark brown oil weighing 18 g. This crude product inhibits the growth of *Staphylococcus aureus* 209P. on agar at dilutions as low as 1 μ g./ml.

In addition to its process aspect, this invention in another of its aspects embraces the antibiotic No. 467 which may, as evidenced by paper chromatographic studies, be in the form of a single substance or a mixture containing a number of say four, components, as well as the components themselves which may be obtained by resolution of No. 467. Information relating to certain of the salient features of the antibiotic No. 467 is provided in the appropriately identified passages which follow.

35 Physical and Chemical Properties of No. 467

No. 467 is soluble in most common organic solvents such as ethyl acetate, chloroform,

methylene chloride (and other halogenated hydrocarbons) and lower alkanols (e.g. methanol and ethanol); it is only very sparingly soluble in water and is substantially insoluble in petroleum ether and hexane. From fermentation broth at a pH of 2—9.5, the antibiotic is easily extracted by butyl alcohol, ethyl acetate, methylene chloride, chloroform, carbon-tetrachloride, benzene or ether.

When subjected to the following qualitative group tests, No. 467 gives the results noted:

Ninhydrin and Sakaguchi tests . . . Negative;
Reducing sugars (with ammoniacal silver nitrate) . . . positive;
Reduction of ferricyanide . . . positive.

Antibiotic 467 in methanol solution is absorbant in the ultra-violet in the range of 210—400 $m\mu$ with the following specific characteristics: It has a rather non-descript ultra-violet absorption curve with inflection points at 264 $m\mu$ and 302 $m\mu$; the point at 264 $m\mu$ having $E^{1\%} = 183$, and 302 $m\mu$ having $E^{1\%} = 116.2$.

Antibiotic No. 467 is stable to acid and to heat.

Total analysis yields the following results: C, 60.73%, H 7.53%, N 3.70%, O 27.20%, Cl 0.04%, S 0.80%.

The infra-red spectrum of No. 467, suspended in Nujol (Nujol is a trade mark for a mineral oil on a hydrocarbon basis sufficiently pure for infra-red spectroscopy obtainable from Plough Inc., Memphis, Tenn.) is reproduced in the accompanying drawing, wherein λ represents the wave lengths in μ , ν represents the

5 frequency in cm^{-1} , and A represents the absorption. The absorption peaks and band characteristics are summarized in Table II wherein the abbreviations have the following meanings (W=weak, M=moderate, M-S=moderate to strong, S=strong, sh=shoulder). By way of explanation intended to avoid pos-

sible confusion, it is mentioned that the peaks which occur at the following wavelengths (3.5—3.6, 6.75—6.85, 7.23 and 13.80—13.90 10 are caused by the mineral oil Nujol and, consequently, they have been omitted from the table.

TABLE II

$\lambda(\mu)$	Peak Strength
2.98—3.15	S (broad)
3.65	M—W (broad)
5.77	W—M
5.83	W—M
6.02	M—S
6.05	M—S
6.07	M—S
6.13	Sh
6.18	S
6.30	Sh
6.37	M
6.45	M
6.58	M-S
7.85—7.95	S (broad)
8.05	S
8.20	Sh
8.35	Sh
8.45	Sh
8.52	M—S
8.65	Sh
9.43	M—S
9.55	M—S
9.80	Sh
10.25	W—M
11.15—11.25	W (broad)
11.95	W
13.15	M—S

Paper Chromatographic Properties

5 The paper chromatograms are made in different solvent systems, and R_F values are determined by a standard bioautographic method, which involves developing and drying a paper chromatogram prior to laying it on an agar plate seeded with *Staphylococcus aureus*. After a contact time of 15 minutes,

the paper is removed from the plate which is then incubated at 37° C. for 16—20 hours. Observations of the location of inhibition zones permits determination of R_F values.

In Table III which follows, there is set forth R_F values of 467 in eight different solvent systems (A—H).

TABLE III

System:	RF Value of Major Spots:
A-descending	0.0, 0.06, 0.40, 0.66
B-ascending	0.0, 1.0
C-ascending	0.78, 1.0
D-ascending	0.95
E-ascending	1.0
F-ascending	1.0
G-ascending	1.0
H-descending	0.0, 0.1, 0.9

Systems:

20 A—Benzene: chloroform (93:7 by volume) saturated with formamide; chromatographic paper, prior to use, is dipped in 25% methanolic formamide, blotted and air dried for 5 minutes to remove methanol, 2 hours.

B—Benzene: methanol (9:1).

25 C—Methanol: water (80:20) containing 3% sodium chloride; the paper is buffered with a solution of 0.95 M sodium sulfate+0.5 M sodium bisulfate and dried prior to developing, 4—6 hours.

30 D—Propanol: acetic acid: water (50.5:40), 18 hours.

E—Butanol: acetic acid: water (4:1:5), 18 hours.

35 F—Propanol: pyridine: acetic acid: water (15:10:3:12), 18 hours.

G—Phenol: water (80 g: 20 ml.), 18 hours.

H—Benzene: petroleum ether: acetone (10:2.5:5), 1—2 hours.

40 From the paper chromatograms obtained using systems D—G it would appear that the antibiotic No. 467 is a single substance. On the other hand, the paper chromatograms obtained using the other systems suggest that the antibiotic No. 467 is a mixture containing, in the case of systems B and C, two components, three components in the case of system H and four components in the case of system A. Accordingly, in certain instances, the antibiotic No. 467 can be resolved chromatographically into two or more components and

these components, which are included in the product aspect of the invention are antibiotically active. The observed R_F values, set forth in Table III, differentiate the antibiotic No. 467 from antibiotics such as oleanomycin, novobiocin, penicillin G, erythromycin and gentamycin.

Biological Properties of No. 467

The antibiotic No. 467 possesses a broad range of activity against gram-positive organisms. It has particular value in combatting manifestations of penicillin resistant microorganisms, since this novel antibiotic is capable of inhibiting or destroying such organisms. It is also useful in treating diseases caused by *Staphylococcus aureus*, *Diplococcus pneumoniae*, *Streptococcus pyogenes* and other pathogenic microorganisms. In addition this antibiotic is useful as a laboratory sterilization aid in eliminating overgrowth of gram-positive organisms from equipment being used to study growth of gram-negative organisms.

The comparative in-vitro activity of No. 467 against a variety of gram-positive organisms is set forth in Table IV below. The susceptibility of the microorganisms to the antibiotic was determined by the standard tube dilution method as follows: In each instance a 10⁻⁵ dilution of a 24-hours broth culture was employed as inoculum with the end points taken after incubation for 24 hours at 37° C. Except where indicated otherwise, the growth medium consisted of tryptose/phosphate broth. The antibiotic used in these

- tests is that obtained by Example 1(D) and had a potency of 18 000 dilution units per milligram. (Indications of potency in dilution serve as a base line for activity; a potency of "18 000 dilution units/mg." means that 1 mg of the product, when dissolved in 1 ml. of the solvent and brought an an 18 000-fold dilution, still inhibits the growth of *Staphylococcus aureus* (ATCC 6538P) in typtose/phosphate broth medium, using the inoculum previously described).

TABLE IV
Antibiotic Spectrum of No. 467
Microorganisms Tested

Systematic Name	Strain Designation	Minimal Inhibiting Concentration ($\mu\text{g/ml}$)
<i>Bacillus cereus</i> var. <i>mycoides</i>	ATCC—7064	0.57
<i>Bacillus cereus</i> var. <i>mycoides</i>	*DA—30	>0.78
<i>Bacillus megatherium</i>	DA—31	0.57
<i>Bacillus sphaericus</i>	DA—32	0.57
<i>Bacillus sphaericus</i>	DA—33	0.28
<i>Staphylococcus aureus</i>	ATCC—6538	0.03
<i>Staphylococcus aureus</i>	ATCC—6538P	0.03
<i>Staphylococcus aureus</i>	ATCC—12715	0.14
<i>Staphylococcus aureus</i>	ATCC—1163	0.14
<i>Staphylococcus aureus</i>	ATCC—9996	0.52
<i>Staphylococcus aureus</i> var. Gray	—	0.14
<i>Staphylococcus aureus</i> var. Smith	DA—141	0.20
<i>Staphylococcus epidermidis</i>	DA—41	0.03
<i>Sarcina lutea</i>	ATCC—9341	0.07
<i>Streptococcus pyogenes</i> C	DA—21	0.14

*DA refers to the private collection of Schering Corp., Bloomfield, New Jersey.

- The in-vivo activity of No. 467 against certain bacterial infections in mice has also been tested. The mice were infected with an inoculum of the particular bacterial administered by intraperitoneal injection. They were then treated by subcutaneous injections of the antibiotic (potency of 18 000 dilution units/mg.) suspended in 0.25% aqueous carb-oxymethyl-cellulose; the injection being administered in two divided dosages. In this manner, the PD_{50} against *Staphylococcus aureus* was determined as 20 mg./kg. There is also a significant delay in death in *Streptococcus* infected mice, following administration subcutaneously or orally of 50 mg./kg. Against *Diplococcus pneumoniae* full protection is obtained with less than 20 mg/kg subcutaneously.
- The acute toxicity (LD/50) for No. 467 (potency = 18 000 dilution units/mg.) measured in the standard manner utilizing mice weighing 18 to 20 g., and in the dosage form of a suspension in 0.25% aqueous carb-oxymethyl-cellulose is as follows: subcutaneous 2500 mg./kg., and intraperitoneal 1500 mg./kg.
- Therapeutic Forms of the Antibiotic No. 467
- When intended for therapeutic purposes the antibiotic No. 467, or a component thereof, may, if necessary or desired, be treated or

processed to bring it into an acceptable form. For instance, it may be combined, as by admixture, with a carrier to provide a therapeutic composition.

5 WHAT WE CLAIM IS:—

1) A process for the production of the antibiotic identified as No. 467 which comprises incubating the micro-organism *Micromonospora halophytica* var. *nigra* or an anti-
10 biotic No. 467—producing mutant thereof in contact with an aqueous nutrient medium containing assimilable carbon and nitrogen until a product having substantial antibiotic activity is produced, and thereafter isolating
15 the biotic No. 467 therefrom.

2) A process as claimed in claim 1, wherein the *Micromonospora halophytica* var. *nigra* utilized is the strain designed as NRRL 3097.

3) A process as claimed in claim 1 or 2, wherein the *Micromonospora halophytica* var. *nigra* is incubated under submerged aerobic conditions at a temperature from 25° to 40° C. and a pH from 6.5 to 8.

4) A process as claimed in claim 3, wherein the pH is from 6.8 to 7.8.

5) A process as claimed in any one of the preceding claims, wherein, after incubation, the mycelium is separated from the broth, the antibiotic is removed from the broth by
30 solvent extraction and the antibiotic is then isolated from the extract.

6) A process as claimed in claim 5, wherein the solvent extraction is effected with a water-immiscible solvent.

7) A process as claimed in claim 6, wherein the solvent is butyl alcohol, ethyl acetate, methylene-chloride, chloroform, carbon tetra-
35 chloride, benzene or ether.

8) A process as claimed in any of claims 5 to 7, wherein the antibiotic is isolated from the extract by evaporation of the solvent
40 therefrom.

9) A process as claimed in any one of the preceding claims, followed by the puri-

45 fication, say by washing and/or by recrystallization from a suitable organic solvent, of the isolated antibiotic.

10) A process as claimed in any one of the preceding claims, wherein the antibiotic
50 is resolved into components.

11) A process as claimed in claim 10, wherein resolution of the antibiotic is accomplished by chromatography.

12) A process for the production of the antibiotic identified as No. 467 substantially
55 as herein described and as shown with reference to Example 1 or 2.

13) The antibiotic identified as No. 467 and components thereof, when produced by a process as claimed in any one of the pre-
60 ceeding claims.

14) The antibiotic identified as No. 467 which has an elementary analysis as follows, C=60.73%, H=7.53%, N=3.7%, O=27.20%, Cl=0.04%, S=0.80%, is absorbant
65 to ultra-violet radiation in the range of 210 to 400 mμ with inflection points at 264 mμ and 302 mμ, the point at 264 mμ having E_{1%}=183 and the point at 302 mμ having E_{1%}=116.2, has an infra-red spectrum when
70 suspended in the hydrocarbon mineral oil (Nujol) substantially as shown in the accompanying drawing; and has an in-vitro antibacterial spectrum including the bacteria
75 enumerated in Table IV herein.

15) The antibiotic No. 467 and its components, substantially as identified herein and mixtures thereof.

16) Therapeutic compositions comprising the antibiotic No. 467 or a component thereof
80 in admixture with a carrier.

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Agents for the Applicants.

processed to bring it into an acceptable form. For instance, it may be combined, as by admixture, with a carrier to provide a therapeutic composition.

5 WHAT WE CLAIM IS:—

1) A process for the production of the antibiotic identified as No. 467 which comprises incubating the micro-organism *Micromonospora halophytica* var. *nigra* or an antibiotic No. 467—producing mutant thereof in contact with an aqueous nutrient medium containing assimilable carbon and nitrogen until a product having substantial antibiotic activity is produced, and thereafter isolating the biotic No. 467 therefrom.

2) A process as claimed in claim 1, wherein the *Micromonospora halophytica* var. *nigra* utilized is the strain designed as NRRL 3097.

3) A process as claimed in claim 1 or 2, wherein the *Micromonospora halophytica* var. *nigra* is incubated under submerged aerobic conditions at a temperature from 25° to 40° C. and a pH from 6.5 to 8.

4) A process as claimed in claim 3, wherein the pH is from 6.8 to 7.8.

5) A process as claimed in any one of the preceding claims, wherein, after incubation, the mycelium is separated from the broth, the antibiotic is removed from the broth by solvent extraction and the antibiotic is then isolated from the extract.

6) A process as claimed in claim 5, wherein the solvent extraction is effected with a water-immiscible solvent.

7) A process as claimed in claim 6, wherein the solvent is butyl alcohol, ethyl acetate, methylene-chloride, chloroform, carbon tetrachloride, benzene or ether.

8) A process as claimed in any of claims 5 to 7, wherein the antibiotic is isolated from the extract by evaporation of the solvent therefrom.

9) A process as claimed in any one of the preceding claims, followed by the purification, say by washing and/or by recrystallization from a suitable organic solvent, of the isolated antibiotic.

10) A process as claimed in any one of the preceding claims, wherein the antibiotic is resolved into components.

11) A process as claimed in claim 10, wherein resolution of the antibiotic is accomplished by chromatography.

12) A process for the production of the antibiotic identified as No. 467 substantially as herein described and as shown with reference to Example 1 or 2.

13) The antibiotic identified as No. 467 and components thereof, when produced by a process as claimed in any one of the preceding claims.

14) The antibiotic identified as No. 467 which has an elementary analysis as follows, C=60.73%, H=7.53%, N=3.7%, O=27.20%, Cl=0.04%, S=0.80%, is absorbant to ultra-violet radiation in the range of 210 to 400 mμ with inflection points at 264 mμ and 302 mμ, the point at 264 mμ having E₁%=183 and the point at 302 mμ having E₁%=116.2, has an infra-red spectrum when suspended in the hydrocarbon mineral oil (Nujol) substantially as shown in the accompanying drawing; and has an in-vitro antibacterial spectrum including the bacteria enumerated in Table IV herein.

15) The antibiotic No. 467 and its components, substantially as identified herein and mixtures thereof.

16) Therapeutic compositions comprising the antibiotic No. 467 or a component thereof in admixture with a carrier.

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