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AMENDED SPECIFICATION

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DRAWINGS ATTACHED

Inventors ROBERT QUINCY THOMPSON, WILLIAM MAX STARK
and CALVIN EUGENE HIGGENS

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Tenebrimycin Antibiotics

We, ELI LILLY AND COMPANY, a corporation organised and existing under the laws of the State of Indiana, United States of America, of 740 South Alabama Street, City of Indianapolis, State of Indiana, United States of America, 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 novel antibiotic substance and its preparation.
The present invention provides an antibiotic substance comprising a tenebrimycin complex or tenebrimycin I, tenebrimycin I', tenebrimycin II, tenebrimycin III, tenebrimycin IV, tenebrimycin VI or mixtures thereof or the acid addition salts thereof with pharmaceutically acceptable acids.
The present invention also provides a pharmaceutical composition comprising an antibiotic substance as defined above and a pharmaceutically acceptable carrier or diluent.
Also, the present invention provides a method of producing an antibiotic tenebrimycin complex, tenebrimycin I, I', II, III, IV, V, VI, or mixtures thereof which comprises cultivating a tenebrimycin-producing strain of *Streptomyces tenebrarius* micro-organism in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic conditions until a substantial amount of the desired antibiotic is produced by said micro-organism in said culture medium, and isolating said tenebrimycin from culture medium.
The novel antibiotic complex provided by this invention comprises at least seven factors, each having antibiotic activity. The complex,

as the free base, is a white solid substance which is soluble in water and dimethyl sulphoxide, slowly soluble in methanol, and insoluble in most other organic solvents such as acetone, the higher alcohols, dioxane, ethyl acetate, diethyl ether, acetonitrile, methyl isobutyl ketone and hydrocarbon solvents. The free base complex is stable at refrigerated temperatures, room temperature, and 37°C. for at least eight weeks over a pH range of pH 1 to pH 11.

The infrared absorption spectrum for the antibiotic complex, obtained from a mineral oil mull thereof, is shown in Figure 1. The distinguishable bands in the infrared absorption spectrum over the range of 2—15 microns are as follows: 3.02 (broad), 3.15, 5.82, 6.26, 7.43, 8.8 (broad), and 9.7 (very broad) microns.

In a series of chemical tests carried out with the complex, positive reactions were obtained in the ninhydrin, anthrone, and Elson-Morgan tests. The Lowry protein test yielded a slightly positive reaction. The biuret and Sakaguchi tests were negative.

As noted above, the antibiotic complex isolated from said culture medium comprises at least seven distinct factors. In the nomenclature employed herein, the term tenebrimycin will be used to designate the antibiotic complex, while various factors constituting the complex will be designated as tenebrimycin I, tenebrimycin I', tenebrimycin II, tenebrimycin III, tenebrimycin IV, tenebrimycin V, and tenebrimycin VI. In the complex as commonly obtained, tenebrimycin I, tenebrimycin I', and tenebrimycin III are present in small amounts. Of the remaining tenebrimycins, the most prevalent appears to be

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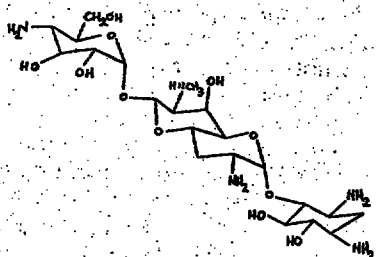
tenebrimycin II, which constitutes about 45—50 percent of the complex, and tenebrimycin V, which constitutes about 30 percent of the complex. Tenebrimycin IV is present to the extent of about 15—20 percent, while tenebrimycin VI constitutes about 10 percent of the complex. The more abundant tenebrimycins have been separated and characterized, descriptions of the properties thereof being contained in the paragraphs which follow.

Tenebrimycin II is a white solid substance. Electrometric titration in water indicates the presence of titratable groups having pK'a values of about 5.7, 6.7, 7.7, and 8.7. The specific rotation of sodium D light at a temperature of 25°C. by this factor is +159° when the concentration of the antibiotic is 1 percent on a weight per volume basis in aqueous solution. Micro-analysis indicates that tenebrimycin II has approximately the following percentage composition: C, 44.90; H, 8.20; N, 12.53; O, 33.24 (direct). The apparent molecular weight, as indicated by titration data, is about 454. The empirical formula which best fits these data is $C_{16}H_{26}N_4O_9$.

The infrared absorption spectrum of tenebrimycin II as a mineral oil mull is shown in Figure 2 of the accompanying drawings. The distinguishable bands in the infrared absorption spectrum over the range of 2—15 microns are as follows: 3.02, 3.14, 6.05, 6.23, 7.4, 8.5, 8.75, 9.17, 9.65, 10.11, 11.15, 11.7, and 12.6 microns.

Tenebrimycin II forms an acetyl derivative which has a specific rotation, $[\alpha]_{D^{25}}$, of about +130° when the concentration of the compound is 1 percent on a weight per volume basis in aqueous solution. The derivative has no remaining titratable groups. The elemental composition as determined by microanalysis indicates that four acetyl groups have been added to the tenebrimycin II structure.

Tenebrimycin II is a compound having the structural formula

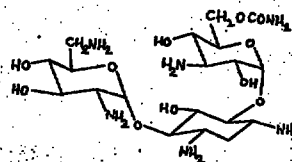


Tenebrimycin IV is a basic substance with titratable groups having pK'a values of about 5.3, 6.8, 7.8, and 9.0 as indicated by electrometric titration in water. The specific rotation of tenebrimycin IV, determined upon a 1 percent aqueous solution at a temperature of 25°C. is +114°. Microanalysis indicates the following percentage composition for tenebrimycin IV: C, 41.66; H, 7.78; N, 15.02; O, 34.93 (direct). The apparent molecular weight determined from the titration data is about 478. These data suggest an empirical formula of $C_{16}H_{26}N_4O_{10}$ for tenebrimycin IV.

The infrared absorption spectrum of tenebrimycin IV as a mineral oil mull is shown in Figure 3 of the drawings. The distinguishable bands in the infrared absorption spectrum over the range of 2—15 microns are as follows: 3.05, 3.17, 5.85, 6.27, 7.46, 8.75, 9.7, 10.5, 11.1, and 12.85 microns.

The tetra-acetyl derivative of tenebrimycin IV is a crystalline solid melting at about 265—267°C. The specific rotation, $[\alpha]_{D^{25}}$, is +109° when the concentration of the derivative is 1 percent in an aqueous solution.

Tenebrimycin IV is a compound having the structural formula



Electrometric titration of tenebrimycin V shows titratable groups having pK'a values of about 5.5, 7.0, 8.0, and 9.1. The specific rotation of sodium D light by a 1 percent aqueous solution of this factor, determined at a temperature of 25°C., is +118°. The elemental compositions as determined by microanalysis is as follows: C, 41.05; H, 7.60; N, 13.62; O, 36.76 (direct). The analytical data and the apparent molecular weight of 424 as determined from titration data suggest an empirical formula for tenebrimycin V of $C_{14}H_{22}N_4O_{10}$.

Figure 4 shows the infrared absorption spectrum of tenebrimycin V as a mineral oil mull, with distinguishable bands in the range of 2—15 microns as follows: 3.05, 3.19, 6.32, 7.45, 8.75, 9.7, 11.15, and 12.25 microns.

Like tenebrimycin IV, tenebrimycin V forms a crystalline tetra-acetyl derivative. The derivative melts with decomposition at about 260°C. and gives a specific rotation, $[\alpha]_{D^{25}}$, of about +109°, when the concentration of the compound is 0.42 percent in water.

Tenebrimycin VI has titratable groups with pK'a values of about 5.6, 7.2, 8.2, and 9.3 as determined by electrometric titration in water. The titration data suggest an apparent molecular weight of 396. The specific rotation of sodium D light by a 1 percent aqueous solution of tenebrimycin VI at a temperature of 25°C. is about +127°. Microanalysis indicates an approximate percentage composition of C, 44.04; H, 8.40; N, 13.78; O, 33.62 (direct). The empirical formula most consistent with the above data is $C_{16}H_{26}N_4O_9$.

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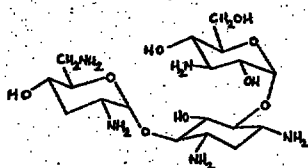
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The infrared absorption spectrum of tenebrimycin VI is shown in Figure 5 of the drawings. The distinguishable bands in the range of 2—15 microns are as follows: 3.05, 3.19, 6.35, 7.47, 8.75, 9.8, 12.3 and 12.95 microns.

A 1 percent aqueous solution of the tetra-acetyl derivative of tenebrimycin VI has a specific rotation of about +95° when determined at 25°C. upon a 1 percent aqueous solution.

Tenebrimycin VI is a compound having the structural formula:



Acid addition salts of tenebrimycin or of the separate antibiotic components thereof can be prepared by conventional methods. Either organic or inorganic acids can be employed for salt formation. A convenient method for the preparation of such salts comprises the addition of a solution of the salt-forming acid to an aqueous solution of the antibiotic. In the case of insoluble acid addition salts, the salt precipitates from solution and is readily

separated by conventional techniques such as filtration or centrifugation. In the event that the desired acid addition salt does not precipitate readily, the precipitation can be aided, as by concentrating the solution to smaller volume or by adding a water miscible solvent such as acetone or the like. The acid addition salt can be conveniently reconverted to the free base form of the antibiotic by passing an aqueous solution of the salt over an anionic exchange resin in the hydroxyl cycle.

The various factors making up the tenebrimycin complex can be distinguished from each other and from other antibiotics having similar properties by paper chromatographic techniques, such as the following. Chromatography was carried out in the conventional descending manner at 73°F. $\pm 1^\circ\text{F}$. on Whatman No. 1 paper having dimensions of 7½ inches by 18½ inches. The results are shown in the following table. The migration constant for each of the antibiotics is expressed as an R_f constant rather than the more conventional R_f value. The R_f value expresses the ratio of the distance traversed by the antibiotic with respect to the end of the tape rather than the solvent front. This method of expressing the constant was chosen in view of the fact that the solvent front had progressed beyond the end of the tape during the time interval employed with most of the solvent systems.

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TABLE I
Paper Chromatography of the Tenebrimycins and
Known Antibiotics with Similar Properties

	Solvent System ¹ and R _F Values						
	A	B	C	D	E	F	G
Tenebrimycin complex	—	—	—	0.65	0.32	0	0
Tenebrimycin I	0.20	—	—	—	—	—	—
Tenebrimycin I'	0.27	—	—	—	—	—	—
Tenebrimycin II	0.40	0.17	0.32	—	—	—	—
Tenebrimycin III	0.04	0.34	0.41	—	—	—	—
Tenebrimycin IV	0.49	0.20	0.32	—	—	—	—
Tenebrimycin V	0.55	0.22	0.35	—	—	—	—
Tenebrimycin VI	0.71	0.31	0.38	—	—	—	—
Kanamycin	0.35	0.35	0.41	0.65	0.42	0	0
Kanamycin B	0.57	0.26	0.32	0.70	0.29	0	0.05
		0.51	0.39				
Gentamycin	0.83	0.75	0.62	—	0.51	0	0.1
		0.95					
Catenulin	0.44	0.25	0.34	0.65	0.35	0	0
Neomycin	0.60	0.12	0.19	—	—	0	0.05

¹The solvent systems employed and the duration of chromatography are indicated below :

A = n-Butanol saturated with water, plus 2 percent p-toluenesulfonic acid, for 40 hours.

B = 80 percent aqueous ethanol plus 1.5 percent sodium chloride, for 40 hours on paper buffered with 0.95 M sulfate-bisulfate.

C = Propanol, pyridine, acetic acid and water (15:10:3:12) for 40 hours.

D = Water saturated with methylisobutyl ketone, plus 1 percent p-toluenesulfonic acid for 6 hours.

E = Propanol and water (1:1), for 24 hours on paper buffered with 0.75 M phosphate, pH 4.0.

F = n-Butanol saturated with water, for 18 hours.

G = n-Butanol saturated with water, plus 2 percent p-toluenesulfonic acid and 2 percent piperidine, for 18 hours.

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The chromatographic data shown above complex with the separation of Tenebrimycin

The chromatographic data shown above indicate that while with any single solvent system several of the factors comprising the tenebrimycin complex appear to be identical to each other or to other known antibiotics, chromatography with a variety of solvent systems clearly establishes non-identity, with the exception of Tenebrimycin V and Kana-

mycin B. These results, considered with other data, such as physical and physiochemical properties, microbiological activities, toxicities, and the like support the conclusion that the individual factors comprising the tenebrimycin complex, with the exception of Tenebrimycin V, constitutes a series of hitherto unknown antibiotic substances.

The tenebrimycin complex and the individual factors comprising the said complex have an inhibitory action against the growth of microorganisms which are pathogenic to animal and plant life. The inhibitory concentrations of the complex and of the individual tenebrimycins against a number of organisms are shown in the following table. The minimum inhibitory concentrations were determined by the agar dilution method.

TABLE II

Test Organism	Minimum Inhibitory Concentration (mcg./ml.)					
	Tenebrimycin ^a	Tenebrimycin II	Tenebrimycin IV	Tenebrimycin V	Tenebrimycin VI	
<i>Staphylococcus aureus</i> 3055	6.25	6.25	6.25	6.25	3.125	
<i>Bacillus subtilis</i>	<1.56	<1.56	<1.56	<1.56	<1.56	
<i>Mycobacterium avium</i>	<1.56	<1.56	<1.56	6.25	<1.56	
<i>Streptococcus faecalis</i>	6.25	3.12	<1.56	3.12	3.12	
<i>Lactobacillus casei</i>	25	50	25	25	12.5	
<i>Leuconostoc citrovorum</i>	25	50	25	25	12.5	
<i>Escherichia coli</i> No. 1	25	25	12.5	12.5	12.5	
<i>Escherichia coli</i> No. 2	25	50	12.5	25	12.5	

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TABLE II (continued)

Test Organism	Minimum Inhibitory Concentration					
	Tenebrimycin ^a	Tenebrimycin II	Tenebrimycin IV	Tenebrimycin V	Tenebrimycin VI	
<i>Proteus</i> sp. No. 1	50	100	50	50	25	25
<i>Proteus</i> sp. No. 2	25	25	12.5	25	12.5	12.5
<i>Pseudomonas</i> sp. No. 2	25	25	100	100	6.25	6.25
<i>Pseudomonas</i> sp. No. 5	6.25	12.5	6.25	3.12	<1.56	<1.56
<i>Klebsiella-Aerobacter</i> No. 14	6.25	12.5	3.12	12.5	6.25	6.25
<i>Klebsiella-Aerobacter</i> No. 15	12.5	12.5	6.25	12.5	6.25	6.25
<i>Salmonella</i> sp. No. 1	25	25	25	25	12.5	12.5
<i>Vibrio metschnikovii</i>	12.5	12.5	12.5	6.25	6.25	6.25
<i>Agrobacterium tumefaciens</i>	50	25	25	50	50	50
<i>Erwinia amylovora</i>	<1.56	<1.56	<1.56	<1.56	<1.56	<1.56
<i>Pseudomonas solanacearum</i>	25	25	12.5	12.5	12.5	12.5
<i>Xanthomonas phaseoli</i>	12.5	12.5	12.5	6.25	3.12	3.12

^aMinimum inhibitory concentrations for the individual tenebrimycins were determined with the free bases. The tenebrimycin complex was employed as a sulfate salt.

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The acute toxicity of the tenebrimycin complex and of the relatively more abundant constituents thereof has been determined in mice. The LD_{50} values for tenebrimycin sulfate are about 300 mg./kg. when the antibiotic is injected intravenously and about 350 mg./kg. when the drug is administered intraperitoneally. At subcutaneous doses of 750 mg./kg. of oral doses of 5600 mg./kg. all mice survived. No significant irritation was observed when one eye of each of four rabbits was treated with one drop of a 50 percent aqueous solution of tenebrimycin sulfate three times daily for five days. The LD_{50} values, expressed as mg./kg. of the free base, for the individual tenebrimycins administered intravenously to mice are as follows: tenebrimycin II about 385, tenebrimycin IV about 220, tenebrimycin V about 140, and tenebrimycin VI about 120.

Because of the uncertainty of taxonomic studies with the *Streptomyces* group of organisms, there is always an element of doubt associated with the classification of a newly discovered organism. However, as nearly as can be determined, the organism employed in the production of the antibiotic complex provided by this invention does not sufficiently resemble any of the streptomyces species described by Waksman in *The Actinomycetes*, Vol. II, The Williams and Wilkins Company (1961), to warrant cultural comparisons. The organism therefore is described and characterized as a new species.

The organism producing the antibiotic complex is a spiral-forming, thermo-durant, aerobic to microaerophilic *Streptomyces* with oblong smooth-walled spores. It is unique in being inhibited by relatively low intensities of artificial light. Because of the latter property, the new species name *Streptomyces tenebrarius* sp. n. has been selected for this organism.

The organism was isolated from a soil sample by suspending portions of the soil sample in sterile distilled water and streaking the suspensions on nutrient agar. The seeded nutrient agar plates were incubated at 25–35°C. until growth was secured. At the end of the incubation period, colonies of the antibiotic-producing organisms were transferred with a sterile platinum loop to agar slants. The agar slants were then incubated to provide suitable quantities of inoculum for the production of the antibiotic. The strain of the organism employed for the production of the antibiotic complex has been placed on permanent deposit with the American Type Culture Collection at Washington, D.C. and has been assigned the culture No. ATCC 17920. The said strain is employed in the production of all factors of the antibiotic complex; under the preferred fermentation conditions, tenebrimycins II, IV, V and VI predominate.

The methods employed in the taxonomic

studies of *S. tenebrarius* ATCC 17920 are those commonly used in the taxonomy of actinomycetes. Cultural characteristics were observed after 14 days' incubation. Morphology was determined on Czapek's peptone agar and Bennett's agar during 2 to 7 days' incubation. Action on milk and the reduction of nitrate were observed at 7 and 14 days, hydrogen sulfide production at 24 and 48 hours, and carbon utilization at 10 days. Unless otherwise noted, the cultures were incubated at 37°C. Carbon utilization tests were carried out according to the method described by Pridham and Gottlieb, *J. Bact.*, 56, 107 (1948).

The results of the taxonomic studies are summarized in the paragraphs which follow. The figures in parentheses refer to color blocks in Maerz and Paul, *Dictionary of Color*, McGraw Book Company, (1950). The colors represented by the color blocks have been translated into the ISCC—NBS color names as found in Circular 553, U.S. Department of Commerce, National Bureau of Standards, 1955.

Microscopic Morphology, Cultural Characteristics and Physiology of *S. tenebrarius* ATCC 17920.

Morphology — On Czapek's-peptone agar; branching spore forms are formed in random clusters on the aerial mycelium. Isolated spores are rare. The intact aerial mycelium is easily detached from the substrate. Mature spore chains usually form 5–6 open spirals. Spores are oblong to cylindrical. When observed with the aid of the electron microscope the spores appear smooth and measure 0.7 to 1.3 by 2.0 to 2.1 microns. Sclerotia were observed by Bennett's medium.

Colony characteristics on:

Czapek's agar — Amount of growth sparse; aerial mycelium sparse, pale orange-yellow (11–A2); sporulation fair; reverse pale orange-yellow (11–A2); soluble pigment slight pink (1–B1).

Czapek's peptone — Growth abundant; aerial mycelium abundant, light yellowish brown with white areas (11–A4); sporulation abundant; reverse light grayish red (4–H1); soluble pigment grayish pink (4–B1).

Calcium malate agar — Growth moderate, aerial mycelium moderate, pale orange-yellow (11–A2); sporulation moderate; reverse grayish pink (4–D1); soluble pigment grayish pink (4–B1).

Tyrosine agar — Growth scant, aerial mycelium scant, pale orange-yellow (9–B2); scant sporulation; reverse pale orange-yellow (10–B3); no soluble pigment.

Inorganic salts—starch agar — Growth moderate, aerial mycelium moderate, brownish pink with white areas (11–A4); sporulation abundant; reverse pale yellow

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	(11—B2); soluble pigment pale yellow (11—B2).	50° — Growth, aerial mycelium and sporulation all abundant.	45
	<i>Glucose—asparagine agar</i> — Growth moderate, aerial mycelium moderate, pale yellow (9—D2) with white areas (10—A1); moderate sporulation; reverse pale yellow (11—B2); no soluble pigment.	55° — Scant growth.	
5	<i>Tomato paste—oatmeal</i> — Growth abundant, aerial mycelium abundant, light yellowish brown (12—B5); abundant sporulation; reverse dark grayish reddish brown (48—J2); soluble pigment dark purplish red (47—H1).	60° — No growth.	
10	<i>Yeast extract</i> — Abundant growth, abundant aerial mycelium, pale orange-yellow (11—A2) with white areas; abundant sporulation; reverse moderate yellow (11—J6); no soluble pigment.	<i>Thermal death point</i> — Spores from a spore suspension heated to 75°C. for 1 minutes remain viable. When the spore suspension was heated to 100°C. for 15 minutes, no spores were viable.	50
15	<i>Nutrient agar</i> — Growth sparse, sparse white (10—A1) aerial mycelium; sparse sporulation; reverse grayish greenish yellow (12—I2); no soluble pigment.	<i>Oxygen tension</i> — Growth is either aerobic or microaerophilic in stab cultures.	
20	<i>Bennett's agar</i> — Growth moderate, moderate white (10—A1) to (10—B1) aerial mycelium; moderate sporulation; reverse pale yellow (11—C2); no soluble pigment.	<i>Effect of ferric ion</i> — A red soluble pigment is produced only in the presence of ferric ion and the intensity of pigmentation is proportional to the concentration of ferric ion within a given range.	55
25	<i>Action on milk</i> — Heavy yellow ring of growth on surface. Coagulation and peptonization observed.	<i>Effect of hydrogen ion concentration, observed on Czapek's peptone agar</i> — No growth below pH 5.0; from pH 5.0 to 6.0 growth and aerial mycelium are fair. Growth and sporulation are abundant at pH 7.0; from pH 8.0 to 8.6 growth and aerial mycelium are fair. The soluble pigment is most intense at pH 5.0 to 6.0 and is slight to absent from pH 6.5 to 8.6.	60
30	<i>Nitrate reduction</i> — Positive.	<i>Reaction to light, observed on Czapek's agar</i> — Growth and sporulation are abundant in the dark. When plate cultures are incubated 15 inches from a 15 watt standard cool white fluorescent light source, growth is sparse and aerial mycelium is absent. Growth and aerial mycelium are moderate when the plate cultures are incubated 15 inches from a 60 watt frosted tungsten lightbulb.	70
	<i>H₂S production</i> — Negative.	In the table summarizing the results of the carbon utilization test carried out with <i>S. tenebrarius</i> ATCC 17920, the symbols employed are interpreted as follows:	
	<i>Nutrient gelatin</i> — Complete liquefaction after 14 days.	++ = positive	75
35	<i>Temperature requirements on Czapek's peptone agar:</i>	(+) = probable	
	20° — No growth.	(-) = questionable	80
	26° — Fair growth, no aerial mycelium.	- = none	85
	30° — Growth and aerial mycelium moderate but no sporulation.		
40	37° — Growth, aerial mycelium and sporulation all abundant.		
	43° — Growth, aerial mycelium and sporulation all abundant.		

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TABLE III
Carbon Utilization by *S. tenebrarius* Strain ATCC 17920

Carbon Source	Response	Carbon Source	Response
L (+) Arabinose	-	D (+) Trehalose	+
L (+) Rhamnose	-	L (+) Raffinose	-
D (-) Ribose	+	Cellulose	-
D (+) Xylose	-	Inulin	(-)
D (-) Fructose	+	i-Inositol	+
D (+) Mannose	+	Mannitol	-
D (+) Glucose	+	d-Sorbitol	(-)
Lactose	(-)	Salicin	(+)
Maltose	+	Control (no carbon)	(-)
Sucrose	(+)		

S. tenebrarius ATCC 17920 also produces the known antifungal antibiotic caerulomycin, which is also produced by *Streptomyces caeruleus* [Can. J. Microbiol., 5, 317, (1959)]. Investigation of a culture of the latter organism revealed that it does not produce tenebrimycin under the conditions described for its culture, nor can it be induced to do so under varied conditions.

A second strain of the new species *Streptomyces tenebrarius* sp. n., which strain produces only three of the factors of the antibiotic complex, has also been isolated and characterized. This strain of *S. tenebrarius*, which produces only tenebrimycins I, I', and II, has been assigned the ATCC accession number ATCC 17921. Strain ATCC 17921 differs from strain ATCC 17920 principally in that it fails to produce an aerial mycelium or spores, and on most media it produces no soluble pigment. The characterization data for this strain of the organism is summarized in the paragraphs which follow. The methods employed in the taxonomic studies of *S. tenebrarius* ATCC 17921 are the same as those referred to above in the description of the characterization of *S. tenebrarius* ATCC 17920, and the symbols employed have the same significance as above.

Microscopic Morphology, Cultural Characteristics and Physiology of *S. tenebrarius* ATCC 17921.

Morphology — Neither aerial mycelium nor spores are present; the vegetation mycelium fragments in slide amounts.

Colony characteristics on:

Czapek's agar — Vegetative growth fair, yellowish white (9—B1); no aerial mycelium; no soluble pigment.

Czapek's peptone — Vegetation growth moderate, grayish yellow (12—B2); no aerial mycelium; slight brownish soluble pigment.

Calcium malate agar — Vegetative growth moderate, grayish yellow (12—B2); no aerial mycelium; slight grayish brown soluble pigment.

Tyrosine agar — Vegetative growth moderate, grayish yellow (11—B2); neither aerial mycelium nor soluble pigment produced.

Inorganic salt—starch agar — Vegetative mycelium fair, light yellow-brown (13—C6); no aerial mycelium; light brown soluble pigment.

Glucose—asparagine agar — Vegetative growth moderate, grayish yellow (11—D2); no aerial mycelium or soluble pigment.

Tomato paste—oatmeal — Vegetative mycelium moderate, pale yellow (10—B2); no aerial mycelium or soluble pigment.

Yeast extract — Vegetative mycelium moderate, light yellow-brown (12—E5); no aerial mycelium or soluble pigment.

Nutrient agar — Vegetative growth moderate, light yellow (9—K4); no aerial mycelium or soluble pigment.

Bennett's agar — Vegetative growth moderate, grayish yellow (12—E4); no aerial mycelium or soluble pigment.

Action on milk — Buff coloured ring of growth on surface. Coagulation and peptonization observed.

Nitrate reduction — Positive.

H₂S production — Negative.

Nutrient gelatin — Complete liquefaction in seven days.

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Oxygen tension — Growth in either aerobic or microaerophilic in stab cultures.

Temperature requirements on Bennett's medium — Growth is moderate at 26, 30, 43, 49, and 55°C. and is most abundant at 37°C.;

no growth occurs at 60°C. The vegetative mycelium is pale yellow-brown at and below 37°C. and is slightly reddish brown at and above 43°C. Neither aerial mycelium nor soluble pigment are observed at any temperature.

TABLE IV
Carbon Utilization by *S. tenebrarius* Strain ATCC 17921

Carbon Source	Response	Carbon Source	Response
L (+) Arabinose	-	Sucrose	(-)
L (+) Rhamnose	-	Trehalose	(+)
D (-) Ribose	+	Raffinose	-
D (+) Xylose	(-)	Cellulose	-
D (-) Fructose	+	i-Inositol	+
D (+) Mannose	(+)	Mannitol	(-)
D (+) Glucose	+	d-Sorbitol	(-)
Lactose	-	Salicin	(-)
Maltose	+	Control (minus carbon)	-

Although this invention is described in detail with particular reference to the newly found organisms *S. tenebrarius* ATCC 17920 and ATCC 17921, it is to be understood that the production of the tenebrimycin complex or of the various factors comprising the said complex by tenebrimycin-producing mutants of *Streptomyces tenebrarius* are within the scope of this invention. The proportions of the various tenebrimycin factors produced by the tenebrimycin-producing mutants would not necessarily be the same as herein indicated. Such tenebrimycin-producing mutants can be produced or obtained by known procedures, by subjecting *S. tenebrarius* ATCC 17920 or ATCC 17921 to X-ray or ultra-violet irradiation or to chemical agents such as, for example, the nitrogen mustards.

The culture medium employable in the fermentation of the organism can be any one of several media since, as it apparent from the above-described utilization tests, the organisms are capable of utilizing a variety of energy sources. However, for economy of production, maximum yield of antibiotic, and ease of isolation, certain culture media containing relatively simple nutrient sources are preferable. For example, the media which are useful in the production of tenebrimycin include an assimilable source of carbon such as glucose, fructose, mannose, maltose and starch. A highly preferred source of carbon is glucose. In addition, the employable media

include a source of assimilable nitrogen, such as peptones, hydrolyzed casein, yeast and amino acids. Presently preferred sources of nitrogen are peptones, hydrolyzed casein and glutamine.

Mineral salts, for example those providing calcium, magnesium, sodium, potassium, chloride, sulfate, phosphate, and carbonate ions can be incorporated in the media with beneficial results, although an excess of phosphate should be avoided since it appears to depress yields of the antibiotic. A source of growth factors such as yeast or yeast extract can also be beneficially included in the medium.

As is necessary for the growth and development of other microorganisms, essential trace elements should also be included in the culture medium for growing the organisms employed in this invention. Such trace elements are commonly present as impurities incidental to the addition of other constituents of the medium.

Submerged aerobic culture conditions are the conditions of choice for the production of antibiotic activity. For preparation of relatively small amounts, shake flasks and surface culture can be employed, but for the preparation of large amounts, submerged aerobic culture in sterile tanks is preferred. The medium in the sterile tank can be inoculated with a sporulated suspension, but because of the growth lag experienced when a sporu-

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lated suspension is used as the inoculum, the vegetative form of the culture is preferred. By thus avoiding the growth lag, more efficient use of the fermentation equipment is realized. Accordingly, it is desirable first to produce a vegetative inoculum of the organism by inoculating a relatively small quantity of culture medium with the spore form of the organism and, when a young active vegetative inoculum has been obtained, to transfer the vegetative inoculum aseptically to the large tank. Suitably, an aliquot of the vegetative inoculum, equal to about 4 percent of the volume of the medium into which the inoculum is induced, is employed. The fermentation medium in which the vegetative inoculum is produced can be either the same as or different from the medium utilized for the large scale production.

As is evident from the above detailed temperature requirements for the organism, the organism will grow over a relatively wide range of temperatures. However, the organisms appear to grow best at temperatures in the range of about 30 to 50°C. Optimal production of antibiotic activity appears to occur at a temperature of about 37 to 43°C. The organisms are sensitive to light and do not grow well in the presence thereof. Accordingly, fermentations employing the organisms are desirably carried out in the absence of visible light.

In accord with the customary practice in aerobic submerged culture processes, sterile air is blown through the culture medium. For efficient growth of the organisms and antibiotic production the volume of air employed in the tank fermentation process is upwards of 0.1 volume of air per minute per volume of culture medium, and is preferably substantially higher. Efficient growth of the organisms and optimal yields of antibiotic activity are obtained when the volume of air employed is at least one volume of air per minute per volume of culture medium.

The concentration of antibiotic activity in the fermentation medium can readily be followed during the course of the fermentation by testing samples of the culture medium for their inhibitory activity against the growth of organisms known to be inhibited in the presence of tenebrimycin. Two of the organisms so employed are *Klebsiella pneumoniae* and *Mycobacterium butyricum*. The former organism is generally employed in the well-known turbidimetric technique, while the latter is utilized in the cup plate method.

In general, maximum production of the antibiotic occurs within about 4 to 7 days after inoculation of the culture medium when submerged aerobic culture or shake flask culture is employed and within a somewhat longer time when surface culture is used.

The mycelium and undissolved residues are removed from the fermentation broth by con-

ventional means such as filtration or centrifugation. The antibiotic activity is contained in the filtered broth and can be recovered therefrom by employing adsorption techniques. The adsorbents which can be employed most advantageously are the cation exchange resins, for example those of the type available commercially under the trade mark "Amberlite IRC-50".

In general, the procedure for recovering tenebrimycin from the fermentation broth is as follows. The whole broth is filtered with the help of a filter aid after the pH of the mixture has been lowered to about pH 2 by the addition of an acid, such as sulfuric acid, phosphoric acid, hydrochloric acid, and the like. The pH of the filtrate is adjusted to about pH 5.5 by the addition of a concentrated base and the mixture is again filtered. The filtrate is passed through an ion exchange column packed with Amberlite IRC-50 in the ammonium cycle. The column is then washed with distilled water and the antibiotic activity is eluted with dilute acid. The fractions containing the antibiotic activity are pooled and concentrated to about 1/20 of the original volume. The pH of concentrate is raised to about pH 11 by the addition of concentrated base and the basic concentrate is poured into about six volumes of acetone, mixed well and chilled. The microbiologically inactive precipitate which separates is removed by filtration. The pH of the filtrate is lowered to about pH 3.5 by the addition of 20 percent sulfuric acid with thorough mixing. The tenebrimycin complex is precipitated in the form of the sulfate salt, while the caeruleomycin which is coproduced in the fermentation remains in the supernatant layer which is discarded. The tenebrimycin sulfate is dissolved in a minimum quantity of water and the aqueous solution is passed through a Dowex 1 x 1 column. ("DOWEX" is a Trade Mark). The aqueous solution of tenebrimycin sulfate is followed on the column with distilled water and the effluent from the column is collected in fractions. The active fractions are pooled and concentrated to a heavy syrup and are then dried to yield the free base form of tenebrimycin.

The major factors comprising the tenebrimycin complex can, if desired, be obtained as single antibiotics by further fractionation of the tenebrimycin obtained as described above. For the fractionation the tenebrimycin is employed in the form of an acid addition salt, suitably the sulfate. A solution of tenebrimycin sulfate is conveniently obtained by preparing a 20 percent aqueous solution of tenebrimycin and adjusting the pH of the solution to about pH 4.5 by the addition of sulfuric acid. Colored impurities can be removed by stirring the solution thus prepared without 5 percent (weight/volume) of an activated carbon such as, for example, Darco

G-60 ("Dacro" is a Trade Mark). Effective decolorization is usually obtained by stirring the mixture containing the carbon for about one hour. The mixture is then filtered and the filtrate which contains the tenebrimycin sulfate is loaded onto a column packed with Amberlite IRC-50 in the ammonium cycle. The column is washed with water and the individual antibiotics comprising the tenebrimycin complex are fractionally eluted with 0.1 N ammonium hydroxide. Generally speaking, the elution is accomplished extremely slowly, and a very large number of fractions is usually necessary in order to effect fractionation of the complex. Thus, for example, the complete fractionation of about 250 g. of tenebrimycin requires about 700 fractions, each comprising about 900 ml. of eluate.

The first active fractions obtained contain tenebrimycins I, I', and II. These are followed by a series of active fractions in which tenebrimycin II is the only antibiotic present. A number of inactive fractions follow the elution of tenebrimycin II before the fractions containing tenebrimycin IV are eluted. The first fractions containing tenebrimycin IV appear to contain traces of tenebrimycin III. Tenebrimycin V is obtained from the column in the active fractions which follow tenebrimycin IV. In order to obtain tenebrimycin VI, the column is further eluted with a more concentrated ammonium hydroxide solution after all of the tenebrimycin V has been recovered from the column. A suitable eluate for the removal of tenebrimycin VI is 0.3 N ammonium hydroxide.

EXAMPLE 1.

Fermentation of *S. tenebrarius* ATCC 17920.

A sporulated culture of *S. tenebrarius* ATCC 17920 is produced by growing the organism on a modified Bennett's agar slant medium having the following composition:

Dextrine	10 g.
Yeast extract (Difco)	1 g.
Hydrolyzed casein (NZ-Amine A, sold by Sheffield Farms)	2 g.
Beef Extract (Difco)	1 g.
CoCl ₂ ·6H ₂ O	0.01 g.
Washed agar	20 g.
Deionized water, q.s. to	1000 ml.

The pH of the medium is adjusted to pH 7 before autoclaving.

The slant is inoculated with spores of *S. tenebrarius* ATCC 17920 and is incubated in the absence of visible light for five days at 37°C. The culture growth on the slant is covered with water and the slant is scraped gently to remove the spores to provide an aqueous spore suspension.

The spore suspension so obtained is em-

ployed to inoculate 800 ml. of a medium having the following composition:

Dextrose	0.05%	65
Nutrisoy flour (Sold by Archer-Daniels-Midland Company—contains 35—45 percent of dispersible protein)	1.5%	
Dextrin 700 (A low chloride potato dextrin made by the Morningstar-Paisley Company)	1%	70
Potassium chloride	0.1%	
NZ-Amine A	0.3%	75
KH ₂ PO ₄	0.05%	
MgSO ₄ ·7H ₂ O	0.5%	
CaCl ₂ ·2H ₂ O	0.025%	
Deionized water		

The inoculated vegetative medium is incubated at about 37°C. for 16 hours on a rotary shaker operating at 250 rpm. and having a 2½ inch throw.

A 50-ml. portion of the vegetative culture is employed to inoculate a 44-liter seed tank containing an aqueous medium having the following composition:

Dextrose	1%	
Soybean grits	1.5%	
KH ₂ PO ₄	0.05%	90
MgSO ₄	0.5%	
KCl	0.1%	
CaCl ₂ ·2H ₂ O	0.025%	
Antifoam agent	0.025%	

The seed tank medium is sterilized at 120°C. for about 30 minutes. The inoculated seed tank medium is incubated at 37°C. for 12 hours. Stirring at a speed of 370 rpm. is begun immediately after inoculation and aeration at the rate of 0.8 cubic feet per minute is maintained throughout the incubation period. At the end of the incubation period the contents of the seed tank are utilized to inoculate a 250 gallon fermentor containing a medium having the following composition:

Dextrose	4%	
Refined soybean oil	3%	
Soybean flour	3%	
NH ₄ Cl	0.5%	110
CaCl ₂	0.3%	
MgSO ₄	0.2%	
NH ₄ NO ₃	0.1%	
NZ-Amine A	0.5%	115
Antifoam agent	0.2%	
Deionized water		

Prior to inoculation, the fermentation medium is sterilized for 30 minutes at 120°C. The fermentation is carried out at 37°C. with aeration at the rate of 17 cubic feet per minute throughout the period from inoculation to

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harvest. Stirring is begun at 125 rpm. and is increased to 180 rpm. after 12 hours. The fermentation is continued for five days.

The fermented culture broth is filtered to remove the mycelium and other undissolved solids. The filtered broth contains antibiotic activity at a concentration of about 680 units per milliliter.

EXAMPLE 2.

10 Fermentation of *S. tenebrarius*.

The procedure described in Example 1 is followed using *S. tenebrarius* ATCC 17921 as the antibiotic-producing organism. The filtered fermentation broth so obtained contains predominantly tenebrimycin II with minor proportions of tenebrimycons I and I'.

EXAMPLE 3.

Isolation of Tenebrimycin Complex.

The pH of 880 liters of antibiotic fermentation broth, obtained as described in Example 1, is lowered to about pH 2 by the addition of about 10—15 litres of 20 percent aqueous sulfuric acid. After 45 kg. of a commercial filter aid are added to the acidified broth, the mixture is mixed thoroughly and filtered. The filter cake is washed with water and the wash solution is added to the filtrate.

The pH of the filtrate is adjusted to pH 5.5 by the addition of 50 percent aqueous sodium hydroxide. About 4 kg. of a commercial filter aid are added and the mixture is stirred thoroughly and filtered. The filtrate is passed through an Amberlite IRC—50 column having dimensions of 4 inches by 6 feet. The column is packed with the resin in the ammonium cycle to a bed height of 44 to 45 inches. After all of the filtrate has passed through the column, the column is washed with about 200 liters of distilled water. The antibiotic activity is then eluted over a period of about 12 to 15 hours with about 150 to 175 liters of 0.1 N sulfuric acid, the eluate being collected in 10-liter fractions. Antibiotic activity becomes detectable in the eluate when the pH thereof decreases to about pH 4 or slightly lower. As the elution progresses, the pH of the eluate continues to drop until it reaches a level of about pH 1.5. The fractions containing antibiotic activity are pooled and concentrated under reduced pressure to about 1/20 of the original volume.

The pH of the concentrated eluate is adjusted to pH 11 by the addition of 50 percent aqueous sodium hydroxide and the basic concentrate is then added to about six volumes of acetone. The mixture is chilled and set aside in order to permit a precipitate devoid

of antibiotic activity to separate. The mixture is filtered and the filter cake, containing inactive precipitate is washed with water and discarded.

The pH of the filtrate is adjusted to pH 3.5 by the addition, with thorough mixing, of 20 percent aqueous sulfuric acid. The tenebrimycin complex precipitates as the sulfate during this treatment and is substantially completely precipitated when the pH of the solution reaches about pH 4 or slightly less. The mixture is filtered and the filtrate, containing the caerulomycin which was coproduced in the fermentation, is discarded.

The filter cake comprising the tenebrimycin sulfate is dissolved in a minimum quantity of water and chilled. Any solids which do not dissolve readily are discarded, as are any solids which precipitate or crystallize during the chilling. The clear solution of tenebrimycin sulfate is passed over a 4-inch by 7-foot bed of Dowex 1 × 1 resin in the basic cycle. The antibiotic solution is followed on the column by about 40 to 50 liters of distilled water. The effluent is collected in 10-liter fractions and the active fractions are combined and concentrated under vacuum to a heavy syrup which is pan dried to give dry tenebrimycin complex in free base form.

EXAMPLE 4.

Separation of the Tenebrimycin Factors.

Tenebrimycin sulfate is obtained by adjusting the pH of a 20 percent aqueous solution containing 258 g. of the tenebrimycin complex in free base form to pH 4.5 by the addition of concentrated sulfuric acid. The solution of tenebrimycin sulfate so prepared is decolorized by stirring for about one hour with 5 percent (weight/volume) of Darco-G—60. The solution is filtered and the filtrate is passed through a 9 × 105 cm. column packed with about 7 liters of Amberlite IRC—50 resin in the ammonium cycle. After the filtrate containing the antibiotic salt has passed through the column, the column is washed with about 17 liters of deionized water. Elution of the antibiotic activity retained by the column is begun with 0.1 N ammonium hydroxide solution at a flow rate of about 20 ml. per minute. The eluate is collected in fractions of about 900 ml. and each fraction is assayed for microbiological activity. The fractions containing the same antibiotic factor are pooled and lyophilized to obtain the individual dried tenebrimycons. The individual tenebrimycons are distributed among the active fractions as follows:

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Fraction	Antibiotic Present
1-34	Inactive
35-53	Tenebrimycin I, I' and II
54-77	Tenebrimycin II
78-153	Inactive
154-172	Tenebrimycin IV with a trace of Tenebrimycin III
173-249	Tenebrimycin IV
250-256	Inactive
257-634	Tenebrimycin V
635-668 ¹	Inactive
669-689	Tenebrimycin VI

¹At this point the eluate is changed to 0.3 N ammonium hydroxide.

EXAMPLE 5.

Preparation of Helianthates of the Tenebrimycins.

5 The preparation of the helianthates of the individual tenebrimycins is illustrated by the following procedure for the preparation of tenebrimycin II helianthate.

10 A solution of 2 g. of tenebrimycin II in 22 ml. of water is prepared and adjusted to pH 5 by the addition of 50 percent sulfuric acid. A hot 10 percent aqueous solution of methyl-orange is then added to the antibiotic solution. The helianthate of tenebrimycin II separates as a precipitate and is removed by filtration. The solid salt is washed thoroughly with hot water and dried under vacuum to yield 3.5 g of tenebrimycin II helianthate.

EXAMPLE 6.

Final Purification of Tenebrimycin Factors.

20 The final purification of the individual tenebrimycin factors is illustrated by the following procedure for the purification of tenebrimycin VI.

25 The helianthate of tenebrimycin VI is prepared according to a procedure analogous to that described in Example 5. Four grams of the salt so prepared are slurried with about 200 ml. of water adjusted to pH 2 by the addition of sulfuric acid. The mixture is stirred for about 4 hours and is then filtered through a medium porosity sintered glass funnel to remove helianthic acid. The filtrate is decolorized by passage through a thin layer of activated carbon and is then passed over a 1 x 32 cm. column packed with Dowex 1 x 2 resin in the hydroxyl cycle. The filtrate is followed on the column by distilled

water to complete the elution of anti-
biotic activity. The effluent is collected
in fractions and the active fractions are
pooled and lyophilized to give a dry residue
comprising the antibiotic. The residue is dis-
solved in about 15 ml. of water and is then
passed over a 1 x 25 cm. column packed
with Bio Rad resin AG11A8. (This is a
retardion type resin prepared by polymerizing
acrylic acid within a Dowex 1 resin, sold
by Bio Rad Laboratories, Richmond, Cali-
fornia.) The antibiotic activity is eluted from
the column with distilled water and the active
fractions are again pooled and lyophilized to
yield about 700 mg. of highly purified tene-
brimycin VI.

Darco G-60 is a finely divided, highly
adsorptive carbon.

Amberlite IRC-50 is a weakly acidic poly-
methacrylate carboxylic acid resin.

Dowex 1 x 1 is a styrene-divinylbenzene
polymer to which quaternary ammonium
exchange groups are attached. The par-
ticular designation indicates that 1 per-
cent of divinylbenzene cross-linking agent
is present in the polymer.

Dowex 1 x 2 is the same resin as that
described immediately above except that
the divinylbenzene cross-linking agent is
present to the extent of 2 percent.

WHAT WE CLAIM IS:—

1. An antibiotic substance comprising a
tenebrimycin complex or tenebrimycin I,
tenebrimycin I', tenebrimycin II, tenebrimycin
III, tenebrimycin IV, tenebrimycin VI or
mixtures thereof or the acid addition salts
thereof with pharmaceutically acceptable
acids.

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Fraction	Antibiotic Present
1-34	Inactive
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173-249	Tenebrimycin IV
250-256	Inactive
257-634	Tenebrimycin V
635-668 ¹	Inactive
669-689	Tenebrimycin VI

¹At this point the eluate is changed to 0.3 N ammonium hydroxide.

EXAMPLE 5.

Preparation of Helianthates of the Tenebrimycins.

5 The preparation of the helianthates of the individual tenebrimycins is illustrated by the following procedure for the preparation of tenebrimycin II helianthate.

10 A solution of 2 g. of tenebrimycin II in 22 ml. of water is prepared and adjusted to pH 5 by the addition of 50 percent sulfuric acid. A hot 10 percent aqueous solution of methyl-orange is then added to the antibiotic solution. The helianthate of tenebrimycin II separates as a precipitate and is removed by filtration. The solid salt is washed thoroughly with hot water and dried under vacuum to yield 3.5 g. of tenebrimycin II helianthate.

EXAMPLE 6.

Final Purification of Tenebrimycin Factors.

20 The final purification of the individual tenebrimycin factors is illustrated by the following procedure for the purification of tenebrimycin VI.

25 The helianthate of tenebrimycin VI is prepared according to a procedure analogous to that described in Example 5. Four grams of the salt so prepared are slurried with about 200 ml. of water adjusted to pH 2 by the addition of sulfuric acid. The mixture is stirred for about 4 hours and is then filtered through a medium porosity sintered glass funnel to remove helianthic acid. The filtrate is decolorized by passage through a thin layer of activated carbon and is then passed over a 1 x 32 cm. column packed with Dowex 1 x 2 resin in the hydroxyl cycle. The filtrate is followed on the column by distilled

water to complete the elution of anti-
biotic activity. The effluent is collected
in fractions and the active fractions are
pooled and lyophilized to give a dry residue
comprising the antibiotic. The residue is dis-
solved in about 15 ml. of water and is then
passed over a 1 x 25 cm. column packed
with Bio Rad resin AG11A8. (This is a
retardation type resin prepared by polymerizing
acrylic acid within a Dowex 1 resin, sold
by Bio Rad Laboratories, Richmond, Cali-
fornia.) The antibiotic activity is eluted from
the column with distilled water and the active
fractions are again pooled and lyophilized to
yield about 700 mg. of highly purified tene-
brimycin VI.

Darco G-60 is a finely divided, highly
adsorptive carbon.

Amberlite IRC-50 is a weakly acidic poly-
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Dowex 1 x 1 is a styrene-divinylbenzene
polymer to which quaternary ammonium
exchange groups are attached. The par-
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cent of divinylbenzene cross-linking agent
is present in the polymer.

Dowex 1 x 2 is the same resin as that
described immediately above except that
the divinylbenzene cross-linking agent is
present to the extent of 2 percent.

WHAT WE CLAIM IS:—

1. An antibiotic substance comprising a
tenebrimycin complex or tenebrimycin I,
tenebrimycin I', tenebrimycin II, tenebrimycin
III, tenebrimycin IV, tenebrimycin VI or
mixtures thereof or the acid addition salts
thereof with pharmaceutically acceptable
acids.

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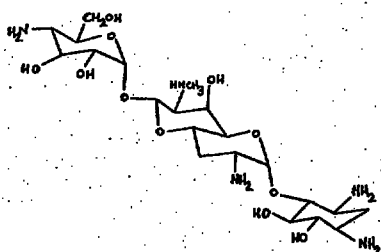
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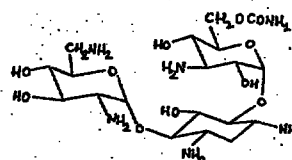
2. The antibiotic tenebrimycin complex being characterized as follows: a white solid substance which is soluble in water and dimethylsulfoxide, slowly soluble in methanol and insoluble in most other organic solvents; is stable over a pH range of pH 1 to pH 11; gives a positive reaction in the ninhydrin, anthrone and Elson-Morgan tests, a slightly positive reaction in the Lowry protein test, and negative reactions in the biuret and Sakaguchi tests; and has an infrared absorption spectrum characterized by distinguishable maxima at 3.02, 3.15, 5.82, 6.26, 7.43, 8.8, and 9.7 microns as illustrated in Figure 1.

3. The antibiotic tenebrimycin II having the structural formula



being further characterized as follows: a white solid substance having titratable groups of pK'a values of about 5.7, 6.7, 7.7, and 8.7 as determined by electrometric titration in water; has an apparent molecular weight of about 454 as determined from the electrometric titration data; has a specific rotation, $[\alpha]_D^{25}$, of $+159^\circ$ ($c = 1$, weight per volume); has the approximate percentage composition of C 44.90, H 8.20, N 12.53, and O 33.24; and has infrared spectral absorption maxima, as a mineral oil mull, of 3.02, 3.14, 6.05, 6.23, 7.4, 8.5, 8.75, 9.17, 9.65, 10.11, 11.15, 11.7, and 12.6 microns as illustrated in Figure 2.

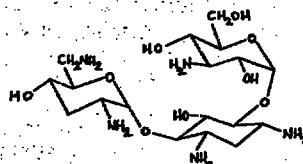
4. The antibiotic tenebrimycin IV having the structural formula



being further characterized as follows: a white solid substance having titratable groups of pK'a values of about 5.3, 6.8, 7.8, and 9.0 as determined by electrometric titration in water; has an apparent molecular weight of about 478 as determined from the electrometric titration data; has a specific rotation, $[\alpha]_D^{25}$, of $+114^\circ$ ($c = 1$, weight per volume); has the approximate percentage composition of C 41.66, H 7.78, N 15.02, and O 34.93;

and has infrared spectral absorption maxima, as a mineral oil mull, of 3.05, 3.17, 5.85, 6.27, 7.46, 8.75, 9.7, 10.5, 11.1, and 12.85 microns as illustrated in Figure 3.

5. The antibiotic tenebrimycin VI having the structural formula



being further characterized as follows: a white solid substance having titratable groups of pK'a values of about 5.6, 7.2, 8.2, and 9.3 as determined by electrometric titration in water; has an apparent molecular weight of about 396 as determined from the electrometric titration data; has a specific rotation, $[\alpha]_D^{25}$, of $+127^\circ$ ($c = 1$, weight per volume); has the approximate percentage composition of C 44.04, H 8.40, N 13.78, and O 33.62; and has infrared spectral absorption maxima, as a mineral oil mull, of 3.05, 3.19, 6.35, 7.47, 8.75, 9.8, 12.3, and 12.95 microns as illustrated in Figure 5.

6. A method of producing an antibiotic tenebrimycin complex, of tenebrimycin factor I, I', II, III, IV, V, VI, or mixtures thereof which comprises cultivating a tenebrimycin-producing strain of *Streptomyces tenebrarius* microorganism in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic conditions until a substantial amount of the desired antibiotic is produced by said microorganism in said culture medium and then isolating said tenebrimycin complex, factor, or mixture thereof, from said culture medium.

7. The method of claim 7 wherein the culture medium is maintained at a temperature between about 37°C . and about 43°C . and the growth of the microorganism is carried out for about 4 to about 7 days.

8. The method of claim 7 or 8 wherein the growth of the microorganism is carried out in the absence of visible light.

9. The method of claim 6, 7 or 8 wherein the microorganism is *Streptomyces tenebrarius* ATCC 17920 or ATCC 17921.

10. The method of claim 9 wherein *Streptomyces tenebrarius* ATCC 17920 is cultivated, the tenebrimycin complex recovered from said culture medium and tenebrimycin II, tenebrimycin IV, tenebrimycin V, or tenebrimycin VI isolated from the complex.

11. The method of claim 9 which comprises cultivating *Streptomyces tenebrarius* ATCC 17921 and producing tenebrimycin II containing lesser quantities of tenebrimycin I and tenebrimycin I'.

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12. A method for preparing the antibiotic as defined in claim 1, or tenebrimycin V, substantially as herein described with particular reference to any one of the specific examples.
- 5 13. The antibiotic substance of claim 1 or tenebrimycin V, or a pharmaceutically acceptable acid-addition salt thereof obtained by the method of any of claims 6 to 12.
- 10 14. A pharmaceutical composition comprising an antibiotic substance as claimed in

claim 1, or by tenebrimycin V or a pharmaceutically acceptable acid-addition salt thereof as claimed in claim 13, and a pharmaceutically acceptable carrier or diluent.

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