

PATENT SPECIFICATION

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(54) ANTIBIOTIC A—4696

(71) We, ELI LILLY AND COMPANY, a Corporation of the State of Indiana, United States of America, of 307 East McCarty Street, 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:—

10 The present invention provides antibiotic A—4696 and the pharmaceutically acceptable acid addition salts thereof.

15 Tooth decay and gum disease are among the important health problems with which man is continuously struggling. The evidence is convincing that dentobacterial plaques are conducive to tooth caries, or periodontal lesions, or both. There is a need for prophylactic programs which will control the formation of dentobacterial plaque or keep such deposits below the level at which toxic reactions occur. Antibiotics which inhibit the growth of plaque-forming microorganisms have been developed, but there remains a need for more effective agents useful in the prevention and treatment of tooth decay and gum disease.

25 The efficient production of animal proteins for human consumption is a continuing problem in agriculture. Many agents have been found, among them numerous antibiotics, which are effective as feed additives in producing additional weight gain in growing chickens and swine. There is a never ending need for improved materials which are safe and economic and can be added to animal feed to increase the weight gain and to improve the feed efficiency. The discovery of agents which will accomplish this purpose represents a real advance in the art.

40 The antibiotic of this invention is produced by culturing the organism *Actinoplanes* sp., strain ATCC 23342, in an aqueous nutrient medium under submerged aerobic fermentation conditions. The antibiotic is separated from the filtered fermentation broth by

adsorption onto an activated adsorbent and eluted therefrom with an acidic solvent. Antibiotic A—4696 is purified as a crystalline compound by adsorption on an acidified chromatographic adsorbent and eluted therefrom with an acidic solvent. Preferably, the antibiotic is converted to the hydrochloride or sulfate salt in an aqueous methanol solution and obtained as a pure crystalline salt by adding acetone thereto and filtering off the precipitate that forms. Antibiotic A—4696 possesses antibacterial and growth promotant activity.

The antibiotic of this invention is a basic compound capable of forming salts with suitable acids. The characterization data presented below are for antibiotic A—4696 in the form of its hydrochloride salt. The antibiotic is conveniently isolated and characterized as the hydrochloride salt, although other pharmaceutically acceptable salts can be prepared by employing methods well-known in the art.

Antibiotic A—4696, as the hydrochloride salt, is a white crystalline compound with a melting point greater than 220° C. It is soluble in water, and insoluble in solvents such as methanol, acetone, ether, chloroform, pyridine, benzene, and aliphatic hydrocarbons. It is very stable in solution over a pH range of from 1.0 to 10.0, at temperatures up to about 27° C.

Electrometric titration of A—4696 hydrochloride in water or in dimethylformamide: water (weight ratio 2:1) produces a curve approximating to a straight line with a slope of about 0.14 from pH 6.0 to 13.0.

An average of several microanalyses has shown A—4696 hydrochloride to have approximately the following percent elemental composition by weight: C, 51.33; H, 5.79; N, 5.46; O, 30.96; Cl, 6.72 percent. The apparent molecular weight as determined by the vapor pressure osmotic method is 1158.

The specific rotation $[\alpha]_D$ of A—4696 hydrochloride at 25° C. is -42.3° (C=1, H₂O).

[Price 25p]

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The ultraviolet absorption spectrum of A—4696 hydrochloride in acidic and neutral solutions shows a single absorption maximum at 276 $m\mu$, with an extinction coefficient, $E_{1\text{ cm}}^{1\%}$ of 45. In a basic solution A—4696 hydrochloride exhibits a single absorption maximum of 300 $m\mu$, with an extinction coefficient, $E_{1\text{ cm}}^{1\%}$ of 65.

The infrared absorption spectrum of A—4696 hydrochloride in a mineral oil mull is shown in the accompanying drawing. The observed distinguishable absorption maxima over the range 2.0 to 15.0 μ are as follows: 3.0, 5.8, 5.9, 6.03, 6.15, 6.28, 6.63, 6.85, 7.27, 7.75, 8.1, 8.25, 8.9, 9.4, 9.9, 10.1 microns.

The paper chromatographic profile of A—4696 hydrochloride is shown by the R_f values in Table I below. The values were obtained in each instance using Whatman (Trade Mark) No. 1 paper and the indicated solvent system. The location of the antibiotic on the chromatogram was determined by bioautograph using *Bacillus subtilis* as the organism.

TABLE I.

Paper Chromatography of A—4696 Hydrochloride	
Solvent System	R_f Values*
1	0.88
2	0.72
3	0.80
4	0.59
5	0.35
6	0.77
7	0.80
8	0.74
9	0.87
10	0.59
11	0.77

* R_f value is defined as the ratio of the distance traveled by the antibiotic from the origin to the distance traveled by the solvent front from the origin.

Key to Solvent Systems:

1. Water saturated with butanol plus 1% *p*-toluenesulfonic acid.
2. Water saturated with methylisobutyl ketone plus 1% *p*-toluenesulfonic acid.
3. Water saturated with methylisobutyl ketone plus 1% *p*-toluenesulfonic acid and 1% piperidine.
4. Water: methanol: acetone (12:3:1). The solution is adjusted to pH 10.5 with NH_4OH and then lowered to pH 7.5 with H_3PO_4 .

5. Methanol: 0.1N HCl (3:1).
6. One percent methylisobutyl ketone plus 0.5% NH_4OH in water.
7. Seven percent sodium chloride plus 2.5% methylisobutyl ketone in water.
8. Ten percent propanol in water.
9. Butanol: ethanol: water (150:15:13.5).
10. Propanol: pyridine: acetic acid: water (15:10:3:12).
11. Water: ethanol: acetic acid (70:24:6).

The pharmaceutically acceptable salts of antibiotic A—4696 can be prepared with mineral acids such as hydrochloric, hydrobromic, sulfuric, and phosphoric, and also with organic acids such as citric, tartaric, maleic, *p*-toluenesulfonic, salicylic, fumaric, acetic, and propionic. The antibiotic salts of such acids can be prepared, for example, by acidifying a solution of the antibiotic free-base with the desired acid and precipitating the salt thus formed by introducing ten volumes of acetone to the solution containing the A—4696 acid salt. The salts can likewise be prepared in certain instances by ion exchange on an ion exchange column. Other commonly used methods for the preparation of antibiotic salts can also be employed.

The antibiotic of this invention has an inhibiting action against the growth of many microbial organisms which are pathogenic to man, animals and plant-life, and is, therefore, useful in suppressing the growth of such organisms. The levels at which A—4696 hydrochloride shows inhibition against the growth of the illustration organisms are set forth numerically in Table II, below. The inhibition levels were determined by either the agar-dilution test or the broth-dilution test, and are stated in terms of the minimum inhibitor concentration (MIC), microgram(s) per milliliter (mcg./ml.). (Identified in Table II by the letter "ad" and "bd", respectively).

In the agar-dilution test the test organism is streaked or implanted on agar plates containing various concentrations of A—4696 hydrochloride in the agar. The test plates are incubated at 37° C. for 48 hours, and the MIC is determined as the plate at the lowest concentration of the antibiotic where growth of the test organism is inhibited.

In the broth-dilution test a series of tubes containing nutrient broth and varying concentrations of A—4696 hydrochloride are inoculated with the test organism and incubated at 37° C. for 24 hours. The MIC is determined as the lowest antibiotic concentration where no growth is present in the tube.

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TABLE II

Test Organism	Minimum Inhibitory Concentration (mcg./ml.)
<i>Staphylococcus aureus</i> 3055	12.5 ad 6.25 bd
<i>Bacillus subtilis</i>	0.78 ad
<i>Mycobacterium avium</i>	0.4 ad
<i>Streptococcus faecalis</i>	3.12 ad
<i>Trichophyton mentagrophytes</i>	0.2 ad
<i>Vibrio coli</i>	12.5 bd
<i>Mycoplasma gallisepticum</i>	50.0 bd
<i>Staphylococcus aureus</i> (penicillin resistant)	10.0 bd
<i>Staphylococcus aureus</i> (methicillin resistant)	5.0 ad
<i>Diplococcus pneumoniae</i>	3.12 bd
<i>Clostridium perfringens</i>	1.25 bd
<i>Clostridium tetani</i>	2.5 bd
<i>Corynebacterium gravis</i>	1.25 bd
<i>Lactobacillus casei</i>	>100 ad
<i>Leuconostoc citrovorum</i>	>100 ad
<i>Escherichia coli</i>	>100 ad
<i>Proteus</i> sp.	>100 ad
<i>Pseudomonas</i> sp.	>100 ad
<i>Salmonella</i> sp.	>100 ad
<i>Vibrio metschnikovii</i>	>100 ad
<i>Saccharomyces pastorianus</i>	>100 ad
<i>Candida albicans</i>	>100 ad

Antibiotic A—4696 hydrochloride when given by subcutaneous injection to mice has *in vivo* antimicrobial activity against infectious organisms; for example: the ED₅₀ values (effective dose to protect 50% of the test animals) in illustrative infections are as follows when two doses are employed: *Staphylococcus aureus*, 9.2 mg./kg.; *Streptococcus pyogenes*, 0.68 mg./kg.; and *Diplococcus pneumoniae*, 0.76 mg./kg.

The antibiotic of this invention is also effective in inhibiting the growth of microorganisms

which contribute to the development of periodontal disease and tooth decay. For example, a solution of A—4696 hydrochloride exhibits antimicrobial activity against plaque forming organisms as illustrated by the following test system: Tubes of nutrient broth containing 5% sucrose are inoculated with a cariogenic microorganism. Glass rods are immersed in the medium and the tubes are incubated at 37° C. overnight after which the layer of plaque (primarily cells and dextran) forms on the surface of the rods. The rods are

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- then transferred to solutions containing varying concentrations of A—4696 hydrochloride and allowed to remain in contact with the antibiotic for 5, 10, and 15 minutes. After the appropriate time has elapsed, the rods are rinsed with sterile, deionized water, and immersed in uninoculated medium containing sucrose. After incubating overnight at 37° C. bromthymol blue is added to each medium.
- 10 Growth is detected by observing the color change from blue to yellow due to the acid production of the organisms when grown in a sucrose containing medium.

- 15 A solution of A—4696 hydrochloride at a concentration of 0.01% was effective against an unidentified cariogenic *Streptococcus* sp. when in contact with the plaque encrusted rods for ten minutes. The growth of this organism was prevented by A—4696 hydrochloride at a 0.1% concentration when the solution was in contact with the test rod for 5 minutes.
- 20 Moreover, A—4696 hydrochloride inhibits the growth of the plaque-forming organism *Odontomyces viscosus* at a concentration of 0.25 mcg./ml. in a broth-dilution test.

- 25 The incorporation of A—4696 or one of its acid addition salts into an appropriate

toothpaste, gel, or powder, or a suitable mouthwash, or other oral hygiene preparation, can provide an effective method for inhibiting the development of dental caries and periodontal disease. Alternatively, a solution of A—4696, or one of its acid addition salts at an appropriate concentration can be applied to the surface of the gums and teeth with a suitable swab.

Antibiotic A—4696 has also been found to be effective in promoting growth in animals. When A—4696 hydrochloride is added to the basal ration of growing chicks at a rate of 45.4 gm./ton, the weight gains and the feed efficiencies are shown to be improved over chicks receiving no antibiotic. Table III, below, shows the data from four separate experiments conducted to determine the effectiveness of A—4696 hydrochloride in chicken weight gain tests. All tests were carried out in chick batteries located in quarters maintained at about 25° C. The birds used in the various tests varied from 1 to 5 days of age at the start of the experiment. Test periods were varied from 10 to 17 days in length. Continuous lights and *ad libitum* feeding were used throughout the trials.

TABLE III
Weight Gains and Feed Efficiency in Young Chicks Fed A—4696 Hydrochloride in the Feed Ration

TABLE III

Weight Gains and Feed Efficiency in Young Chicks Fed A-4696 Hydrochloride in the Feed Ration

Feed Ration	A-4696 Hydrochloride g./ton of feed	No. of Days on Treatment	No. of Birds in Treatment	Average Gain, g./Bird	Feed Conversion Efficiency Pound Feed/Sound Gain
Basal ration	0	10	40	147	1.53
Basal ration plus A-4696 Hydrochloride	45.4	10	40	146	1.47
Basal ration	0	10	20	146	1.50
Basal ration plus A-4696 Hydrochloride	45.4	10	20	153	1.45
Basal ration	0	17	80	295	1.56
Basal ration plus A-4696 Hydrochloride	45.4	17	80	320	1.46
Basal ration	0	17	80	300	1.51
Basal ration plus A-4696 Hydrochloride	45.4	17	80	318	1.43
Basal ration	0	17	80	287	1.58
Basal ration plus A-4696 Hydrochloride	45.4	17	80	307	1.47

The effect of feeding A-4696 hydrochloride to weanling pigs and its influence on the average daily weight gain and feed efficiency was tested. Weanling pigs at 4 to 5 weeks of age were maintained on a diet which contained no antibiotics for approximately one week before initiating the experiment. The pigs were then weighed and assigned to experimental groups on the basis of weight and litter. Pigs were housed in enclosed buildings with adequate heat and ventilation, and feed

and water were available *ad libitum* at all times. In each test the pigs were divided into two groups, one of the groups received the basal ration with no antibiotic added, and the other group received the same basal ration plus antibiotic A-4696 hydrochloride. Table IV, shown below, lists the improvement in average daily gain and feed efficiency in those pigs which were fed the A-4696 hydrochloride as contrasted with those which received only the basal ration.

TABLE IV

Average Daily Weight Gain and Feed Efficiency in Weanling Pigs Fed Antibiotic A-4696 Hydrochloride as a Part of the Daily Diet

Feed Ration	A-4696 Hydrochloride g./ton	No. of Pigs in Test	No. of Days on Test	Average Daily Gain Pounds	Feed Conversion Efficiency Pound Feed/Pound Gain
Basal ration	0	11	28	0.67	1.91
Basal ration plus A-4696 Hydrochloride	20	11	28	0.95	1.77
Basal ration	0	11	34	1.10	2.41
Basal ration plus A-4696 Hydrochloride	50	11	34	1.23	2.17

The acute toxicity of A-4696 hydrochloride, determined in mice and expressed as LD₅₀, is 2,400 mg./kg. when administered intraperitoneally.

The antibiotic of this invention is produced by culturing a newly discovered strain of an *Actinoplanes* organism under aerobic conditions in a suitable culture medium until the culture medium contains substantial antibiotic activity. The antibiotic can be recovered by employing various isolation and purification procedures commonly used and understood in the art. When the antibiotic is to be incorporated in feed-stuff for animal use, a lesser degree of purification is required than that which is necessary if the antibiotic is to be used for medicinal purposes.

The microorganism used according to this invention for the production of antibiotic A-4696 has been identified as a strain of a species of *Actinoplanes* of the family Actinoplanaceae. The actinoplanaceae are a new family of microorganisms of the order Actinomycetales, having been first described by Dr. John N. Couch, Jour. Elisha Mitchell Sci. Soc.,

65, 315-318 (1949); and 66, 87-92 (1950); Trans. New York Acad. Sci., 16, 315-318 (1954); Jour. Elisha Mitchell Sci. Soc., 71, 148-155 and 269 (1955); Bergey's Manual of Determinative Bacteriology, 7th Edition, 825-829 (1957); and Jour. Elisha Mitchell Sci. Soc., 79, 53-70 (1963).

The *Actinoplanes* sp. useful for the production of antibiotic A-4696 has been deposited and made a part of the stock culture collection of the American Type Culture Collection, Rockville, Maryland, from which it is available to the public without restriction under the number ATCC 23342.

The *Actinoplanes* sp. useful for the production of A-4696 was isolated from a sample of soil obtained from the Cascade mountain area in the state of Washington. This organism has been designated number 581 in the collection of Dr. John N. Couch at the University of North Carolina.

Actinoplanes sp., strain ATCC 23342, is characterized by the physical and cultural properties set forth in the following paragraphs. The system of Ridgeway, Color Standards and

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Nomenclature, (1912), is employed for the naming of the colors.

Microscopic Morphology and General Cultural Characteristics of *Actinoplanes* sp. ATCC 23342.

- 5 *Microscopic Morphology*—Mycelia are rather spare on *Liquidamber* (Sweet gum tree) pollen in water. The hyphae are branched, septate, 0.2—1.5 μ . in diameter. Hyphae fill the pollen grain, extending out in the water to a distance about equal to the diameter of the grain. Sporangiophores project above the surface of the water; sporangial stalks, usually single, at times branched to bear two, or rarely more, sporangia on the same sporangiophore; stalks 1.0—1.5 μ ., thick, septate. Sporangia are rather small, 4—11 μ . in diameter, subglobose, rarely globose, usually with an irregular wall. Mature spores are arranged in one or more indistinct coils in the sporangium. Sporangial dehiscence is produced by the swelling of an intersporal substance which causes the sporangium to enlarge and assume an almost smooth spherical shape. Spores are motile, about 1.0—1.5 μ ., in diameter; globose to subglobose.

Cultural Characteristics on:

- 30 *Czapek Agar* (S. A. Waksman, The Actinomycetes, 1950). Growth is good; the point inoculum about 2 cms. in diameter after eight weeks. The central part is mound-like; the smear flattened and minutely bumpy. The color of the mycelia is a brilliant zinc orange; the agar is pale buff to distinctly yellowish. Sporangia are rarely formed. *Peptone Czapek Agar* (5 g. of peptone substituted for 2 g. of sodium nitrite). Growth is similar to that observed on Czapek Agar, but furrows, ridges and bumps are more distinct than on Czapek Agar. No sporangia are formed.

- 40 As previously noted, *Actinoplanes* sp., strain ATCC 23342, can be grown in a culture medium to produce antibiotic A—4696. The culture medium can be any one of a number of different media. However, for economy in production, maximum yield, and ease of isolation of the antibiotic certain culture media are preferred. Thus, for example, starch is one of the preferred sources of carbohydrate, and soybean meal is one of the preferred nitrogen sources. Other carbohydrate sources which can be used include molasses, glucose, dextrin, glycerol, and the like. Nitrogen sources also include amino acid mixtures, peptones, and the like.

- 55 Nutrient inorganic salts to be incorporated in the culture media can include the customary salts capable of yielding sodium, potassium, ammonia, calcium, phosphate, chloride, sulfate, acetate and carbohydrate moieties.
- 60 Additionally, sources of growth factors, such as distillers' solubles and yeast extracts, can be included with beneficial effect on the production of A—4696 antibiotic. 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 *Actinoplanes* sp. employed in this invention. Such trace elements are commonly supplied as impurities incidental to the addition of the other constituents of the medium.

The organism used to produce A—4696 can be grown over a relatively wide pH range. However, it is desirable to adjust the pH of the culture medium to between the pH 6.5 and pH 7.2 before inoculation with the organism. As with other Actinomycetes, the pH of the growing medium gradually changes during the growth period; the pH at the end of the fermentation period usually ranging from 7.0 to 7.8.

Submerged aerobic culture conditions are preferred for the production of A—4696. Relatively small amounts of the antibiotic can be produced by shake flask culture; however, for the preparation of large amounts, submerged aerobic culture in sterile tanks is preferred. The culture medium in the sterile tank can be inoculated with a sporulated or mycelial fragment suspension; but because of the time lag experienced when a sporulated suspension is used, the preferred inoculum is the vegetative form of the culture. More efficient use of fermentation equipment is realized by avoiding the time lag in the growth cycle.

Accordingly, it is desirable to produce a vegetative inoculum of the organism by inoculating a relatively small quantity of culture medium with the spores or mycelial fragments of the organism, and when a young active vegetative inoculum is obtained aseptically transfer it to the large tank. The medium in which the vegetative inoculum is grown can be the same as that utilized for large-scale fermentation of A—4696, although other media can be employed.

The A—4696 producing *Actinoplanes* sp., strain ATCC 23342, grows at temperatures between 20° and 40° C. The largest amounts of A—4696 appear to be produced at a temperature of about 30° C.

Sterile air is blown through the culture medium in the submerged aerobic culture process. The volume of air sparged into the culture medium varies from 0.1 to 1.0 volume of air per minute per volume of culture medium. The most efficient growth and antibiotic production are achieved when the volume of air is at least 1/2 volume of air per minute per volume of culture medium.

The rate of production of A—4696 and the concentration of antibiotic activity in the culture medium can be followed during the growth period by testing samples of the fermentation broth for antibiotic activity against organisms known to be susceptible to the antibiotic. One such assay organism useful in the present invention is *Bacillus subtilis*. The bioassay can be carried out by the standard turbi-

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dimetric or cup-plate methods, or by the paper disc assay on agar plates.

Generally, maximum production of the antibiotic occurs within about 4 to 6 days in shake flasks or submerged aerobic culture fermentations.

Antibiotic A—4696 can be recovered from the culture medium and separated from other substances which may be present by adsorptive and extractive techniques. Adsorptive techniques are preferred because such procedures avoid the use of large volumes of solvents required in extraction processes.

After fermentation, the antibiotic activity is present in both the broth and mycelium. The culture medium is filtered to separate the fermentation broth from the solid mycelia. The mycelial cake is washed with water and then slurried in water adjusted to a pH of 10.5 with 5.0N NaOH, agitated vigorously for 30 minutes and filtered. The two filtrates and the wash water are combined and the antibiotic activity adsorbed therefrom on a suitable adsorptive agent, such as activated carbon, activated acid alumina, polyamide resin, cellulose, silica gel, and the like. The adsorption can be accomplished by passing the antibiotic activity-containing solution over a bed of the adsorbent, or by adding the adsorbent to the solution, mixing thoroughly for 20 to 30 minutes and filtering off the exhausted solvent. The antibiotic adhering to the adsorbent can be recovered as impure A—4696 by customary elution procedures.

The crude A—4696 can be purified by the preparation of the picrate salt and the conversion thereof to the hydrochloride salt, or by adsorption and elution procedures using an appropriate adsorbent or ion exchange resin. Suitable adsorbents include Polyamide resin (M. Woelm, Eschwege, Germany), acidic alumina, neutral alumina, basic alumina, silica gel, Amberlite XAD—2 (Rohm and Haas, Philadelphia), Amberlite XAD—4 (Rohm and Haas, Philadelphia), and Dowex 50 (H⁺) (Dow Chemical, Midland, Michigan). ("Amberlite" and "Dowex" are Trade Marks.)

This invention is further illustrated by the following examples:

EXAMPLE 1.

A. Shake Flask Fermentation of Antibiotic A—4696.

Mycelial fragments of *Actinoplanes* sp., strain ATCC 23342, were inoculated on a nutrient agar slant having the following composition:

Ingredient	Amount
Pre-cooked oatmeal	60 g.
Yeast	2.5 g.
K ₂ HPO ₄	1.0 g.
Dried distiller's solubles	5.0 g.
Czapek's mineral stock*	5.0 ml.
Agar	25 g.
Water, deionized	1 l.

*Czapek's mineral stock has the following composition:

KCl	100 g.	
MgSO ₄ ·7H ₂ O	100 g.	
FeSO ₄ ·7H ₂ O	2 g.	
(Dissolve in two mls. conc. HCl)		70
Water, deionized	1 l.	

The slant was inoculated with ATCC 23342 and incubated for 6 days at 30° C. The culture does not normally sporulate on this medium, and it is necessary to macerate the mycelial mat with a flattened, sharpened, inoculating needle in order to increase the number of potential growth centers. The macerated mature culture was covered with sterile distilled water and scraped carefully with a sterile rod to obtain a mycelial suspension.

The suspension thus obtained was used to inoculate 100 ml. of a sterile vegetative medium having the following composition:

Ingredient	Amount	
Glucose	5.0 g.	
Dextrin	20.0 g.	
Soybean meal	15.0 g.	
Yeast extract	2.5 g.	90
Calcium carbonate	1.0 g.	
Water, tap	1 l.	

The inoculated vegetative medium was grown for 48 hours at 30° C. on a rotary shaken operating at 250 rpm.

Ten ml. of the incubated vegetative medium was inoculated into 100 ml. of a sterile "bump" medium of the same composition as given next above. The thus inoculated "bump" medium was incubated for 24 hours at 30° C. with constant shaking on a rotary shaker operating at 250 rpm.

Four-tenths ml. of the incubated "bump" medium was inoculated into 100 ml. portions of a production medium of the composition shown below contained in 500 ml. Erlenmeyer flasks, and sterilized at 120° C. for 30 minutes:

Ingredient	Percent	
Dextrose	1.0	110
Dextrin	3.0	
Peptone	1.5	
Soybean meal	0.5	
MgSO ₄ ·7H ₂ O	0.2	
Molasses, beat sugar	1.5	115
Corn steep liquor	0.5	
Betaine	0.1	
K ₂ HPO ₄	0.05	
Water, deionized, q.s.	25 ml.	

The pH of the medium was adjusted to 7.5 with 5N sodium hydroxide solution before sterilization. After sterilization the pH was approximately 6.9.

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5 The production fermentation was shaken for about 96 hours at a temperature of 30° C. on a rotary shaker operating at 250 rpm. The pH at the end of the fermentation cycle was about 7.2.

B. 40-liter tank fermentation of antibiotic A-4696.

10 The preparation of the inoculum proceeded through the incubation of the "bump" medium detailed under section A, above. Twenty-five liters of a production medium as outlined above, with .02% Dow Corning (Trade Mark) antifoam added, was sterilized by autoclaving at 120° C. for 30 minutes and charged into a 40 l. fermentation tank. One-hundred milliliters of incubated "bump" medium was inoculated into the sterile production medium. The inoculated production medium contained in the 40 l. tank was allowed to ferment for 4 days at 30° C. The fermentation was aerated with sterile air in an amount of about one-half volume of air per volume of culture medium per minute. The fermenting production medium was agitated with a mixer utilizing an impeller of a proper size and turning at an appropriate rpm to insure adequate mixing of air with the medium. The pH of the culture medium gradually increased from an initial level of about 6.9 to about 7.2 as the fermentation proceeded.

C. Isolation of Antibiotic A-4696.

35 The whole broth obtained from an A-4696 fermentation, as described above, was filtered with the aid of a commercial filter aid. The filtrate was set aside. The mycelial cake was washed with 32 l. of water and the wash water set aside. The mycelial cake was then suspended in an additional 32 l. of water and the pH of the mixture adjusted to pH 10.5 with 5N sodium hydroxide solution. The mycelial cake water suspension was stirred for 45 minutes and the mixture was filtered. This filtrate and the water wash were combined with the original filtrate from the fermentation broth and the pH of the combined filtrates was adjusted to pH 4.0 with H₂SO₄. The acidified combined filtrates were passed through a carbon column utilizing 1 kg. of activated carbon, (Pittsburgh, 12×40). The activated carbon column was washed until the effluent was colorless. The A-4696 activity was adsorbed on the carbon column. The A-4696 activity was eluted from the carbon column utilizing a 1% H₂SO₄ solution in acetone: H₂O (1:1). Two liters of the acidified acetone-water solution was sufficient to elute the A-4696 activity from the carbon column. The eluate containing the A-4696 activity was treated with a saturated barium hydroxide solution, in order to form a precipitate of barium sulfate, thus removing the sulfate ions from the solution.

The mixture was filtered and the barium

sulfate precipitate was discarded. The filtrate containing the A-4696 activity was concentrated under vacuum to dryness. The resulting residue comprising the A-4696 activity amounted to approximately 80 g.

D. Conversion of A-4696 Activity to Crude A-4696 Hydrochloride.

70 The approximately 80 g. of A-4696 activity was taken up in a volume of 5 l. of water, and then treated with 500 g. of activated carbon (Darco (Trade Mark) G-60, Atlas Chemical, Wilmington, Del.). This mixture was stirred for 1 hour and then filtered. The filtrate was discarded. The carbon filter cake containing the A-4696 activity was washed with 1 l. of water and the water wash was discarded. The carbon filter cake was further washed with 1 l. of 0.05N aqueous hydrochloric acid. The acid wash was discarded. The washed carbon cake was eluted by stirring 30 minutes with 500 ml. of an aqueous hydrochloride acid-acetone solution (0.05N HCl: acetone [7:3]). The mixture was filtered and the filtrate set aside. The elution of the activated carbon was repeated 4 times in the same manner, each time setting aside the filtrate. The five eluates containing the A-4696 activity were combined. The combined eluates were then concentrated under vacuum to a volume of approximately 100 ml. Two-hundred milliliters of methanol was added to the aqueous concentrate containing the A-4696 activity. Two liters of acetone was added to this aqueous-methanol solution. A precipitate, consisting of crude A-4696 hydrochloride, formed in the acetone-aqueous methanol solution. After filtering and drying the precipitate, 60.9 g., of crude A-4696 hydrochloride was recovered.

E. Preparation of Crystalline A4696 hydrochloride.

105 Twenty-five grams of A-4696 hydrochloride, prepared according to the procedure outlined in Section D of this example, was dissolved in 20 ml. of water. The A-4696 hydrochloride solution was passed over a water washed Polyamide resin bed, (M. Woelm, Eschwege, Germany), contained in a glass column of approximately 7×60 centimeters. The effluent was set aside. The Polyamide resin column was washed with water at a flow rate of approximately 8-10 ml. per minute. The antimicrobial activity of the column effluent was measured by conventional procedures. The effluents containing antimicrobial activity were combined and concentrated to dryness under vacuum. The residue was dissolved in a mixture of 25 ml. water and 50 ml. methanol. This aqueous methanol solution of A-4696 hydrochloride was acidified to a pH of 2.0 with 5N HCl. Approximately 1.5 l of acetone was added to the aqueous methanol solution to precipitate the hydrochloride salt

of A-4696. The mixture was filtered and the A-4696 hydrochloride precipitate recovered therefrom.

The filter cake containing the A-4696 hydrochloride was dissolved in the minimum quantity of water. An amount of ethanol equal to twice the volume of water was then added and the mixture was heated to approximately 60° C. The mixture was then cooled and the hydrochloride salt of A-4696 crystallized therefrom. The crystals were filtered and dried. Approximately 9 g. of crystalline A-4696 hydrochloride was recovered.

EXAMPLE 2.

15 Purification of A-4696 Over Acid-Washed Alumina.

Two and three-tenths grams of crude A-4696, obtained as described in Example 1 through section D above, was dissolved in 20 ml. of 50 percent aqueous methanol and run over a 2×4 cm. column of acid-washed alumina (Alcoa (Trade Mark)). The column was immediately washed with methanol. The activity was eluted from the acid alumina with 50% aqueous methanol. Aliquots of the elution were collected and the antimicrobial activity determined on each fraction. The fractions which exhibited antimicrobial activity were combined, concentrated to approximately 100 ml. and acetone added to precipitate the A-4696. The precipitate was filtered and dried *in vacuo*, yielding approximately 930 mg. of pure crystalline A-4696.

The acid-washed alumina used in the purification described above was prepared as follows: Alcoa (Trade Mark) activated alumina F-20 was washed with water adjusted to pH 3 with H₂SO₄ by stirring for 6 hours maintaining the pH at 3.0. The alumina was filtered off, washed with 50% aqueous methanol, and dried in an oven at 100° C.

EXAMPLE 3.

45 Purification of A-4696 by the Preparation of the Picrate Salt and Conversion thereof to the Hydrochloride Salt.

Five-hundred milligrams of crude A-4696, obtained as described in Example 1 through section D above, was dissolved in 25 ml. of water and 25 ml. of a saturated aqueous solution of picric acid were added with stirring. The mixture was allowed to stand overnight at 5° C. and a precipitate formed. The precipitate was centrifuged off and dried. The precipitate thus obtained was dissolved in 25 ml. methanol, the pH was adjusted to 1.5 with HCl, and added to 500 ml. of diethyl ether to precipitate the A-4696 hydrochloride salt. The hydrochloride salt was centrifuged off, washed with diethyl ether and dried *in vacuo* yielding approximately 305 mg. of A-4696 hydrochloride as a white crystalline salt.

EXAMPLE 4.

Preparation of A-4696 Sulfate Salt.

Two grams of A-4696 hydrochloride, obtained as described in Example 1 through section E above, was dissolved in 300 ml. of water, the pH adjusted to 7.5 with 5N sodium hydroxide, and 30 g. of Darco G-60 carbon was added. The mixture was stirred for 30 minutes; filtered with the aid of a commercial filter aid, and the filter cake washed successively with 300 ml. each of water and 0.05N H₂SO₄.

The activity was eluted from the filter cake by adding the filter cake to 500 ml. of a mixture consisting of 70% 0.05N H₂SO₄ and 30% acetone, stirring for 30 minutes and filtering to remove the carbon. The filtrate was concentrated to approximately 10 milliliters. About 20 ml. of methanol was added to the concentrate and the thus formed solution was added to 600 ml. of acetone to precipitate the A-4696 sulfate. The precipitate was filtered off; washed with acetone and dried *in vacuo*, yielding 862 mg. of A-4696 sulfate as a white crystalline salt.

It will be understood that the present invention includes within its scope a pharmaceutical composition comprising the antibiotic of the invention and a pharmaceutical carrier or excipient. Also included within the scope of the invention is a method of treating bacterial infections in warm-blooded animals, excluding man, which comprises the administration to the animal of the antibiotic of the invention or a pharmaceutical composition as described above. The scope of the invention also extends to a method of producing weight gain in growing poultry which comprises feeding to the poultry a poultry feed composition comprising the antibiotic of the invention and a suitable carrier. Additionally the invention includes within its scope a method of producing weight gain in growing warm-blooded animals, excluding man, which comprises feeding the animals with an animal feed composition comprising the antibiotic of the invention and a suitable carrier.

WHAT WE CLAIM IS:—

1. The antibiotic substance A-4696, or a pharmaceutically acceptable acid addition salt thereof, which in the form of its hydrochloride salt is a white, crystalline substance soluble in water and insoluble in the common organic solvents; has a melting point greater than 220° C.; has an electrometric titration curve in a solution of 66 percent by weight dimethylformamide in water approximating to a straight line with a slope of about 0.14 from pH 6.0 to pH 13.0; has the approximate composition by weight of 51.33 percent carbon, 5.79 percent hydrogen, 5.46 percent nitrogen, 30.96 percent oxygen, and 6.72 percent

of A-4696. The mixture was filtered and the A-4696 hydrochloride precipitate recovered therefrom.

- 5 The filter cake containing the A-4696 hydrochloride was dissolved in the minimum quantity of water. An amount of ethanol equal to twice the volume of water was then added and the mixture was heated to approximately 60° C. The mixture was then cooled and the hydrochloride salt of A-4696 crystallized therefrom. The crystals were filtered and dried. Approximately 9 g. of crystalline A-4696 hydrochloride was recovered.

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35 The acid-washed alumina used in the purification described above was prepared as follows: Alcoa (Trade Mark) activated alumina F-20 was washed with water adjusted to pH 3 with H₂SO₄ by stirring for 6 hours maintaining the pH at 3.0. The alumina was filtered off, washed with 50% aqueous methanol, and dried in an oven at 100° C.

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The activity was eluted from the filter cake by adding the filter cake to 500 ml. of a mixture consisting of 70% 0.05N H₂SO₄ and 30% acetone, stirring for 30 minutes and filtering to remove the carbon. The filtrate was concentrated to approximately 10 milliliters. About 20 ml. of methanol was added to the concentrate and the thus formed solution was added to 600 ml. of acetone to precipitate the A-4696 sulfate. The precipitate was filtered off; washed with acetone and dried *in vacuo*, yielding 862 mg. of A-4696 sulfate as a white crystalline salt.

It will be understood that the present invention includes within its scope a pharmaceutical composition comprising the antibiotic of the invention and a pharmaceutical carrier or excipient. Also included within the scope of the invention is a method of treating bacterial infections in warm-blooded animals, excluding man, which comprises the administration to the animal of the antibiotic of the invention or a pharmaceutical composition as described above. The scope of the invention also extends to a method of producing weight gain in growing poultry which comprises feeding to the poultry a poultry feed composition comprising the antibiotic of the invention and a suitable carrier. Additionally the invention includes within its scope a method of producing weight gain in growing warm-blooded animals, excluding man, which comprises feeding the animals with an animal feed composition comprising the antibiotic of the invention and a suitable carrier.

WHAT WE CLAIM IS:—

1. The antibiotic substance A-4696, or a pharmaceutically acceptable acid addition salt thereof, which in the form of its hydrochloride salt is a white, crystalline substance soluble in water and insoluble in the common organic solvents; has a melting point greater than 220° C.; has an electrometric titration curve in a solution of 66 percent by weight dimethylformamide in water approximating to a straight line with a slope of about 0.14 from pH 6.0 to pH 13.0; has the approximate composition by weight of 51.33 percent carbon, 5.79 percent hydrogen, 5.46 percent nitrogen, 30.96 percent oxygen, and 6.72 percent

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- chlorine; has an apparent molecular weight of 1158, as determined by the vapor pressure osmotic method; has a specific rotation, $[\alpha]_D^{25} -42.3^\circ$ ($c=1$, H₂O); has an ultra-violet absorption spectrum in acidic and neutral solutions at 276 mμ., with an extinction coefficient, $E_{1\text{ cm}}^{1\%}$ of 45, and in a basic solution a single absorption maximum of 300 mμ. with an extinction coefficient $E_{1\text{ cm}}^{1\%}$ of 65; and has
- 5 the following distinguishable bands in its infrared absorption spectrum when determined in a mineral oil mull: 3.0, 5.8, 5.9, 6.03, 6.15, 6.28, 6.63, 6.85, 7.27, 7.75, 8.1, 8.25, 8.9, 9.4, 9.9 and 10.1 microns.
- 10 2. The hydrochloride salt of antibiotic A—4696 as defined in claim 1.
- 15 3. The sulfate salt of antibiotic A—4696 as defined in claim 1.
- 20 4. A method of producing the antibiotic of claim 1 which comprises cultivating the organism *Actinoplanes* sp., strain ATCC 23342, in a liquid culture medium containing assimilable sources of carbon, nitrogen and inorganic salts under submerged aerobic conditions until
- 25 a substantial amount of antibiotic activity is imparted by said organism to said culture medium.
- 30 5. The method of claim 4 which additionally comprises adsorbing the antibiotic activity produced onto an adsorbent; eluting said antibiotic activity from said adsorbent with an acidic solvent; adsorbing said antibiotic activity on an acidified chromatographic adsorbent; and eluting said antibiotic from said
- 35 chromatographic adsorbent.
- 40 6. A composition for inhibiting the growth of microorganisms which contribute to the development of periodontal disease and tooth decay comprising the antibiotic of any one of claims 1 to 3 and a suitable carrier.
7. An animal feed composition comprising the antibiotic of any one of claims 1 to 3 and a suitable carrier.
8. A poultry feed composition comprising

the antibiotic of any one of claims 1 to 3 and a suitable carrier. 45

9. The method of producing antibiotic A—4696 and its pharmaceutically acceptable acid addition salts as claimed in claim 4 substantially as described in Examples 1 to 4. 50

10. Antibiotic A—4696 and its pharmaceutically acceptable acid addition salts when produced by the method claimed in claim 9.

11. A method according to claim 4 wherein the initial pH of the culture medium is from 6.5 to 7.2. 55

12. A method according to either of claims 4 and 11 in which the organism is cultivated at a temperature of from 20° to 40° C.

13. A method according to any of claims 4, 11 and 12, wherein sterile air is sparged through the culture medium at a rate of from 0.1 to 1.0 volume of air per minute per volume of culture medium. 60

14. Antibiotic A—4696 or a pharmaceutically acceptable acid addition salt thereof which has been obtained by a method according to any of claims 4, 5, 11 to 13. 65

15. A pharmaceutical composition comprising the antibiotic of any of claims 1 to 3 and a pharmaceutical carrier or excipient. 70

16. A method of treating bacterial infections in warm-blooded animals, excluding man, which comprises the administration of the antibiotic of any of claims 1 to 3 or a pharmaceutical composition as claimed in claim 15 to the animal. 75

17. A method of producing weight gain in growing poultry which comprises feeding to the poultry a composition according to claim 8. 80

18. A method of producing weight gain in growing warm-blooded animals, excluding man, which comprises feeding the animals with a composition according to claim 7. 85

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