

PATENT SPECIFICATION

(11) 1 433 417

1 433 417

- (21) Application No. 36111/73 (22) Filed 30 July 1973
 (31) Convention Application No. 276 546
 (32) Filed 31 July 1972 in
 (33) United States of America (US)
 (44) Complete Specification published 28 April 1976
 (51) INT CL² C12D 9/14; C07D 498/08; C07G 11/00//A01N 9/22;
 A61K 31/42; (C07D 498/08, 263/00, 273/00)
 (52) Index at acceptance
 C2A 1B 1C1A 1C1B 1C1C 1C1D1 2F 3A1 3B
 C2C 1377 214 247 255 25Y 306 30Y 351 352 360 362 36Y 388
 633 645 672 761 762 767 790 791 79Y TR
 (72) Inventors ROBERT L. HAMILL and WILLIAM MAX STARK



(54) ANTIBIOTIC A—2315 AND PROCESS FOR PREPARATION THEREOF

(71) We, ELI LILLY AND COMPANY, a corporation of the State of Indiana, United States of America, having a principal place of business at 307 East McCarty 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:—

The present invention relates to a novel antibiotic substance and to a process for its production. In particular, this invention relates to the neutral, nitrogenous antibiotic arbitrarily designated herein as antibiotic A—2315.

Antibiotic A—2315 is produced by culturing the newly characterized *Actinoplanes philippinensis* NRRL 5462 under submerged aerobic fermentation conditions until a substantial amount of antibiotic activity has been produced. The antibiotic is isolated from the filtered fermentation broth as an amorphous solid, preferably by extraction with an organic solvent and purification by column chromatography over activated alumina or silica gel. Antibiotic A—2315 is a useful antibacterial agent, particularly against gram-positive streptococci. In one aspect, antibiotic A—2315 is effective in the inhibition of organisms responsible for dental caries and for periodontal disease. Antibiotic A—2315 also promotes growth when incorporated in the diet of poultry; additionally, it is a plant-fungicidal agent.

Antibiotic A—2315 is a white, amorphous solid, having a molecular weight of 503.2638, as determined by mass spectrum, and an elemental analysis as follows: 61.51 percent carbon, 7.51 percent hydrogen, 8.69 percent nitrogen, and 21.53 percent oxygen. Based on elemental analysis and molecular weight, an empirical formula of $C_{26}H_{37}N_3O_5$ is assigned to A—2315. Electrometric titration of A—2315 in 66 percent dimethylformamide in water did not indicate the presence of titratable groups. The specific rotation of antibiotic A—2315 is -132° when determined at a temperature of 27°C . in methanol in which the concentration of the antibiotic is 0.375 percent on a weight per volume basis.

The infrared absorption curve of A—2315 in chloroform is shown in the accompanying drawing. The following distinguishable absorption maxima are observed (w = weak; m = medium; s = strong): 2.81 (w), 2.99 (w—m), 3.38 (m), 5.82 (m), 6.02 (s), 6.20 (w), 6.30 (m), 6.40 (w), 6.66 (m—s), 6.82 (w), 6.94 (w—m), 7.05 (w), 7.30 (w—m), 7.71 (w), 8.88 (w—m), 9.11 (w—m), 10.41 (m), 10.89 (w—m), 11.14 (w—m) microns.

The ultraviolet absorption spectrum of antibiotic A—2315 in 95 percent ethanol shows an absorption maximum at $214\text{ m}\mu$ (ϵ 40,200) with an absorptivity value ($E_{1\%}^{1\text{cm}}$) of 799.

Antibiotic A—2315 is soluble in methanol, ethanol, higher alcohols and most organic solvents, but is only slightly soluble in water. In the paper chromatography systems indicated below, using *Sarcina lutea* as a detection organism, antibiotic A—2315 has the following R_f values:

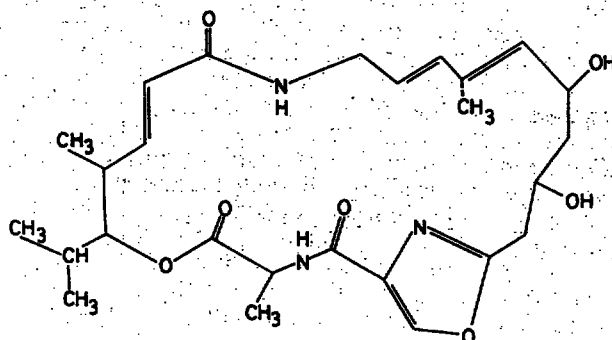
Rf Value

Solvent System

0.19	Methyl ethyl ketone (MEK):benzene:water (1:5:1) upper layer
0.91	Butanol saturated with water
0.91	Butanol saturated with water plus 2% <i>p</i> -toluene-sulfonic acid (<i>p</i> -TSA)
0.56	Methyl isobutyl ketone (MIBK) saturated with water
0.60	MIBK saturated with water plus 2% <i>p</i> -TSA
0.83	Water:methanol:acetone (12:3:1), adjusted to pH 10.5 with NH_4OH and then lowered to pH 7.5 with H_3PO_4
0.75	1% MIBK, 0.5% NH_4OH in water
0.72	17.4 g. K_2HPO_4 , 30 ml. ethanol per liter of water
0.65	7% NaCl plus 2.5% MEK in water
0.84	Propanol:water (1:9)
0.90	Butanol:ethanol:water (13.5:15:150)

When subjected to amino-acid analysis, antibiotic A—2315 releases a large amount of alanine. Antibiotic A—2315 contains two hydroxyl groups and is capable of forming ester derivatives in a conventional manner, for example, by acetylation with acetic anhydride in pyridine. These ester derivatives are also useful as antibiotics.

Based upon observed physical data and upon structure elucidation studies, a proposed tentative structure for A—2315 can be devised. The structure has not been determined with certitude, however; and it is to be understood that the structure presented herein represents merely a working hypothesis. The following structure is postulated for antibiotic A—2315:



Antibiotic A—2315 has an inhibitory action against the growth of certain microorganisms. The levels at which antibiotic A—2315 inhibits the growth of organisms were determined using various testing procedures.

Disc-Plate Screening Procedure

Agar plates, inoculated with the test organism, are used; 6 mm. discs (0.02 ml. capacity) are saturated from log 2 dilutions of the antibiotic solution. Disc content is 1/50 of the concentration of the solution used, i.e. disc content of 30 $\mu\text{g.}$ is obtained from a solution of 1500 $\mu\text{g./ml.}$ concentration.

The results obtained from disc-plate tests of antibiotic A—2315 are given in Table I.

(10) (17)

TABLE I

DISC PLATE ACTIVITY OF A-2315

Test Organism	Minimal Inhibitory Concentration (MIC (μ g./disc))
<i>Staphylococcus aureus</i> 3055*	0.025
<i>Staphylococcus aureus</i> 3130***	0.2
<i>Staphylococcus aureus</i> 3074**	0.4
<i>Mycobacterium avium</i>	3.13
<i>Streptococcus pyogenes</i>	0.012
<i>Bacillus subtilis</i>	50.
<i>Sarcina lutea</i>	0.008
<i>Proteus</i> sp.	100.
<i>Escherichia coli</i>	12.5
<i>Salmonella typhimurium</i>	12.5
<i>Salmonella typhosa</i>	12.5
<i>Shigella flexneri</i>	1.56
<i>Klebsiella pneumoniae</i>	12.5
<i>Klebsiella pneumoniae</i>	50.0
<i>Candida albicans</i>	100.0
<i>Saccharomyces pastorianus</i>	>100.0
<i>Trichophyton mentagrophytes</i>	>100.0
<i>Fusarium moniliforme</i>	>100.0
<i>Neurospora</i> sp.	>100.0

*benzylpenicillin-susceptible

**benzylpenicillin-resistant

***benzylpenicillin-resistant, methicillin-resistant

The results of confirmation broth-dilution susceptibility tests of antibiotic A—2315 against pathogenic gram-positive organisms, prior to use for therapy of experimental infection in mice, are summarized in Table II.

TABLE II

BROTH-DILUTION SUSCEPTIBILITY OF A—2315

Test Organism	MIC (μ g./ml.)
<i>Staphylococcus aureus</i> 3055*	3.13
<i>Streptococcus pyogenes</i> C203*	0.4
<i>Diplococcus pneumoniae</i> Park I	25.0

* benzylpenicillin-susceptible

12

Antibiotic A—2315, when given by subcutaneous injection to mice, has *in vivo* antimicrobial action against experimental bacterial infections. When two doses of A—2315 are administered to mice in illustrative infections, the ED₅₀ values (effective dose to protect 50 percent of the test animals; see Warren Wick, *et al.*, *J. Bacteriol.* 81, 233—235 (1961)) are as follows:

<i>Staphylococcus aureus</i> 3055	90 mg./kg.
<i>Streptococcus pyogenes</i>	83 mg./kg.
<i>Diplococcus pneumoniae</i> (Park I)	>166 mg./kg.

The acute toxicity of antibiotic A—2315, administered either orally or subcutaneously to mice, expressed as LD₅₀, is greater than 500 mg./kg. The acute toxicity of A—2315, administered intraperitoneally to mice, expressed as LD₅₀, is greater than 400 mg./kg.

Another useful property of antibiotic A—2315 is its activity against *Mycoplasma gallisepticum*, an organism pathogenic for poultry. Of particular interest is the activity of A—2315 against strains of this organism that are resistant to tylosin. The minimal inhibitory concentrations, as determined by *in vitro* broth-dilution studies, are summarized in Table III below:

TABLE III

SENSITIVITY OF MYCOPLASMA GALLISEPTICUM (MG) TO A—2315

MG Isolate	MIC (μg./ml.)
34159 (tylosin resistant)	0.195
41313 (tylosin resistant)	0.78
42854 (tylosin resistant)	0.195
43480 (tylosin resistant)	<0.097
38502 (tylosin sensitive)	<0.097

Further evaluation of antibiotic A—2315 in chickens infected with *Mycoplasma gallisepticum* indicated some weight advantage and lowered incidence of air-sac lesions in surviving chickens. The antibiotic was prepared in polyethylene glycol 200 and injected subcutaneously in the neck. No signs of toxicity were seen, except for slight hemorrhage in the neck area in some of the treated chicks that died early. Results of this test are summarized in Table IV.

TABLE IV

ACTIVITY OF A—2315 IN EMBRYO CHICKS INFECTED WITH MYCOPLASMA GALLISEPTICUM

Treatment Groups	Dead Chicks		Average Weight	Survivors ^a Air-Sac Lesions	MG Antibody
	No. Died No. in Group	Air-Sac Lesions			
Infected Controls	13/20	11/13 ^b	100 gm.	7/7	7/7
Normal Controls	0/20	—	214 gm.	0/20	0/20
A—2315 (3 mg./chick)	12/20	9/9 ^c	133 gm.	0/8	8/8

^a Data collected at 14 days

^b 2/13 not examined due to decomposition

^c 3/9 not examined due to decomposition

13

In one embodiment, therefore, this invention provides an agent useful against resistant *Mycoplasma* in poultry. Protection against *Mycoplasma* is obtained when antibiotic A—2315 is administered parenterally or orally to animals in a dosage range of from about 50 to about 500 mg./kg. of animal body weight. When used to afford either prophylactic or therapeutic protection against this respiratory disease, a suitable concentration of antibiotic A—2315 is conveniently incorporated into the normal food ration of animals.

Antibiotic A—2315 has the ability to inhibit the growth of microorganisms contributing to the development of periodontal disease. In broth-dilution tests, A—2315 inhibits the growth of the cariogenic organism *Streptococcus mutans* at concentrations of 1—2 μ g./ml. and inhibits the growth of cariogenic filamentous rods at a concentration as low as 0.1 μ g./ml.

A solution of antibiotic A—2315 exhibits inhibitory activity against cariogenic organisms as illustrated by the following test system: Tubes of nutrient broth containing 5 percent sucrose are inoculated with cariogenic microorganisms. Narrow glass rods, which extend below the surface of the nutrient broth, are inserted into the tubes. After incubation at 37°C. overnight, a layer of artificial plaque (primarily cells and dextran) forms on the surface of the rods. The rods are then transferred to solutions containing varying concentrations of A—2315 and are allowed to remain in contact with the antibiotic solutions for periods of 5, 10, and 15 minutes. After the appropriate time has elapsed, the rods are rinsed by dipping in sterile, deionized water; the rods are then incubated overnight at 37°C. in uninoculated medium containing sucrose and bromothymol blue. Growth is detected by observing the color change of bromothymol blue from green to yellow, due to acid production by the organisms when grown in a sucrose-containing medium. No growth indicates that the antibiotic has destroyed the organism.

Using this test, a solution of antibiotic A—2315 at a concentration of 1.0% was effective against a cariogenic *Streptococcus sp.* when the solution was in contact with the plaque-encrusted rods for as little as five minutes.

Because of its activity against cariogenic organisms and those organisms implicated in periodontal disease, antibiotic A—2315 is suitable for incorporation, in inhibitory concentrations, in preparations employed in oral hygiene, such as toothpastes, powders or gels and mouthwashes. In general, preparations containing from about one to about 15 percent of A—2315 are useful for the control of cariogenic organisms.

Still another useful property of antibiotic A—2315 is its ability to improve growth performance in animals. When A—2315 was added to the diet of growing chicks at a level of 45.4 grams/ton, the average weight gain after ten days was 159 grams, as compared with an average weight gain of 148 grams for the control group. The feed-conversion efficiency (feed/gain) for chickens fed antibiotic A—2315 was 1.44; in contrast, the feed-conversion efficiency for the control group was 1.53.

The ability of antibiotic A—2315 to stimulate weight gains makes it especially useful for this purpose. When used as a growth-promoting agent, suitable concentrations of the antibiotic are conveniently incorporated into the normal food ration of the animals. Antibiotic A—2315 typically is effective as a growth-promoting agent when administered to animals at levels of from about 20 to about 50 grams/ton of feed ration.

In yet another aspect of the invention, antibiotic A—2315 is useful as a plant-fungicidal agent. At concentrations of 400 ppm, antibiotic A—2315 protected bean plants which had been inoculated with bean rust (*Uromyces phaseoli* var. *typica*) and gave moderate protection to cucumber plants which had been inoculated with anthracnose (*Colletotrichum lagenarium*). Only slight phytotoxicity was observed in either case.

In order to protect plants, a fungicidally effective amount of antibiotic A—2315 may be applied in different ways, for example, in the form of liquid sprays or dusting powders, using suitable solvents, carriers, emulsifiers, wetting agents or surface-active agents as needed. Although individual conditions must be considered, A—2315 is typically useful in the control of fungi when administered to plants in amounts of from about 200 to about 500 ppm.

As is apparent from the foregoing properties, antibiotic A—2315 is useful for suppressing the growth of pathogenic organisms. In addition to the foregoing applications, solutions containing as little as two percent of the antibiotic are useful for disinfecting glassware, dental and surgical instruments, and surfaces

such as walls, floors, and tables in areas where maintenance of sterile conditions is important, for example, in hospitals and food-preparation areas.

The novel antibiotic of this invention is produced by culturing an A—2315-producing strain of an *Actinoplanes* organism under submerged aerobic conditions in a suitable culture medium until the culture medium contains substantial antibiotic activity. The antibiotic is recovered by employing various isolation and purification procedures commonly used and understood in the art.

The microorganism useful for the preparation of antibiotic A—2315 has been taxonomically characterized as a new strain of *Actinoplanes philippinensis* Couch. The genus *Actinoplanes* is a member of the *Actinoplanaceae*, a family of microorganisms of the order *Actinomycetales*. The *Actinoplanaceae* family was first described by Couch [J. *Elisha Mitchell Sci. Soc.*, 65, 315—318 (1949); *ibid.*, 66, 87—92 (1950); *Trans. New York Acad. Sci.*, 16, 315—318 (1954); J. *Elisha Mitchell Sci. Soc.*, 71, 148—155 and 269 (1955); "Bergey's Manual of Determinative Bacteriology," Seventh Edition, 825—829 (1957); J. *Elisha Mitchell Sci. Soc.*, 79, 53—70 (1963)].

The methods employed in the taxonomic studies of the A—2315-producing strain of *Actinoplanes* are similar to those recommended by the International *Streptomyces* Project along with other supplementary tests commonly used in taxonomy [E. B. Shirling and D. Gottlieb: "Methods for Characterization of *Streptomyces* Species." *International Bull. Systemic Bacteriol.*, 16, 313—340 (1966)].

MORPHOLOGY.

Vegetative Mycelium.

The hyphae on synthetic and semisynthetic agar media are at first short, slightly branched, septate and 0.2 to 1.2 μ m. in diameter. After about two weeks, the colonies may consist of well-defined masses of vertical hyphae arising from extensive substrate hyphae buried more deeply in the agar medium; or, alternatively, they may consist of slightly mucoid masses of shorter hyphae which obscure the vertical hyphae. In the colonies with a well-defined mass of vertical hyphae, the mycelia form a compact, slightly raised leathery growth. In the colonies with the shorter hyphae, the mycelia form a predominantly dome-shaped growth. No aerial hyphae are produced on any media. Sporangia are produced in large numbers on the surface of the dome-shaped colonies.

Sporangia.

Sporangia are formed in abundance on semisynthetic Anio-Henssen agar. They appear after about two to four weeks of incubation at 17 to 19°C. The sporangia are borne singly on very short sporangiophores (terminal portion of hyphae above the agar surface). The sporangia may be spherical, subspherical or slightly fan-shaped and are relatively small, having diameters of 4 to 10 μ m. The sporangia contain 20 to 40 subspherical sporangiospores which are arranged in one or more indistinct coils in the sporangia. The sporangiospores are approximately 1.0 μ m. in diameter at maturity. Sporangial dehiscence appears to be by the complete or partial disintegration of the sporangium. Ten or 15 minutes after being placed in water, the sporangia begin to swell to a typically spherical shape and then to liberate spores by wall disintegration. The spores are usually not immediately motile, but become actively motile by means of polar flagellae in about five minutes.

APPEARANCE ON MEDIA.

Czapek Solution Agar:

Good growth; a point inoculum produces a colony about 0.75 cm. in diameter in four weeks. No aerial hyphae are observed. The colony is generally flattened with a slight central peak. The colony color is a moderate orange. Sporangia are only rarely observed.

Peptone Czapek Solution Agar:

Good growth; a point inoculum produces a colony about 1.0 cm. in diameter in four weeks. No aerial hyphae are observed. The colony is slightly raised and somewhat convoluted or bumpy. The colony color is a dull red brown to orange brown. No sporangia are produced.

Anio-Henssen Agar:

Growth fair; a point inoculum produces a colony about 0.5 cm. in diameter in

four weeks. No aerial hyphae are observed. The colony is mounded with a round, cruciform or star-shaped central depression or crevasse. The colony color is a dull gray to gray white. Sporangia are formed in large numbers in colonies incubated at 17 to 19°C, and in small numbers on colonies incubated at higher temperatures.

Cultural characteristics on various media are described in Table V. The designations ICP refer to International *Streptomyces* Project media (Shirling and Gottlieb).

TABLE V
CULTURE CHARACTERISTICS OF A-2315 ON VARIOUS MEDIA
(Incubation Temperature 30°C.)
Reverse Color

Medium	Vegetative Mycelium Amount	M P Code* ICNB Name**	Soluble Pigment
Emerson's	3+	13K8; strong yellowish br.	Brown
Glycerol Glycine	4+	9L8; strong orange	Slight brown
Glucose Asparagine	3+	10F7; moderate orange	None
Inorganic salts-starch agar (ICP 4)	3+	12A8; moderate orange	Slight brown
Glycerol asparagine-agar (ICP 5)	4+	12A9; brownish orange	None
Calcium Malate	2+	44B1; reddish gray	Slight Purple
Bennett's	3+	12C7; light brown	Slight brown
Oatmeal agar (ICP 3)	2+	44B1; reddish gray	None
Yeast extract-malt extract agar (ICP 2)	3+	12D7; light brown	None
Nutrient	1—2+	12E6; light yellow brown	None
Czapek's	3+	11C9; moderate orange	None
Yeast Extract	3+	13H7; yellow beige	None

Medium	Vegetative Mycelium Amount	M: P Code* ICNB Name**	Soluble Pigment
Tyrosine	1+	No assignment because of poor reverse	None
TPO	2—3+	12E7; light brown	Brown

* Refers to A. Maerz and M. Rea Paul, "A Dictionary of Color," McGraw-Hill, New York, N.Y.

** "The ISCC—NBS Method of Designating Colors and a Dictionary of Color Names," National Bureau of Standards Circular 553, U.S. Government Printing Office

CELL WALL CHEMISTRY.

Actinoplanes philippinensis NRRL 5462. possesses a cell-wall chemistry most similar to *Actinoplanes philippinensis* (Szanişzlo and Gooder, 1967). Automatic amino-acid and amino-sugar analyses of cell-wall residues remaining after 18 hours of hydrolysis with 1 ml. of 6 N hydrochloric acid at 100°C. revealed that glucosamine, muramic acid, glycine, alanine and 2,6-diaminopimelic acid (DAP) are present in the walls in large concentrations. 2,6-Diamino-3-hydroxy-pimelic acid (HDAP) is noticeable absent. A comparison of the cell-wall amino acids of *Actinoplanes philippinensis* NRRL 5462 and *A. philippinensis* (Szanişzlo) is presented in Table VI.

TABLE VI

MOLAR RATIOS* OF PRINCIPAL CELL-WALL AMINO ACIDS

Organism	Glutamic	Glycine	Alanine	HDAP	DAP
<i>A. philippinensis</i> NRRL 5462	1.00	1.14	0.59	—	1.10
<i>A. philippinensis</i> (Szanişzlo and Gooder)	1.00	1.08	0.57	—	1.06

* Glutamic acid is considered as unity.

Paper chromatographic analyses of residues remaining after 2 hours of hydrolysis with 2 ml. of 2 N sulfuric acid at 100°C. revealed that the monosaccharides glucose, galactose, mannose, arabinose, xylose and rhamnose are present in the cell walls in detectable quantities. These sugars were also found in the cell walls of *A. philippinensis* (Szanişzlo and Gooder).

Based on the foregoing taxonomic description, the A—2315-producing organism has been classified as a novel strain of *Actinoplanes philippinensis* Couch. The culture herein described differs from the published description of that species in that it does not produce sporangia on glucose asparagine agar media; also slight color differences are observed on three media. These differences indicate the variations in strains, but do not constitute a difference in species.

The *Actinoplanes* culture useful for the production of antibiotic A—2315 has been deposited without restriction as to availability and made a part of the stock culture collection of the Agricultural Research Service, Northern Marketing and Nutrition Research Division, U.S. Dept. of Agriculture, Peoria, Illinois 61604, from which it is available to the public under the number NRRL 5462.

As previously noted, *Actinoplanes philippinensis* NRRL 5462 can be grown in a culture medium to produce antibiotic A—2315. The culture medium can be any one of a number of media; however, for economy of production, maximum yield,

and ease of isolation of the antibiotic, certain culture media are preferred. Thus, for example, glucose is one of the preferred sources of carbohydrate, and soybean meal is one of the preferred nitrogen sources.

Nutrient inorganic salts to be incorporated in the culture medium can include the customary salts capable of yielding sodium, potassium, ammonium, calcium, phosphate, chloride, sulfate, acetate, carbonate, and like ions. Additionally, sources of growth factors such as distiller's solubles and yeast extracts can be included with beneficial results.

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* employed in this invention. Such trace elements are commonly supplied as impurities incidental to the addition of the other constituents of the medium.

The initial pH of the culture medium can be varied widely. Prior to inoculation with the organism, however, it is desirable to adjust the pH of the culture medium to between pH 6.5 and 7.3, depending on the particular medium employed. The final pH is determined, at least in part, by the initial pH of the medium, the buffers present in the medium, and the period of time for which the organism is permitted to grow.

Preferably, submerged aerobic fermentation in large tanks is used for the production of substantial quantities of antibiotic A—2315. Small quantities of the antibiotic are obtained by shake-flask culture. Because of the time lag in antibiotic production commonly associated with the inoculation of large tanks with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of the culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to the larger tank. The medium used for the growth of the vegetative inoculum can be the same as that employed for larger fermentations, although other media can be employed.

The A—2315-producing organism can be grown at temperatures between about 20°—40°C. Optimal A—2315 production appears to occur at a temperature of about 30°C.

As is customary in aerobic submerged culture processes, sterile air is blown through the culture medium. For efficient growth of the organism and production of A—2315, the volume of air employed in the tank production of A—2315 preferably is above 0.1 volume of the air per minute per volume of culture medium. Optimum growth occurs when the volume of air employed is between 0.2 and 0.8 volumes of air per minute per volume of culture medium.

The production of antibiotic can be followed during the fermentation by testing samples of the broth for antibiotic activity against organisms known to be sensitive to the antibiotic. One assay organism useful in testing the antibiotic of the present invention is *Sarcina lutea*. The bioassay is conveniently performed by paper-disc assay on agar plates.

Generally, maximum production of the antibiotic occurs within two to six days in large-tank or shake-flask fermentation. Commonly, maximum production of antibiotic activity is realized within 20 to 96 hours.

Antibiotic A—2315 can be recovered from the culture medium and separated from other substances which may be present by extractive and adsorptive techniques. Extractive processes for the initial recovery of A—2315 are preferred. Chloroform is a suitable solvent for separating the antibiotic from the filtered, basified culture broth, although other commonly used solvents are satisfactory. For further purification of A—2315, adsorption and elution procedures using adsorptive materials, such as carbon, polyamide resin, silica gel, Sephadex LH—20, Amberlite XAD polymeric adsorbent and the like, can be advantageously employed. ("Sephadex" and "Amberlite" are registered Trade Marks).

This invention is further illustrated by the following examples, but is not to be construed as limited thereby.

EXAMPLE 1.

A. Shake Flask Fermentation of A—2315.

A culture of *Actinoplanes philippinensis* NRRL 5642 was prepared and maintained on an agar slant having the following composition:

18

10

1,433,417

10

Ingredient	Amount
Pre-cooked dried oatmeal	60.0 gm.
Debittered dried brewer's yeast	2.5 gm.
K ₂ HPO ₄	1.0 gm.
Czapek's mineral stock*	5.0 ml.
Agar	25.0 gm.
Deionized water	1100.0 ml.

* Czapek's mineral stock has the following composition:

Ingredient	Amount
FeSO ₄ ·7H ₂ O (Dissolved in 2 ml. conc. HCl)	2.0 gm.
KCl	100.0 gm.
MgSO ₄ ·7H ₂ O	100.0 gm.
Deionized water	q.s. to 1 liter

The pH of the medium was adjusted from about pH 6.2 to pH 7.3 with sodium hydroxide solution. After sterilization by autoclaving at 120°C. for about 30 minutes at 15—20 pounds pressure, the pH of the medium was 6.7.

The slant was inoculated with *Actinoplanes philippinensis* NRRL 5462 and incubated at 30°C. for ten to fourteen days. The culture does not normally sporulate on this medium; it is necessary, therefore, 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 beef serum and was scraped carefully with a sterile rod to obtain a mycelial suspension. This suspension was divided into six tubes for lyophilization. One of the lyophilized pellets was used to inoculate 50 ml. of a vegetative medium having the following composition:

Ingredient	Amount
Glucose	10.0 gm.
Potato starch	30.0 gm.
Soybean flour	20.0 gm.
Defatted cottonseed flour	20.0 gm.
CaCO ₃	2.0 gm.
Tap water	1.1 liter

The inoculated vegetative medium, in a 250 ml. flask, was incubated at 30°C. for 72 hours on a rotary shaker operating at 250 rpm.

Ten milliliters of the incubated vegetative medium was used to inoculate 200 ml. of a second-stage vegetative growth medium of the same composition as that of the vegetative medium described above. This second-stage medium, in a one-liter flask, was incubated at 30°C. for 24 hours on a rotary shaker operating at 250 rpm.

B. Tank Fermentation of A—2315.

The second-stage vegetative medium (200 ml.), prepared as described above, was used to inoculate 25 liters of a sterile production medium of the following composition:

Ingredient	Percent
Glucose	1.0
Potato starch	4.0
Molasses	0.5
Soluble meat peptone	4.0
Enzyme-hydrolyzed casein	0.4
Soybean flour	1.0
MgSO ₄	0.3
CaCO ₃	0.2
Antifoam (Dow Corning)	0.02
Tap water	q.s. 25 liters

The pH of the medium was 6.4 after sterilization by autoclaving at 120° for 30 minutes at 15—20 pounds pressure. In a 40-liter fermentation tank, the inoculated production medium was allowed to ferment for four days at a temperature of 30°C. The fermentation medium was aerated with sterile air at the rate of one-half volume of air per volume of culture medium per minute. The fermentation medium was stirred with conventional agitators at 400 rpm.

C. Isolation of A—2315.

The whole fermentation broth from a 25-liter tank grown according to the procedure described in the previous section was filtered, using a filter aid. The filtrate was adjusted to pH 8.5 with 2 N sodium hydroxide, and the resulting solution was extracted with 2/3 volume of chloroform. The chloroform extract was concentrated under vacuum to a low volume and was added slowly with stirring to 20 volumes of petroleum ether (Skellysolve F.). The precipitate was separated by filtration and was dried *in vacuo* to give 3.13 gm. of crude antibiotic.

This crude antibiotic, dissolved in ethyl acetate (100 ml.), was applied to a 2 x 100 cm. column of basic alumina (M. Woelm, Eschwege, Germany). After the column was washed with 300 ml. of ethyl acetate, elution with ethyl acetate:ethanol (19:1) gave fractions containing A—2315 activity. These fractions were combined and evaporated to dryness under vacuum. A solution of the residue in a small amount of chloroform was added to 20 volumes of petroleum ether (Skellysolve F) to precipitate the active material. The precipitate was separated by filtration and dried under vacuum to give 1.63 gm. of antibiotic A—2315.

The elution of activity was monitored by paper-disc agar-plate assay, using *Sarcina lutea* as the test organism, and by thin-layer chromatography on silica gel, using ethyl acetate:ethanol (9:1) as the developing system and *Sarcina lutea* as the bioautograph organism; alternatively, iodine vapors or sulfuric acid spray were used for detection purposes.

EXAMPLE 2.

PURIFICATION OF ANTIBIOTIC A—2315 USING SILICA GEL.

The procedure described in Example 1, Part C, for the isolation of antibiotic A—2315 was followed through the precipitation with petroleum ether to give 5 gm. of crude product. This product was dissolved in ethyl acetate (approximately 10 ml.) and sufficient methanol to produce a clear solution. The resulting solution was added to a 5 x 90 cm. column containing 300 gm. of silica gel (Merck), packed in ethyl acetate. The column was then washed with 2.5 liters of ethyl acetate to remove impurities. The A—2315 activity was eluted from the column with ethyl acetate:ethanol (95:5). These active fractions were combined and concentrated to dryness under vacuum to give 2.0 gm. of A—2315 as a white powder. Further elution with this solvent system gave an additional 0.8 gm. of A—2315 as a light yellow powder.

WHAT WE CLAIM IS:—

1. The antibiotic A—2315, said antibiotic being a white amorphous solid having a specific rotation $[\alpha]^{27}_D$ of -132 (c = 0.375 in methanol); having an approximate elemental composition of 61.51% carbon, 7.51% hydrogen, 8.69% nitrogen, and 21.53% oxygen; having an approximate molecular weight of 503 as determined by mass spectrum; having an empirical formula of $C_{26}H_{37}N_3O_7$; having in chloroform the following distinguishable bands in its infrared absorption spectrum: 2.81, 2.99, 3.38, 5.82, 6.02, 6.20, 6.30, 6.40, 6.66, 6.82, 6.94, 7.05, 7.30, 7.71, 8.88, 9.11, 10.41, 10.89, and 11.14 microns; having in 95% ethanol solution an ultraviolet absorption maximum at 214μ with an absorptivity value ($E^{1\%}_{1cm}$) of

approximately 799; being soluble in methanol and ethanol, but being only slightly soluble in water; releasing alanine upon amino-acid analysis; and containing hydroxyl groups which are capable of esterification.

2. A process for producing the antibiotic of Claim 1 which comprises cultivating *Actinoplanes philippinensis* NRRL 5462 in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic conditions until a substantial amount of antibiotic A—2315 is produced by said organism in said culture medium.



* C L M S *

~~18~~ 19

11

1,433,417

11

The pH of the medium was 6.4 after sterilization by autoclaving at 120° for 30 minutes at 15—20 pounds pressure. In a 40-liter fermentation tank, the inoculated production medium was allowed to ferment for four days at a temperature of 30°C. The fermentation medium was aerated with sterile air at the rate of one-half volume of air per volume of culture medium per minute. The fermentation medium was stirred with conventional agitators at 400 rpm.

C. Isolation of A—2315.

The whole fermentation broth from a 25-liter tank grown according to the procedure described in the previous section was filtered, using a filter aid. The filtrate was adjusted to pH 8.5 with 2 N sodium hydroxide, and the resulting solution was extracted with 2/3 volume of chloroform. The chloroform extract was concentrated under vacuum to a low volume and was added slowly with stirring to 20 volumes of petroleum ether (Skellysolve F.). The precipitate was separated by filtration and was dried *in vacuo* to give 3.13 gm. of crude antibiotic.

This crude antibiotic, dissolved in ethyl acetate (100 ml.), was applied to a 2 x 100 cm. column of basic alumina (M. Woelm, Eschwege, Germany). After the column was washed with 300 ml. of ethyl acetate, elution with ethyl acetate:ethanol (19:1) gave fractions containing A—2315 activity. These fractions were combined and evaporated to dryness under vacuum. A solution of the residue in a small amount of chloroform was added to 20 volumes of petroleum ether (Skellysolve F) to precipitate the active material. The precipitate was separated by filtration and dried under vacuum to give 1.63 gm. of antibiotic A—2315.

The elution of activity was monitored by paper-disc agar-plate assay, using *Sarcina lutea* as the test organism, and by thin-layer chromatography on silica gel, using ethyl acetate:ethanol (9:1) as the developing system and *Sarcina lutea* as the bioautograph organism; alternatively, iodine vapors or sulfuric acid spray were used for detection purposes.

EXAMPLE 2.

PURIFICATION OF ANTIBIOTIC A—2315 USING SILICA GEL.

The procedure described in Example 1, Part C, for the isolation of antibiotic A—2315 was followed through the precipitation with petroleum ether to give 5 gm. of crude product. This product was dissolved in ethyl acetate (approximately 10 ml.) and sufficient methanol to produce a clear solution. The resulting solution was added to a 5 x 90 cm. column containing 300 gm. of silica gel (Merck), packed in ethyl acetate. The column was then washed with 2.5 liters of ethyl acetate to remove impurities. The A—2315 activity was eluted from the column with ethyl acetate:ethanol (95:5). These active fractions were combined and concentrated to dryness under vacuum to give 2.0 gm. of A—2315 as a white powder. Further elution with this solvent system gave an additional 0.8 gm. of A—2315 as a light yellow powder.

WHAT WE CLAIM IS:—

1. The antibiotic A—2315, said antibiotic being a white amorphous solid having a specific rotation $[\alpha]^{27}_D$ of -132 ($c = 0.375$ in methanol); having an approximate elemental composition of 61.51% carbon, 7.51% hydrogen, 8.69% nitrogen, and 21.53% oxygen; having an approximate molecular weight of 503 as determined by mass spectrum; having an empirical formula of $C_{26}H_{37}N_3O_7$; having in chloroform the following distinguishable bands in its infrared absorption spectrum: 2.81, 2.99, 3.38, 5.82, 6.02, 6.20, 6.30, 6.40, 6.66, 6.82, 6.94, 7.05, 7.30, 7.71, 8.88, 9.11, 10.41, 10.89, and 11.14 microns; having in 95% ethanol solution an ultraviolet absorption maximum at 214μ with an absorptivity value ($E^{1\%}_{1cm}$) of

approximately 799; being soluble in methanol and ethanol, but being only slightly soluble in water; releasing alanine upon amino-acid analysis; and containing hydroxyl groups which are capable of esterification.

2. A process for producing the antibiotic of Claim 1 which comprises cultivating *Actinoplanes philippinensis* NRRL 5462 in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic conditions until a substantial amount of antibiotic A—2315 is produced by said organism in said culture medium.



12



1,433,417

12

3. A process as claimed in Claim 2, which comprises additionally recovering antibiotic A—2315 from the culture medium.

4. A process for producing the antibiotic A—2315 as defined in Claim 1 substantially as hereinbefore described with particular reference to Examples 1 and 2.

5. The antibiotic A—2315 as defined in Claim 1 substantially as hereinbefore described.

K. W. H. McVEY,
Chartered Patent Agent,
Erl Wood Manor,
Windlesham, Surrey,
England.
Agent for the Applicants.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1976.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from
which copies may be obtained.