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(54) THE ANTIBIOTIC A204 AND METHOD OF PRODUCTION

(71) We, ELI LILLY AND COMPANY, a Corporation organised 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 concerns novel antibiotic agents comprising the compounds denominated herein by the names A204I, A204II and the salts thereof, and a process for preparing same.

The compound A204I is more specifically described as a white crystalline solid melting at 96—98°C. when crystallized from ethyl ether; which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether and ketones, is slightly soluble in alcohols and is very slightly soluble in water; which is acidic, having one titratable group of $pK'a$ equals 6.1; which has an apparent molecular weight of about 900 as determined from titration data; which has the approximate composition of 61.74 percent carbon, 9.37 percent hydrogen, and 28.38 percent oxygen; which has a specific optical rotation, $(\alpha)_D^{25}$, of $+68.12^\circ$ ($C=1$ percent, w./v. in methanol); which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 2.95; 3.43; 5.95; 6.87; 7.26; 7.80; 8.52; 8.95; 9.17; 9.34; 9.59; 9.82; 10.07; 10.24; 10.51; 10.77; 11.40; and 11.80 microns, and which has no characteristic ultraviolet absorption spectrum. The infrared absorption spectrum of the compound A204I in chloroform is shown in Figure 1 of the accompanying drawings.

The compound A204II is more specifically described by the mixed sodium-potassium salt

thereof being a white crystalline solid which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether, and ketones, is slightly soluble in alcohols and is very slightly soluble in water; which has one titratable group of $pK'a=6.3$ in a 66 percent dimethylformamide-water solution, which has a specific rotation $(\alpha)_D^{25}$ of $+42.3^\circ$ ($C=1$ percent w./v. in methanol); and which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 3.20; 3.45; 6.26; 6.90; 7.30; 7.68; 7.80; 8.20; 8.50; 8.80; 9.22; 9.39; 9.59; 9.70; 9.82; 10.08; 10.26; 10.48; 10.83; 10.96; 11.30; 11.55; and 11.78 microns.

The specific optical rotation was determined of the crystalline free acid compound dried at room temperature *in vacuo* over anhydrous calcium chloride for about 15 hours; the titration with electrometric titration in a 66 percent dimethylformamide-water solution; and the elemental analyses was determined of the compounds dried *in vacuo* at about 80°C. over phosphorus pentoxide.

Antibiotic A204 I and A204 II are separate compounds produced by culturing an antibiotic A204-producing strain of *Streptomyces albus* under aerobic conditions in a culture medium containing an assimilable source of carbon nitrogen, and inorganic salts.

The organisms were first isolated from soil samples obtained from Perry, Florida, in the United States, by suspending portions of the soil samples in sterile distilled water, and by streaking the suspensions on nutrient agar plates. The seeded nutrient agar plates were incubated at about 30°C. for several days. At the end of the incubation time, colonies of the A204-producing organisms were transferred by means of a sterile platinum loop to agar slants. The inoculated agar slants were

then incubated to provide larger amounts of inoculum for the production of antibiotic A204.

5 The term A204 is used herein to denote the antibiotic compound or compounds obtained from the culture. A204 may consist solely of A204 I or contain in addition some A204 II and possibly some additional closely related components.

10 This organism capable of producing antibiotic A204 has been placed on permanent deposit, without restriction, with the culture collection of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture (Formerly Northern Regional Research Laboratories), Peoria, Illinois, and is available to the public under culture No. NRRL 3384.

20 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, the organisms which produces antibiotic A204 appears to resemble most nearly, in its most important characteristics, the published description of the organisms *Streptomyces albus*, *Streptomyces albidoflavus*, and *Streptomyces flaveolus*. However, we consider the culture to be a strain of *S. albus*, (*Rossia Doria*) Waksman and Henrici, based upon the description of the neotype strain of *S. albus* ATCC 3004 by Lyons and Pridham, [*J. Bacteriol* 83: 370—380 1962)], and the *S. albus* strain of IMRU 3005 described by Waksman, [*The Actinomycetes*, Vol. II "Classification, Identification, and Description of Genera and Species," Williams and Wilkins Co., Baltimore (1961)].

35 Despite the similarities, however, sufficient differences exist to distinguish the novel

organism employed in this invention from all previously described strains of *S. albus*. Therefore, we have classified the organism NRRL 3384 as a novel strain of *Streptomyces albus*.

45 The detailed descriptions contained herein are made with particular reference to the newly found organism NRRL 3384. However, it is to be understood that the production of antibiotic A204 by the growing mutants of this A204-producing organisms is within the scope of this invention. Such other mutants can be produced by known procedures as by subjecting the above strain to the action of x-ray, ultraviolet radiation, or chemical agents such as nitrogen mustards.

55 The methods employed in the taxonomic studies of the antibiotics A204-producing strain of *S. albus* were those recommended for the International Streptomyces Project described by Shirling and Gottlieb, "Methods for Characterization of *Streptomyces* Species," *Intern. Bull. Systematic Bacteriol*: 16 313—340 (1966), along with other supplementary tests. Carbon utilization tests were carried out according to the method described by Pridham and Gottlieb, *J. Bac.* 56, 107 (1948). The data obtained from the taxonomic studies are shown in tabular form below. Color names were assigned according to the ISCC—NBS Method of Designating Colors and a Dictionary of Color Names." U.S. Dept. of Commerce Circ. 553. Washington, D.C.). Figures in parentheses refer to Tresner and Backus color series (Tresner and Backus [1966], "System of Color Wheels for Streptomyces Taxonomy," *Appl. Microbiol*, 11: 335—338). Maerz and Paul color blocks are enclosed in brackets. (Maerz and Paul, 1950, *Dictionary of Color*. McGraw-Hill, New York).

TABLE I

Description of Culture NRRL 3384

Property Observed	Characteristics
Morphology	Spiral sporophores produced containing 3 to 50 spores per chain, usually 10 to 50; spores ovoid to elongate, $1.9\mu \times 1.3\mu$; smooth spore surfaces as observed in electron micrographs.
CULTURAL CHARACTERISTICS ON:	
Calcium malate	Growth abundant, reverse pale yellow green [10B1]; sporulation and aerial mycelium abundant, (7) pale yellow, 2ba [11C1]; no soluble pigment; medium slightly cleared.

TABLE 1 (Continued)

Description of Culture NRRL 3384

Property Observed	Characteristics
Tomato paste oatmeal	Abundant growth, grayish yellow reverse [11B2]; sporulation and aerial mycelium abundant (W) white <i>a</i> ; no soluble pigment.
ICP No. 2 ¹ Yeast-Malt Agar)	Abundant growth, grayish yellow reverse [11B2]; sporulation and aerial mycelium abundant (Y) pale yellow, <i>2ba</i> [11C1]; no soluble pigment.
ICP No. 3 ¹ Oatmeal Agar)	Fair growth, reverse white; sporulation and aerial mycelium fair (W) white <i>a</i> ; no soluble pigment.
ICP No. 4 (Inorganic Salts-Starch Agar)	Abundant growth, reverse grayish yellow [11B2]; abundant sporulation and aerial mycelium (Y) pale yellow <i>2ba</i> [11C1]; no soluble pigment.
ICP No. 5 (Glycerol-Asparagine Agar)	Abundant growth, reverse pale yellow green [10B1]; abundant sporulation and aerial mycelium (W) white, <i>a</i> ; no soluble pigment.
<i>Czapek's</i> agar	Good growth, reverse white; good sporulation and aerial mycelium (W) white <i>a</i> ; no soluble pigment.
<i>Tyrosine</i> agar	Very poor growth; no colors assigned.
<i>Glucose asparagine</i>	Fair growth, reverse yellow [11C1]; fair sporulation and aerial mycelium (Y) pale yellow <i>2ba</i> [11C1]; no soluble pigment.
<i>Emerson's</i> agar	Abundant growth, reverse grayish-yellow [12B3]; abundant sporulation and aerial mycelium (GY) yellowish-gray, <i>2dc</i> [10A2]; no soluble pigment.
<i>Bennett's</i> agar	Very poor growth; no colors assigned.
<i>Nutrient's</i> agar	Fair growth, reverse grayish-yellow [11B2]; fair sporulation and aerial mycelium (W) white <i>a</i> ; no soluble pigment.
Action on milk	No change after 14 days.
Nitrate reduction	Positive

TABLE I (Continued)
Description of Culture NRRL 3384

Property Observed	Positive	Characteristics
Melanine production	Positive	
Peptone-iron agar	Negative	
Tryptone yeast extract	Negative	
Gelatin liquefaction	None at 14 days	
Temperature requirements	Growth and sporulation—fair at 26°; good at 30°; vegetative growth at 37°C.; a trace of growth, vegetative only at 43—49°. No growth at 55°.	
Response of substrate mycelium to pH change	All media but ICP No. 4 unaffected. Change on ICP No. 4 from brown to white or colorless when treated with either 0.05N HCl or 0.05N NaOH.	
Response of soluble pigment to pH change	No soluble pigment.	

Carbon Utilization

L-arabinose	+
Sucrose	(—)
D-Xylose	(+)
D-Fructose	(+)
Glucose	+
Rhamnose	+
Raffinose	+
I-Inositol	+
D-Mannitol	+
minus carbon (control)	—

Utilization Code:

+ = positive

(+) = probable utilization

(—) = questionable utilization

— = no utilization

The culture medium employable in producing antibiotic A204 by cultivation of the above-described organism can be any one of several media, since, as is apparent from the above-described utilization tests, the organism is capable of utilizing different energy sources. However, for economy of production, maximum yield of antibiotic, and ease of isolation of the antibiotic, certain relatively simple nutrient sources are preferable. For example, the media which are useful in the production of the antibiotic include an assimilable source of carbon such as glucose, fructose, starch, glycerine, molasses, dextrin, brown sugar, and the like. The preferred source of carbon is glucose. Additionally, employable media include a source of assimilable nitrogen such as soybean meal, corn steep solids, yeast, cottonseed meal, beef extract, peptones (meat or soy), casein, amino acid mixtures and the like. Preferred sources of nitrogen are peptones, soybean meal, amino acid mixtures, and the like. Among the nutrient inorganic salts which can be incorporated in the culture media are the customary salts capable of yielding sodium, potassium, ammonium, calcium, phosphate, sulfate, chloride, carbonate, and like ions.

Minor elements necessary for optimum growth and development of the organism used for the production of antibiotic A204 should also be included in the culture medium. Such trace elements commonly occur as impurities in the other constituents of the medium in amounts sufficient to meet the growth requirements of the actinomycetes employed in this invention.

The initial pH of the culture medium can be varied. However, it has been found desirable that the initial pH of the medium be between 6.5 and 7.5. As has been observed with other actinomycetes, the pH of the medium gradually increases throughout the growth period of the organism while the antibiotic is being produced, and may attain a level of from 7.0 to 7.6 or above, the final pH being dependent at least in part on the initial pH of the medium, the buffers present in the medium, and the period of time the organism is permitted to grow.

Submerged, aerobic cultural conditions are the conditions of choice for the production of antibiotic A204. For preparation of relatively small amounts, shake flask and surface culture in bottles 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 sporulated 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. The 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 of antibiotic A204.

The organism which produces antibiotic A204 will grow over a wide temperature range between 25—37°C. and normally the growth of the organism is carried out for a period of 24 to 72 hours. Optimal production of A204 seems to occur at temperatures of 26—30°C. In general, maximum production of the antibiotic occurs within about 48—72 hours after inoculation of the culture medium.

As is customary in aerobic, submerged culture processes, sterile air is blown through the culture medium. For efficient growth of the organism and antibiotic A204 production, the volume of air employed in the tank production of A204 is from 0.2 to 0.4 volume of air per minute per volume of culture. The preferred volume is 0.35 volume of air per minute per volume of culture medium.

The concentration of antibiotic activity in the culture medium can be followed readily during the fermentation period by testing samples of the culture medium for their inhibitory activity against the growth of organisms known to be inhibited by the presence of antibiotic A204. The organisms *Staphylococcus aureus*, *Sarcina lutea*, *Bacillus subtilis*, and *Mycobacterium avium* have been found useful for this purpose. The testing of the samples can be carried out by the well-known turbidometric or cup-plate methods.

In general, maximum production of A204 occurs within two to three days after inoculation of the culture medium in submerged aerobic culture or shake flask culture processes.

The antibiotic activity produced during the fermentation of A204 can occur either in the antibiotic broth or in the mycelium or in both places. Accordingly, isolation techniques employed in the production of A204 are designed to permit maximum recovery of the antibiotic from either or both sources. Thus, for example, the fermentation broth as obtained may be filtered and the filtrate extracted with a suitable solvent to recover the antibiotic activity not retained by the mycelial cake. In addition, the antibiotic present in the mycelial cake is recovered by thorough extraction with a suitable solvent. In either event, the antibiotic can be recovered from the extracting solvent by the ordinary methods commonly employed in the art.

Chemical and physical analyses of crystalline A204 indicate the presence of 4—5 methoxy groups.

Paper chromatography of antibiotic A204I on Whatman No. 1 paper (Whatman is a registered Trade Mark) gave the following R_f values; $R_f=0.14$ in a solvent of water, methanol, acetone, and benzene in a volume ratio of 72:24.5:3:0.5; $R_f=0.87$ in a solvent of 10 percent aqueous *n*-propanol; $R_f=0.33$ in a solvent containing water, methanol and acetic acid in a ratio of 12:3:1 (solution was adjusted to pH 10.5 with NH_4OH and then to pH 7.3 with H_3PO_4). In determining the foregoing values, the antibiotic was applied to the paper in a methanolic solution. Bioautographs were obtained by placing the paper chromatogram on agar plates seeded with *Bacillus subtilis* as the test organism.

When A204I is subjected to thin-layer chromatography on silica gel plates in an ethyl acetate solvent, utilizing a vanillin or sulfuric acid spray as a detector, it has an R_f value of about 0.8.

The sodium salt of antibiotic A204I is a white, crystalline solid, melting at 144–145°C., and having a molecular weight of about 960 as determined by x-ray analysis. The sodium salt is soluble in esters, benzene, halogenated hydrocarbons, DMF, DMSO, ether, and ketones, and is slightly soluble in alcohols. The specific optical rotation $(\alpha)_D^{25}$ of the crystalline sodium salt of antibiotic A204I, dried at room temperature *in vacuo* over anhydrous calcium chloride for about 15 hours, was found to be +54.95° (C=2 percent, w./v. in methanol).

The infrared absorption spectrum of the sodium salt of antibiotic A204I in chloroform is shown in Figure 2 of the accompanying drawing. The distinguishable bands in the infrared spectrum over the range of 2.0 to 15.0 microns are as follows: 3.1–3.2 (broad); 3.44; 6.26; 6.87; 7.29; 7.67; 7.79; 8.45; 8.96; 9.11; 9.18; 9.36; 9.56; 9.82; 10.05; 10.26; 10.54; 10.95; and 11.77 microns.

The molecular weight of the hydrated crystalline silver salt of antibiotic A204I, determined from x-ray data, is about 1099. Crystals of the silver salt are colourless but are sensitive to light and x-rays, turning dark brown after two days irradiation in air. The observed density of the silver salt, measured by flotation in aqueous ZnCl_2 is 1.293 g./cm³. The only extinction rule is $hkl: h+k=Zn$, which indicates the noncentric monoclinic space group C_2 . There are four asymmetric monoclinic units and one molecule per asymmetric unit in a unit cell which has the dimensions: $a=25.977 \text{ \AA}$, $b=14.517 \text{ \AA}$, $C=14.418 \text{ \AA}$, $B=91.94 \text{ \AA}$.

As is the case with many antibiotics, antibiotic A204 occasionally comprises several closely related components. The second component is not found in all fermentations and, when present, is usually present in amounts up to 5 percent of the total yield. This

second, minor, occasionally observed component has been arbitrarily designated as A240II. Thus far, it has only been isolated as the mixed sodium-potassium salt. The mixed sodium-potassium salt of A204II has been found to have activity comparable to antibiotic A204I.

An average of several elemental analyses of the crystalline mixed sodium-potassium salt of A204II dried *in vacuo* at about 80°C. over phosphorus pentoxide gave the values of 60.66% carbon, 8.76% hydrogen and 27.09% oxygen.

Several atomic absorption analyses indicated that the compound was a mixed sodium-potassium salt of various proportions, containing one mole of the mixed cation per mole of A204II.

The mixed sodium-potassium salt of A204II has no characteristic ultraviolet absorption pattern.

Thin-layer chromatography of the mixed sodium-potassium salt of factor II on silica gel plates gave an R_f value of 0.75 in an ethyl acetate system using a sulfuric acid spray for detection.

The antibiotic compounds of the present invention have industrial, pharmaceutical, human and veterinary applications, the term industrial being used to distinguish from the human and veterinary applications. The compounds are usually combined by known methods with suitable carriers to provide the desired pesticide.

Antibiotic A204 and its salts exhibit excellent activity against coccidiosis infections when incorporated into the diet of chickens. A level of from 9 to 45 grams of antibiotic A204 or its salts per ton of feed has been effective in controlling single infections of *E. tenella*, *E. acervulina*, and *E. maxima*. The same levels were effective in controlling a mixed infection of *E. brunetti* and *E. tenella* as well as a mixed infection of eight species of coccidi (Coccivac type D).

The compounds of this invention also exhibit insecticidal and acaricidal activity. Also they exhibit inhibitory action against the growth of microbial organisms including gram-positive bacteria and fungi, which are pathogenic to animal and plant life. Hence the antibiotics are also useful, when incorporated into disinfectant solutions, for sterilizing surfaces around swimming pools, farm buildings housing livestock and the like, and for maintaining such surfaces free of bacterial and fungal contaminants.

The compounds of this invention also exhibit activity against bean powdery mildew, and maize dwarf virus at concentrations of from 16 to 400 ppm., and hence are also useful in controlling such plant viruses and fungi.

The following examples are provided by way of illustration. Examples 1 to 10 are examples of the invention claimed and Ex-

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amples 11 to 13 illustrate the biocidal activity of the antibiotics.

EXAMPLE 1

Shake Flask Production of Antibiotic A204I

The production of antibiotic A240I in shake flask cultures is illustrated by the following example:

Spores of *Streptomyces albus* NRRL 3384 were inoculated on a nutrient agar slant made up of 10 g. of dextrin, 2 g. of an enzyme digested casein, 1 g. of beef extract, 1 g. of yeast extract, 20 g. of agar and sufficient water to make a total volume of 1 liter. The slants were inoculated with spores of *Streptomyces albus* NRRL 3384 and incubated for four to five days at 30°C. The slants were covered with sterile distilled water and gently scraped to remove the organisms and provide an aqueous suspension thereof. One milliliter of the resulting spore suspension was used to inoculate each 100 ml. portion of the vegetative medium.

The vegetative culture medium was prepared by combining 15 g. of glucose, 15 g. of soybean meal, 5 g. of corn steep solids, 5 g. of sodium chloride, 2 g. of calcium carbonate, and sufficient tap water to make the total volume 1 liter. The vegetative inoculum was shaken for 48 hours at 30°C. on a reciprocal shaker having a 2-inch stroke at 108 r.p.m. The inoculum so prepared was then utilized in the production of antibiotic A204I as follows.

A production medium was prepared having the following composition:

Soy bean meal	15 g./l.
Casein	1 g./l.
NaNO ₃	3 g./l.
Glucose syrup	20 g./l.
CaCO ₃	2.5 g./l.
Tap water	

One hundred milliliter portions of the production medium were placed in five hundred milliliter Erlenmeyer flasks, which were then sterilized at 120°C. for 30 minutes. When cooled, each flask was inoculated with a 5 percent vegetative inoculum prepared as previously described.

The production flasks were shaken for 48 hours at 30°C., on a rotary shaker operating at 250 r.p.m. The pH of the uninoculated medium varied over a pH range of 6.5 to 7.5. The harvest pH at the end of the fermentation cycle was between 7.0 to 7.6. The antibiotic activity was found in both the broth and the mycelium. This activity was determined by assaying the broth and the mycelium against *Bacillus subtilis*, using known disc or cup-plate methods.

The whole broth (25 liters) obtained by the above procedure, was filtered *in vacuo* with the aid of diatomaceous earth. The mycelial cake was extracted three times with one-half

volume of 50 percent aqueous methanol. The three mycelial extracts were combined and concentrated *in vacuo* to remove the methanol. The filtered broth and the aqueous concentrate of the mycelial extract solutions were combined, the pH of the mixture adjusted to pH 3 with hydrochloric acid, and the solution extracted two times with one-half volume of ethyl acetate. The ethyl acetate extracts were combined and concentrated to dryness, redissolved in chloroform (10 ml. for each gram of solids), and the chloroform solution passed through a 12 inch X 40 inch column of Pittsburgh carbon containing 2 g. of carbon for each gram of solids. The column was then eluted with 5 to 10 times the original volume of chloroform.

The chloroform eluate fractions were combined, concentrated to dryness *in vacuo*, dissolved in a small amount of warm methanol, chilled, and the resulting crystals filtered. Recrystallization produced 8.9 g. of antibiotic A204I crystals having a potency of from 1,200 to 1,400 microbiological units/mg. The crystals were identified as antibiotic A204I by NMR, IR, thin-layer chromatographic, and paper chromatographic studies, as described above, and having a melting point of 96—98°C.

EXAMPLE 2

Production of Antibiotic A204I

Antibiotic A204I was produced according to the process of Example 1, but utilizing a production medium having the following composition:

Glucose	15 g.
Soybean meal	15 g.
Cornsteep solids	5 g.
NaCl	5 g.
CaCO ₃	2 g.

and sufficient tap water to bring the total volume to 1 liter. The antibiotic produced was identified as A204I and had a melting point of 96—98°C.

EXAMPLE 3

Antibiotic A204I was produced according to the process of Example 1, but utilizing a production medium having the following composition:

Glucose	20 g.
(NH ₄) ₂ SO ₄	5 g.
CaCO ₃	8 g.
KCl	4 g.
KH ₂ PO ₄	0.4 g.
Soybean meal	5 g.

and sufficient tap water to bring the total volume to 1 liter. The antibiotic produced was identified as A204I and had a melting point of 96—98°C.

EXAMPLE 4

Antibiotic A204I was produced according to the process of Example 1, but utilizing a production medium having the following composition:

Glucose	10 g.
Edible molasses	20 g.
Peptone	5 g.
CaCO ₃	2 g.

and sufficient tap water to bring the total volume to 1 liter. The antibiotic produced was identified as A204I and had a melting point of 96—98°C.

EXAMPLE 5

Pilot Plant Production of Antibiotic A204I

The production of antibiotic A204I by submerged fermentation on a pilot plant scale is illustrated by the following procedure:

A 250 ml. flask containing 50 ml. of a vegetative medium comprising 10 g./l. dextrose, 25 g./l. soluble starch, 15 g./l. nutrisoy grits, 10 g./l. cornsteep liquor, and 2 g./l. CaCO₃ in tap water, having an adjusted pH of 6.5 (NaOH), was inoculated with a 5 percent suspension obtained according to the method described in Example 1 from a nutrient agar slant culture comprising 10 g. glucose, 10 g. yeast extract, 5 g. NaCl, 0.01 g. FeSO₄·7H₂O, 0.25 g. MgSO₄·7H₂O, and 20 g. Meer agar (washed three times) in one liter of deionized water. The inoculated vegetative medium was incubated at about 30°C. for 48 hours on a rotary shaker having a 2-inch diameter arc, operating at 250 r.p.m. A 16 ml. portion of the vegetative culture so obtained was employed to inoculate a 1 liter flask containing 200 ml. of the vegetative medium. The inoculated vegetative medium was incubated at about 30°C. for 30 hours on a rotary shaker having a 2-inch diameter arc, operating at 250 r.p.m. A 200 ml. portion of the vegetative culture so obtained was employed to inoculate a 40 liter fermenter containing 25 liters of a sterilized aqueous fermentation medium comprising 0.2 g./l. of Dow Corning Antifoam, 25 g./l. dextrose, 15 g./l. nutrisoy grits, 3 g./l. blackstrap molasses, 1 g./l. casein and 2.5 g./l. CaCO₃ in tap water. The fermentation was maintained at a temperature of 30°C. for 30 hours after inoculation. Agitation at the rate of 420 r.p.m. was carried out throughout the fermentation. Aeration throughout the fermentation was at a rate of 0.4 cubic feet of air per cubic feet of medium per minute. Isolation of the antibiotic was carried out as described in Example 1. The antibiotic produced was identified as A204I and had a melting point of 96—98°C.

EXAMPLE 6

Preparation of Salts of Antibiotic A204 from Broth and Mycelium

The alkali metal, ammonium, and amine salts of antibiotic A204 are prepared from the broth and mycelium according to the method of Example I by adjusting the pH of the combined filtered broth and aqueous concentrate of the mycelial extract to at least pH 8.5 with the appropriate alkali metal, ammonium, or amine hydroxide, extracting two times with one-half volume of ethyl acetate, and then proceeding as described in Example I.

The acid and its salts can also be crystallized from a number of ketones, esters, alcohols, and benzene-hydrocarbon mixtures, or can be purified by other methods known in the art such as column chromatography, and the like.

EXAMPLE 7

Preparation of the Sodium Salt of Antibiotic A204I

To 100 mg. of antibiotic A204I, obtained in Example 1 and dissolved in one ml. acetone, was added one drop of 5N NaOH and one ml. water. The mixture was clarified by heating slightly, and allowed to stand at -15°C. until crystallization occurred. The crystals were filtered and recrystallized from aqueous alcohol to yield 72 mg. of the sodium salt of antibiotic A204I and having a melting point of 144—145°C.

EXAMPLE 8

Preparation of the Potassium Salt of Antibiotic A204I

The potassium salt is obtained by substituting one drop of 5N KOH for the 5N NaOH of the above method.

EXAMPLE 9

Preparation of the Silver Salt of Antibiotic A204I

Six hundred and seventy milligrams of the sodium salt of antibiotic A204I were dissolved in 40 ml. methanol, mixed with a solution of 270 mg. of silver nitrate in 2 ml. water, and allowed to stand at room temperature for 3 hours in the dark. The solution was evaporated to dryness and washed with water to remove the excess silver nitrate. The residue was dissolved in 10 ml. of methanol with heat, filtered through a sintered glass funnel while hot, and allowed to stand 16 hours at 50°C. Large white rectangular crystals of the silver salt of antibiotic A204I were obtained, and were recrystallized from aqueous acetone to yield 450 mg. of the silver salt of antibiotic A204I. The [density] molecular weight of the salt was determined by x-ray data as being about 1099.

EXAMPLE 10

Isolation of Antibiotic A204II

Mixed sodium potassium antibiotic A204 salt (100 g.), obtained by the method described in Example 6 was dissolved in 500 ml. of a benzene:ethylacetate (7:3) mixture. The solution was chromatographed over a column containing 2000 g. of silica gel (Grace-Davison Chemical Co. Grade 62). The elution was carried out with a benzene:ethylacetate (7:3) mixture, and the elution of the fractions was followed by silica gel thin layer chromatography using ethyl acetate as the developing solution and an H₂SO₄ spray for detection. A204I was eluted first. After approximately 32 litres of solvent was collected, the solvent was changed to a benzene:ethylacetate (1:1) solution. The elution was continued until no further spots were detected on the silica gel-TLC plates.

The fractions containing high concentrations of A204II were combined, concentrated to dryness, and crystallized from an acetone-water solution. The resulting crystals (3 g.) were rechromatographed on a column containing 71 g. of silica gel in a benzene:ethyl

acetate (7:3) mixture and again following the elution by TCL. The fractions containing only A204II were combined, concentrated to dryness, and crystallized in an acetone-water solution to yield 788 mg. of crystalline A204II (m.p., 177—179°C., having an activity equal to 1550 units of A204/ml.).

EXAMPLE 11

The insecticidal and acaricidal activity of antibiotic A204 is summarized in Table II.

The following rating scale is utilized in Table II.

Percent Dead	Rating	
0—10	0	40
11—20	1	
21—30	2	
31—40	3	
41—50	4	
51—60	5	45
61—70	6	
71—80	7	
81—90	8	
91—100	9	

TABLE II

Efficacy of Antibiotic A204 as an Insecticide

Insect	Route	Rating (ppm.)					
		1,000	500	250	100	50	25
Mexican Bean Beetle	Stomach	8	4	0	—	—	—
Southern Army Worm	Stomach	8	9	8	8.5	3.5	3
Melon Aphid	Contact	3.5	2	1	—	—	—
	Systemic	7	0	0	—	—	—
Two-Spotted Spider Mite	Contact	9	7	7	8	7	6
Milkweed Bug	Contact	9	9	4	3	1	2

EXAMPLE 12

The inhibitory levels were determined by the agar dilution test. In the agar dilution test, the test organism was streaked on a series of agar plates containing various concentra-

tions of antibiotic A204 to determine the minimum concentration of antibiotic A204 in mcg./ml. (micrograms per milliliter) in the agar substrate which inhibited the growth of the organism over a period of 24 hours.

TABLE III

Test organism (strain)	Minimum Inhibitory Concentration mcg./ml. 24 hours
<i>Staphylococcus aureus</i> (3055)	6.25
<i>Bacillus subtilis</i> (X12—1)	6.25
<i>Mycobacterium avium</i> (X—85)	1.56
<i>Streptococcus faecalis</i>	1.56
<i>Lactobacillus casei</i>	1.56
<i>Leuconostoc citrovorum</i>	0.39
<i>Trichophyton mentagrophytes</i>	25.00
<i>Xanthomonas phaseoli</i>	100.00
<i>Botrytis cinerea</i>	100.00
<i>Collectotrichum pisi</i>	100.00
<i>Helminthosporium sativum</i>	100.00
<i>Pullularia</i> sp.	100.00
<i>N. gruberi</i> (HB—1) in MK ₂ tissue culture	100.00

The sodium salt of the free acid antibiotic A204I exhibits comparable in vitro activity.

EXAMPLE 13

- 5 The mixed sodium and potassium salt of A204II exhibits inhibitory action as illustrated

in Table IV against the growth of a number of microbiological organisms. The inhibitory levels were determined as described herein-above.

TABLE IV

Test Organism	Minimum Inhibitory Concentration mcg./ml.
<i>Staphylococcus aureus</i>	12.50
<i>Bacillus subtilis</i>	3.12
<i>Mycobacterium avium</i>	12.50
<i>Streptococcus faecalis</i>	3.12
<i>Lactobacillus casei</i>	6.25
<i>Leuconostoc citrovorum</i>	6.25
<i>Alternaria solani</i>	100.00
<i>Botrytis cinerea</i>	50.00
<i>Collectotrichum pisi</i>	50.00
<i>Helminthosporium sativum</i>	50.00

WHAT WE CLAIM IS:—

1. Antibiotic agent selected from the compound A204I, A204II, mixtures thereof, and their alkali metal, alkaline earth metal and basic nitrogen salts, said A204I being characterized as a white crystalline solid melting at 96—98°C. when crystallized from ethyl ether; which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether and ketones, is slightly soluble in alcohols, and is very slightly soluble in water; which is acidic, having one titratable group of pK'a equals 6.1; which has an apparent molecular weight of about 900 as determined from titration data; which has the approximate composition of 61.74 percent carbon, 9.37 percent hydrogen, and 28.38 percent oxygen; which has a specific optical rotation, $(\alpha)_D^{25}$, of +68.12° (C=1 percent, w./v. in methanol); which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 2.95; 3.43; 5.95; 6.87; 7.26; 7.80; 8.52; 8.95; 9.17; 9.34; 9.59; 9.82; 10.07; 10.24; 10.51; 10.77; 11.40 and 11.80 microns, and which has no characteristic ultraviolet absorption spectrum, and the mixed sodium-potassium salt of said A204II being characterized as a white crystalline solid which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether, and ketones, is slightly soluble in alcohols and is very slightly soluble in water; which has one titratable group of pK'a=6.3 in a 66 percent dimethylformamide-water solution, which has a specific rotation $(\alpha)_D^{25}$ of +42.3° (C=1 percent

w./v. in methanol); and which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 3.20; 3.45; 6.26; 6.90; 7.30; 7.68; 7.80; 8.20; 8.50; 8.80; 9.22; 9.39; 9.59; 9.70; 9.82; 10.08; 10.26; 10.48; 10.83; 10.96; 11.30; 11.55; and 11.78 microns.

2. The antibiotic A204I of claim 1 being characterized as a white crystalline solid melting at 96—98°C. when crystallized from ethyl ether; which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether and ketones, is slightly soluble in alcohols, and is very slightly soluble in water; which is acidic, having one titratable group of pK'a equals 6.1; which has an apparent molecular weight of about 900 as determined from titration data; which has the approximate composition of 61.74 percent carbon, 9.37 percent hydrogen, and 28.38 percent oxygen; which has a specific optical rotation, $(\alpha)_D^{25}$, of +68.12° (C=1 percent, w./v. in methanol); which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 2.95; 3.43; 5.95; 6.87; 7.26; 7.80; 8.52; 8.95; 9.17; 9.34; 9.59; 9.82; 10.07; 10.24; 10.51; 10.77; 11.40 and 11.80 microns, and which has no characteristic ultraviolet absorption spectrum.

3. The antibiotic A204II of claim 1 wherein the mixed sodium-potassium salt thereof is characterized as a white crystalline solid which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethyl-



TABLE IV

Test Organism	Minimum Inhibitory Concentration mcg./ml.
<i>Staphylococcus aureus</i>	12.50
<i>Bacillus subtilis</i>	3.12
<i>Mycobacterium avium</i>	12.50
<i>Streptococcus faecalis</i>	3.12
<i>Lactobacillus casei</i>	6.25
<i>Leuconostoc citrovorum</i>	6.25
<i>Alternaria solani</i>	100.00
<i>Botrytis cinerea</i>	50.00
<i>Collectotrichum pisi</i>	50.00
<i>Helminthosporium sativum</i>	50.00

WHAT WE CLAIM IS:—

1. Antibiotic agent selected from the compound A204I, A204II, mixtures thereof, and their alkali metal, alkaline earth metal and basic nitrogen salts, said A204I being characterized as a white crystalline solid melting at 96—98°C. when crystallized from ethyl ether; which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether and ketones, is slightly soluble in alcohols, and is very slightly soluble in water; which is acidic, having one titratable group of pK'a equals 6.1; which has an apparent molecular weight of about 900 as determined from titration data; which has the approximate composition of 61.74 percent carbon, 9.37 percent hydrogen, and 28.38 percent oxygen; which has a specific optical rotation, $(\alpha)_D^{25}$, of +68.12° (C=1 percent, w./v. in methanol); which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 2.95; 3.43; 5.95; 6.87; 7.26; 7.80; 8.52; 8.95; 9.17; 9.34; 9.59; 9.82; 10.07; 10.24; 10.51; 10.77; 11.40 and 11.80 microns, and which has no characteristic ultraviolet absorption spectrum, and the mixed sodium-potassium salt of said A204II being characterized as a white crystalline solid which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether, and ketones, is slightly soluble in alcohols and is very slightly soluble in water; which has one titratable group of pK'a=6.3 in a 66 percent dimethylformamide-water solution, which has a specific rotation $(\alpha)_D^{25}$ of +42.3° (C=1 percent

w./v. in methanol); and which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 3.20; 3.45; 6.26; 6.90; 7.30; 7.68; 7.80; 8.20; 8.50; 8.80; 9.22; 9.39; 9.59; 9.70; 9.82; 10.08; 10.26; 10.48; 10.83; 10.96; 11.30; 11.55; and 11.78 microns.

2. The antibiotic A204I of claim 1 being characterized as a white crystalline solid melting at 96—98°C. when crystallized from ethyl ether; which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethylsulfoxide, ether and ketones, is slightly soluble in alcohols, and is very slightly soluble in water; which is acidic, having one titratable group of pK'a equals 6.1; which has an apparent molecular weight of about 900 as determined from titration data; which has the approximate composition of 61.74 percent carbon, 9.37 percent hydrogen, and 28.38 percent oxygen; which has a specific optical rotation, $(\alpha)_D^{25}$, of +68.12° (C=1 percent, w./v. in methanol); which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 2.95; 3.43; 5.95; 6.87; 7.26; 7.80; 8.52; 8.95; 9.17; 9.34; 9.59; 9.82; 10.07; 10.24; 10.51; 10.77; 11.40 and 11.80 microns, and which has no characteristic ultraviolet absorption spectrum.

3. The antibiotic A204II of claim 1 wherein the mixed sodium-potassium salt thereof is characterized as a white crystalline solid which is soluble in esters, benzene, halogenated hydrocarbons, dimethylformamide, dimethyl-

- sulfoxide, ether, and ketones, is slightly soluble in alcohols and is very slightly soluble in water; which has one titratable group of $pK'a=6.3$ in a 66 percent dimethylformamide-water solution, which has a specific rotation $(\alpha)_D^{25}$ of $+42.3^\circ$ ($C=1$ percent w./v. in methanol); and which when measured in a chloroform solution has the following distinguishable bands in its infrared absorption spectrum over the range of 2.0 to 15.0 microns; 3.20; 3.45; 6.26; 6.90; 7.30; 7.68; 7.80; 8.20; 8.50; 8.80; 9.22; 9.39; 9.59; 9.70; 9.82; 10.08; 10.26; 10.48; 10.83; 10.96; 11.30; 11.55; and 11.78 microns.
4. A method of producing the antibiotic A204 as hereinbefore defined which comprises cultivating an antibiotic A204-producing strain of *Streptomyces albus* in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic conditions until a substantial amount of A204 is produced by said organism in said culture medium.
5. A method according to claim 4 wherein the organism is *Streptomyces albus* NRRL 3384.
6. A method according to claim 4 or 5 wherein the culture medium is maintained at a temperature of 25°C. to 37°C. and the growth of the organism is carried out for a period of 24 to 72 hours.
7. A method according to claim 4, or 5 or 6 additionally comprising recovering the A204 from said culture medium.
8. A method according to claim 4, 5 or 6, wherein the alkali metal, alkaline earth metal, or basic nitrogen salt of antibiotic A204 is recovered by treatment of the A204-containing medium with the appropriate alkali metal, ammonium or amine hydroxide.
9. A method according to claim 4, 5, 6 or 7 additionally comprising recovering antibiotic A204I and antibiotic A204II as separate substances, by converting the antibiotic A204 to an antibiotic A204 salt, adsorbing the antibiotic A204 from a solution of said salt upon a suitable resin and selectively eluting antibiotic A204II and A204I free of A204II from said resin.
10. A method of producing the antibiotics of claim 1 substantially as herein described.
11. The antibiotics of claim 1 substantially as herein described.
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