

PATENT SPECIFICATION

DRAWINGS ATTACHED

1021401



Date of Application and filing Complete Specification: Oct. 16, 1962.

No. 39130/62.

Application made in United States of America (No. 152,262) on Nov. 14, 1961.

Application made in United States of America (No. 198,414) on May 23, 1962.

Application made in United States of America (No. 211,153) on July 16, 1962.

Complete Specification Published: March 2, 1966.

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Index at acceptance:—C2 A(1B, 1C1A, 1C1B, 1C1D1, 1C2A, 1C2C, 2A3); A5 B(1R2, 1S, 2R2, 2S)

Int. Cl.:—C 07 g // A 61 k

COMPLETE SPECIFICATION

Novel Antibiotics and processes for their manufacture

We, SCHERICO LIMITED, of Falkengasse 2, Lucerne, Switzerland, a body corporate constituted under the laws of Switzerland, do hereby declare the invention for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to new antibiotics and to methods for their microbiological preparation, and for concentrating, isolating and purifying them.

According to this invention, new antibiotics are supplied by fermentation of certain species of the genus *Micromonospora* (order Actinomycetales), namely *Micromonospora purpurea* and *M. echinospora* (inclusive their mutants* and variants), until at least one antibiotically active product has formed, and by isolation from the so-obtained substrate of at least one of the antibiotically active fractions. The said fractions may be further separated, and the so-obtained antibiotics may be transformed into their usual derivatives such as their salts with acids their reaction products with bisulfite addition compounds of aldehydes and ketones, their N-acyl and O-acyl derivatives, and their Schiff's bases.

In particular, this invention relates to the basic antibiotics below designated as "gentamycin" and "the antibiotics coproduced with gentamycin" or "BA—3".

Cultures of the living organisms have been deposited and made a part of the stock culture collection of the United States Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois, from which they are available under their respective NRRL identifications.

THE PREFERRED MICROORGANISMS.

Of the microorganisms found to be useful for the preparation of gentamycin and its coproduced antibiotics, one has been designated

as *Micromonospora purpurea* n.sp., (hereinafter called *M. purpurea*), has been assigned the number NRRL 2953, and was originally isolated from a sample of park loam in Syracuse, New York.—*M. purpurea* is characterized by a fine mycelium averaging 0.5 μ in diameter; does not produce a true aerial mycelium; produces single spores which average 1.0 μ in diameter, at the ends of simple sporophores; is non-acid-fast; gram-positive; digests certain types of protein and starch; is aerobic, grows well between 22—37°C. but not at 46°C. or above. The colony form and orange color on most rich organic agar media are typical. On other agar media, low in nitrogen, the color of the colony is reddish purple to purple. While this reddish purple pigment is characteristic of the fresh soil isolate, it might be temporarily or permanently lost in strains obtained by repeated isolation and transfer.

Another species, essentially equivalent to *M. purpurea* in the production of antibiotics, has been designated as *Micromonospora echinospora* n. sp. (hereinafter called *M. echinospora*). This species was isolated from a mud sample in Jamesville, New York, and has been assigned the number NRRL 2985.—*M. echinospora*, when grown in a medium consisting of 0.05% yeast extract (Difco) ("Difco" is a registered trade mark), 1% dextrose and 0.1% calcium carbonate in distilled water and incubated on a rotary shaker at 28°C. for 30 days, is characterized by a long, branched, regular, non-septate mycelium approximately 0.5 μ in diameter. The growth is purple in color. Spores are abundantly produced singly

* The term "mutants" includes natural and artificial mutants, e.g. mutants produced from the described microorganisms by mutating agents such as high frequency radiation (X-ray, ultraviolet), actinophages and nitrogen mustards,

as terminal enlargements of the hyphal tip. Both spores and sporophores are characteristically clavate. The mature spore is somewhat spherical having a diameter of about 1.0—1.5 μ . By phase contrast microscopy ($\times 1000$) the spores appear rough-walled. Electronmicrographs indicate the roughness to be due to spines which regularly protrude beyond the spore surface, occasionally attaining a length of 0.2 μ . (In this broth medium under the aforementioned conditions, *M. purpurea* does not appear to produce spores.) *M. echinospora* digests certain types of protein and starch, is aerobic and also grows well in the range of 22—37°C. but not above about 50°C. When grown in a medium consisting of 0.3% beef extract, 0.5% tryptose, 0.1% dextrose, 2.4% soluble starch, 0.5% yeast extract, 0.1% calcium carbonate and 1.5% agar, all in tap water, shaking at 28°C. for 30 days, a purple diffusible pigment may be produced. This diffusible pigment characteristic may be temporarily lost in strains obtained by repeated isolation and transfer. (*M. purpurea* on the immediately aforedescribed medium generally does not produce such a diffusible pigment.)

For isolating the microorganisms, a portion of the soil sample is shaken in sterile distilled water, and after making suitable dilutions the suspension is plated on screen agar medium. The medium should desirably contain 1% glycerol, 0.2% sodium nitrate, 0.1% dipotassium hydrogen phosphate, 0.05% magnesium sulfate, 0.05% potassium chloride, 0.001% ferrous sulfate, and 1.5% agar; preferably it contains 0.5% tryptose, 2.0% soluble starch, 0.3% calcium propionate and 2.0% agar; all in distilled water.

The cultures are tested for antibiotic activity by first growing in the following medium for up to 60 days at 26°C.: 0.3% beef extract, 0.5% tryptose, 0.1% dextrose, 2.4% soluble starch, 0.5% yeast extract, 1.5% agar, all in tap water. The whole aqueous agar is then extracted with butanol and the butanol-water extract is concentrated. Sufficient antibiotic is extracted by the butanol-water mixture to provide a concentrate which, by disc test, inhibits the growth of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Mycobacterium smegmatis*. Antibiotic activity against the same organisms is observed after growth in submerged fermentation for 96 hours in an aqueous medium containing suitable nutrients, such as one containing 1% yeast extract, 1% cerelose ("Cerelose" is a registered trade mark) and 0.1% calcium carbonate or one containing the components described earlier in this paragraph.

Tables I to V compare the above-mentioned novel species with certain known species of Micromonospora. Where dashes appear, data are missing or have not been entered in the Tables.

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

In Table II is shown data relative to colonies incubated for 14 days at 26°C. on various media. The data of the known species, with which ours are compared, have been taken from Bergey's "Manual of Determinative Bacteriology", 7th Edition, 1957, published by The Williams and Wilkins Company, Baltimore, Maryland.

In Tables IIIA and IIIB, growth and colony color of our novel species in various media are compared with those exhibited by three presently available species of Micromonospora. The media employed are those commonly used for Streptomyces determinations. The color of the colony is identified by the two systems employed for the data in Table I.

Our microorganisms are capable of utilizing various carbon and nitrogen sources.

In Table IV, their carbon utilization is compared with that of certain known Micromonospora species by a visual estimate of growth on agar plates in media consisting of 0.5% yeast extract Difco ("Difco" is a registered trade mark) (a product of Difco Laboratories Inc., Detroit, Michigan), 1.5% agar and 1% of the respective test carbohydrate, all in distilled water.

In Table V, the nitrogen utilization of our microorganisms is compared with that of other Micromonospora species by visual estimate of growth on agar plates in a medium consisting of 1% glucose, 1.5% agar and the particular nitrogen source used (its concentration as indicated), all in distilled water. The color code used in Table V is in conformity with that set forth for Table I.

We have stated above that we envision as within our inventive concept the use of gentamycin and/or BA—3-producing mutants of our novel species for the production of antibiotics as well as the use of their gentamycin and/or BA—3-producing variants. Specific examples of such variants, which differ from the described species in a minor fashion such as a morphological or biochemical property, are two variants of *M. echinospora*, being designated as *M. echinospora* var. *ferruginea* and

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antibiotic

M. echinospora var. *pallida*. Cultures of them have also been deposited and made a part of the stock culture collection of the United States Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois, from which they are available under their respective NRRL identifications, namely NRRL 2995 and NRRL 2996, respectively. These variants were also originally isolated from mud samples near Jamesville, New York, and are taxonomically the same as *M. echinospora* but with the following differences:

M. echinospora var. *ferruginea* does not reduce nitrates; grows well on ribose (both *M. echinospora* and *M. echinospora* var. *pallida* grow poorly on this pentose); its colonies tend to be reddish or yellowish brown in color (occasionally turning reddish or purplish-brown on aging), maroon pigments are occasionally encountered in the mycelium. Sporulation is generally poor, however, on some media (for example, tomato paste-oatmeal agar plus 0.1% sodium carbonate) in old cultures, both spore and sporophores have been found possessing the characteristic roughened wall. In the latter medium and on Bennett's agar growth is good; the colony is plicate; and the color is red-mahogany—g6 ½ PI, deep reddish brown—41.

M. echinospora var. *pallida* fails to produce a dark purple mycelial pigmentation on agar media. Its colony color on most agar media varies in a range from pale orange to light ("milk coffee") tan. On Bennett's agar and tomato paste-oatmeal agar + 0.1% sodium carbonate, growth is good; the colony plicate. The colony color on the former medium is apricot—g4GA, light orange—52; in the latter medium the periphery is of the aforementioned color with the center turning black.

These variants are equivalent to the above described species for the purpose of producing antibiotics in general and gentamycin in particular.

FORMATION AND ISOLATION OF THE ANTIBIOTICS.

The novel microorganisms described above and the other microorganisms envisaged by the general concept of this invention, by the fermentation methods essentially as described below, produce antibiotic substances. After the fermentation (incubation), the antibiotic products are found in both the mycelium and the broth; following separation of the broth from the mycelium, we prefer to utilize the mycelium as the source of antibiotic products. Thereby, mixtures of antibiotics are initially obtained.

In particular, from paper chromatographic studies it appears that the antibiotic substances produced by *M. purpurea* and *M. echinospora* and equivalents thereof, comprise at least three components. To the main component has been assigned the name gentamycin. The other

components which have been isolated are referred to as BA—3 (Fraction A) and BA—3 (Fraction B), respectively.

In order to form the antibiotic substances, the microorganisms may be grown at a temperature of about 25°C. to 40°C. under submerged aerobic conditions in an aqueous nutrient medium containing an assimilable carbon and nitrogen source. Suitable carbon sources are carbohydrates such as starch, dextrin, certain sugars and the like. Suitable nitrogen sources are both organic and inorganic nitrogen, preferably the former, such as soybean meal, peptones and the like.

The fermentation is preferably carried out at a pH in the range of 6 to 8 suitably for a period of about 24 to 48 hours, although longer or shorter times may be employed. At about the end of this period, peak antibiotic production has been attained.

Since most of the activity resides in the mycelium, the latter may be filtered off and the filtrate may be discarded. The antibiotics produced (which in view of the quantitative conditions, may simply be called "crude gentamycin") may be separated from the mycelium by acid extraction with mineral acid at a pH of about 2. The acid extracts may be neutralized to a pH of about 6 and may be either passed through an ion exchange resin, preferably of the IRC—50 "Amberlite" type ("Amberlite" is a registered trade mark); or precipitated by formation of an insoluble salt, e.g. helianthate, Reineckate, dodecylbenzenesulfonate ("Santomerose"—S salt) ("Santomerose" is a registered trade mark). Examples of Amberlite type resins employed herein, both anionic and cationic exchange, are found in the "Handbook of Chemistry and Physics", 42nd Edition, Chemical Rubber Publishing Co., Cleveland, Ohio (1960).

In a typical application of the "resin technique", the afore-mentioned solution of the "crude gentamycin" (see above) at pH 6 is passed through the ion exchange resin and the crude gentamycin is eluted from the resin with dilute sulfuric acid. The eluate is made alkaline to a pH of about 10 with sodium hydroxide.

Upon the addition of acetone, inorganic salts are precipitated. The supernatant liquor is adjusted to pH 4.5 and concentrated in vacuo to give a concentrated solution of crude gentamycin sulfate. Methanol is added to this solution and crude gentamycin sulfate is precipitated.

In a typical application of the "insoluble salt method", the neutralized (pH 6) mycelial extract is treated with Santomerse S and the precipitated salts removed by filtration. The precipitate is washed with water and dissolved in methanol. The methanolic solution is filtered and then passed through an anionic exchange resin, of the Amberlite IRA—401 S type. The eluate is concentrated, adjusted to



pH 4.5 with sulfuric acid resulting in a concentrated solution essentially of gentamycin sulfate. The said sulfate is obtained by adding methanol to the solution and separating the precipitated salt by filtration. It may be further purified by reprecipitation as an insoluble salt followed by regeneration.

The crude gentamycin as isolated by the resin technique may either be used as it is, or may be further divided into gentamycin itself and its co-produced antibiotics. If only gentamycin is intended to be prepared, this is preferably done by fractional precipitation of its Santomerse S salt (sodium dodecylbenzene sulfonate).

If the co-produced antibiotic components BA—3 (Fractions A and B) are to be isolated as well, they are preferably separated from a concentrated methanolic solution of crude gentamycin by addition of acetone (fractionate precipitation). [Advantage is taken of the greater solubility of gentamycin in acetone-methanol, as compared with BA—3 (Fraction A) and BA—3 (Fraction B).] After washing with acetone, the precipitate is dried and consists essentially of a mixture of BA—3 (Fractions A and B).—An alternate method for isolating the said mixture from gentamycin is one which takes advantage of the greater solubility of the dodecylbenzene sulfonate salts of these two components, as compared with that of the respective salt of gentamycin. The dodecylbenzene sulfonate salt of gentamycin is precipitated by the addition of a solution of Santomerse S to a solution of crude gentamycin sulfate. After filtering and washing the filter cake (which may be worked up to yield purified gentamycin or a salt thereof), the filtrates are now combined and represent a solution of the dodecylbenzene sulfonate salts of BA—3 (Fraction A) and BA—3 (Fraction B), from which solution the said components may be isolated.

From the mixture obtained according to one of the preceding alternative methods, Fraction A and Fraction B can be separated from each other by paper chromatographic methods.

In the examples which follow are illustrated suitable methods for fermenting the micro-organisms preferably used, extracting the antibiotic substances from the mycelium, and resolving them into their major components. In some of the examples an assay value of the produced antibiotic is expressed in terms of units per milligram as an indication of activity. The assay is effected microbiologically by a standard disc-type agar diffusion assay technique [compare Donald C. Grove and William A. Randell "Assay Methods of Antibiotics—A Laboratory Manual", New York (1955)], using *Staphylococcus aureus* (ATCC 6538P) as test organism. A reference curve is prepared by plotting the dosage-response of the antibiotic diluted in phosphate buffer at pH8 in a medium consisting of

peptone	0.6%
pancreatic digest of casein	0.4%
yeast extract	0.3%
beef extract	0.15%
dextrose	0.15%
agar	1.5%
pH	6.6

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The used suspension of the assay organism (*Staphylococcus aureus*) is standardized to provide 20% transmission at 660 μ in a colorimeter. The potency of the sample is determined from the reference curve and expressed in terms of units per milligram; a unit being that amount of test substance which is required to produce a 18 mm. zone of inhibition with a 13.5 mm. disc.

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EXAMPLE 1.

Preparation of Gentamycin by Fermentation of *M. purpurea* in Laboratory Scale.

A. Germination stage:

Add a lyophilized culture of *M. purpurea* to a 300 ml. shake flask containing 100 ml. of the following sterile medium.

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Bacto-beef extract	3 g.
Tryptose	5 g.
Dextrose	1 g.
Soluble Starch	24 g.
Yeast extract	5 g.
Tap Water	1000 ml. *)

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Incubate the flask and its contents for 5 days at 37°C. on a rotary shaker (280 RPM, 5 cm. stroke).

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B. Fermentation stage:

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

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Yeast extract	10 g.
Dextrose	10 g.
Calcium carbonate	1 g.
Tap water	1000 ml.

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Incubate the flasks and their contents for 5 days at 26°C. on a rotary shaker. Pool the contents of the flasks. Acidify an aliquot of the whole broth to pH 2 with 6N sulfuric acid and centrifuge. Collect the supernatant broth and neutralize with 6N sodium hydroxide. The neutral broth aliquot is tested

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*) In this and similar composition data throughout this specification, the solvent (usually water) is sometimes indicated in terms of absolute quantities, sometimes it follows to the expression "q.s.". While these are indeed two different disclosures, they may, within the accuracy required for these compositions, be replaced with each other, as desired.

for activity against *Staphylococcus aureus* by agar diffusion technique. Broths obtained as described assay at 15—20 units/ml. of anti-biotic.

5 C. Isolation of Crude Gentamycin (as Sulfate) from Fermentation Substrate:

Adjust the pH of the whole broth from part B of this Example (about 2 liters) to 2 with 6 N. sulfuric acid. Stir and filter using a filter aid. Adjust the pH of the filtrate to 7.0 with 10 6 N. sodium hydroxide. Pass the neutral broth through a cationic exchange resin column (IRC—50 Amberlite, 1—10 g. resin/liter of broth). Discard the eluate. Elute the column 15 with sulfuric acid at a pH of 2.0. Neutralize the eluate with 6 N. sodium hydroxide to pH 7. Concentrate the solution in vacuo to about one-tenth volume. Adjust pH to 10.0 with 20 6 N. sodium hydroxide solution and add 4 volumes of acetone while stirring. Chill the mixture and filter. Adjust the filtrate to pH 5.0 with 6 N. sulfuric acid and concentrate in vacuo at room temperature until the activity of the concentrate approximates 25 20 000 units/ml. Add 10 volumes of methanol while stirring and filter the precipitated sulfate of gentamycin. Dry in vacuo. Total yield: about 100 mg., assaying at 343 units/mg. by agar diffusion against *Staphylococcus aureus*.

30 D. Purification of the Isolated Crude Gentamycin:

Dissolve 15 g. of the crude salt obtained as previously described (assaying at about 340—450 units/mg.) in 50 ml. of water. Pass 35 through an anionic exchange resin (Amberlite IRA—400, OH form) in a column of 38 mm. diameter and 61 cm. height. Wash column with 2 liters water. Concentrate the eluate to dryness in vacuo and dissolve the residue in 40 50 ml. of methanol. While stirring add the solution of 1 liter of acetone. Filter and wash the precipitate with acetone. Evaporate the filtrate to dryness and take up in 50 ml. of methanol. Add the solution to 500 ml. of ether 45 while stirring. Filter and wash the precipitate with ether. Evaporate the filtrate to a residue consisting of purified gentamycin, about 3 grams, assay: 961 units/mg.

EXAMPLE 2.

50 Preparation of BA—3 (Mixture of Fractions A and B) in Laboratory Scale.

Follow Example 1 (parts A to C). Following then part D of Example 1, the addition of acetone to the methanolic solution of the concentrated eluate causes precipitation of BA—3 55 (mixture of fractions A and B). The precipitate after washing with acetone and drying is sufficiently pure for use.

EXAMPLE 3.

Preparation of Gentamycin and BA—3 (Fractions A and B) by Fermentation of *M. echinospora* or its Variants in Laboratory Scale.

By replacing the lyophilized culture of *M. purpurea* used for Example 1 (Part A), with a corresponding culture of *M. echinospora* (or of one of the variants *M. echinospora* var. *ferruginea* and *M. echinospora* var. *pallida*), and following essentially the procedure of Examples 1 and/or 2, gentamycin and/or BA—3 60 65 70 (Fractions A and B), respectively, may likewise be obtained.

EXAMPLE 4.

Preparation of Gentamycin by Fermentation of *M. purpurea* in Technical Scale.

A. Germination stage:

Carry out the germination in the manner described in Example 1 (part A).

B. Inoculum Preparation Stage:

Transfer 25 ml. of the first inoculum obtained from the previous germination stage, to each of four 2-liter flasks, each containing 500 ml. of the sterile medium utilized for germination. Incubate the flasks and contents for 5 days at 28°C. on a rotary shaker (80 rotations/minute, 5 cm. stroke). Pool the contents of the flasks. Add 25 ml. of the so obtained second inoculum to each of twenty 2-liter flasks, each containing 500 ml. of the following medium:

Soybean meal	30 g.
Dextrose (cerelose)	40 g.
Calcium carbonate	1 g.
Tap water	1000 ml.

Incubate the flasks and their contents for 3—5 95 days at 28° C. on a rotary shaker (280 rotations per minute, 5 cm stroke). Pool and aseptically transfer the broth into a sterile inoculum flask having a side arm (total volume: about 10 liters).

C. Tank Fermentation Stage:

Aseptically transfer the 10 liters of inoculum to a 240 liter fermenter containing about 185 liters of the following sterile medium:

Bacto-beef extract	600 g.	105
Bacto-tryptose	1000 g.	
Dextrose (cerelose)	200 g.	
Soluble Starch	4800 g.	
Yeast extract	1000 g.	
Antifoamer on silicone basis*	100 ml.	110
Tap water	qu.s 200 liters	

* the trade product "Anti-foamer GE 60" of General Electric Co. was used, but other anti-foamers are also suitable.



Cap



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liters



Cap

Adjust the pH to 6.9 to 7.0 before sterilization. Aerobically ferment for 20 to 30 hours

(until the packed cell volume is about 10 to 15%) under the following conditions:

- 5 Temperature 37°C.
 Sterile air input about 150 liters/minute
 Pressure " 0.5 atm. above norm. atm. pressure
 Agitation 180 rotations per minute



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atm



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- 10 Aseptically transfer the contents of the fermenter to a 2500 liter fermentation tank containing about 1665 liters of a sterile medium having the following composition:

15	Yeast extract	17 kg.
	Dextrose	17 kg.
	Calcium carbonate	1.7 kg.
	Anti-foamer	400 ml.
	Water	qu.s. 1655 liters

Ferment at 35°C. while agitating at 120 rotations/minute and introducing air at about 0.5 ~~atm.~~ above normal atmospherical pressure in an amount of about 425 liters/minute, for 24 to 31 hours.

- 20 At the end of this period, the potency of the produced antibiotics reaches a peak which remains substantially constant. During the fermentation the pH remains substantially in the range of 6.6 to 7.4. The packed cell volume reaches a constant value of 2 to 2.5 ml. The potency of the produced antibiotic is approximately 25 units/ml.

D. Isolation of Crude Gentamycin (as Sulfate) from Tank Fermentation:

- 25 Add 25 kg. of filter-aid ("Celite") ("Celite" is a registered trade mark), to the about 1850 liters of fermentation broth from part C of this Example, and filter. Suspend the filter-aid and the adhering mycelium in about 370 liters water, adjust the pH to 2 with 6 N. sulfuric acid and stir for 15 minutes.
- 30 Filter and discard the mycelial cake. Adjust the pH of the filtrate to 7.0 by adding 4 N. sodium hydroxide. Charge the neutralized filtrate to a cationic exchange adsorption column which contains 18 to 20 liters of Amberlite IRC-50, in its sodium form. Discard the eluate, wash the resin with water and elute with 2 N. sulfuric acid, pooling the acid eluates. Continue elution until pH of eluate is about 0.5. Neutralize the pooled eluates to a pH of about 7.5 with 2 N. sodium hydroxide. Concentrate to about 3.7 liters in vacuo at a temperature below 45°C. Render the concentrate to pH 10.5 with 2 N. sodium hydroxide solution. Add 5 volumes of acetone, cool, filter the precipitated salts and wash them with acetone; then combine with filtrate and washings. Adjust the pH of the combined filtrate to 4.5 with 2 N. sulfuric acid, concentrate in vacuo below 45°C. to 100 ml.
- 55 Add one liter of methanol, filter and discard

filtrate. Dissolve the precipitate (crude gentamycin sulfate) in sufficient water (100 ml.) to make a 20% solution. Adjust pH to 10.5 with 2 N. sodium hydroxide and add 5 volumes of acetone. Cool, filter and discard the precipitated salts. Adjust pH of filtrate to 4.5 with 2 N. sulfuric acid, and concentrate in vacuo to 50 ml. Add 500 ml. of methanol, filter and wash precipitate with cold methanol. Dry the solid in vacuo over phosphorus pentoxide, to yield about 20 to 30 grams of crude gentamycin sulfate (activity: approximately 500 units/mg.), which consists substantially of the sulfate of gentamycin in admixture with the sulfates of BA-3 (Fraction A) and BA-3 (Fraction B).

E. Alternative Method for the Isolation of Crude Gentamycin (as Sulfate):

Filter the broth from part C of this Example and suspend the mycelium in 350 liters of water. While stirring, add 6 N. sulfuric acid to adjust pH to 2. Filter and wash mycelium with water. Combine both filtrate and washings. Adjust pH to 6.5 with 4 N. sodium hydroxide. Now add 2 kg. of diatomaceous earth filter-aid and 750 ml. of a 30% aqueous solution of Santomerse S (sodium dodecylbenzene - sulfonate). Stir vigorously for 15 minutes, filter and wash the filter cake with water. Partially air-dry the filter cake and then suspend it in 40 liters of methanol. Stir for 10 minutes and filter. Extract the cake again with 40 liters of methanol and combine the methanolic extracts. Pass the methanol solution (about 100 liters) through an anionic exchange adsorption column which contains 5.0 liters Amberlite 401 S in methanol (resin in hydroxy form). Wash column with 20 liters of methanol. Combine eluates, concentrate in vacuo to about 10 liters and filter. Adjust pH of filtrate to 7.0 with 2 N. sulfuric acid, concentrate to 400 ml. and filter. Adjust pH of filtrate to 4.5 with 2 N. sulfuric acid and add 5 to 10 volumes of methanol. Filter the precipitated crude gentamycin sulfate, wash with methanol and dry at 40-60°C. in vacuo. Yield: about 56 g. (assay: 625 units/mg.).

F. Purification of the isolated Crude Gentamycin Sulfate:

Dissolve 55 g. of crude gentamycin sulfate, as obtained by part D or E of this Example, in 10 liters of water, adjust the pH

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to 6.5 with 2 N. sodium hydroxide and add 300 g. of diatomaceous earth filter aid. While stirring add 250 ml. of a 30% aqueous solution of Santomerse S. Filter, wash the precipitate with water and air dry. Suspend the filter cake in 10 liters of methanol, stir and filter. Repeat the methanol extraction of the filter cake and combine the filtrates. Pass the filtrates through an anionic exchange column containing 5 liters of Amberlite IRA-401 S (OH form) in methanol, at a flow rate of about 500 ml./minute. Wash the column with 10 liters of methanol and combine the eluates. Concentrate the combined eluates in vacuo to 1 liter, add dilute sulfuric acid until precipitation is complete. (According to an advantageous modification, the sulfuric acid is added until a pH of 4.5 has been reached, then while stirring add 18 g. of activated pulverized charcoal, continue stirring for 15 minutes, filter, wash the carbon cake with water, combine filtrate and washings and add to 10 volumes of methanol.) Filter the thus formed gentamycin sulfate and dry. Yield: about 37 g.; assay: about 780 units/milligram.

G. Preparation of Gentamycin from the Gentamycin Sulfate:

Dissolve 10 g. of purified gentamycin sulfate (from part F of this Example) in 1500 ml. of water and pass the solution through an anionic exchange column containing 100 ml. of Amberlite IRA-400 (OH form). Concentrate the eluate and lyophilize to yield about 6.7 g. of gentamycin with an assay value of about 1120 units/milligram.

EXAMPLE 5.

Preparation of BA-3 (Mixture of Fractions A and B) in Technical Scale.

Follow Example 4 (parts A to C, then D or E). Following then part F of Example 4, the filtrate from the precipitate obtained by addition of the Santomerse S solution is combined with the washing water from the filter cake and passed through a column of a freshly regenerated anionic exchange resin (preferably Amberlite IRA-401 S, OH form). The eluate is concentrated to 150 ml. and added dropwise to 10 volumes of acetone while stirring whereupon salts are precipitated, which are filtered off, washed with acetone and discarded. The combined filtrate is concentrated so as to eliminate acetone, resulting in an aqueous solution of BA-3 (Fraction A) and BA-3 (Fraction B). The solution is adjusted to pH 4.0 with 2 N. sulfuric acid and filtered. There is added ten volumes of methanol and the resulting precipitate is filtered, washed with methanol, and dried. The dried amorphous material consists essentially of the sulfates of BA-3 (Fraction A) and BA-3 (Fraction B).

EXAMPLE 6.

Preparation of Gentamycin and BA-3 (Fractions A and B) by Fermentation of *M. Echinospora* or its Variants in Technical Scale.

By carrying out the germination referred to in part A of Example 4 with lyophilized culture of *M. echinospora* (or of one of the variants *M. echinospora* var. *ferruginea* and *M. echinospora* var. *pallida*) instead of *M. purpurea*, and following then essentially the procedure of Examples 4 and/or 5, gentamycin and/or BA-3 (Fractions A and B), respectively, may likewise be obtained.

EXAMPLE 7.

Preparation of Gentamycin Hydrochloride.

Dissolve 10 g. of purified gentamycin sulfate (from part F of Example 4) in 1500 ml. of water, absorb as described in part G of Example 4 and adjust the eluate to pH 4.5 with hydrochloric acid. Concentrate the solution to dryness in vacuo. Dissolve the residue in 125 ml. of methanol and add 750 ml. of acetone. Collect the precipitated gentamycin hydrochloride on a filter, wash with acetone and dry in vacuo at 40-60°C. Yield: about 8.9 grams; assay: about 820 units/milligram.

EXAMPLE 8.

Preparation of Gentamycin Helianthate.

Dissolve 6.4 g. of crude gentamycin sulfate (obtained according to either of parts D and E of Example 4) in 65 ml. of water. Add a hot solution (75°C.) of 32 g. of Methyl Orange in about 300 to 350 ml. of water. Stir and heat the mixture to 75°C. and filter hot. Wash the precipitated helianthate with water and dry in vacuo over phosphorus pentoxide. Recrystallize from hot aqueous methanol obtaining about 12.4 grams of reddish-brown needles, m.p.=265°C. (dec.).

Analysis: C 52.74%, H 6.43%, N 13.61%, O 19.42%, S 7.64%.

The helianthate so formed from crude gentamycin sulfate may contain minor quantities of the respective salts of the co-produced antibiotics BA-3 (Fraction A) and BA-3 (Fraction B). However, the preparation of the helianthate in the above manner does represent a purification, since upon conversion to the hydrochloride (by dissolving in 2 N. hydrochloric acid; filtering through a bed of charcoal; concentrating of the filtrate; adding of six volumes of acetone; and separating of the hydrochloride thus precipitated by filtration, and drying), a product with an assay value of approximately 760 units/milligram can be obtained.

EXAMPLE 9.

Preparation of Gentamycin Reineckate.

Dissolve 2.2 g. of crude gentamycin sulfate (from either of parts D and E of Example 4) in 22 ml. of water. Add 82 ml. of a saturated aqueous Reinecke Salt solution while stirring. Stir the mixture at room temperature for one hour and then allow to stand at about 5°C. overnight. Filter the precipitate, wash with ice water and dry in vacuo over phosphorus pentoxide. Recrystallize the Reineckate from hot aqueous methanol obtaining purple needles, m.p.=270°C. (dec.).

Analysis: C 24.50%, H 4.49%, N 22.10%, S 27.92%, Cr 9.52%.

The Reineckate salt so obtained may contain minor quantities of the corresponding salts of co-produced antibiotics but still may be utilized as a purification, since upon reconversion to the sulfate [by dissolving of the Reineckate in aqueous methanol; adsorbing on to a strongly basic anion exchange resin (Amberlite IRA-401 S); concentrating of the eluate; adjusting of the pH to 4.5 with dilute sulfuric acid; adding of five volumes of methanol; and filtering of the precipitated, purified gentamycin sulfate], a product with an assay value of about 700 units/milligram can be obtained.

EXAMPLE 10.

Preparation of the Sodium Salt of Gentamycin-Poly-N-Methylenesulfonic Acid.

Dissolve 16.4 g. of gentamycin sulfate (from part F of Example 4), in 108 ml. of water. Add 30 g. of sodium formaldehyde-bisulfite in 100-200 mg. portions. Stir for 30 minutes, add triethylamine until the pH rises to 6.8 and continue stirring for 18 hours. Add the reaction mixture to 5 volumes of methanol and cool to 5°C. Filter and wash the precipitate with methanol and then with ether. Air dry. Yield colorless amorphous powder.

A higher grade product is obtainable as follows: Dissolve 200 g. of gentamycin sulfate [obtained according to example 4 (F)] in 1.3 liters of water. Add 366 g. of sodium formaldehyde bisulfite in small portions over a period of 20 minutes. Adjust the pH to 6.7 with 135 ml. triethylamine and stir for 16 hours at room temperature. Add the solution to 7.5 liters of methanol while agitating. Filter the precipitate and wash with methanol. Combine filtrate and methanol washes and add to 9.8 liters of acetone while stirring. Filter the precipitate, wash with acetone and dry. Yield: 178 g. of an amorphous white powder; activity 580 units/milligram.

Analysis: C 26.44%, H 6.18%, N 5.54%, S 14.66%, Na 7.49%; Total ash content 22.2%.

PHYSICAL AND CHEMICAL PROPERTIES OF THE ANTIBIOTICS PREPARED FROM THE ABOVE PREFERRED MICROORGANISMS.

GENTAMYCIN, as produced above, is a white amorphous powder having the following physical and chemical characteristics:

I. Melting point (Kofler block): softening at 102°C.; complete melt at 108°C.

II. Analysis:

a) Elementary analysis:

C 50.20%, H 8.52%, N 13.47%, O 27.81% (by difference) no other elements are present.

b) Qualitative and quantitative group tests:

- | | | |
|---|----------|----|
| 1. Methoxy groups | none | |
| 2. N-methyl groups | 2.75% | |
| 3. C-methyl groups | 2.97% | |
| 4. Van Slyke nitrogen | 10.18% | |
| 5. Ninhydrin test | positive | 80 |
| 6. Elson-Morgan test (amino sugars) | positive | |
| 7. Maltol reaction test (no maltol formation after heating with alkali) | negative | 85 |
| 8. Furfural test (no volatile ultraviolet absorbing products formed after heating with 40% aqueous sulfuric acid at 100°C.) | negative | 90 |
| 9. Sakaguchi test (for guanidines) | negative | |

III. Molecular weight:

Calculated (in the assumption of one N-methyl group per molecule) 545;

Found (ebullioscopic in methanol) 543.

IV. Optical Rotation:

$[\alpha]_D^{25} = +146^\circ$ (1% in water) 100

V. Solubility:

Very soluble in water; soluble in polar media such as pyridine and dimethylformamide; as well as in acidic media with salt formation; very soluble in methanol; moderately soluble in ethanol; insoluble in ether, benzene and halogenated hydrocarbons. 105

VI. Ultraviolet Spectrum:

Completely transparent in the range of from 220-400 mμ.

VII. Infrared Spectrum:

Figure 1 of the accompanying drawings shows the spectrum in Nujol* whereby λ represents the wave length in μ, ν represents the frequency in cm⁻¹ and A means absorption. 115

*) Nujol is a trade mark for a mineral oil on hydrocarbon basis sufficiently pure for infrared spectroscopy, obtainable from Plough Inc., Memphis, Tennessee.



Stir

The absorption peaks (w=weak, m= strong, vs=very strong, sh=shoulder) are moderate, m→s=moderate to strong, s= located at the following wave lengths:

λ (μ)	Peak strength
3.00	m—s
3.10	sh
3.25	sh
3.42	s
4.30	w
6.35	m
6.85	s
7.25	m—s
7.50	sh (broad)
7.80	sh
8.77	w
9.06	m (broad)
9.52	s
9.78	s
10.45	m—s
11.58	w

VIII. Salt formation:

Gentamycin is a moderately strong base (The salts are obtained by concentration of which readily forms salts with any strong their aqueous solutions and precipitation with organic and inorganic acid. In general, the a water miscible organic solvent, preferably a mineral acid salts are completely water soluble. lower aliphatic alcohol or ketone.)

15 A. Hydrochloride:

Melting point 194—209°C. (dec.)
Optical Rotation $[\alpha]_D^{25} = +113^\circ$ (1% in water)

Elementary Analysis:

C 35.47%, H 7.27%, N 9.69%, O 22.46%, Cl 24.90%.

20 Solubility: Very soluble in water and methanol; slightly soluble in ethanol, insoluble in other common organic solvents.

Infrared spectrum (Nujol)—(See figure 2 of the drawings)

λ (μ)	Peak strength
3.00	m—s
3.45	s
4.27	sh
4.95	w
6.27	m—s
6.64	sh
6.85	s
7.27	s
7.58	w
7.95	sh
8.27	w
8.95	w (broad)
9.20	m (broad)
10.25	sh (broad)
10.98	w
11.49	w (broad)
13.90	m

B. Sulfate:

Melting point 218—237°C. (dec.)

Elementary Analysis:

C 31.22%, H 6.57%, N 8.46%, O 41.63%; SO₄ 31.22%.5 Optical Rotation: $[\alpha]_D^{25} = +102^\circ$ (1% in water)

Infrared Spectrum (in Nujol)—(See figure 3 of the drawings):

λ (μ)	Peak strength
2.95→3.25.....	s (broad)
3.45	vs
4.27	sh
4.80	w (broad)
6.14	m—s
6.55	m—s
6.86	s
7.26	s
7.45	sh
7.77	w
8.70→9.75.....	vs (broad)
10.27	m
11.38	w (broad)
13.88	m

C. Other Salts:

10 Gentamycin by the action of the appropriate acids (or of soluble salts thereof) in aqueous media is convertible into insoluble or slightly soluble acid addition salts. Where the acid anion is pharmaceutically acceptable, the salt formed with gentamycin becomes suitable for incorporation in an aqueous or oil suspension formulation. Such preparations, administered parenterally, provide depot effects with slow release of the antibiotic. In general, the fatty acids having eight or more carbon atoms provide such salts of gentamycin wherein the solubility has been reduced. Representative of such acids are lauric, stearic, palmitic, oleic and the like. Indeed, other acids behave in a similar fashion in providing salts with gentamycin wherein its hydrophylic properties are counteracted upon by a hydrophobic component provided by the acid anion. Other types of acids which may be employed are aryl substituted alkanolic acids, such as phenylbutyric acid; aromatic carboxylic acids, such as naphthalene-1-carboxylic acid; substituted sulfuric and sulfonic acids, such as lauryl-sulfuric and dodecyl-benzenesulfonic acid, respectively; further cholic acid, Fernholz acid, and related compounds such as tannic acid.

IX. Miscellaneous Properties:

40 1. No reaction with 1.8 N. methanolic hydrogen chloride at reflux after two hours; quantitative recovery of starting material.
2. Antibiotic activity of gentamycin completely destroyed by hydrolysis with 6N hydrochloric acid at 140°C. in 15 minutes. With

2N hydrochloric acid at 100°C., 7 hours reaction time necessary to completely destroy activity.

3. Stability: The activity of gentamycin is not significantly altered when an aqueous solution of the antibiotic is subjected to a temperature of 100°C. for 30 minutes throughout the pH range of from 2 to 12.

4. Other Derivatives: Gentamycin like other antibiotics having primary amino groups, is transformed into N-methylenesulfonic acid derivatives by the action of sodium formaldehyde bisulfite (preferably upon gentamycin sulfate). The extent of conversion of the primary amino groups of gentamycin to the N-methylenesulfonic acid derivative is governed by the amount of formaldehyde-sodium bisulfite addition compound used in the reaction. The pH of the medium from which the product is isolated determines whether the product is a methane sulfonic acid or a salt thereof. Other analogous sulfonate derivatives are prepared by the action upon gentamycin of the addition compounds of aliphatic or aromatic ketones or aldehydes with bisulfite. These derivatives exhibit altered therapeutic indices as compared with the parent antibiotic. The derivative of gentamycin wherein all the primary amino groups appear to have been converted to their respective N-methylenesulfonic acids is prepared as in Example 10. The properties of this derivative are as follows: Soluble in water, insoluble in methanol; $[\alpha]_D^{25} = +49.7$ (1% in water); infrared absorption bands at 2.90 μ (s), 6.08 μ (m—s), 6.50 μ (sh), 8.72 μ (vs) and 9.62 μ (s).

X. Paper chromatographic properties:

Gentamycin, as determined by paper chromatograms, is different from other basic antibiotics. In obtaining the chromatogram, an ascending system is employed. Table VI sets forth the comparative R_F values (those values connected by a hyphen indicate a streak while those unconnected indicate separate and discrete spots) in seven different systems.

The antibiotics produced by known Microspora species differ grossly from gentamycin in that they are not bases, they are not broad spectrum in their antibiotic activity, and they are not extractable with an IRC-50 type Amberlite resin as described in this case.

BA-3 (MIXTURE OF FRACTIONS A AND B), prepared as described above, exhibits the following physical and chemical properties:

I Analysis:

a) Qualitative elementary analysis:

C, H, N, O; no other elements present.

b) Qualitative group tests:

- | | |
|-------------------|----------|
| 1. Ninhydrin | positive |
| 2. Elson-Morgan | positive |
| 3. Maltol | negative |
| 4. Furfural | negative |
| 5. Sakaguchi | negative |
| 6. Methoxy groups | negative |

II. Solubility:

Similar to that of gentamycin.

III. Ultraviolet spectrum:

Transparent in the range from 220 to 400 m μ .

IV. Salt formation:

Sulfate: White amorphous powder; soluble in water, insoluble in ethanol, methanol, acetone and other common organic solvents.

V. Paper chromatography: Exhibits the same R_F values as gentamycin in systems A, B, C, D, F and G (see Table VI). In system E, a resolution is observed with BA-3 (Fraction A) exhibiting an R_F value of 0.15 and BA-3 (Fraction B) exhibiting an R_F value of 0.23, as compared with the R_F value of gentamycin in this system of 0.45.

BIOLOGICAL PROPERTIES OF THE ANTIBIOTICS OBTAINED FROM THE EXAMPLES.

GENTAMYCIN possesses a broad antibacterial and anti-rickettsial spectrum. It is a useful anti-infective agent capable of effectively inhibiting certain disease manifestations caused by *Staphylococcus aureus*, *Klebsiella pneumoniae* and other pathogenic organisms. It is also useful in treating mastitis in cattle.

It is particularly effective in combatting urinary tract infections caused by gram-negative organisms such as species of *Proteus* and

Pseudomonas. It is known that the number of hospital infections due to gram-negative organisms is increasing. Gentamycin appears particularly useful in treating such chronic gram-negative infections of the kidney and bladder even in refractory patients. The high antibiotic activity of gentamycin against *Proteus* and *Pseudomonas* infections easily permits clinical utilization. As a particular example of such effect, gentamycin has been successfully employed in treating a chronic *Pseudomonas* kidney infection at a dose of 1mg./kg., administered three times daily. This infection which did not respond well to other therapy was completely eliminated from the urine after one day of treatment and did not recur even after 10 days. Similar responses have been elicited against several *Proteus* infections. It thus appears that gentamycin possesses an advantageous combination of unique types of activity in conjunction with low toxicity permitting its use in preference to other antibiotics.

The antibiotic activity of gentamycin is 1120 units/mg.; that of its hydrochloride is 820, and of its sulfate is 800 units/mg.

The comparative in vitro activity of gentamycin against a variety of gram-positive, gram-negative and acid-fast organisms is set forth in Table VII. The susceptibility of the microorganisms to the antibiotic was determined by the standard tube dilution method as follows: in each instance 10^{-3} dilutions of 24-hour broth cultures were employed as inoculum with the end points taken after the incubation for 24 hours at 37°C. Except where indicated otherwise, the growth medium consisted of a brain-heart infusion broth (Difco). In the table, the sulfates of the particular antibiotics were utilized in the tests against the microorganisms enumerated therein. The minimal inhibitory concentrations, however, are expressed in terms of the pure base antibiotic.

The in vivo activity against certain bacterial infections in mice has also been tested. The mice were infected with an inoculum of the particular bacteria administered by intraperitoneal injection. They were then treated by subcutaneous injections of gentamycin sulfate (724 units/mg.) dissolved in water, said injection administered in two equally divided daily dosages. The protecting dose for varying percentages of injected animals was determined as shown in Table VIII.

The acute toxicity of gentamycin in mice has been determined in the standard manner by a variety of routes. Utilizing the above gentamycin sulfate in mice weighing 18 to 20 grams, the following data was obtained:

Mode of Administration
subcutaneous
intraperitoneal
intravenous
oral

LD₅₀ (mg/kg. mouse)

675

550

75

> 10 000.

- Gentamycin in addition exhibits in vivo antiviral activity against *Rickettsia akari*. Groups of 6-day old embryonated eggs were each infected with 50 times the LD₅₀ of *Rickettsia akari*. A single dose of 3.0 mg. of gentamycin, administered 3 hours prior to infection, gave a 50% protection (PD₅₀). A 20% protection was obtained when treatment with gentamycin commenced 2 hours after infection.—In mice infected intracerebrally with approximately 450 times the LD₅₀ of *Rickettsia akari*, 100% protection was afforded by 5 mg. of gentamycin, administered subcutaneously 4 hours prior to infection and continued for 4 days at 5 mg. per day in two divided daily doses. When treatment with gentamycin was delayed 48 hours after intracerebral infection, 100% protection was still obtained by subcutaneous administration of 10 mg. per day (2 doses of 5 mg. each) for five days.

- Gentamycin and its salts exhibit a therapeutic effect when tested in mice against the H 37 RV strain of *Mycobacterium tuberculosis*.

- BA—3: Utilizing a solution of the mixture, the said solution assaying at 10 units/ml. (based upon the assay method applied for gentamycin), antibiotic activity is demonstrable against *Staphylococcus aureus*, *Streptococcus fecalis*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella schottmuelleri*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The mixture appears to be inactive against *Rickettsia akari* in mice at 10 mg./day. Its antibiotic activity (tested as for gentamycin) is about 200 units/milligram.

PHARMACEUTICAL APPLICATION FORMS OF THE ABOVE ANTIBIOTICS.

- The antibiotics according to the invention for administration to the patients are brought into such dosage forms as are commonly used for antibiotics. For the preparation of the said dosage forms usual pharmaceutical carriers may be used.

- Below are attached examples of such dosage forms containing gentamycin (in the form of its sulfate) as active ingredient. As is evident, the gentamycin sulfate may be replaced by other derivatives of gentamycin, by free gentamycin, by other antibiotics according to the invention (inclusive their usual derivatives), or by mixtures of such antibiotics and/or derivatives.

EXAMPLE A.

Injectable.

For the preparation of a suitable injectable solution, 50 g. gentamycin (in the form of its sulfate) are dissolved in sufficient injection water, and the solution obtained is filled up with injection water, so as to give 1.0 liter solution. Filter the solution through a bacteria-retentive filter and fill aseptically into sterile ampules. Autoclave the ampules at 121°C. for 30 minutes.

EXAMPLE B.

Topical Ointment.

An ointment may be prepared from the following ingredients:

Gentamycin (as sulfate)	5.0 g.	70
Methyl p-hydroxy-benzoate	0.2 g.	
Butyl p-hydroxy-benzoate	1.8 g.	
Mineral oil	100.0 g.	
Petrolatum	qu.s. 1 kg.	

For preparing the ointment, of the above ingredients, the petrolatum is melted and mixed at 70 to 90°C. with the methyl and butyl p-hydroxy-benzoate. After everything has dissolved, allow the solution to cool to approximately 40°C.—Separately disperse the gentamycin sulfate in the mineral oil and pass the dispersion through a colloid mill. Add the milled dispersion to the above ointment base and allow the mixture to cool spontaneously to room temperature under continuous agitation.—Fill the product into appropriate tubes or jars. The so-obtained ointment may also be used as ophthalmic ointment.

EXAMPLE C.

Ophthalmic Drops.

Ophthalmic drops containing gentamycin are obtained from the following ingredients:

Gentamycin (as sulfate)	5.0 g.	
Phenyl-ethyl alcohol	5.0 g.	
Purified water	qu.s. 1 liter	95

For preparing the drops, dissolve the phenyl-ethyl alcohol in approximately 90% of the water, then dissolve the gentamycin sulfate in the phenyl-ethyl alcohol solution and add sufficient water to make the product measure 1.0 liter. Filter the solution through a bacteria-retentive filter and fill aseptically into sterile dropper bottles.

TABLE I
Comparative Taxonomy of Micromonospora species
PART A: Medium contains 0.1% NZ Amine Type A, 1% dextrose, 1.5% Agar

Organism	Observations	
	Macroscopic	Microscopic
<i>M. purpurea</i>	No aerial mycelium; colony slightly raised, ridged, waxy; fair growth; no diffusible pigments produced.— Color: Surface: dark wine-g7PI, dark reddish brown-44; Reverse: wine-g7PG, dark red-16.	Mycelium long, branched, regular, non-septate, 0.5 μ in diameter; few sporophores and spores present. spores 1.0 μ in diameter.
<i>M. chalybea</i> ATCC 12452	No aerial mycelium; colony slightly raised, ridged, waxy; fair growth; no diffusible pigment produced.— Color: Surface: coffee-g3PN, dark olive brown-96; Reverse: coffee-g3PN, dark olive brown-96	Mycelium long, branched, regular, non-septate, very fine — less than 0.5 μ in diameter; spores abundant, spherical to ellipsoidal, brown, 1.0 μ in diameter, spores mostly free but a few are seen attached singly to long sporophores.
<i>M. fusca</i> NRRL B—943	No aerial mycelium; colony slightly raised, moist, granular to ridged; fair growth; very slight yellowish brown diffusible pigment produced.— Color: Surface: russet orange-g4PC, deep orange-51; Reverse: luggage tan-g4NE, strong brown-55.	Mycelium short, irregular, producing many polymorphic elements, 0.8—2.0 μ in diameter, spores 1.0 μ in diameter, difficult to find among numerous dark walled (olive brown) polymorphic forms.
<i>M. sp.</i> ATCC 10026	No aerial mycelium; colony slightly raised, dry, granular to ridged; no diffusible pigment produced.— Color: Surface: periphery coffee-g3PN, dark olive brown-96, center russet orange-g4PC, deep orange 51; Reverse: periphery coffee-g3PN, dark olive brown-96, center russet orange-g4PC, deep orange 51.	Mycelium long, branched, regular, non-septate, 0.5 μ in diameter; spores very abundant, in clusters and borne singly, 1.0 μ in diameter, some polymorphic forms present, both spores and polymorphic forms dark walled.

TABLE I (Continuation)

PART B: Medium contains 3% NZ-Amine Type A, 1% dextrose, 1.5% Agar

Organism	Observations	
	Macroscopic	Microscopic
<i>M. purpurea</i> NRRL 2953	No aerial mycelium; colony raised, convolute, waxy; abundant growth; no diffusible pigment formed.— Color: Surface: terra cotta-g5PE, brown-55, Reverse: russet-5PG, strong brown-55.	Mycelium long, branched, regular, non-septate, 0.5 μ in diameter; sporophores single, spores borne terminally, spherical to ellipsoidal, 1.0 μ in diameter.
<i>M. echinospora</i> NRRL 2985	No aerial mycelium; colony raised, crenate-convolute, waxy; good growth; slight amber diffusible pigment.— Color: Surface: deep reddish brown-g61/2PL, dark reddish brown-44; Reverse: russet-5PG, strong brown-55	Mycelium long, branched, regular, non-septate, 0.5 μ in diameter; no spores.
<i>M. chalybea</i> ATCC 12452	No aerial mycelium; colony raised, ridged, waxy, fair growth; no diffusible pigment. Color: Surface: copper brown-g5PI, strong brown-55; Reverse: russet brown-g4PI, strong brown-55.	Mycelium long, branched, regular, non-septate, 0.5 μ in diameter; spores abundant, borne singly and in clusters, spherical to ellipsoidal, 1.0 μ in diameter.
<i>M. fusca</i> NRRL B-943	No aerial mycelium; colony raised, rough granular, dull; good growth; very slight yellowish brown diffusible pigment. Color: Surface: terra cotta-g5PE, strong brown-55; Reverse: russet-g5PG, strong brown 55.	Mycelium irregular, many polymorphic elements present, 0.5–2.0 μ in diameter, olive brown in color; true spores, 1.0 μ in diameter, grading into polymorphic forms 0.5–2.0 μ in diameter.
<i>M. sp.</i> ATCC 10026	No aerial mycelium; colony raised, dense granular, dry; good growth.— Color: Surface: periphery russet orange-g4PC, strong orange-50, center: chocolate brown-g4PN, dark grayish brown-62; Reverse: dark spice brown g4PL, dark brown-59.	Mycelium of two types: one long and regular, the other short and irregular, producing many polymorphic elements, 0.8 to 2.0 μ in diameter; true spores abundant, borne in clusters, 1.0 μ in diameter, spherical to ellipsoidal.



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TABLE II

Medium Tested	<i>M. Purpurea</i> NRRL 2953	<i>M. chalybea</i>	<i>M. fusca</i>	<i>M. parva</i>	<i>M. globosa</i>	<i>M. coerulea</i>
Glucose-asparagine-agar	No aerial mycelium. Growth: fair. No soluble pigment. Color: bright peach-g51A, moderate reddish orange-37. No dark colored spore layer detected.	No aerial mycelium. Growth: vigorous. Soluble pigment. Color: orange, changing to deep brown, with grayish to brownish black spore layer.	Growth, vigorous. Soluble pigment. Color: orange, changing to deep brown, with grayish to brownish black spore layer.	Growth poor.	Growth poor.	Growth poor.
Gelatin	Weak liquefaction*	Weak liquefaction	Weak liquefaction	Liquefaction	Liquefaction	Rapid liquefaction
Milk	Slowly digested*	Peptonized; occasionally coagulated	Slowly digested; not coagulated	Unchanged	Coagulated and peptonized	May be coagulated; digestion very slight
Sucrose	Utilized*	Inverted	Inverted	Not inverted	Inverted	Inverted
Starch	Hydrolyzed*	Hydrolyzed	Hydrolyzed	Hydrolyzed	Hydrolyzed	Hydrolyzed
Cellulose	Attacked to slight extent*	Rapidly decomposed	Attacked to slight extent	Not decomposed	Not decomposed	Not decomposed
Nitrate Reduction	Variable*	Nitrites produced	Variable	No reduction	Nitrites produced	No reduction
Temperature	Good growth at 22°—37° C.; no growth at 47° C.	Optimum Growth between 30—35° C.	—	—	—	—
Aerobic or Anaerobic growth	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic

* the properties of *M. echinospora* are identical with regard to these media.

TABLE IIIA

Medium	<i>M. purpurea</i> NRRL 2953	<i>M. echinospora</i> NRRL 2985	<i>M. chalybea</i> ATCC 12452	<i>M. fusca</i> NRRL B-943	<i>M. sp.</i> ATCC 10026
Bennett's Agar	Growth: good. Color: terra cotta-m5PE, brownish orange-54.	Growth: good. Color: deep maroon-g71/2PL, dark grayish reddish brown-47.	Growth: good. Color: periphery terra cotta-g5PE, strong brown-55m, center: chocolate brown-g4PN, dark brown-59.	Growth: good. Color: terra-cotta-g5PE, strong brown-55.	Growth: good. Color: periphery terra cotta-g5PE, strong brown-55, center: clove brown 3-PL, dark yellowish-brown-78.
Emerson's Agar	Growth: good. Color: burnt orange-g5NC, strong reddish orange-35.	Growth: good. Color: tile red-g5NE, strong brown-55.	Growth: good. Color: bitter-sweet-g5PC, deep orange-51.	Growth: good. Color: tangerine-g5PA, vivid orange.	Growth: good. Color: oak brown-g4PL, strong-brown-55.
Tomato Paste-Oatmeal-Agar	Growth: good. Color: burnt orange-g5NC, strong reddish orange-35.	Growth: fair. Color: dusty orange-g4LC, moderate orange-53.	Growth: good. Color: periphery terra cotta-g5PE, strong brown-55, center: chocolate brown-4PN, dark brown-59	Growth: fair. Color: bright peach-g5IA, moderate reddish orange-37.	Growth: fair. Color: bright peach-g5IA, moderate reddish orange-37.
Glucose-Asparagine-Agar	Growth: fair. Color: bright peach-g5IA, moderate reddish orange-37.	Growth: poor.	Growth: good. Color: terra cotta-g5PE, strong brown-55.	Growth: fair. Color: sun orange-g5LA, strong orange-50.	Growth: poor.
Glucose-Yeast Extract-Agar	Growth: good. Color: deep brown-g5PL, dark brown-59.	Growth: good; colony crenate-granular. Color: maple-g4LE, light brown-57.	Growth: good. Color: deep brown-g4PL, dark brown-59.	Growth: good. Color: terra cotta-g5PE, strong brown-55.	Growth: good. Color: oak brown-g4PL, strong brown-55.

TABLE IIIB

Medium	<i>M. purpurea</i> * NRRL 2953	<i>M. chalybea</i> ATCC 12462	<i>M. fusca</i> NRRL B-943	<i>M. sp.</i> ATCC 10026
Potato Slice	Growth: poor.	Growth: good. Color: barn red-g6PG, moderate reddish brown-43.	Growth: good. Color: bitter-sweet-g5PC, deep orange-51.	Growth: good. Color: bitter-sweet-g5PC, deep orange-51.
Carrot Slice	Growth: poor.	Growth: good. Color: chocolate brown-g4PN, dark brown-59.	Growth: poor.	Growth: fair. Color: burnt orange-g5NC, strong reddish orange-35.
Sucrose-Nitrate Agar. (Czapek's Agar)	Growth: poor.	No growth.	No growth.	No growth.
Tyrosine Agar (Observations at 2, 7 and 14 days; after Gordon & Smith, J. Bact. 69: 147).	Growth: fair. Color: no diffusible pigment.	Growth: good. Color: black diffusible pigment.	Growth: fair. Color: no diffusible pigment.	Growth: poor. Color: no diffusible pigment.
Peptone-Iron Agar (Observations at 2, 7, and 14 days)	Growth: good. Color: no reaction.	Growth: good. Color: no reaction.	Growth: fair. Color: no reaction.	Growth: fair. Color: no reaction.
Bromocresol purple-milk.	Growth: good. Digestion. Color: no pH change.	Growth: good. Digestion. Color: no pH change.	Growth: fair. Digestion. Color: no pH change.	Growth: good. Digestion. Color: no pH change.

* data for *M. echinospora* are identical with these.



underline



identical

TABLE IV

Test Carbo- hydrate Used	<i>M. purpurea</i> NRRL 2953	<i>M. echinospora</i> NRRL 2985	<i>M. chalcea</i> ATCC 12452	<i>M. fusca</i> NRRL B-943	<i>M. sp.</i> ATCC 10026
Arabinose	good	good	good	good	good
Cellulose	poor	poor	poor	poor	poor
Glucose	good	good	good	good	good
Galactose	fair	fair	good	good	fair
Lactose	poor	poor	fair	poor	poor
Levulose	poor	poor	fair	poor	poor
Mannose	fair	fair	good	good	good
Raffinose	poor	poor	poor	poor	poor
Rhamnose	poor	good	poor	poor	poor
Starch	good	good	good	good	good
Sucrose	good	good	good	good	good
Xylose	good	good	good	poor	poor
Inositol	poor	poor	poor	poor	poor
Mannitol	poor	poor	poor	good	good
Sorbitol	poor	poor	poor	poor	poor
Control 0.5% yeast extract	poor	poor	poor	poor	poor

TABLE V

Nitrogen Source	<i>M. purpurea</i> * NRRL 2953	<i>M. chalybea</i> ATCC 12452	<i>M. fusca</i> NRRL B-943	<i>M. sp.</i> ATCC 10026
0.5% Difco Yeast Extract	Growth: good. Color: tile- red-g5NE, strong brown-55.	Growth: good. Color: barn red-g6PG, moderate reddish brown-43.	Growth: good. Color: russet orange-g4PC, strong orange-50. sweet-g5PC, deep orange-51.	Growth: good. Color: bitter
1.0% NZ Amine Type A	Growth: good. Color: burgundy g7½PL, dark grayish reddish brown-47.	Growth: good. Color: henna- g5PG, strong brown-55.	Growth: good. Color: henna- g5PG, strong brown-55.	Growth: good. Color: terra cotta-g5PE, strong brown-55.
1% Asparagine	Growth: fair. Color: bur- gundy-g7½PL, dark grayish reddish brown-47.	Growth: fair. Color: clove brown-g3PL, dark yellowish brown-78.	Growth: poor.	Growth: poor.
1% Glutamic Acid	Growth: fair. Color: bur- gundy-g7½PL, dark grayish reddish brown-47.	Growth: fair. Color: clove brown g3PL, dark yellowish brown-78.	Growth: poor.	Growth: poor.
1% Sodium Nitrate	Poor to no growth.	No growth.	No growth.	No growth.
1% Ammonium Nitrate.	Poor to no growth.	No growth.	No growth.	No growth.

* data for *M. echinospora* are identical with these.

TABLE VI
Comparison of R_F values of Gentamycin in various systems
with those of other basic antibiotics

Antibiotic	System* and R_F Value						
	A	B	C	D	E	F	G
Gentamycin	0.59	0.26	0.1	0.3	0.45	0.0—0.25	0.0—0.48
Neomycin	0.22	0.12	0.29	0.0	0.05 0.20	0.0—0.13	0.0—0.50
Kanamycin	0.30	0.18	0.25	0.0—0.35	0.15 0.25	0.02	0.76
Neamine	0.30	—	—	—	0.30	0.05	0.75
Streptomycin	0.57	0.40	0.21	0.06	0.03 0.19	0.0	0.0—0.33
Streptothricin	0.36	0.30	0.27	0.27	0.09	0.0	0.18
Paromomycin	0.33	0.11	0.38	0.0	0.03—0.29	0.03	0.25
Viomycin	0.27	0.19	0.51	—	0—0.25	0.0	0.43—0.65
Dihydro- streptomycin	0.53	0.36	0.18 0.45	—	0.03—0.3	0.0	0.22

* Systems:

- A. 80 methanol (in water) + 3% NaCl, using paper buffered to pH 2.3 with sulfate — bisulfate.
- B. Propanol-pyridine — acetic acid — water. (15:10:3:12).
- C. Propanol — water — acetic acid. (50:40:5).
- D. 80% Aqueous phenol.
- E. Water-saturated n-butanol containing 2% *p*-toluenesulfonic acid.
- F. Butanol — water (84:16) + 2% piperidine.
- G. Water — butanol (94:6) + 0.25% *p*-toluenesulfonic acid.

TABLE VII
Antibiotic Spectrum (In-vitro-Activity) of Gentamycin
as Compared with Kanamycin and Neomycin

Microorganisms Tested		Minimal Inhibiting Concentration ($\mu\text{g/ml.}$)		
Systematic Name	Strain Designation*	Gentamycin	Kanamycin	Neomycin
<u>Gram Positive</u>				
<i>Bacillus cereus</i> var. <i>mycoides</i>	DA 30	0.5	8.0	0.5
<i>Bacillus cereus</i> var. <i>mycoides</i>	DA 34	0.5	1.5	0.5
<i>Bacillus cereus</i> var. <i>mycoides</i>	ATCC 7064	0.75	3.0	2.0
<i>Bacillus megatherium</i>	DA 31	0.075	0.25	0.075
<i>Bacillus subtilis</i>	ATCC 6633	0.015	0.035	0.015
<i>Bacillus sphaericus</i>	DA 32	0.1 **	0.75	0.25
<i>Bacillus sphaericus</i>	DA 33	0.05 **	0.75	0.075
<i>Brucella abortus</i>	DA 70	0.25	0.75	0.5
<i>Staphylococcus aureus</i>	ATCC 6538P	0.075	0.75	0.1
<i>Staphylococcus aureus</i>	ATCC 12715	0.035	0.5	0.035
<i>Staphylococcus aureus</i>	ATCC 6538	0.175	0.75	0.375
<i>Staphylococcus aureus</i>	ATCC 9996	0.25	1.5	0.75
<i>Staphylococcus aureus</i>	ATCC 1163	0.175	0.75	0.375
<i>Staphylococcus aureus</i>	Gray	0.375	1.5	0.25
<i>Staphylococcus aureus</i>	Smith	0.25	0.75	0.375
<i>Staphylococcus aureus</i>	DA 40	0.25	1.5	0.25
<i>Staphylococcus epidermidis</i>	DA 41	0.375	0.25	0.25
<i>Sarcina lutea</i>	ATCC 9341A	0.25	0.75	0.25
<i>Sarcina lutea</i>	ATCC 9341	0.25	3.0	0.25
<i>Streptococcus pyogenes</i>	DA 21	6.0 ***	32.0	16.0
<i>Streptococcus fecalis</i>	DA 20	12.0	> 100.0	16.0
<i>Streptococcus fecalis</i>	ATCC 10541	12.0	> 100.0	48.0
<i>Micrococcus flavus</i>	DA 60	0.075	6.0	0.75
<i>Corynebacterium simplex</i>	DA 80	20.5	—	0.25

TABLE VII

Microorganisms Tested		Minimal Inhibiting Concentration ($\mu\text{g./ml.}$)		
Systematic Name	Strain Designation*	Gentamycin	Kanamycin	Neomycin
<u>Gram Negative</u>				
<i>Aerobacter aerogenes</i>	DA 90	0.75	94.0	24.0
<i>Aeromonas salmonicida</i>	DA 100	0.25	3.0	0.25
<i>Alcaligenes</i> sp.	ATCC 10153	0.025	0.5	0.75
<i>Alcaligenes fecalis</i>	ATCC 8750	1.5	3.0	1.5
<i>Escherichia coli</i>	DA 110	1.25	12.0	3.0
<i>Escherichia coli</i>	ATCC 10586	1.5	12.0	6.0
<i>Escherichia intermedia</i>	DA 111	0.375	1.5	1.5
<i>Klebsiella pneumoniae</i>	DA 1	0.35	0.75	0.375
<i>Proteus hydrophila</i>	DA 120	0.5	12.0	12.0
<i>Proteus vulgaris</i>	DA 121	6.0	16.0	12.0
<i>Pseudomonas aeruginosa</i>	ATCC 10197	0.1	0.25	0.25
<i>Pseudomonas aeruginosa</i>	ATCC 9027	0.25	12.0	1.5
<i>Pseudomonas aeruginosa</i>	ATCC 9721	0.25	6.0	1.0
<i>Pseudomonas aeruginosa</i>	ATCC 10145	0.25	12.0	3.0
<i>Pseudomonas aeruginosa</i>	ATCC 8709	0.25	12.0	1.5
<i>Salmonella schottmuelleri</i>	DA 10	1.5	12.0	1.5
<i>Salmonella typhimurium</i>	DA 11	6.0	12.0	12.0
<i>Salmonella typhimurium</i>	ATCC 9187	3.0	12.0	12.0
<u>Acid Fast</u>				
<i>Mycobacterium smegmatis</i>	DA 150	0.15†	—	—
<i>Mycobacterium tuberculosis</i>	H 37 RV	0.20††	—	—

* DA refers to Schering Corporation Collection No.

** 1% Yeast Extract, 1% Dextrose Medium

*** 5% Human Serum Added

† Yeast-beef broth (Difco)

†† Dubos — Medium + "tween 80" (= poly-oxyethylene-sorbitan monooleate)
("tween" is a registered trade mark)

TABLE VIII
In Vivo Activity of Gentamycin

Infecting Microorganism	Dosage gentamycin μg/mouse/day	Percentage Survival
<i>Klebsiella pneumoniae</i>	0 (Control saline solution)	0
	20	20
	30	50
	40	80
	50	100
<i>Staphylococcus aureus</i> (Gray)	40	40
	60	50
	80	100
<i>Salmonella schottmuelleri</i>	80	50

WHAT WE CLAIM IS:—

1. A process for the production of the antibiotic identified as gentamycin and/or the antibiotics identified as BA—3 (fractions A and B), which comprises incubating a micro-organism of the species *Micromonospora purpurea* or the species *Micromonospora echinospora*, or a gentamycin and/or BA—3 producing mutant or variant thereof, until at least one compound having substantial antibiotic activity is produced and isolating at least one of the antibiotic compounds thus produced.

2. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora purpurea* NRRL 2953.

3. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora echinospora* NRRL 2985.

4. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora echinospora* var. *ferruginea* NRRL 2995.

5. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora echinospora* var. *pallida* NRRL 2996.

6. A process according to any of claims 1 to 5, wherein the micro-organism is incubated under submerged aerobic conditions at a temperature from 25° to 40°C, and a pH from 6 to 8 in an aqueous nutrient medium containing assimilable carbon and nitrogen.

7. A process according to any of claims 1 to 6, wherein, after incubation, the mycelium of the culture is extracted with acid and the gentamycin and/or BA—3 recovered from the acid extract by adsorption or fractionate

salt precipitation or a combination of both methods.

8. A process according to any of claims 1 to 7, followed by the conversion of an antibiotic compound thus produced into a salt with an acid, into a reaction product with a bisulphite addition compound of a ketone or an aldehyde particularly formaldehyde, into an N-acyl or O-acyl derivative or into a Schiff's base.

9. A process according to any of claims 1 to 8, substantially as hereinbefore described and exemplified in Examples 1 to 10.

10. An antibiotic compound or a derivative thereof, obtainable by a process according to any of claims 1 to 9.

11. The antibiotic identified as gentamycin, a basic organic substance, having a melting point of 102° to 108°C. with decomposition; a specific optical rotation $[\alpha]_D^{25} = +146^\circ$ (1% in water); an elementary analysis, C=50.40%, H=8.52%, N=13.47%, O=27.81%, with no other elements present and a molecular weight of 543 when determined ebullioscopically in methanol; being very soluble in water, aqueous acid media, and methanol, and substantially insoluble in ether, benzene and halogenated hydrocarbons; having an infra-red adsorption spectrum when suspended in hydrocarbon oil substantially as shown in Figure 1 herein; being transparent to ultra-violet radiation in the range 220 to 400 mμ; and having an in-vitro antibacterial spectrum including the bacteria enumerated in Table VII herein.

TABLE VIII
In Vivo Activity of Gentamycin

Infecting Microorganism	Dosage gentamycin μg/mouse/day	Percentage Survival
Klebsiella pneumoniae	0 (Control saline solution)	0
	20	20
	30	50
	40	80
	50	100
Staphylococcus aureus (Gray)	40	40
	60	50
	80	100
Salmonella schottmuelleri	80	50

WHAT WE CLAIM IS:—

1. A process for the production of the antibiotic identified as gentamycin and/or the antibiotics identified as BA—3 (fractions A and B), which comprises incubating a micro-organism of the species *Micromonospora purpurea* or the species *Micromonospora echinospora*, or a gentamycin and/or BA—3 producing mutant or variant thereof, until at least one compound having substantial antibiotic activity is produced and isolating at least one of the antibiotic compounds thus produced.
2. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora purpurea* NRRL 2953.
3. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora echinospora* NRRL 2985.
4. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora echinospora* var. *ferruginea* NRRL 2995.
5. A process according to claim 1, wherein the incubated micro-organism is *Micromonospora echinospora* var. *pallida* NRRL 2996.
6. A process according to any of claims 1 to 5, wherein the micro-organism is incubated under submerged aerobic conditions at a temperature from 25° to 40°C, and a pH from 6 to 8 in an aqueous nutrient medium containing assimilable carbon and nitrogen.
7. A process according to any of claims 1 to 6, wherein, after incubation, the mycelium of the culture is extracted with acid and the gentamycin and/or BA—3 recovered from the acid extract by adsorption or fractionate

salt precipitation or a combination of both methods.

8. A process according to any of claims 1 to 7, followed by the conversion of an antibiotic compound thus produced into a salt with an acid, into a reaction product with a bisulphite addition compound of a ketone or an aldehyde particularly formaldehyde, into an N-acyl or O-acyl derivative or into a Schiff's base.

9. A process according to any of claims 1 to 8, substantially as hereinbefore described and exemplified in Examples 1 to 10.

10. An antibiotic compound or a derivative thereof, obtainable by a process according to any of claims 1 to 9.

11. The antibiotic identified as gentamycin, a basic organic substance, having a melting point of 102° to 108°C. with decomposition; a specific optical rotation $[\alpha]_D^{25} = +146^\circ$ (1% in water); an elementary analysis, C=50.40%, H=8.52%, N=13.47%, O=27.81%, with no other elements present and a molecular weight of 543 when determined ebullioscopically in methanol; being very soluble in water, aqueous acid media, and methanol, and substantially insoluble in ether, benzene and halogenated hydrocarbons; having an infra-red adsorption spectrum when suspended in hydrocarbon oil substantially as shown in Figure 1 herein; being transparent to ultra-violet radiation in the range 220 to 400 mμ; and having an in-vitro antibacterial spectrum including the bacteria enumerated in Table VII herein.



12. Salts of gentamycin with acids, reaction products of gentamycin with bisulphite addition compounds of aldehydes and ketones, N- and O-acyl derivatives of gentamycin and Schiff's bases derived from gentamycin. 5
13. Gentamycin sulphate.
14. Gentamycin hydrochloride.
15. Gentamycin helianthate.
16. Gentamycin reineckate.
- 10 17. Sodium salt of gentamycin-poly-N-methylene sulphonie acid.
18. The antibiotics identified as BA—3 (fractions A and B), as identified herein.
- 15 19. Salts of BA—3 (fractions A and B) with acids, including the sulphate, and reaction products of BA—3 (fractions A and B) with bisulphite addition compounds of aldehydes and ketones.
20. A pharmaceutical composition comprising a compound claimed in any of claims 10 to 19 in admixture with a pharmaceutically acceptable carrier. 20
21. A pharmaceutical composition according to claim 20 comprising gentamycin and/or gentamycin sulphate in admixture with pharmaceutically acceptable carrier. 25
22. A pharmaceutical composition according to claim 20 or 21 substantially as hereinbefore described with reference to Examples A to C. 30

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Leamington Spa: Printed for Her Majesty's Stationery Office, by the Courier Press
(Leamington) Ltd.—1966. Published by The Patent Office, 25 Southampton Buildings,
London, W.C.2, from which copies may be obtained.



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