

PATENT SPECIFICATION

(11) 1302 142

DRAWINGS ATTACHED

- (21) Application No. 20147/71 (22) Filed 19 April 1971
 (31) Convention Application No. 4916 (32) Filed 22 Jan. 1970 in
 (33) United States of America (US)
 (44) Complete Specification published 4 Jan. 1973
 (51) International Classification C12D 9/14 // A61K 21/00
 (52) Index at acceptance
 C2A 1B 1C1A 1C1B 1C2A 1C2B 1C2C 2A3
 ASE 1A3A 1A3F 1A3H 1A5A2 1C15B2 1C15F3 1C2A



(72) ANTIBIOTIC 67—694 AND METHODS FOR PRODUCTION THEREOF

(71) We, SCHERICO LTD., of Topferstrasse 5, 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 novel antibioticly active substances, to processes for their production, isolation and purification and to compositions containing such substances. More specifically, this invention relates to novel antibioticly active substances obtainable by the cultivation of a hitherto undescribed species of the genus *Micromonospora* of the order *Actinomycetales*.

In one of its aspects, the present invention provides a novel antibioticly active substance herein designated Antibiotic 67—694 or an antibioticly active substance which contains or is contained in the said Antibiotic 67—694, said substance being in free form or in the form of a pharmaceutically acceptable functional derivative.

The novel antibioticly active substance is obtainable by cultivation of a hitherto undescribed species of *Micromonospora* herein designated *Micromonospora rosaria*.

In another of its aspects, the present invention provides a process for the production of an antibioticly active substance designated Antibiotic 67—694 or an antibioticly active substance which contains the said Antibiotic 67—694, which comprises cultivating a micro-organism of the species *Micromonospora rosaria* in a nutrient medium under aerobic conditions until substantial antibiotic activity is imparted to the medium. The process of the present invention includes within its scope the use of variants and mutants, both natural and artificial, of *Micromonospora rosaria* which give rise to Antibiotic 67—694, for example, mutants produced from the specified micro-organism by mutating agents such as high frequency radiation (including X-ray and ultra-

violet radiation), actinophages and nitrogen mustards.

The preferred micro-organism employed in the process of the present invention is an Antibiotic 67—694 producing species of the genus *Micromonospora* which species has been designated *Micromonospora rosaria* (sometimes hereinafter referred to as *M. rosaria*). This species was isolated from a soil sample taken from a park (Little Thicket) in San Jacinto County, Texas. One of its characteristics is its ability to produce the Antibiotic 67—694. A culture of the living organism has 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, where it has been assigned accession number NRRL 3718. Sub-cultures of *Micromonospora rosaria* NRRL 3718 are available to the public upon request to the aforementioned agency.

M. rosaria is aerobic and grows well on a variety of solid and liquid nutrient media. It grows especially well under submerged aerobic conditions.

M. rosaria may be distinguished from the other species of *Micromonospora* by a variety of taxonomical parameters. Thus, macroscopic examination of a 14 day culture at 24—26° C. of *M. rosaria* (NRRL 3718) on an agar medium comprising 3% NZ Amine Type A (a peptone resulting from the enzymatic digestion of casein which serves as a source of nitrogen and is available from the Sheffield Chemical Company, Norwich, New York), 1% dextrose and 1.5% agar reveals that growth is fair with no apparent aerial mycellium, colonies are slightly raised and are granular to weakly folded. No diffusible pigment is observed.

The color of the colony surface, described according to the "Descriptive Color Name Dictionary", Taylor, Knoche and Granville, published by the Container Corporation of America, 1950, U.S.A., together with the color

- hip number according to the "Color Harmony Manual", 4th Edition, 1958, published by the same company, is chest-nut brown g4ni. This color corresponds to the synonym, or near synonym, moderate brown 58, described according to the National Bureau of Standards Circular No. 553, November 1, 1955, U.S.A. (The color designations hereinafter specified are designated in accordance with the same two color reference systems). It will be appreciated that, as with other micro-organisms, the color of the colony surface and formation of pigment as herein described, although generally characteristic for the particularly micro-organism, may be temporarily or permanently changed by sub-culturing.
- Microscopic examination of the culture from the aforementioned medium shows that the mycelium is branched, has an average length of 10 to 20 μ and a diameter of approximately 0.6 μ . Numerous chlamydospores are produced, the spores being up to 2.0 μ in diameter. Conidia are not observed when the above agar medium is employed.
- M. rosaria* grows well at 27—37° C. but no growth has been observed at 50° C. A growing colony of *M. rosaria* will hydrolyse gelatin, milk and starch but does not reduce nitrate to nitrite. The test procedures employed in determining the foregoing characteristics are those described by Gordon et al in J. Bacteriology 69, 147 (1956) and 73, 15 (1957).
- Further growth characteristics of *Micromonospora rosaria* on specified media are set forth in Table I below. Unless otherwise stated, the reported growth characteristics were observed for cultures grown for 14 days at 24—26° C.

TABLE I

Medium	Growth Characteristics
Bennett's Agar	Growth: Good, plicate; wine red diffusible pigment produced. Color: Dark brown-g5pn; dark brown-59 to black.
Emerson's Agar	Growth: Fair, flat to furrowed; wine red diffusible pigment produced. Color: Dark brown mahogany-M6pn; brownish gray-64 to black.
Tomato Paste — Oatmeal Agar	Growth: Fair, plicate; no diffusible pigment produced. Color: Dark brown-g4pn; dark brown-59
Glucose-Asparagine Agar	Growth: Poor to no growth
Glucose-Yeast Extract Agar	Growth: Good, plicate, wine red diffusible pigment produced.
Czapek's Agar (Glucose)	Growth: Fair, granular; faint reddish brown, diffusible pigment produced. Color: Deep brown-g4pl; dark brown-59
Calcium Malate Agar	Growth: Poor, non-descriptive.
Ordinary Agar (Water Agar)	Growth: Poor, non-descriptive.

TABLE I (Continued)

Medium	Growth Characteristics
Nutrient Agar	Growth: Fair, slightly raised above the agar surface; reddish brown diffusible pigment produced. Color: Brick red-g6NG; moderate reddish brown-43.
Löffler's Serum Agar (Difco*)	Growth: Good, substrate mostly decomposed and cleared. Color: Dark luggage tan-g4PG; strong brown-55.
Potato Plug	Growth: Poor, non-descriptive.
Peptone-Glucose Agar	Growth: Fair to good: reddish brown diffusible pigment produced. Color: Chestnut brown-g4NI; moderate brown-58
Egg Agar (Dorset Egg Medium — Difco*)	Growth: Fair, no decomposition or clearing of substrate: diffusible lavender-colored pigment produced. Color: Colonies of two colors:— 1) Dusty orange-g4LC moderate orange-53, and 2) Deep eggplant-g10PL; very dark reddish purple-243 Diffusible pigment: Dusty orchid-g10GE; grayfish purple-228
Gelatin Medium	Hydrolysis: Positive
Starch Agar	Growth: Good, wine red diffusible pigment produced. Color: Brick red-g6PN: moderate reddish brown-43. Hydrolysis: Positive
Tyrosine Agar	Growth: Poor; no reaction.
Litmus Milk (Difco*)	Coagulated and partially peptonised with an acid reaction.
Cellulose Medium	Cellulose decomposed very slowly, requiring 3 to 6 months or longer. Pale rose pink diffusible pigment produced in the medium.

* "Difco" is a registered trade mark (Difco Laboratories Inc., Detroit Michigan).

Micromonospora rosaria is capable of utilizing a variety of carbon and nitrogen sources.

Table II below, sets forth observations on carbohydrate utilization in terms of observed growth characteristics of *M. rosaria* on a

medium containing the specified carbohydrate. In each case, the medium consisted of 1% of the specified carbohydrate, 0.5% yeast extract and 1.5% agar, remainder distilled water.

10

TABLE II
Carbohydrate Utilization

Carbohydrate	Growth Characteristics	
D-Arabinose	++	black, flat, dark red diffusible pigment produced.
L-Arabinose	++	black, flat, dark red diffusible pigment produced.
D-Glucose	+++	furrowed, dark brown, wine red diffusible pigment produced.
D-Galactose	+	flat, brown, wine red diffusible pigment produced.
β -Lactose	+	brown, faint reddish diffusible pigment produced.
D-Levulose	++	flat to granular, dark brown, wine red diffusible pigment produced.
D-Ribose	++	dark, brown, flat, wine red diffusible pigment produced.
α -Melibiose	$\pm$	
D-Melizitose	$\pm$	faint pinkish diffusible pigment produced.
L-Rhamnose	++	dark brown, flat, dark red diffusible pigment produced.
Starch	++	brown, no diffusible pigment produced.
Sucrose	+++	black, granular, dark wine red diffusible pigment produced.
D-Xylose	++	brown, granular, wine red diffusible pigment produced.

TABLE II (Continued)

Carbohydrate	Growth Characteristics
D-Mannitol	++ dark brown, flat, dark red diffusible pigment produced.
Growth on media containing, as the sole carbohydrate source, glycerol, raffinose, inositol, dulcitol or salicin is erratic and generally poor.	
Control 0.5% yeast extract	±

Key to Table II

+++ = very good growth
 ++ = good growth
 + = fair growth
 ± = poor growth
 — = very poor growth

Table III below, sets forth observations on In each case, the medium consisted of the nitrogen utilization in terms of observed stated amount of the nitrogen source, 1% glutamine containing the specified nitrogen source.

TABLE III

Nitrogen Utilization

Nitrogen Source	Growth Characteristics
0.5% Yeast Extract (Difco*)	Growth good; wine red diffusible pigment produced.
1.0% NZ Amine Type A	Growth good; no diffusible pigment produced.
1% Asparagine	Growth poor; faint pinkish diffusible pigment produced.
1% Glutamic Acid	Growth poor; pinkish diffusible pigment produced.
1% Sodium Nitrate	Growth poor; faint pinkish diffusible pigment produced.
1% Ammonium Nitrate	Growth poor; no diffusible pigment produced.

* "Difco" is a registered trade mark (Difco Laboratories Inc., Detroit, Michigan).

10 THE ANTIBIOTIC

(1)—Production of the Antibiotic

15 Cultivation of *M. rosaria* in a nutrient medium under aerobic conditions gives rise to the antibiotically active substance herein designated Antibiotic 67—694. In addition, the co-production of other antibiotically active

substances on cultivation of *M. rosaria* has been observed. For convenience, however, the production of an antibiotically active product is hereinafter described primarily with reference to Antibiotic 67—694.

20 Production of Antibiotic 67—694 is suitably effected by cultivating the micro-organism

M. rosaria in an aqueous nutrient medium under aerobic, preferably submerged aerobic conditions. For the production of small amounts (such as microgram to milligram quantities) of Antibiotic 67—694, surface culture in bottles or shake flasks may be conveniently employed. For larger scale production, submerged culture of the micro-organism in shake flasks, or, in particular, by tank fermentation will generally be more appropriate.

The nutrient medium (sometime hereinafter referred to as the fermentation medium) employed in producing Antibiotic 67—694 is typically a liquid containing an assimilable carbon source, such as a carbohydrate, and an assimilable nitrogen source, such as a proteinaceous material. For promoting good growth of the microorganism, and hence good antibiotic production, a combination of a carbon source and a nitrogen source upon which the micro-organism grows well, will be employed. Accordingly, preferred carbon sources include glucose, mannitol, levulose, sucrose, starch and ribose, and the preferred nitrogen sources include corn steep liquor, yeast extract, soya bean meal, meat peptones, casein hydrolysate and beef extract. Other carbon and nitrogen sources of like suitability may similarly be employed, the suitability of a particular source being readily ascertained by observation of the growth characteristics of *M. rosaria* with that source.

The media A, B and C given below are specific examples of media which may be employed in the production of Antibiotic 67—694:

Medium A

	Beef Extract	3 g.
	Tryptose	5 g.
40	Yeast extract	5 g.
	Dextrose	1 g.
	Potato starch	24 g.
	Calcium carbonate	2 g.
	Tap water	1000 ml.

Medium B

45	Yeast extract	5 g.
	Corn steep solids	1 g.
	Starch	30 g.
	Calcium carbonate	1 g.
50	Tap water	1000 ml.

Medium C

	Yeast extract	7 g.
	Fish solubles	1 g.
	Corn steep liquor solids	1 g.
55	Potato starch	30 g.
	Tap water	1000 ml.

Production of Antibiotic 67—694 is effected at temperatures conducive to satisfactory growth of the microorganism. Suitably, the temperature employed lies within the range

of from 20° C. to 40° C. and preferably within the range of from 27° to 37° C. During growth of the micro-organism and attendant fermentation, the pH of the medium should lie within the range of from 6.0 to 8.5, preferably within the range of from 7.0 to 8.0. The fermentation medium may be adjusted to the required pH prior to sterilisation and inoculation of the medium and then adjusted periodically during subsequent fermentation, as and when required. Alternatively, buffering agents such as calcium carbonate may be included in the fermentation medium to maintain the medium at the required pH, or to facilitate such maintenance. In general, a growth period of from 2 to 7 days will usually result in optimum production of the Antibiotic 67—694.

Production of Antibiotic 67—694, particularly on a technical scale as is exemplified by tank fermentation, is desirably carried out in a two stage process. In the first stage, a germination stage, a vegetative inoculum of *M. rosaria* is produced by inoculating a suitable medium with a slant culture or lyophilized culture of *M. rosaria* and then culturing the medium for a period of time sufficient to give a vegetative inoculum, this period usually being from 24 to 120 hours, with 72 to 96 hours being a preferred period. In the second stage, a fermentation stage, the vegetative inoculum obtained in the germination stage is introduced into the selected nutrient medium in an amount equivalent to about 5 per cent by volume, based on the volume of the nutrient medium to be employed in this fermentation stage.

The medium and conditions employed in the germination stage will, in general, be similar to those employed in the fermentation stage. Any medium and conditions conducive to good growth of the micro-organism can, however, be employed. Where, in the germination stage or the fermentation stage, undue foaming is experienced, this may be controlled by using a suitable anti-foam agent such as that sold under the trade designation Dow Corning B (The Dow Chemical Company, Midland, Michigan).

In the fermentation stage, growth of the micro-organism will usually be maintained for a period sufficient to give optimum or peak production of Antibiotic 67—694. Production of the antibiotic can readily be followed by, for example, periodically sampling the medium and assaying the samples. Peak or optimum production of the antibiotic will usually be reached within a period of from 24 to 100 hours.

As previously stated, cultivation of *M. rosaria* with attendant production of Antibiotic 67—694 is effected under aerobic conditions. Such conditions may be achieved in any convenient manner. Thus, for example, in tank fermentation where submerged growth of the microorganism is required, the necessary aerobic conditions may be conveniently

thin
ring
ant
lie
fer- 65
The
the
la-
dic- 70
and
ents
led
the
ate
period 75
opti-
t.
icu-
by
to a 80
ua-
ria
um
of
for 85
ta-
om
g a
ver-
ob- 90
ed
ant
ne,
um
the 95
lar
ge.
od
er, 100
age
is
ing
old
B 105
nd,
the
or
ak 110
ed
the
or
ill 115
om
M.
di-
on- 120
in
de,
th
ry
ily 125

achieved by passing air through the nutrient medium.

(2)—Isolation and purification of the Antibiotic

5 Isolation of the antibiotically active product from the fermented medium may be effected by extracting the medium under alkaline conditions, preferably at a pH of about 9.5, with a water-immiscible organic solvent. The required degree of alkalinity can be achieved by adding to the fermented medium any suitable base such as an alkali metal, or alkaline earth metal, hydroxide, preferably sodium hydroxide. Typical water-immiscible organic solvents 15 which may be used are benzene, toluene, *n*-butanol, methylene chloride, chloroform, ethyl acetate, amyl acetate and the like, with ethyl acetate being a preferred solvent.

The total volume of solvent employed will, of course, be dependent upon such factors as the relative solubility of the antibiotic, the amount of antibiotic which is required to be extracted and the manner of effecting the extraction, and this amount can readily be ascertained for any particular case. For the purpose of illustration, however, it may be mentioned that in a typical case where ethyl acetate is the solvent employed, a total volume equivalent to 4 to 10 times the volume of the fermented medium, used in successive portions equivalent to twice the volume of the fermented medium, has been found to give satisfactory separation.

The antibiotic product may be recovered from the organic extract by, for instance, the following method:—The organic solvent extract is concentrated, preferably *in vacuo*, to a convenient volume (suitably, about one fiftieth of the volume of the fermentation medium) and then extracted with an aqueous 0.1N sulfuric acid solution. In this manner a partial or substantial separation of antibiotic product from the plurality of minor components co-produced during fermentation is achieved. The effectiveness of this separation can be followed by determining the relative amount of co-produced minor components in the product using chromatographic procedures, and the extraction procedure repeated, if necessary, until a substantial separation is achieved. In repeating the extraction procedure, the initial sulfuric acid extract is rendered alkaline, the resulting mixture extracted with the organic water-immiscible solvent and then the organic extract in turn extracted with 0.1N sulfuric acid. Once the antibiotic product has been substantially separated from the co-produced minor components, the sulfuric acid extract is rendered alkaline, re-extracted with the organic water-immiscible solvent and the extract then concentrated *in vacuo* (suitably to a volume equivalent to about one thousandth of the volume of the original fermentation medium).

The concentrated extract is then added 65 under vigorous stirring to ten times its volume of a diethyl ether-hexane mixture consisting of, by volume, 5 parts diethyl ether and 4 parts of hexane. The precipitated material so-produced is filtered off, washed sparingly with a further portion of the diethyl ether-hexane mixture and discarded. The filtrate and washings are combined and evaporated to a residue. The residue is dissolved in an organic water-immiscible solvent, the solution washed several 70 times with water and then dried over a suitable drying agent such as anhydrous sodium sulphate. After filtration, the solution is evaporated to a residue, the residue dissolved in a minimal amount of diethyl ether, the solution filtered and then added, under stirring, to petroleum ether (boiling point 30°–60° C.). The precipitate obtained is Antibiotic 67–694 product and is usually off-white to buff in color. This precipitate is filtered off, washed 85 sparingly with a portion of petroleum ether and then dried, *in vacuo*, at approximately 40° C.

Using the above procedure we have obtained Antibiotic 67,694 products assaying, in the assay method hereinafter described, at a potency of about 700 µg/mg. 90

The assay method employed in the present specification for determining the relative potency of an antibiotically active product is a cylinder cup assay method using *Bacillus subtilis* ATCC 6633 as the test organism. 95

The method is substantially that described for erythromycin in "Assay Methods of Antibiotics"—Grove and Randall, Medical Encyclopedia Inc., New York (1955), pages 96–103, using a base layer of 21 ml. and a seed layer of 4 ml. of Antibiotic Medium No. 5. A standard curve is plotted using the following concentrations of antibiotic (µg/mg) in a 0.1M phosphate buffer (pH 8.0):—0.64, 0.8, 1.0, 1.25 and 1.56. A potency of 1000 µg/mg is assigned to that material for which one µg/mg in 1.0 ml. of 0.1M phosphate buffer gives a zone of inhibition of 17.8 ± 1.0 mm. 110 against the test organism *B. subtilis* ATCC 6633.

The crude Antibiotic 67–694 assaying at approximately 700 µg/mg may be further purified by, for instance, the following procedure. The Antibiotic 67–694 is dissolved in a mixture consisting of, by volume, 9 parts of a halogenated lower alkane, such as chloroform, and 1 part of an alcohol, such as methanol. The solution is passed through a column containing a solid adsorbent such as silicic acid, alumina, magnesium silicate, diatomaceous earth or cellulose and the like, and then the column is eluted with a mixture consisting of, by volume, 4 parts of a chlorinated lower alkane, such as chloroform, and 1 part of a lower alcohol, such as methanol. Fractions of the eluate are collected, and the presence of antibiotically active material in the frac- 125

tions detected by disc testing a sample from the fraction against *Staphylococcus aureus*. In this manner, the output from the column is monitored.

- 5 Those fractions containing antibiotically active material are further tested by chromatographing two samples per fraction on silica gel thin-layer plates using the solvent mixture employed in the column chromatographic separation. The plate on which the first sample is chromatographed is sprayed with a suitable spray such as a methanol-sulfuric acid mixture and then heated to reveal the presence and location of organic matter on the chromatogram (as a dark spot). The second sample and plate are used for bioautography against *Sar-*
 10 *cina lutea* to detect the presence and location (expressed herein as an R_f value) of antibiotically active material. In this latter test, using a chloroform-methanol mixture (4:1 by volume) and running the chromatogram for about 1 hour, Antibiotic 67—694 has an R_f value of from 0.4 to 0.5. Chromatography of crude Antibiotic 67—694 reveals the presence
 15 of at least two co-produced antibiotics. In the chloroform-methanol (4:1 by volume) system, two antibiotics having R_f values of 0.39 and 0.58 respectively have been observed.

- Fractions containing antibiotically active material of like R_f value are combined and evaporated *in vacuo* to a residue. The residue obtained from fractions containing material having an R_f of from 0.4 to 0.5 is dissolved in acetone and then added to petroleum ether.
 35 The precipitate so-obtained is removed by filtration or by centrifuging and discarded, while the filtrate or supernatant liquor is evaporated to dryness to give a solid residue which is Antibiotic 67—694 assaying, in the hereinbefore described assay method, at a potency of
 40 1000 $\mu\text{g}/\text{mg}$.

In an alternative procedure for isolating

and purifying Antibiotic 67—694, the initial extract obtained on extracting the fermented medium with the water-immiscible organic solvent is evaporated to give a residue which is taken up in a suitable solvent system and chromatographed. The fractions containing the Antibiotic 67—694 are then treated in the manner hereinbefore described to give the purified Antibiotic 67—694.

Crude Antibiotic 67—694 may also be purified by prepared a suitable derivative of the antibiotic, such as an ester or salt, isolating the derivative and, if required, further purifying the derivative. Where the free antibiotic is desired, this is then regenerated from the derivative. Examples of suitable salts are those formed with acids such as hydrochloric, sulfuric, phosphoric, acetic, succinic, and the like acids. Examples of suitable esters are acetates, butyrates, octanoates, benzoates, succinates and the like esters. Suitably, such esters may be prepared from the corresponding acid anhydrides or acid chlorides.

(3)—Chemical and physical properties of Antibiotic 67—694

For Antibiotic 67—694 having a potency of 1000 $\mu\text{g}/\text{mg}$ in the hereinbefore described assay method, the following chemical and physical characteristics have been observed.

I. Chromatographic constants— R_f values.

The observed R_f values for Antibiotic 67—694 in specified solvent systems and as determined against *S. lutea* are set forth in Table IV below. The Antibiotic 67—694 was chromatographed on thin layer (250 microns in thickness) silica gel (GF) plates available under the trade name "Uniplat" (Analtech Inc., Wilmington, Delaware) using agar seeded with *S. lutea*. In Table IV the quoted R_f values are expressed in terms of the inhibition zone.

TABLE IV

Solvent system (on volume basis)	R_f value of inhibition zone against <i>S. lutea</i>
Chloroform:methanol:17% ammonia 2:1:1	0.98
Butanol:acetic acid:water 3:1:1	0.37
Chloroform; ethanol 4:1	0.45
Chloroform:methanol 3:2	0.48

II. Specified chemical and physical data.

1. Elemental Analysis	Found	Calculated for $C_{31}H_{51}NO_9$
Carbon	63.18	64.00
Hydrogen	8.86	8.84
Nitrogen	2.29	2.41
Oxygen (by difference)	25.67	24.75
2. Empirical formula	: $C_{31}H_{51}NO_9$	
3. Molecular weight	: 581 (as determined by mass spectrometry)	
4. Optical rotation	: $[\alpha]_D^{25} = -33.4^\circ$ (0.3% in ethanol)	
5. Melting point	: 110—114°C	
6. pK_a	: 8.7	
7. Neutralisation equivalent	: 599	
8. Ultraviolet absorption spectrum in methanol:	: $E_{1cm}^{1\%} = 238$ (at 240 m μ)	

III. Infra-red absorption spectrum.

5 The recorded infra-red absorption spectrum of Antibiotic 67—694 as a mineral oil mull is reproduced in Figure 1 of the accompanying drawing in which λ represents the wavelength in microns, ν represents the frequency in cm^{-1} and T represents the percentage transmittance.

10 The major peaks and bands are set forth in Table V below in which the abbreviations have the following significance: S = strong, M = medium, W = weak and brd. = broad; the figures quoted are in microns. In Table V, the peaks attributable to mineral oil have been 15 omitted.

TABLE V.

2.87	(M)		
3.70	(W)	8.57	(S)
5.80	(S)	9.00	(S)
5.92	(M—S)	9.32	(S)
6.17	(M—S)	9.55	(S)
		9.73	(M)
		10.17	(M—S)
7.11	(W)	10.37	(M)
7.60	(M)	11.15	(W—M)
7.82	(M)	11.48	(W)
		11.87	(W—M)
7.98	(W)	11.95	(W)
8.44	(S)	12.62	(W)
		13.95	(W,brd.)

IV. Nuclear magnetic resonance spectrum.

The nuclear magnetic resonance spectrum of Antibiotic 67—694 is reproduced in Figure 2 of the accompanying drawing. The spectrum was obtained with a Varian A—60—A spectrometer (Varian Associates, California) for a solution of 40 mg of Antibiotic 67—694 dissolved in 0.4 ml of deuterated chloroform containing a small quantity of deuterium oxide. ("Varian" is a Registered Trade Mark). The spectrum is recorded, in terms of δ and τ values, in parts per million (PPM) from tetra-

methylsilane, the internal standard. In Figure 2, the insert at about 6.8 PPM in the partial spectrum (which appears above the full spectrum) would normally appear at about 9.8 PPM but would be outside the range of the recorded spectrum. In order to record the peak, the spectrometer was offset 180 Hertz (cycles per second) which is equivalent to about 3 PPM. In view of the intensity of the peak, it is considered to be material to an accurate interpretation of the spectrum.

V. Solubility.

Solvent	Solubility *
Water	Slightly soluble
Methanol	Very soluble
Acetone	Very soluble
Chloroform	Very soluble
Benzene	Very soluble
Diethyl ether	Sparingly soluble

* Terminology employed is that given in U.S. Pharmacopeia, 17th revision (1965), page 8.

VI. pH Stability.

No substantial change in potency was observed when Antibiotic 67—694 was dissolved in ethanol, diluted with an appropriate buffer and heated to a temperature of 100° C. for 30 minutes for a pH range of 2 to 10.

VII. Stability to enzymes.

Antibiotic 67—694 has been tested for

stability against trypsin, chymotrypsin, pepsin and α -amylase. Solutions of the enzymes were prepared at 1 mg./ml. in McIlvaine's buffers at the optimal pH for the enzyme (i.e. trypsin and chymotrypsin pH 8; pepsin pH 2.2; and α -amylase pH 4). Two hundred (200) μ g. of antibiotic was dissolved in 0.5 ml of water, mixed with 0.5 ml. of enzyme solution and in-

cubated at 37°C. for 24 hours. The solutions were disc assayed against *Escherichia coli* and *Staphylococcus aureus* after 0, 1, 2.5 and 24 hours incubation. The enzymes caused no substantial loss in potency of the antibiotic over the 24 hour interval.

VIII. Color Tests

Antibiotic 67—694 gives a positive color reaction in the Molisch Starch—KI and Elson Morgan tests; and a negative color reaction in the biuret, ninhydrin and Sakaguchi tests.

From the infra-red absorption spectrum (Figure 1), the nuclear magnetic resonance spectrum (Figure 2) and the hereinbefore quoted chemical and physical data, Antibiotic 67—694 appears to be of the macrolide class having one tertiary amino-group. Further, it has at least two esterifiable hydroxy groups, one of which may be esterified selectively. The nuclear magnetic resonance spectrum indicates the presence of an aldehyde group.

Thus the antibiotically active substance provided in accordance with the invention and which is designated Antibiotic 67—694 may be characterised as having, in its free form, an infra-red absorption spectrum in mineral oil substantially as shown in Figure 1 of the accompanying drawing and a nuclear magnetic resonance spectrum in deuterated chloroform substantially as shown in Figure 2 of the accompanying drawing.

Alternatively the antibiotically active substance provided in accordance with the invention and which is designated Antibiotic 67—694 may be characterised as having, in its free form, the following characteristics, namely an elemental analysis corresponding to the empirical formula of $C_{31}H_{51}NO_8$, a molecular weight as determined by mass spectrometry of 581, a melting point of 110—114°C., a specific optical rotation as measured by the D-line of sodium and at 25°C. in ethanol at 0.3 per cent concentration of -33.4° , an ultra-violet absorption maximum at 240 m μ

with an $E_{1\text{cm}}^{1\%}$ of 238, an infra-red absorption spectrum in mineral oil substantially as shown in Figure 1 of the accompanying drawing, a nuclear magnetic resonance spectrum in deuterated chloroform substantially as shown in Figure 2 of the accompanying drawing, and an

antibacterial spectrum substantially as shown in Table VI herein.

The present invention includes within its scope pharmaceutically acceptable (i.e. non-toxic) functional derivatives of Antibiotic 67—694 such as pharmaceutically acceptable salts and pharmaceutically acceptable esters. Typical salts are acid-addition salts derived from inorganic acids such as hydrochloric, sulfuric or phosphoric acid, or reactive derivatives thereof such as an acid salt, or organic acids such as acetic, succinic, citric, lactic, malic, malonic, maleic, tartaric, glutaric, lauric, adamantonic, cinnamic, camphoric, palmitic, stearic, undecylenic and the like acids. Such salts may be prepared by reacting Antibiotic 67—694 or an antibiotically active substance which contains or is contained in the said Antibiotic 67—694, or a reactive derivatives thereof, with the appropriate acid, or a reactive derivative thereof, and isolating the resulting salt. Typical pharmaceutically acceptable esters are those derived from hydrocarbon carboxylic acids such as acetic, propionic, butyric, valeric, hexanoic, octanoic, stearic, succinic, benzoic, phthalic, phenylacetic, phenoxyacetic, adamantonic and the like acids including isomers thereof (such as isobutyric acid), such esters being suitably obtained by esterifying the Antibiotic 67—694 or an antibiotically active substance which contains or is contained in the said Antibiotic 67—694, or a reactive derivative thereof, using the appropriate acid anhydride, acid chloride or other equivalently acting agent. The pharmaceutically acceptable esters may be in the form of monoesters or diesters which latter term includes mixed diesters.

(4)—Biological properties of Antibiotic 67—694

I. *In vitro* Activity.

The *in vitro* activity of Antibiotic 67—694 having a potency of 775 $\mu\text{g}/\text{mg}$ was ascertained by conventional tube dilution procedures using yeast beef broth medium at pH 7.4. The tubes were incubated for 18 hours at 37°C. prior to being observed. The results of the determination are set forth in Table VI below and demonstrate that the antibiotic exhibits a somewhat broad antibacterial spectrum *in vitro* with a greater effect upon gram-positive bacteria.

TABLE VI

Organism	No. Strains	Minimal Inhibitory Concentration ($\mu\text{g/ml}$) Range*
<i>Staphylococcus aureus</i>	6	0.03—3.0
<i>Streptococcus pyogenes</i>	7	0.75—3.0
<i>Enterococcus sp.</i>	2	0.03—0.08
<i>Diplococcus pneumoniae</i>	3	0.03—0.08
<i>Escherichia coli</i>	3	3.0
<i>Klebsiella pneumoniae</i>	4	0.3—3.0
<i>Aerobacter aerogenes</i>	3	0.75—7.5
<i>Proteus sp.</i>	3	0.3
<i>Pseudomonas aeruginosa</i>	4	0.75—3.0
<i>Salmonella schottmuelleri</i>	3	0.3—3.0

* Antibiotic 67-694 of potency 775 $\mu\text{g/mg}$ employed, and results corrected to a potency of 1000 $\mu\text{g/mg}$. Figures quoted in Table VI are thus for a potency of 1000 μg .

II. *In Vivo* Activity

The protective activity of Antibiotic 67—694 having a potency of 1000 $\mu\text{g/mg}$ was tested in male CF—1 (Carworth Farms) mice weighing approximately 20 grs. each. The antibiotic was given as a suspension or a solution in an aqueous vehicle containing 0.5% carboxymethylcellulose in two doses; one shortly before and one 4 hours after intraperi-

toneal infection with the bacteria. Infected non treated mice (controls) generally died within 18 hours. Survival rate in the treated group was determined 48 hours after infection. The results of the *in vivo* tests using a representative gram-positive and a representative gram-negative organism are tabulated in Table VII below:

TABLE VII

Organism	Route	Protective Activity of Mice
		PD ₅₀ (mg/kg)
<i>Staphylococcus aureus</i> gray	Subcutaneous	50
	Oral	200
<i>Streptococcus pyogenes</i> C	Subcutaneous	50
	Oral	200

20 III. *Acute Toxicity*

The acute toxicity, expressed as LD₅₀, of Antibiotic 69—694 is given in Table VIII below:

Table VIII	
Route of Administration	LD ₅₀ (mg/kg)
Intraperitoneal	350
Subcutaneous	625
Intravenous	155

Antibiotic 67—694 and pharmaceutically acceptable functional derivatives thereof may be used alone or in combination with other antibacterial agents to prevent the growth of, or to reduce the number of, susceptible organisms, especially the gram-positive organisms set forth in Table VI above. Thus, Antibiotic 67—694 and pharmaceutically acceptable functional derivatives thereof may be used

in wash solutions for sanitary purposes, as in the cleansing of laboratory glassware and equipment, and may be used for laundering purposes, for example as a bacteriostatic rinse for laboratory uniforms. Additionally, Antibiotic 67—694 and pharmaceutically acceptable functional derivatives thereof may be used in the treatment of laboratory and domestic animals that are infected with susceptible organisms.

Examples

The invention will now be further illustrated by way of the following examples, in which Examples 1 and 2 illustrate the cultivation of *Micromonospora rosaria* (specifically, that assigned NRRL 3718 was employed) and production of Antibiotic 67—694, Examples 3 to 5 illustrate the isolation and purification of Antibiotic 67—694, Examples 6 to 11 illustrate the preparation of specified derivatives of Antibiotic 67—694 and Examples 12 to 15 illustrate compositions containing Antibiotic 67—694.

Example 1

A. Shake Flask Fermentation

Under aseptic conditions, add a loopful (e.g. 0.5 ml.) of a *Micromonospora rosaria* culture from an agar slant or a lyophilised culture of *M. rosaria* to 100 ml. of a sterile medium contained in a 300 ml. Erlenmeyer flask and having the composition:

	Bacto - beef extract (Difco)	3 g.
	Bacto-tryptose (Difco)	5 g.
35	Bacto-yeast extract (Difco)	5 g.
	Dextrose	1 g.
	Potato starch	24 g.
	Calcium carbonate	2 g.
	Tap water	1000 ml.

40 Incubate the contents of the flask at 35° C. for 72 hours with agitation on a rotary shaker.

B. Fermentation Stage

45 Transfer aseptically 5 ml. of the inoculum produced in the above germination stage to each of a number of 500 ml. Erlenmeyer flasks containing 100 ml. of a sterile medium having the composition:—

	Bacto-yeast extract (Difco)	5 g.
	Corn steep solids	1 g.
50	Starch	30 g.
	Calcium carbonate	7 g.
	Tap water	1000 ml.

55 Incubate the contents of the flasks at 28° C. for from 72 to 100 hours with agitation on a rotary shaker.

After the first 24 hours, samples are periodically taken from the fermented media and assayed for antibiotic activity so that peak production, that is the stage at which the anti-

biotic activity of the fermented media reaches a peak, can be ascertained. When fermentation is effectively complete, as evidenced by peak production, the contents of the flasks may be conveniently combined for subsequent isolation of the antibiotically active product.

During germination and fermentation, the pH of the nutrient medium is adequately maintained within the region of the preferred pH range by the buffering action of the calcium carbonate component. Cultivation is effected under submerged aerobic conditions.

Example 2

Tank Fermentation

A. Germination Stage

Under aseptic conditions, add 25 ml. of inoculum, prepared as described in Example 1A, to 500 ml. of a sterile medium of composition given in Example 1A and contained in a 2 litre Erlenmeyer flask. Incubate the contents of the flask at 28° C. for 72 hours with agitation on a rotary shaker.

B. Fermentation Stage

Transfer aseptically the whole of the product from the above germination stage to a 14 litre fermentor containing 9.5 litres of the medium employed in Example 1B.

Add 6 ml. of anti-foam agent (such as that sold under the trade designation Dow-Corning B) and adjust the pH of the mixture to a pH of about 7.0 using either dilute (1N) aqueous sodium hydroxide or dilute (1N) aqueous sulphuric acid as required. Ferment the mixture at 28° C. until peak antibiotic activity is attained, which usually occurs after fermenting for between 72 and 100 hours. During fermentation aerobic conditions are suitably maintained by passing air through the fermentation medium (specifically, passage of air at a rate of approximately 3.5 l./min. has been found to be satisfactory).

Example 3

Isolation of Antibiotic 67—694

Adjust the pH of a fermented medium obtained by the method of Example 2 to about 9.5 with 1N sodium hydroxide solution and then extract the medium with successive 20 l. portions of ethyl acetate until the antibiotically active product is effectively removed (2 to 5 extractions are usually adequate). Combine the ethyl acetate extracts and concentrate, *in vacuo*, to about 200 ml. Back-extract the biologically active material from the ethyl acetate extract with about 200 ml. of 0.1N aqueous sulfuric acid. Adjust the pH of the acid extract to about 9.5 using dilute sodium hydroxide and re-extract with ethyl acetate. Combine the extracts and evaporate, *in vacuo*, to about 10 ml. Add the concentrate to about 100 ml. of a vigorously stirred mixture of 6 volumes of diethyl ether and 4 volumes of hexane. Filter off the precipitate, wash spar-

ingly with a fresh portion of the diethyl ether-hexane mixture and discard the precipitate. Combine the diethyl ether-hexane filtrate and washings and evaporate, *in vacuo*, to a residue. Dissolve the residue in about 5 ml. of diethyl ether, filter the solution and then add under stirring to 50 ml. of petroleum ether (b.p. 30—60° C.). Filter off the resulting off-white to buff colored precipitate and wash sparingly with petroleum ether. Dry the precipitate at 40° C. *in vacuo*. The antibiotically active product (crude Antibiotic 67—694) obtained in this manner usually assays at a potency of about 700 µg/mg. in the hereinbefore described assay method.

Example 4

Purification of Antibiotic 67—694

Dissolve the Antibiotic 67—694 product obtained in Example 3 in a mixture of chloroform (9 parts by volume) and methanol (1 part by volume). Add the solution so-obtained to a column containing 250 g. of silicic acid and then elute the column with a mixture of chloroform (8 parts by volume) and methanol (2 parts by volume). Collect 5 ml. fractions at the rate of approximately 0.5 ml/min. Sample each fraction and disc test against *Staphylococcus aureus*. Chromatograph a portion of each sample on silica gel GF plates (thickness 250 microns) commercially available from Analtech Inc., Wilmington, Delaware, under the Registered Trade Mark of Uniplate. Fractions containing Antibiotic 67—694 have an R_f value of approximately 0.4 to 0.5. Combine the fractions having an R_f value within this range and evaporate the combined fractions, *in vacuo*, to a residue. Dissolve the residue in acetone and then add diethyl ether. Separate the resulting precipitate by filtration or by centrifuging and discard the precipitate. Evaporate the filtrate or supernatant liquor, *in vacuo*, to a solid residue. The antibiotically active product so obtained is Antibiotic 67—694 assaying at a potency of 1000 µg/mg in the hereinbefore described assay method and having the following physical constants:—

m.p. = 110—114° C.

$[\alpha]_D^{25} = -33.4^\circ$ (Concentration = 3% in ethanol)

$E_{1\%}^{1\text{cm.}} = 238$ (at 240 mµ)

Infra-red spectrum — As shown in Figure 1.

Nuclear magnetic resonance spectrum — as shown in Figure 2.

Other physical and chemical properties for such a product are as hereinbefore described in the passage entitled "The Antibiotic".

Example 5

Alternative isolation and purification procedure for Antibiotic 67—694

Adjust the pH of a fermented medium prepared as in Example 2 to a pH of approximately 9.5 using 1N aqueous sodium hydroxide solution. Extract the fermented medium with successive 20 l. portions of ethyl acetate until the antibiotically active product is effectively removed (2 to 5 extractions are usually adequate) and then evaporate the combined ethyl acetate extracts, *in vacuo*, to a residue. Dissolve the residue in a chloroform-methanol mixture (9 parts by volume of chloroform and 1 part by volume of methanol) and add the solution to a silicic acid column. Then elute the column with a chloroform-methanol mixture consisting of 8 parts by volume of chloroform and 2 parts by volume of methanol. Collect 5 ml. fractions and sample each fraction. Disc test a portion of each sample against *Staphylococcus aureus* and chromatograph a second portion of each sample on a Uniplate (Registered Trade Mark).

Combine fractions having similar R_f values. Those containing Antibiotic 67—694 have an R_f value of approximately 0.4 to 0.5 and these fractions are combined and then evaporated *in vacuo*, to a residue. Dissolve the residue in acetone and then add diethyl ether. Separate the resulting precipitate by filtration or by centrifuging and discard the precipitate. Evaporate the filtrate or supernatant liquor, *in vacuo*, to a solid residue. The antibiotically active product so-obtained is Antibiotic 67—694 assaying at a potency of about 1000 µg/mg and having physical constants substantially as specified in Example 4.

Example 6

Antibiotic 67—694 Potassium Dihydrogen Phosphate Salt

Add 500 mg. of Antibiotic 67—694 to 13 ml. of water. Add 130 mg. of potassium dihydrogen phosphate and stir at room temperature for 2 hours. Add about 50 mg. of decolorising charcoal and stir for an additional 15 minutes. Filter the solution and lyophilize to obtain the title product.

m.p. 118—121° C.

$[\alpha]_D^{20} = -21.2^\circ$ (0.3% in water)

By utilizing substantially the procedure of this example and by substituting equivalent quantities of acids having substantial solubility in water such as hydrochloric, sulfuric, phosphoric, acetic, succinic, citric, lactic, malic, malonic, maleic and the like, the respective pharmaceutically acceptable acid-addition salts

60	may be prepared. Generally, such salts exhibit <i>in vitro</i> and <i>in vivo</i> antibacterial spectra that are quite similar, if not identical, to that of the free nitrogen base. Where differences do appear, they are usually attributable to differences in solubility. By virtue of their solubility in aqueous media, the foregoing salts are particularly well suited for utilization in aqueous preparations and solutions for oral and parenteral administration.	amples 8 and 9 with an equivalent quantity of an acid anhydride or acid chloride derived from an acid such as propionic, butyric, hexanoic, octanoic, stearic, succinic, benzoic, phthalic, phenylacetic, phenoxyacetic, adamantonic and the like acids, and by following the procedures of Examples 8 and 9, other monoesters of Antibiotic 67—694 may be obtained.	60
pre- oxi- side with 65 until velv ade- ethyl Dis- anol form the ure 75 pro- Col- ion. inst aph date	5	Example 10 <i>Antibiotic 67—694 Diacetate</i> Dissolve 5.0 g. of Antibiotic 67—694 in 50 ml. of pyridine; add 2.5 ml. of acetic anhydride and hold the resulting solution at room temperature for about 4 days. Concentrate the pyridine solution <i>in vacuo</i> to a residue keeping the temperature at about 35° C. Dissolve the residue in acetone and dilute the solution with 5% ammonium hydroxide to yield the product of this example as a suspension. Filter the suspension and wash with water. Crystallize the product from aqueous acetone to obtain the title product.	65
ues. an ese ted in rate by By- in ally 7— mg/ ally	10	Example 7 <i>Antibiotic 67—694 Tartrate Salt</i> Dissolve 1 g. of Antibiotic 67—694 in 50 ml. of diethyl ether; add a solution of 250 mg. of tartaric acid in 1.5 ml. of ethanol. Stir the resulting mixture for about 15 minutes then filter the precipitated salt. Wash the precipitate with diethyl ether, dry the precipitate and obtain thereby the title product.	70
13 di- ra- de- nal ize	15	20 m.p. 129—134° C. [α] _D ²⁰ = -12° (0.3% in water)	75
105	20	By utilizing substantially the procedure of this example and by substituting equivalent quantities of acids having substantial solubility in ether-alcohol mixtures such as glutaric, lauric, adamantonic, cinnamic, camphoric, palmitic, stearic, undecylenic and the like, the respective pharmaceutically acceptable acid-addition salts may be prepared. Generally, due to their molecular weight and solubility characteristics, these salts are particularly well suited for utilization in topical preparations such as creams and ointments.	80
of ent lu- ic, lic, ive alts	25	Example 8 <i>Antibiotic 67—694 Monoacetate</i> Dissolve 1.0 g. of Antibiotic 67—694 in 10 ml. of dry acetone and add 0.17 ml. of acetic anhydride. Stir the mixture at room temperature for 4 hours, then slowly and with stirring add 1.2% ammonium hydroxide solution to precipitate the product. Filter the resulting precipitate, wash with dilute aqueous acetone, and then with water, and dry. Crystallize from aqueous acetone to obtain the desired product.	85
110	30	m.p. 104—107° C. [α] _D ²⁰ = -24° (0.3% in ethanol)	90
115	35	Example 11 <i>Antibiotic 67—694 Dibenzoate</i> Dissolve 250 mg. of Antibiotic 67—694 in 5 ml. of pyridine and add 0.25 ml. of benzoyl chloride with stirring. Keep the solution at room temperature for 5 days, then concentrate <i>in vacuo</i> to about 1 ml. and dilute with about 5 ml. of acetone. Add 5% ammonium hydroxide until the product precipitates. Filter the suspension, wash the precipitate with water and dry to obtain Antibiotic 67—694 dibenzoate.	95
	40	m.p. 116—119° C. [α] _D ²⁵ = +20° (0.3% in ethanol)	100
	45	It will be readily apparent that by preparing a particular monoester of Antibiotic 67—694 and then subjecting the monoester to the action of a different acid anhydride or acid chloride one may prepare a mixed diester. Such diesters are within the scope of the present invention.	105
	50	By substituting an equivalent quantity of other acid anhydrides or acid chlorides for those used in Examples 10 and 11 above and by following the procedures of said Examples, other diesters of Antibiotic 67—694 may be obtained. Exemplary of such esters are the dipropionate, divalerate, dihexanoate, distearate, disuccinate, diphtalate, diphenylacetate, diphenoxycetate, diadamantoate, diisopropyl carboxylate and the like.	110
	55	In the forementioned Examples illustrating the preparation of derivatives, and in the following Examples illustrating compositions, the Antibiotic 67—694 is typically Antibiotic 67—694 assaying at about 1000 µg/mg.	115

According to a further aspect of the present invention there are provided antibioticly active therapeutic compositions, which may be in the form of a solid shaped dosage unit, comprising an antibioticly active substance designated Antibiotic 67—694 or an antibioticly active substance which contains said Antibiotic 67—694, the Antibiotic 67—694 being in free form or in the form of a pharmaceutically acceptable functional derivative, and a carrier in association therewith.

The invention also provides a process for preparing an antibioticly active therapeutic composition which comprises bringing an antibioticly active substance designated Antibiotic 67—694 or an antibioticly active substance which contains the said Antibiotic 67—694, said substance being in free form or in the form of a pharmaceutically acceptable functional derivative, into a form suitable for therapeutic administration, preferably by admixing the antibioticly active substance with a suitable pharmaceutical carrier.

Examples 12 to 15 which follow illustrate some of the plurality of dosage forms in which Antibiotic 67—694 and its pharmaceutically acceptable salts and esters may be employed.

The specified formulations, other than the topical formulations, are designed to permit the administration of from about 5 to 50 mg. of antibiotic (as the free base) per kilogram of body weight per day. It is contemplated that the medicaments be administered up to about 8 times in a 24 hour period. The topical formulations are contemplated as being applied to the infected area about 2 to 4 times daily. It is to be noted, however, that the size of the dose administered and the frequency of administration will to a large extent be dependent upon the type of infection, the severity of the infection and the individual characteristics of the animal species being treated.

Example 12

<i>Capsule</i>		
45	Antibiotic 67—694	250.00 mg.
	Lactose	248.75 mg.
	Magnesium Stearate	1.25 mg.
		500.00 mg.

Procedure:

1. Blend the Antibiotic 67—694 and the lactose.
2. Add the magnesium stearate and mix.
3. Fill capsule.

Example 13

Oral suspension (to give a dose of 125 mg/ 5 ml.)			55
	Antibiotic 67—694 tartrate	25.00 g.	
	Magnesium Aluminum Silicate	9.5 g.	
	Sodium Carboxymethylcellulose, U.S.P.	2.5 g.	60
	Sodium Citrate, U.S.P.	25.0 g.	
	Flavor	q.s.	
	Color	q.s.	
	Methylparaben, U.S.P.	0.9 g.	
	Propylparaben, U.S.P.	0.2 g.	65
	Polysorbate 80, U.S.P.	1.0 g.	
	Sorbitol Solution, U.S.P.	500.0 g.	
	Water	q.s.	1000.0 g.

Procedure:

1. Heat 200 ml. of water to boiling, and dissolve in it one half of the parabens. Cool to about 70° C., then mix in the Polysorbate 80. Sprinkle in the silicate, stirring until a uniform smooth suspension results.
2. Heat an additional 200 ml. of water to boiling, and dissolve in it the remainder of the parabens. Disperse the carboxymethylcellulose in this until a smooth gel results. Mix in the Sorbitol Solution. Then introduce and dissolve the sodium citrate.
3. Add 2 to 1 slowly, with constant stirring. Cool the mixture to 25° C. Add the Antibiotic 67—694, flavor and color, mixing thoroughly. Add sufficient quantity of water to make the total volume 1000 ml.

Example 14

Topical Cream

	Antibiotic 67—694 Monoacetate	10 g.	
	Stearic Acid	200 g.	
	Sorbitan Monostearate	104 g.	90
	Sorbitan Monoleate	20 g.	
	Polyoxyethylene Sorbiton Mono-laurate	56 g.	
	Water	q.s.	1000 g.

Procedure:

1. Heat the stearic acid, sorbitan monostearate, sorbitan monoleate and polyoxyethylene sorbitan monolaurate to 65° C.
2. Heat about 90% of the water to 70° C.
3. Add the water to Step 1 and mix to form the cream base.
4. Slurry the Antibiotic 67—694 with about 10% of the water and pass through a colloid mill.
5. Add the milled slurry to the molten base and mix. Allow to cool.

Example 15

Topical Ointment

Antibiotic 67—694 Dibenzoate 10 g.
 Petrolatum 990 g.

- 5 1000 g.
- Procedure:
1. Melt the petrolatum.
 2. Slurry the Antibiotic 67—694 with about 10% of the petrolatum and pass through a colloid mill.
 3. Mix the milled slurry with the remainder of the molten petrolatum. Allow to cool.

WHAT WE CLAIM IS:—

1. An antibiotically active substance designated Antibiotic 67—694, which substance in its free form has an infra-red absorption spectrum in mineral oil substantially as shown in Figure 1 of the accompanying drawing and a nuclear magnetic resonance spectrum in deuterated chloroform substantially as shown in Figure 2 of the accompanying drawing, and which is (a) in free form or (b) in the form of a pharmaceutically acceptable functional derivative.
2. A substance as claimed in claim 1 or claim 2 in the form of a pharmaceutically acceptable functional derivative.
3. A substance as claimed in claim 1 or claim 2 in the form of a pharmaceutically acceptable functional derivative.
4. A substance as claimed in claim 3, wherein the pharmaceutically acceptable functional derivative is a salt.
5. A substance as claimed in claim 3, wherein the pharmaceutically acceptable functional derivative is an ester.
6. A substance as claimed in claim 4, wherein the salt is a potassium dihydrogen phosphate salt.
7. A substance as claimed in claim 4, wherein the salt is a tartrate salt.
8. An antibiotically active substance designated Antibiotic 67—694, which substance in its free form has the following characteristics, namely an elemental analysis corresponding to the empirical formula of $C_{31}H_{31}NO_8$, a molecular weight as determined by mass spectrometry of 581, a melting point of 110—114° C., a specific optical rotation as measured by the D-line of sodium and at 25° C. in ethanol at 0.3 per cent concentration of —33.4°, an ultra-violet absorption maximum at 240 m μ with an $E_{1\%}^{1\text{cm}}$ of 238, an infra-red absorption spectrum in mineral oil substantially as shown in Figure 1 of the accompanying drawing, a nuclear magnetic resonance spectrum in deuterated chloroform substantially as shown in Figure 2 of the accompanying drawing, and an antibacterial spectrum substantially as shown in Table VI herein, and which is (a) in free form or (b) in the form of a pharmaceutically acceptable functional derivative.

8. A substance as claimed in claim 5, wherein the ester is a monoacetate ester.

9. A substance as claimed in claim 5, wherein the ester is a diacetate ester.

10. An antibiotically active substance according to claim 1 and substantially as hereinbefore described.

11. A process for the production of an antibiotically active substance designated Antibiotic 67—694 or an antibiotically active substance which contains the said Antibiotic 67—694, which comprises cultivating a micro-organism of the species *Micromonospora rosaria* in a nutrient medium under aerobic conditions until substantial antibiotic activity is imparted to the medium.

12. A process as claimed in claim 11, wherein the micro-organism of the species *Micromonospora rosaria* is the strain designated NRRL 3718.

13. A process as claimed in claim 11 or claim 12, wherein cultivation is effected under submerged aerobic conditions.

14. A process as claimed in any one of claims 11 to 13, wherein cultivation is effected at a temperature of from 20° to 40° C. and at a pH of from 6.0 to 8.5.

15. A process as claimed in claim 14, wherein the temperature is from 27° to 37° C. and the pH is from 7.0 to 8.0.

16. A process as claimed in any one of claims 11 to 15, which further comprises isolating from the fermented nutrient medium an antibiotically active product, said product being an antibiotically active substance designated Antibiotic 67—694 or an antibiotically active substance which contains the said Antibiotic 67—694.

17. A process as claimed in claim 16, wherein the isolation of the antibiotically active product involves the use of solvent-extraction techniques.

18. A process as claimed in claim 17, wherein the isolation of the antibiotically active product comprises extracting the fermented medium under alkaline conditions with a water-immiscible organic solvent and recovering the antibiotically active product from the organic solvent extract.

19. A process as claimed in claim 18, wherein the organic solvent extract obtained on extracting the fermented medium with the water-immiscible organic solvent is submitted to one or more sequences of the steps of extracting the organic extract with aqueous dilute acid, rendering the aqueous extract alkaline and then re-extracting the aqueous alkaline extract with a water-immiscible solvent, said one or more sequences of steps being followed by recovering the antibiotically active product from the resulting organic solvent extract.

20. A process as claimed in any one of claims 16 to 19, which includes the step of resolving the antibiotically active product into

16

5 mg/ 55

100 g.

9.5 g.

2.5 g. 60

5.0 g.

q.s.

q.s.

0.9 g.

0.2 g. 65

1.0 g.

0.0 g.

0.0 g.

4 dis- 70

Cool

lysor-

ringg

results.

er to 75

er of

ethyl-

results.

intro- 80

ring.

Anti-

ixing

v of 85

ml.

10 g.

00 g.

4 g. 90

0 g.

6 g.

0 g.

95

rate,

or-

Form 100

about

col-

base 105

Example 15

Topical Ointment

Antibiotic 67—694 Dibenzoate

10 g.

Petrolatum

990 g.

1000 g.

Procedure:

1. Melt the petrolatum.
2. Slurry the Antibiotic 67—694 with about 10% of the petrolatum and pass through a colloid mill.
3. Mix the milled slurry with the remainder of the molten petrolatum. Allow to cool.

WHAT WE CLAIM IS:—

1. An antibiotically active substance designated Antibiotic 67—694, which substance in its free form has an infra-red absorption spectrum in mineral oil substantially as shown in Figure 1 of the accompanying drawing and a nuclear magnetic resonance spectrum in deuterated chloroform substantially as shown in Figure 2 of the accompanying drawing, and which is (a) in free form or (b) in the form of a pharmaceutically acceptable functional derivative.
2. 8. An antibiotically active substance designated Antibiotic 67—694, which substance in its free form has the following characteristics, namely an elemental analysis corresponding to the empirical formula of $C_{21}H_{31}NO_9$, a molecular weight as determined by mass spectrometry of 581, a melting point of 110—114° C., a specific optical rotation as measured by the D-line of sodium and at 25° C. in ethanol at 0.3 per cent concentration of —33.4°, an ultra-violet absorption maximum at 240 m μ with an $E_{1\%}^{1\text{cm}}$ of 238, an infra-red absorption spectrum in mineral oil substantially as shown in Figure 1 of the accompanying drawing, a nuclear magnetic resonance spectrum in deuterated chloroform substantially as shown in Figure 2 of the accompanying drawing, and an antibacterial spectrum substantially as shown in Table VI herein, and which is (a) in free form or (b) in the form of a pharmaceutically acceptable functional derivative.
3. A substance as claimed in claim 1 or claim 2 in the form of a pharmaceutically acceptable functional derivative.
4. A substance as claimed in claim 3, wherein the pharmaceutically acceptable functional derivatives is a salt.
5. A substance as claimed in claim 3, wherein the pharmaceutically acceptable functional derivative is an ester.
6. A substance as claimed in claim 4, wherein the salt is a potassium dihydrogen phosphate salt.
7. A substance as claimed in claim 4, wherein the salt is a tartrate salt.

8. A substance as claimed in claim 5, wherein the ester is a monoacetate ester.

9. A substance as claimed in claim 5, wherein the ester is a diacetate ester.

10. An antibiotically active substance according to claim 1 and substantially as hereinbefore described.

11. A process for the production of an antibiotically active substance designated Antibiotic 67—694 or an antibiotically active substance which contains the said Antibiotic 67—694, which comprises cultivating a micro-organism of the species *Micromonospora rosaria* in a nutrient medium under aerobic conditions until substantial antibiotic activity is imparted to the medium.

12. A process as claimed in claim 11, wherein the micro-organism of the species *Micromonospora rosaria* is the strain designated NRRL 3718.

13. A process as claimed in claim 11 or claim 12, wherein cultivation is effected under submerged aerobic conditions.

14. A process as claimed in any one of claims 11 to 13, wherein cultivation is effected at a temperature of from 20° to 40° C. and at a pH of from 6.0 to 8.5.

15. A process as claimed in claim 14, wherein the temperature is from 27° to 37° C. and the pH is from 7.0 to 8.0.

16. A process as claimed in any one of claims 11 to 15, which further comprises isolating from the fermented nutrient medium an antibiotically active product, said product being an antibiotically active substance designated Antibiotic 67—694 or an antibiotically active substance which contains the said Antibiotic 67—694.

17. A process as claimed in claim 16, wherein the isolation of the antibiotically active product involves the use of solvent-extraction techniques.

18. A process as claimed in claim 17, wherein the isolation of the antibiotically active product comprises extracting the fermented medium under alkaline conditions with a water-immiscible organic solvent and recovering the antibiotically active product from the organic solvent extract.

19. A process as claimed in claim 18, wherein the organic solvent extract obtained on extracting the fermented medium with the water-immiscible organic solvent is submitted to one or more sequences of the steps of extracting the organic extract with aqueous dilute acid, rendering the aqueous extract alkaline and then re-extracting the aqueous alkaline extract with a water-immiscible solvent, said one or more sequences of steps being followed by recovering the antibiotically active product from the resulting organic solvent extract.

20. A process as claimed in any one of claims 16 to 19, which includes the step of resolving the antibiotically active product into



fractions using chromatographic techniques.

21. A process as claimed in any one of claims 16 to 20, wherein Antibiotic 67—694 is isolated in free form.

5 22. A process as claimed in any one of claims 16 to 21, wherein Antibiotic 67—694 prior or subsequent to isolation, is converted into a pharmaceutically acceptable functional derivative.

10 23. A process as claimed in claim 22, wherein the pharmaceutically acceptable functional derivative is an ester.

24. A process as claimed in claim 22, wherein the pharmaceutically acceptable functional derivative is a salt.

15 25. A process as claimed in any one of claims 11 to 24, substantially as hereinbefore described and exemplified in any of Examples 1 to 11.

20 26. A fermented nutrient medium of *Micromonospora rosaria* whenever prepared by a process as claimed in any one of claims 11 to 15.

25 27. An antibiotically active substance designated Antibiotic 67—694 or an antibiotically active substance which contains the said Antibiotic 67—694, the Antibiotic 67—694 being in free form or in the form of a pharmaceutically acceptable functional derivative, whenever prepared by a process as claimed in any one of claims 11 to 25.

30 28. An antibiotically active therapeutic composition comprising an antibiotically active substance designated Antibiotic 67—694 or

an antibiotically active substance which contains said Antibiotic 67—694, the Antibiotic 67—694 being in free form or in the form of a pharmaceutically acceptable functional derivative, and a carrier in association therewith.

29. An antibiotically active therapeutic composition as claimed in claim 28, and substantially as hereinbefore described with particular reference to any one of Examples 12 to 15.

30. An antibiotically active therapeutic composition as claimed in claim 28 or claim 29 in the form of a solid shaped dosage unit.

31. A process for preparing an antibiotically active therapeutic composition which comprises bringing an antibiotically active substance designated Antibiotic 67—694 or an antibiotically active substance which contains the said Antibiotic 67—694, said substance being in free form or in the form of a pharmaceutically acceptable functional derivative, into a form suitable for therapeutic administration.

32. A process as claimed in claim 31, wherein the antibiotically active substance or pharmaceutically acceptable functional derivative thereof is admixed with a suitable pharmaceutical carrier.

MATHYS & SQUIRE,
Chartered Patent Agents,
10 Fleet Street,
London, E.C.4.
Agents for the Applicants.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1973.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

ERRATUM

SPECIFICATION No. 1,302,142

Page 17, line 25, for 8. read 2.

THE PATENT OFFICE
26th February, 1973



* C L M S *