

PATENT SPECIFICATION

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(54) ANTICOCCIDIAL SUBSTANCE AND ITS PREPARATION

(71) We, RHONE-POULENC INDUSTRIES, a French Body Corporate of 22 Avenue Montaigne, 75360 Paris Cedex 08, France, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to antibiotic substances, their preparation, and compositions containing them.

The present invention provides a new antibiotic 31,559 RP and its salts with metals and nitrogen-containing bases. It has valuable anticoccidial activity and antibacterial activity. It is also valuable as a growth factor for ruminants and as an agent for counteracting dysentery in pigs.

31,559 RP can be obtained by culturing a new micro-organism more completely identified below, belonging to the *Streptomyces* genus and called *Streptomyces hygroscopicus* DS 24,367 (NRRL 5787).

31,559 RP contains carbon, hydrogen and oxygen. The elementary composition of its sodium salt is approximately:
 C=63.2%, H=8.8%, O=24.3%, Na=2.9%.

Its neutral equivalent weight (measured by titration of the sodium salt in acetic acid with perchloric acid) is 836.

The elementary analysis and NMR spectra of the proton and carbon ¹³C of the sodium salt of 31,559 RP are consistent with the structure indicated below.

The sodium salt of 31,559 RP has the following properties:

it is a white microcrystalline powder;

[using the notation of the Pharmacopée Française, IXth edition, 2nd part, page 13 (1972)] it is practically insoluble in water, sparingly soluble in hydrocarbon solvents such as hexane, soluble in alcohols such as methanol, ketones such as acetone, dimethylformamide, and ethyl acetate, and readily soluble in chlorinated solvents such as methylene chloride and chloroform;

its melting point (measured on a Kofler bench) is 220°C (with decomposition); its optical rotation: (c=1.050 in methanol)

$[\alpha]_D^{20} = +51.2^\circ \pm 1^\circ$, $[\alpha]_{436}^{20} = +101^\circ \pm 1.5^\circ$, $[\alpha]_{565}^{20} = +167^\circ \pm 2^\circ$;

its ultra-violet spectrum (determined on a solution of concentration 0.950 mg./cc. in ethanol) shows no characteristic absorption up to 210 nm.;

its infra-red spectrum (determined on tablets of a mixture with potassium bromide is shown in the single Figure of the accompanying drawing in which the wavelengths expressed in microns (upper scale) and in wave numbers in cm⁻¹ (lower scale) have been plotted as the abscissae and the optical densities have been plotted as the ordinates. The main infra-red absorption bands for this product, expressed in wave numbers (cm⁻¹), are given in Table I below.

8

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2

TABLE I

3,430 S	1,372 sh	1,090 sh	775 sh
3,180 S	1,362 sh	1,072 S	765 sh
2,970 vS	1,350 sh	1,065 sh	748 m
2,935 S	1,335 sh	1,040 S	705 m
2,925 sh	1,298 sh	1,018 S	655 sh
2,880 S	1,292 m	1,000 sh	635 m
2,825 m	1,260 sh	985 S	610 w
2,600 sh	1,238 S	968 vS	590 vw
2,060 vw	1,220 sh	940 S	570 sh
1,945 vw	1,200 S	928 m	565 w
1,850 vw	1,190 sh	912 S	550 w
1,760 vw	1,185 sh	890 m	535 w
1,730 vw	1,170 S	870 S	500 vw
1,630 sh	1,152 w	852 m	482 vw
1,590 S	1,140 sh	828 m	420 m
1,450 S	1,115 S	815 sh	378 w
1,398 S	1,108 sh	792 sh	350 m
1,380 S	1,092 vS	788 m	

vS=very strong

S=strong

m=medium

w=weak

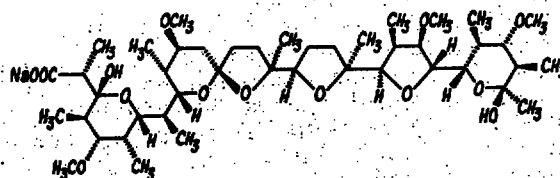
vw=very weak

sh=shoulder

The sodium salt of 31,559 RP can also be characterised by ascending chromatography on a thin layer of silica gel, using two systems of solvent mixtures:

- 1) Ethyl acetate, cyclohexane, water and butanol (50—50—25—5 by volume): in this system, the sodium salt of 31,559 RP has an Rf of 0.5.
- 2) Methylene chloride and methanol (94—6 by volume): in this system, the sodium salt of 31,559 RP has an Rf of 0.7.

The sodium salt of 31,559 RP has been found to have the formula:



Bacteriostatic activity in vitro:

31,559 RP exhibits bacteriostatic activity especially against certain gram-positive bacteria.

The bacteriostatic activity of the sodium salt of 31,559 RP against a certain

number of microorganisms was determined by one of the dilution methods usually employed for this purpose. For each microorganism, the lowest concentration of active substance which, under defined conditions, prevents any visible development in a suitable nutrient broth was determined. The results of the various determinations are given in Table II below, where the minimum bacteriostatic concentrations are expressed in micrograms of substances per cc. of test medium:

TABLE II

Bacterial organism tested	Minimum bacteriostatic concentrations (in $\mu\text{g}/\text{cc.}$)
<i>Staphylococcus aureus</i> , strain 209 P-ATCC 6538 P	0.8
<i>Staphylococcus aureus</i> , Smith strain	2.5
<i>Sarcina lutea</i> -ATCC 9341	1.9
<i>Streptococcus faecalis</i> -ATCC 8043	0.5
<i>Streptococcus pyogenes haemolyticus</i> , Dig 7 strain (Institut Pasteur)	0.8
<i>Diplococcus pneumoniae</i> , Til strain (Institut Pasteur)	0.2
<i>Neisseria catarrhalis</i> (A 152, Institut Pasteur)	50
<i>Bacillus subtilis</i> -ATCC 6633	0.8
<i>Bacillus cereus</i> -ATCC 6630	0.5
<i>Mycobacterium species</i> -ATCC 607	25
<i>Escherichia coli</i> -ATCC 9637	>150
<i>Shigella dysenteriae</i> , Shiga L (Institut Pasteur)	>150
<i>Salmonella paratyphi</i> A (Lacasse strain, Institut Pasteur)	>150
<i>Salmonella schottmuelleri</i> (paratyphi B), (Fougenc strain, Institut Pasteur)	>150
<i>Proteus vulgaris</i>	>150
<i>Pseudomonas aeruginosa</i>	>150

Toxicity:

In chicks, the 50% lethal dose (LD_{50}) of the sodium salt of 31,559 RP is 150 mg./kg. orally as a single dose.

Anticoccidial activity:

The anticoccidial activity of the sodium salt of 31,559 RP was determined in chicks infested with *Eimeria tenella* or *Eimeria acervulina*. Substantial anticoccidial activity is also shown when the sodium salt of 31,559 RP is incorporated into the feedstuff at non-toxic concentrations between 0.005 and 0.04% by weight in the feedstuff.

The organism which produces 31,559 RP is a *Streptomyces* strain which has been isolated from a sample of earth taken in India, which has been given the number DS 24,367. A sample thereof has been filed at the Northern Regional Research Laboratory of the U.S. Department of Agriculture at Peoria, Illinois, (U.S.A.), where it has been recorded under the reference NRRL 5787.

This strain was isolated by the general method which consists of suspending a small amount of earth in sterile distilled water, of diluting the suspension to different concentrations, and of spreading a small volume of each dilution on the surface of Petri dishes containing a nutrient agar medium. After incubation for a few days at 25°C, which allows the microorganisms to develop, the colonies which it is desired to isolate in order to investigate them further are removed and trans-

planted on sloping nutrient agars in order to produce more abundant cultures thereof.

The streptomyces strain DS 24,367 belongs to the species *Streptomyces hygroscopicus*, the essential characteristics of which have been defined by H. D. TRESNER and E.J. BACKUS (Applied Microbiology, 4, 243—250, 1956) and by S. A. WAKSMAN (The Actinomycetes, II, The Williams and Wilkins Company, Baltimore, 1961, page 230—231); consequently it has been denoted by the name *Streptomyces hygroscopicus*, strain DS 24,367.

S. hygroscopicus DS 24,367 possesses the following three properties which correspond to the three characteristics by which H. D. TRESNER and E. J. BACKUS and S. WAKSMAN define the species *S. hygroscopicus*:

a) its sporophores generally end in tight spirals possessing a coil of a few turns; these spiral sporophores are usually grouped along a filament so as to form more or less elongated clusters,

b) when its sporulated aerial mycelium has reached a good stage of development, it shows a dark grey colouration corresponding to that shown by the species *S. hygroscopicus*, and

c) on some culture media which permit good development of the aerial mycelium, shiny black zones with a moist appearance appear, on aging, in the sporulated surfaces.

In the case of *S. hygroscopicus* DS 24,367, the formation of an aerial mycelium frequently occurs only very sparingly and it can even fail to appear on a certain number of culture media. When a grey sporulated aerial mycelium appears, the formation of black zones on aging, when it does take place, generally occurs only in a rather discrete manner and is restricted to small dots or small zones distributed locally on the aerial mycelium rather than over all of its entire surface. It is, however, evident and can be observed in particular on Pridham yeast extract agar, on Pridham oatmeal and tomato paste agar and on glucose-asparagine agar.

The strain of *S. hygroscopicus* mentioned as a reference by S. A. WAKSMAN in "The Actinomycetes" shows, on the few culture media where its morphological appearance is described, some minor differences compared with the strain DS 24,367, the most important of which consist of the fact that it forms an aerial mycelium on nitrated agar containing sucrose and on nutrient agar, whilst on these two media the strain DS 24,367 does not form any aerial mycelium. Moreover, on a medium containing sucrose as a source of carbon, *S. hygroscopicus* does not reduce nitrates whilst, under the same conditions, the strain DS 24,367 reduces nitrates to nitrites to a large extent. However, these few differences are not sufficiently important to distinguish between the strain DS 24,367 and the species *S. hygroscopicus* of which it otherwise possesses the main characteristics.

S. hygroscopicus DS 24,367 forms chains of spores which generally end in tight spirals with a limited number of turns, rarely more than 3 or 4, although, occasionally, it is possible to observe spirals forming a larger number of turns, or also a few chains of spores which are simply curved over in their terminal portion without forming a complete turn, or spirals which are more or less loose and unwound. The sporiferous apparatus possesses a cluster structure, the spiral sporophores, which can themselves possess a few branches, being inserted along a main filament, the length of which can be quite considerable. The spores are oval to cylindrical, measuring 0.8 to 1.0 μ /0.6 to 0.8 μ . Microscopic examinations have shown an identical organisation of the sporiferous apparatus on Bennett agar and on Pridham oatmeal-tomato agar. By its method of sporulation, *S. hygroscopicus* DS 24,367 is placed in the *Spira* section of the Pridham classification.

S. hygroscopicus DS 24,367 develops well at 25°C, a little less well at 37°C, and does not develop at 50°C. In its cultures carried out at 25°C, it possesses the following biochemical properties:

Production of melanin	: negative
Production of H ₂ S	: negative
Tyrosinase	: negative
Liquefaction of gelatine	: positive
Utilisation of cellulose	: positive
Production of nitrites from nitrates	: highly positive
Hydrolysis of starch	: positive
Culture on milk	: peptonisation without coagulation; little variation in the pH over 1 month

The culture characteristics of *Streptomyces hygroscopicus* DS 24,367 are given in the table below. They are those of cultures which have reached a good stage of development, that is to say approximately 2 to 3 weeks at 25°C unless otherwise stated. These characteristics were observed on nutrient agars and broths usually employed to determine the morphological characteristics of *Streptomyces*, the cultures on agar media being carried out on sloping agars. Some of the culture media employed were prepared in accordance with the formulations indicated in "The Actinomycetes" (S. A. WAKSMAN, page 193—197, Chronica Botanica Company, Waltham, Mass., U.S.A., 1950); in this case, they are indicated by the letter W, followed by the number which was given to them in "The Actinomycetes". The references of the other culture media or the constituents in them are as follows:

- Ref. A — "Bennett's Agar" — S.A. WAKSMAN — The Actinomycetes, volume 2, page 331 — No. 30 — The Williams and Wilkins Company, Baltimore, 1961
- Ref. B — "Hickey and Tresner's Agar" — T. G. PRIDHAM and colleagues, Antibiotics Annual, 1956—1957, page 950
- Ref. C — Formulation W—22, to which 2% of agar have been added
- Ref. D — "Yeast Extract Agar" — T. G. PRIDHAM and colleagues, Antibiotics Annual, 1956—1957, page 950
- Ref. E — "Tomato Paste Oatmeal Agar" — T. G. PRIDHAM and colleagues, Antibiotics Annual, 1956—1957, page 950
- Ref. F — "Melanin formation medium" — S. A. WAKSMAN — The Actinomycetes, volume 2, page 333 — No. 42 — The Williams and Wilkins Company, Baltimore, 1961
- Ref. G — W. E. GRUNDY and colleagues, Antibiotics and Chem., 2, 401, 1952
- Ref. H — "Inorganic Salts Starch Agar" — T. G. PRIDHAM and colleagues, Antibiotics Annual, 1956—1957, page 951.
- Ref. I — Corresponds to the formulation W—1, wherein 30 g of sucrose are replaced by 15 g of glucose
- Ref. J — Corresponds to the formulation W—1, wherein 30 g of sucrose are replaced by 15 g of glycerine
- Ref. K — Corresponds to the formulation W—18, wherein 30 g of sucrose are replaced by 15 g of glucose
- Ref. L — Corresponds to the formulation W—18, wherein the sucrose is removed and is replaced by small strips of filter paper partially immersed in the liquid
- Ref. M — "Manual of Methods for Pure Culture Study of Bacteria" — Society of American Bacteriologists, Geneva, N.Y., II₃₀—18
- Ref. N — "Plain Gelatin" — prepared in accordance with the instructions of the "Manual of Methods for Pure Culture Study of Bacteria" — Society of American Bacteriologists, Geneva, N.Y., II₃₀—18
- Ref. P — Commercially available skimmed milk powder, reconstituted in accordance with the manufacturer's instructions
- Ref. Q — Medium indicated for investigation into the production of H₂S by: H. D. TRESNER and F. DANGA — Journal of Bacteriology, 76, 239—244, 1958.

(12)

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6

Culture medium	Degree of development	Vegetative mycelium	Aerial apparatus (comprising the combination of the aerial mycelium and the sporulation)	Soluble pigment	Observations and biochemical properties
Bennett agar (Ref. A)	Good	Brownish-yellow to yellow-brown	Whitish to grey. Very poorly developed	Light yellow-brown	Sporiferous apparatus in clusters. Chains of spores ending in tight spirals. Oval to cylindrical spores measuring 0.8 to 1.0 μ /0.6 to 0.8 μ
Hickey and Tresner agar (Ref. B)	Moderate	Yellow-brown	Whitish. In trace amounts	Light yellow-brown.	
Emerson agar (Ref. C)	Good	Light yellow-brown	Whitish. In trace amounts	None or very light brownish	
Pridham yeast extract agar (Ref. D)	Fairly good	Yellow-brown	Whitish to grey. Very poorly developed. On aging, appearance of small black dots characteristic of the species "S. Hygroscopicus".	Very light yellowish brown	
Pridham oatmeal and tomato paste agar (Ref. E)	Fairly good	Brown-yellow to yellow-brown	Whitish to grey. Rather poorly developed. On aging, appearance of small black dots characteristic of the species "S. hygroscopicus".	Very light brownish.	Sporiferous apparatus in clusters. Chains of spores ending in tight spirals. Oval to cylindrical spores measuring 0.8 to 1.0 μ /0.6 to 0.8 μ
Glucose-peptone agar (W-6)	Moderate	Yellowish grey to yellow-brown.	Whitish. In trace amounts.	Light brownish yellow to light yellow-brown.	

Culture medium	Degree of development	Vegetative mycelium	Aerial apparatus (comprising the aerial mycelium and the sporulation)	Soluble pigment	Observations and biochemical properties
Nutrient agar (W-5)	Moderate	Yellow-brown	None.	None.	
Agar containing tyrosine and yeast extract for the formation of melanin (Ref. F)	Very poor	Yellow-brown to orange-brown.	None.	Violet greyish brown.	Formation of melanin: negative (readings made according to the author's instructions)
Krainsky calcium malate agar (Ref. G)	Very poor	Colourless to greyish white.	None.	None.	Solubilisation of calcium malate: positive but weak.
Ovalbumin agar (W-12)	Very poor	Colourless to greyish white.	None.	None.	
Glucose-asparagine agar (W-2)	Moderate	Slightly greenish-yellow	Whitish to grey. Very moderately developed. On aging, appearance of small black dots characteristic of the species "S. hygroscopicus".	Light greyish yellow	
Glycerine-asparagine agar (W-3)	Moderate	Light yellowish grey to yellow.	Whitish to greyish. In trace amounts.	None or light brownish	
Pridham starch-inorganic salts agar (Ref. H)	Moderate	Yellowish-grey to yellow-brown	Whitish to grey. In trace amounts.	None or very light brownish	Hydrolysis of the starch; positive
Starch-nitrate agar (W-10)	Poor	Light brownish-grey.	None.	None.	
Czapek synthetic agar containing sucrose (W-1)	Poor	Very light brownish yellow-grey.	None.	Light brownish-yellow.	

Culture medium	Degree of development	Vegetative mycelium	Aerial apparatus (comprising the combination of the aerial mycelium and the sporulation)	Soluble pigment	Observations and biochemical properties
Czapek synthetic agar containing glucose (Ref. I)	Poor	Very light brownish yellow.	None.	Light brownish yellow.	
Czapek synthetic agar containing glycerine (Ref. J)	Poor	Light brownish-yellow.	None.	Light brownish yellow.	
Starch-nitrate broth (W-10)	Poor	Whitish.	None.	None.	Formation of nitrites: highly positive.
Czapek synthetic broth containing sucrose (W-18)	Poor	Whitish flocculent culture.	None.	None.	Formation of nitrites: highly positive.
Czapek synthetic broth containing glucose (Ref. K)	Poor	Whitish flocculent culture	None.	None.	Formation of nitrites: highly positive.
Czapek synthetic broth containing cellulose (Ref. L)	Poor	Whitish flocculent culture	Grey. Very moderately developed on the paper emerging from the broth.	None.	Formation of nitrites: highly positive. Utilisation of the cellulose: positive.
Nitrated nutrient broth (Ref. M)	Poor	Yellowish brown ring.	None.	None.	Formation of nitrites: highly positive
Culture on potato (W-27)	Very moderate	Light brownish grey.	Whitish. In trace amounts.	Light brownish grey.	
12% pure gelatine (Ref. N)	Good	Whitish to light brownish	None.	None.	Liquefaction positive, good.

Culture medium	Degree of development	Vegetative mycelium	Aerial apparatus (combination of the aerial mycelium and the sporulation)	Soluble pigment	Observations and biochemical properties
Skimmed milk	Good	Yellowish white ring becoming brownish yellow at the end of 1 month of culture.	None.	—	Peptonisation without coagulation: no appreciable variation in the pH over the course of 1 month or very slight acidification.
Tresner and Danga agar (Ref. P)	Very moderate	Light yellowish brown.	None.	None.	Production of H ₂ S: negative (readings made in accordance with the author's instructions)

The degree of development in the above Table is classified in decreasing order as: very good, good, fairly good, moderate, very moderate, poor, very poor, or nil.

- 5 The ability of *Streptomyces hygroscopicus* DS 24,367 to utilise various sources of carbon and nitrogen for its development was determined in accordance with the Pridham and Gottlieb method (J. of Bact., 56, 107—114, 1948); the degree of development was observed, after a suitable period of incubation at 25°C, on the base medium indicated by the authors, replacing either the glucose by one of the various sources of carbon tested, or the (NH₄)₂SO₄ by one of the various sources of nitrogen tested. The results are given in the table below.

Source of carbon tested	Utilisation	Source of nitrogen tested	Utilisation
D-Xylose	positive, but slow	NaNO ₃	positive
L-Arabinose	positive	NaNO ₂	positive
L-Rhamnose	positive	(NH ₄) ₂ SO ₄	positive
D-Glucose	positive	(NH ₄) ₂ HPO ₄	positive
D-Galactose	positive, but slow	Urea	positive
D-Fructose	positive	L-Asparagine	positive
D-Mannose	positive, but slow	Glucosamine	positive

(15)

Source of carbon tested	Utilisation	Source of nitrogen tested	Utilisation
L-Sorbose	negative	Glycine	positive
Lactose	positive	Sarcosine	positive
Maltose	negative	DL-Alanine	positive
Sucrose	positive	DL-Valine	positive
Trehalose	positive	DL-Aspartic acid	positive
Cellobiose	positive	L-Glutamic acid	positive
Raffinose	positive	L-Arginine	positive
Dextrin	positive	L-Lysine	positive
Inulin	positive	DL-Serine	positive
Starch	positive	DL-Threonine	positive
Glycogen	positive	DL-Methionine	negative
Glycerol	positive	Taurine	negative
Erythritol	negative	DL-Phenylalanine	positive, but slow
Adonitol	positive	L-Tyrosine	positive
Dulcitol	negative	DL-Proline	positive
D-Mannitol	positive	L-Hydroxyproline	positive
D-Sorbitol	negative	L-Histidine	positive
Inositol	negative	L-Tryptophane	positive, but slow
Salicin	negative	Betaine	negative

According to the invention 31,559 RP is prepared by culturing *Streptomyces hygroscopicus* DS 24,367 or a productive mutant thereof (i.e. a mutant capable of producing 31559 RP) aerobically in a culture medium, and isolating the 31559 RP formed during the culture, as such or as a salt.

A culture of the micro-organism *Streptomyces hygroscopicus* DS 24,367 (NRRL 5787) in a synthetic culture medium containing a source of assimilable carbon, a source of assimilable nitrogen, and mineral elements, and substantially free from other micro-organisms is within the scope of the invention.

The culture of *Streptomyces hygroscopicus* DS 24,367 can be carried out by any method of aerobic surface culture or submerged culture, but the latter is to be preferred for reasons of convenience. For this purpose, the various types of apparatus usually employed in the fermentation industry may be used.

In particular, the following sequence can be adopted for carrying out the culture:

Streptomyces hygroscopicus DS 24,367 — stock

↓
culture on agar

↓
culture in a shaken flask

↓
inoculum culture in a fermenter

↓
production culture in a fermenter

The fermentation medium must essentially contain a source of assimilable carbon and a source of assimilable nitrogen, mineral elements, in particular chlorides, and optionally growth factors, and these materials may be introduced as well-defined products or as complex mixtures such as are encountered in biological products of various origins.

Suitable sources of assimilable carbon include carbohydrates such as glucose, dextrans, starch or other carbon sources such as sugar alcohols (including glycerol) and certain organic acids such as lactic or citric acid. Certain animal or vegetable oils such as lard oil or soya oil may replace these various carbon sources or can be added to them.

Suitable sources of assimilable nitrogen are extremely varied. They can be very simple chemical substances such as inorganic or organic ammonium salts, urea and certain amino acids. Nitrogen can also be introduced in complex substances, mainly containing nitrogen in a protein form, such as casein, lactalbumin, gluten and their hydrolysis products, soya flour, groundnut flour, fishmeal, meat extract, yeast extract, distillers' solubles and corn-steep.

Among the mineral elements added, some can have a buffering or neutralising effect such as alkali metal or alkaline earth metal phosphates or calcium or magnesium carbonates. Others provide the ionic equilibrium necessary for the development of *Streptomyces hygroscopicus* DS 24,367 and for the formation of 31,559 RP, such as alkali metal and alkaline earth metal chlorides and sulphates. Finally, some act more especially as activators of the metabolic reactions of *Streptomyces hygroscopicus* DS 24,367; these are zinc, cobalt, iron, copper and manganese salts.

Suitable growth factors are vitamin-type natural products such as riboflavin, folic acid and pantothenic acid.

The pH of the fermentation medium at the start of the culture should be between 5.8 and 7.8, and preferably between 6.2 and 7.4. The optimum temperature for the fermentation process is between 25 and 30°C, but satisfactory production is achieved at temperatures between 23 and 33°C. The aeration of the fermentation can vary between rather wide values. It has, however, been found that a rate of aeration of 0.3 to 3 litres of air per litre of broth per minute is very suitable. The maximum yield of 31,559 RP is obtained after 2 to 8 days of culture, this period of time depending essentially on the medium used.

From these facts, it can be seen that the general conditions for the culture of *Streptomyces hygroscopicus* DS 24,367 for the production of 31,559 RP can vary to a large extent and can be adapted to particular requirements.

31,559 RP can be isolated from the products of the culture in the following way:

The medium is filtered at an acid pH, generally between 3 and 6 and preferably close to 5. The activity retained in the filter cake is extracted therefrom by means of a suitable organic solvent such as a lower alcohol, that is an alcohol containing from 1 to 4 carbon atoms, for example methanol, or a chlorinated solvent such as methylene chloride.

After treatment with a suitable alkali, 31,559 RP can be isolated in the form of (e.g.) the sodium salt from the solutions mentioned above by crystallisation after concentration under reduced pressure optionally followed by dilution with a non-solvent or a poor solvent for the salt, and storing in a cold chamber.

31,559 RP or its sodium salt can be purified by conventional methods, such as crystallisation, chromatography on various adsorbents, or counter-current distribution.

The following Example, illustrates the invention.

EXAMPLE.

A) Fermentation

The following constituents are introduced into a 170 litre fermenter:

Peptone	1,200 g.
Yeast extract	600 g.
Glucose monohydrate	1,200 g.
Agar	240 g.
Tap water, sufficient for	110 litres.

18

12

1,495,858

12

The pH is adjusted to 7.30 by adding 10 N sodium hydroxide solution (70 cc.). The medium is sterilised by bubbling steam at 122°C through it for 40 minutes. After cooling, the volume of the broth is 120 litres because of the condensation of steam during the sterilisation process, and the pH is 6.85. The broth is then inoculated with a culture (200 cc.) of *Streptomyces hygroscopicus* DS 24,367, produced in an agitated Erlenmeyer flask. The culture is developed at 27°C for 25 hours whilst agitating and aerating with sterile air; it is then suitable for inoculating the production culture.

The production culture is carried out in a 800 litre fermenter into which the following substances are introduced:

Distillers' solubles	2 kg.
Bean granules	10 kg.
Glycerine	6 kg.
Sodium chloride	2 kg.
Solution of cobalt chloride hexahydrate, of concentration 20 g/l.	0.4 litres
Tap water,	q.s.p. 365 litres.

The pH of the medium is adjusted to 7.50 by adding 10 N sodium hydroxide solution (200 cc.), and the broth is then sterilised by bubbling steam at 122°C through it for 40 minutes. After cooling, the volume of the broth is 390 litres because of the condensation of steam during the sterilisation process; it is made up to 400 litres by adding a sterile aqueous solution (10 litres) containing:

Glucose monohydrate	4 kg.
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The pH of the medium is 6.65; it is inoculated with the inoculum culture (40 litres) from the 170 litre fermenter described above. The culture is developed at 27°C for 113 hours while stirring with a turbine rotating at 205 revolutions/minute and aerating with sterile air (in a volume of 20 m.³/hour). At the end of the operation, the pH of the culture is 7.65 and the volume is 380 litres.

B) Extraction

The medium obtained as described above (400 litres) is brought to pH 5 by adding a 6 N solution of hydrochloric acid (2.8 litres) and is then stirred for half an hour. After adding a filtration aid (20 kg.), the medium is filtered on a filter press and the filter cake is washed on the filter with water (100 litres), the pH of which is adjusted to 5 with 6 N hydrochloric acid. The filtrate and the wash liquid are removed.

The filter cake is triturated in methanol (300 litres); the mixture obtained is adjusted to pH 7 by adding 6 N sodium hydroxide solution (200 cc.) and is stirred for half an hour. The mixture is filtered on a filter press and the filter cake is washed on the filter with methanol (80 litres). The filtrate and the wash liquid are combined and concentrated under reduced pressure (5 to 10 mm Hg) so as to produce an aqueous concentrate (30 litres). This concentrate is adjusted to pH 3 by adding 6 N hydrochloric acid (290 cc.) and is then stirred for 10 minutes in the presence of methylene chloride (30 litres). The two phases are separated. The aqueous phase is extracted twice more by this process, using methylene chloride (30 litres each time).

The three methylene chloride extracts are combined and stirred with water (9 litres), while acidifying to a pH of 3 in the aqueous phase (20 cc. of 6 N hydrochloric acid). The organic phase is isolated and washed with water (18 litres), adding sodium hydroxide solution until a pH of 9.5 is obtained in the aqueous phase (20 cc. of 6 N sodium hydroxide solution). The methylene chloride phase is concentrated under reduced pressure (5 to 10 mm Hg) to a volume of 1 litre.

C) Purification

The methylene chloride concentrate obtained above is poured slowly into

hexane (10 litres) at ambient temperature. The solution is filtered and inactive insoluble matter (63 g.) is thus removed. The filtrate is concentrated under reduced pressure (5 to 10 mm Hg) to a volume of approximately 0.5 litre. This solution is stirred for 2 hours at a temperature of 0°C and is then left to stand for 24 hours at +4°C. The crystals obtained are isolated by centrifuging the solution and are then washed with hexane at +4°C (150 cc.) and dried under reduced pressure. The sodium salt of 31,559 RP (103 g.) is thus isolated.

D) Recrystallisation

The sodium salt of 31,559 RP obtained above (57 g.) is dissolved in acetone (1.2 litres) and stirred for 15 minutes. Decolorising charcoal (2.5 g.) washed with hydrochloric acid is added, the mixture is stirred again for half an hour and the decolorising charcoal is finally filtered off. Distilled water (750 cc.) is added to the filtrate with is stirred slowly. The sodium salt of 31,559 RP crystallises slowly at a temperature of 0°C.

The crystals are filtered off, washed with a mixture of acetone and water (50—50 by volume) (150 cc.) and dried under reduced pressure at 35°C. The sodium salt of 31,559 RP (35 g.) is obtained.

On concentrating the mother liquors to half their original volume and then cooling them to 4°C, the sodium salt of 31,559 RP (a further 13.2 g.) is obtained.

The invention includes within its scope compositions for feeding to animals which contain 0.0001 to 99.9% by weight of 31,559 RP or a salt thereof with a metal or a nitrogen-containing base, and a substance capable of being consumed by the animals. (Any salt used must, of course, contain a cation which is non-toxic at the concentration and dosage administered.) Such compositions may comprise a mixed feedstuff for animals or a concentrated mixture for feeding animals, 31,559 RP or a salt thereof with a metal or a nitrogen-containing base, and optionally another anticoccidial agent. These compositions are particularly useful for combating coccidiosis in birds and more especially in poultry.

The dose necessary to produce a suitable effect can of course vary within rather wide limits depending on the proportion of the feedstuffs themselves. In general terms, it is sufficient for the feedstuffs fed to the animals to contain 0.005 to 0.04% by weight of 31,559 RP or a salt thereof.

The 31,559 RP or salt thereof can be distributed as a uniform dispersion throughout the complete feedstuffs, at the above concentration.

It can also be distributed, at a dose of .025 to 1%, throughout supplementary feedstuffs, most frequently together with other additives such as vitamins and inorganic salts. These supplementary feedstuffs can either be mixed with the main ration or consumed as they are; they usually represent 5 to 20% of the main ration.

The "premixes", used to prepare the complete rations or supplementary feedstuffs, usually contain 0.05 to 20% of 31,559 RP or a salt thereof, diluted in a feedstuff extender. They form a convenient intermediate which makes it easier to distribute the active product uniformly throughout the feedstuffs. The premixes themselves are generally produced from concentrates which contain 99.9 to 20% of 31,559 RP or a salt thereof, to which edible denaturing agents such as edible dyestuffs, flavouring substances, dispersing agents or agents which prevent agglomeration and feedstuff extenders have been added.

The concentrates and premixes are generally pulverulent. The supplementary feedstuffs and the complete feedstuffs can be either pulverulent or in the form of granules prepared by the usual techniques.

The following Example (in which the percentages are by weight) illustrates compositions according to the invention.

Example A.

A base feedstuff having the following composition is prepared:

Cereal middlings flour	13.41%
Barley flour	13.41%
Maize flour	13.41%
Wheat flour	31.32%
Fishmeal	8.92%

	Soya flour	8.92%	
	Dehydrated fodder flour	4.56%	
	Yeast extract	2.33%	
	Dry milk powder	2.68%	
5	Sodium chloride	0.09%	5
	Calcium chloride	0.89%	
	Mineral elements	0.06%	
	Vitamin-type complex:		
	Vitamin A	4,000 I.U./kg.	
10	Vitamin D ₃	1,000 I.U./kg.	10
	Choline chloride	11.5 mg./kg.	
	Riboflavin	2.24 mg./kg.	

0.02% of the sodium salt of 31,559 RP is added to and distributed uniformly throughout this feedstuff.

The invention also provides compositions based on 31,559 RP or a salt thereof which can be used as a growth factor for ruminants (cattle, sheep and goats) and as an agent for combating dysentery in pigs.

These compositions consist of mixed feedstuffs for ruminants or pigs and of concentrated mixtures for feeding animals, which contain 31,559 RP or a salt thereof.

The dose necessary to produce a suitable effect can of course vary within wide limits depending on the species of animal and on the nutrient value of the feedstuffs themselves.

It is particularly advantageous to provide the animals with a daily amount of 31,559 RP of between 50 and 250 mg. per head of animal.

The 31,559 RP is preferably distributed throughout supplementary feedstuffs which contain 0.01 to 0.1% of the antibiotic, most frequently together with other additives such as vitamins and inorganic salts. These supplementary feedstuffs can be either mixed with the ration or consumed as they are, and usually represent approximately 1 to 10% of the daily ration; 31,559 RP thus corresponds to 0.0001—0.01% of the daily ration.

The "premises", used to prepare the complete rations or supplementary feedstuffs, generally contain 0.1 to 5% of 31,559 RP diluted in a feedstuff extender. They form a convenient intermediate which makes it easier to distribute 31,559 RP uniformly throughout the feedstuffs. The "premises" themselves are generally produced from concentrates which contain 99.9 to 5% of 31,559 RP to which edible denaturing agents such as edible dyestuffs, flavouring substances, dispersing agents or agents which prevent agglomeration and feedstuff extenders have been added.

The concentrates and "premises" are generally pulverulent. The supplementary feedstuffs can be either pulverulent or in the form of granules prepared in accordance with the usual techniques. In these compositions, 31,559 RP or its salts can be in the form of fine particles which are free or covered with a coating.

The following Example illustrates the use of 31,559 RP as a growth factor:

EXAMPLE B.

Twelve young bullocks of the Holstein breed are placed in individual stalls, the average surface area of which is 48 m², and are then weighed. The feedstuffs to be investigated are dispensed from day 0, the day on which the experiment was started. The bullocks are then weighed after 28 days and then after 56 days, and the weight of the feedstuffs consumed during this period is determined. The average gains in weight and the average degree of conversion of the feedstuff are

determined for each feedstuff investigated within the time intervals 0—28 days and 28—56 days.

The animals are fed twice per day. The daily ration is determined in such a way that the bullocks absorb the maximum amount of food without leaving any. In the experiment, the young bullocks are divided into 3 groups. Each animal receives a base feedstuff which consists of maize, hay and a protein supplement.

Each animal of group I receives the base feedstuff. Each animal of group II receives the base feedstuff in which the protein supplement contains 100 mg. of the antibiotic 31,559 RP. Each animal of group III receives the base feedstuff in which the protein supplement contains 200 mg. of the antibiotic 31,559 RP. The average daily ration per animal has the following composition:

	Group I	Group II	Group III
Maize	8.86 kg.	8.96 kg.	8.91 kg.
Hay	1.46 kg.	1.45 kg.	1.47 kg.
Protein supplement*	0.49 kg.	0.5 kg.	0.51 kg.
Antibiotic 31,559 RP	0	100 mg.	200 mg.

* The protein supplement used in "PRO BLEND 50" supplied by Messrs. Landmarck, Inc., which consists of:

Total protein equivalent >50%
Lipids > 1%
Vegetable fibres < 9%
Vitamin A >65,000 units/kg.

The results obtained are given in the following tables:

I—Average weight in kg. (per animal)

	On day 0	After 28 days	After 56 days
Group I	285.1	325.2	374.5
Group II	284.9	325.2	381.1
Group III	286.6	333	387

II-Variation in the average weight and degree of conversion

	0-28 days			28-56 days			0-56 days		
	Group I	Group II	Group III	Group I	Group II	Group III	Group I	Group II	Group III
Average gain in weight, in kg., per animal	40.1	40.3	46.4	49.3	55.9	54	89.4	96.2	100.4
Average gain in weight, in kg., per day per animal	1.43	1.44	1.65	1.76	1.99	1.93	1.59	1.72	1.79
Average consumption, in kg., per day per animal	9.83	9.76	9.84	11.78	12.5	11.94	10.81	10.9	10.89
Degree of conversion	6.86	6.77	5.94	6.70	6.04	6.19	6.77	6.34	6.08

Thus, during the first 28 days, the animals receiving 100 mg. of 31,559 RP per head and per day have a gain in weight of 3% more and a degree of conversion of 1.3% less than those of the control animals, and these percentages are respectively 15.3% and 13.4% in the case of the animals receiving 200 mg. of 31,559 RP per head per day.

During the second period (28-56 days), the animals receiving 100 mg. of 31,559 RP per day overtake those receiving the higher dose. The gains in weight are then 13.4% higher and the degrees of conversion 9.8% lower than those of the controls at the dose of 100 mg./day and these percentages are respectively 9.6% and 7.6% for the dose of 200 mg./day. Over the total period of the experiment (56 days), the average daily gains are improved by 7.8% and 12.2% and the degrees of conversion by 6.4% and 10.2% relative to the controls, at the respective doses of 100 and 200 mg. of 31,559 RP per day.

WHAT WE CLAIM IS:-

1. The antibacterial and antiseptic hereinbefore designated 31,559 RP, and its salts with metals and nitrogen-containing bases, having, in the form of the sodium salt, the properties:
it is a white micro-crystalline powder which is practically insoluble in water, sparingly soluble in hydrocarbon solvents, soluble in alcohols, ketones, dimethyl-formamide and ethyl acetate, and readily soluble in chlorinated solvents:
its elementary composition is approximately:
C=63.2%, H=8.8%, O=24.8%, Na=2.9%;



II-Variation in the average weight and degree of conversion

	0-28 days			28-56 days			0-56 days		
	Group I	Group II	Group III	Group I	Group II	Group III	Group I	Group II	Group III
Average gain in weight, in kg., per animal	40.1	40.3	46.4	49.3	55.9	54	89.4	96.2	100.4
Average gain in weight, in kg., per day per animal	1.43	1.44	1.65	1.76	1.99	1.93	1.59	1.72	1.79
Average consumption, in kg., per day per animal	9.83	9.76	9.84	11.78	12.5	11.94	10.81	10.9	10.89
Degree of conversion	6.86	6.77	5.94	6.70	6.04	6.19	6.77	6.34	6.08

Thus, during the first 28 days, the animals receiving 100 mg. of 31,559 RP per head and per day have a gain in weight of 3% more and a degree of conversion of 1.3% less than those of the control animals, and these percentages are respectively 15.3% and 13.4% in the case of the animals receiving 200 mg. of 31,559 RP per head per day.

During the second period (28-56 days), the animals receiving 100 mg. of 31,559 RP per day overtake those receiving the higher dose. The gains in weight are then 13.4% higher and the degrees of conversion 9.8% lower than those of the controls at the dose of 100 mg./day and these percentages are respectively 9.6% and 7.6% for the dose of 200 mg./day. Over the total period of the experiment (56 days), the average daily gains are improved by 7.8% and 12.2% and the degrees of conversion by 6.4% and 10.2% relative to the controls, at the respective doses of 100 and 200 mg. of 31,559 RP per day.

15 WHAT WE CLAIM IS:-

1. The antibacterial and antioocidial hereinbefore designated 31,559 RP, and its salts with metals and nitrogen-containing bases, having, in the form of the sodium salt, the properties:

it is a white micro-crystalline powder which is practically insoluble in water, sparingly soluble in hydrocarbon solvents, soluble in alcohols, ketones, dimethyl-formamide and ethyl acetate, and readily soluble in chlorinated solvents:

its elementary composition is approximately:
C=63.2%, H=8.8%, O=24.8%, Na=2.9%;

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1,495,858

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its melting point is 220°C (with decomposition);

its optical rotation ($c=1.050$ in methanol)

$[\alpha]_D^{20}=+51.2^{\circ}\pm 1^{\circ}$ $[\alpha]_{436}^{20}=+101^{\circ}\pm 1.5^{\circ}$ $[\alpha]_{435}^{20}=+167^{\circ}\pm 2^{\circ}$;

it does not show any characteristic absorption up to 210 nm in the ultraviolet; its infra-red spectrum (determined on tablets of a mixture with potassium bromide) shows absorption bands at (in wave numbers):

3430 S, 3180 S, 2970 vS, 2935 S, 2925 sh, 2880 S, 2825 m, 2600 sh, 2060 vw, 1945 vw, 1850 vw, 1760 vw, 1730 vw, 1630 sh, 1590 S, 1450 S, 1398 S, 1380 S, 1372 sh, 1362 sh, 1350 sh, 1335 sh, 1298 sh, 1292 m, 1260 sh, 1238 S, 1220 sh, 1200 S, 1190 sh, 1185 sh, 1170 S, 1152 w, 1140 sh, 1115 S, 1108 sh, 1092 vS, 1090 sh, 1072 S, 1065 sh, 1040 S, 1018 S, 1000 sh, 985 S, 968 vS, 940 S, 928 m, 912 S, 890 m, 870 S, 852 m, 828 m, 815 sh, 792 sh, 788 m, 775 sh, 765 sh, 748 m, 705 m, 655 sh, 635 m, 610 w, 590 vw, 570 sh, 565 w, 550 w, 535 w, 500 vw, 482 vw, 420 m, 378 w, 350 m cm^{-1} ;

in ascending chromatography on a thin layer of silica gel using, as solvent, a mixture of ethyl acetate, cyclohexane, water and butanol (50—50—25—5 by volume) it has an R_f of 0.5, and using, as solvent, a mixture of methylene chloride and methanol (94/6 by volume) it has an R_f of 0.7.

2. A process for the preparation of 31,559 RP and its salts, which comprises culturing *Streptomyces hygroscopicus* DS 24,367 (NRRL 5787), or a productive mutant thereof, aerobically in a culture medium, and isolating the 31,559 RP formed during the culture as such or as a salt.

3. Process according to claim 2, in which the pH of the culture medium at the start of the culture is between 5.8 and 7.8.

4. Process according to claim 3, in which the pH of the culture medium at the start of the culture is between 6.2 and 7.4.

5. Process according to any of claims 2 to 4 in which the temperature of the culture is between 23° and 33°C.

6. Process according to claim 5, in which the temperature of the culture is between 25° and 30°C.

7. Process according to any of claims 2 to 6, in which the culture medium is aerated at a rate of 0.3 to 3 litres of air per litre of medium per minute.

8. Process according to any of claims 2 to 7, in which the culture is continued for 2 to 8 days.

9. Process according to any of claims 2 to 8, in which the 31,559 RP is isolated by filtering the culture at a pH of 3 to 6 and extracting the 31,559 RP from the filter cake with an organic solvent therefor.

10. Process according to claim 2 substantially as described in the foregoing Example.

11. 31,559 RP or a salt thereof when produced by the process of any of claims 2 to 10.

12. Composition for feeding to animals, comprising 0.0001 to 99.9% by weight of 31,559 RP (as the acid or as a salt with a metal or a nitrogen-containing base) and a substance capable of being consumed by the animals.

13. Composition according to claim 12, in the form of a concentrate comprising 0.05 to 99.9% by weight of the 31,559 RP and an edible substance suitable for mixing with animal feedstuffs.

14. Composition according to claim 12, in the form of a supplementary feed for animals, comprising 0.005 to 1% of the 31,559 RP.

15. Composition according to claim 12, comprising 0.0001 to 0.1% of the 31,559 RP and a ruminant feedstuff or supplementary feedstuff.

16. Composition according to claim 12, comprising 0.0001 to 0.1% of the 31,559 RP in combination with a pig feedstuff supplementary feedstuff.

17. Composition according to claim 12 substantially as hereinbefore described.

18. Method for treating or preventing coccidiosis which comprises administering to a non-human animal subject thereto an amount of 31,559 RP as such or as a salt sufficient to treat or prevent the coccidiosis.

19. Method for promoting the growth of ruminants which comprises administering to a ruminant a sufficient amount of 31,559 RP as such or as a salt to promote the growth of said ruminant.

20. Method or treating or preventing swine dysentery which comprises administering to pigs an amount of 31,559 RP as such or as a salt sufficient to treat or prevent the swine dysentery.



18

1,495,858

18

21. A culture of the micro-organism *Streptomyces hygroscopicus* DS 24,367 (NRRL 5787) in a synthetic culture medium containing a source of assimilable carbon, a source of assimilable nitrogen, and mineral elements and substantially free from other micro-organisms.

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