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PATENT SPECIFICATION

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DRAWINGS ATTACHED.



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COMPLETE SPECIFICATION.

The New Organic Compound Tylosin, its Hydrolysis Product
Desmycosin and Methods for Preparing Same.

We, ELI LILLY AND COMPANY, a Corporation of the State of Indiana, United States of America, of 740 South Alabama Street, City of Indianapolis, State of Indiana, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to novel organic compounds having antibacterial action. More particularly, it relates to the novel nitrogenous organic compounds Tylosin and Desmycosin and to methods for their preparation.

The invention provides a method of producing the organic compound Tylosin which comprises cultivating a Tylosin-producing strain of *Streptomyces fradiae* in an aqueous culture medium containing assimilable sources of carbon, nitrogen and inorganic salts, preferably under submerged aerobic conditions until a substantial amount of Tylosin is produced by said organism in said aqueous culture medium.

The invention further provides a method of producing Tylosin which comprises cultivating a Tylosin-producing strain of *Streptomyces fradiae* in an aqueous culture medium containing assimilable sources of carbon, nitrogen and inorganic salts, preferably under submerged aerobic conditions until a substantial amount of Tylosin is produced by said organism in said aqueous culture medium, and recovering the Tylosin from said culture medium.

Tylosin may be hydrolysed with dilute acid to produce the new organic compound Desmycosin.

[Price 4s. 6d.]

There are provided by this invention novel organic bases which have been arbitrarily named and are referred to herein as Tylosin and Desmycosin. The novel bases have closely related chemical, pharmacological, and antimicrobial properties. Acid hydrolysis of Tylosin results in the formation of Desmycosin by the removal from the Tylosin molecule of a sugar moiety which is mycarose. Both of the novel bases form acyl derivatives and acid addition salts, and such are included within this invention. The chemical, physical, and other properties of the novel organic bases as hereinafter set forth, identify the compounds and differentiate them from previously known or described compounds.

The free base form of Tylosin has the following characteristics:—It is a white solid. It readily crystallizes as small plates from a number of crystallizing solvents such as aqueous acetone, aqueous lower alcohols, having from 1 to 4 carbon atoms; such as aqueous ethanol, and water. The free base which exhibits inverse solubility can be crystallized from water by dissolving it in water at a temperature of about 2° C., and gradually warming the solution to room temperature or slightly above whereupon the Tylosin separates in crystalline form. The dried crystals of Tylosin melt at 127—132° C.

The free base of Tylosin is soluble in slightly acidic, aqueous solutions, for example, 5 per cent aqueous acetic acid. Additionally, it is soluble in most polar organic solvents, illustrative examples of which are lower ketones, having from 3 to 6 carbon atoms, such as acetone, methyl ethyl ketone, and methyl isobutyl ketone.

lower alcohols such as methanol and ethanol esters such as ethyl acetate and ethyl formate, halogenated hydrocarbons such as methylene chloride and chloroform, ethers such as diethyl ether, nitrogen-containing organic solvents such as dimethyl formamide, pyridine and triethylamine, and heterocyclic organic solvents such as tetrahydrofuran and thiophene. It is soluble in benzene, but is relatively insoluble in non-polar solvents of the aliphatic hydrocarbons class, for example, hexane and heptane. It is slightly soluble in water and in mildly alkaline aqueous solutions.

The free base of Tylosin is relatively stable in aqueous solution over a pH range of pH 4 to pH 9, but is unstable in strongly acidic and strongly basic solutions. When Tylosin is treated with an acid solution having a pH lower than about pH 4, degradation of the free base soon occurs, yielding Desmycosin, and a carbohydrate fragment, which as mentioned before, is mycarose.

An electrometric titration of Tylosin in dimethyl formamide-water solution (2:1 parts by volume) reveals the presence of one titratable group of $pK^a=7.0$.

The molecular weight as determined by the titration data is 930 ± 30 .

A solubility analysis of the crystalline base in water showed Tylosin to be soluble to the extent of 6.0 mg./ml. at 26.0°C .

An average of several elemental analyses of the crystalline base dried *in vacuo* at about 80°C . over phosphorus pentoxide gave the following values:—

Carbon	...	...	59.68 per cent
Hydrogen	...	...	8.53 per cent
Nitrogen	...	...	1.55 per cent
Oxygen (by difference)	...	...	30.24 per cent

formula

The above data indicate an empirical data of $\text{C}_{16}\text{H}_{17}\text{NO}_{11}$, but as will be realized, this formula is necessarily only an approximation, and may be subject to slight revision if and when the complete molecular structure of the compound is elucidated.

The infra-red absorption curve of the crystalline free base in chloroform is shown in Figure I of the accompanying drawings. The distinguishable bands in the infra-red spectrum over the range of 2.0μ to 15.0μ are as follows: 2.85, 3.36, 3.41 (shoulders at 3.47, 3.52, and 3.58), 3.68, 5.80, 5.94, 6.27, 6.88, 7.09, 7.27, 7.36, 7.61, 7.90, 8.45, 8.62, 8.75, 8.95, 9.28, 9.53, 9.85, 10.04, 10.13, 10.40, 10.80, 11.10, 11.53, and 11.91.

An ultraviolet absorption spectrum of Tylosin water shows an intense absorption maximum at about $288 \text{ m}\mu$ having a molar extinction of $E_{1\%}^{1\text{cm}}=241$.

The specific optical rotation of a sample of the crystalline free base of Tylosin dried

at room temperature *in vacuo* over anhydrous calcium chloride for about 15 hours is as follows $[\alpha]_{\text{D}}^{25} = -46.3^\circ$; $C=2$ per cent in methanol (weight per volume).

A powder X-ray diffraction pattern using unfiltered chromium radiation and α wavelength value of $2.2896 \text{ \AA}$. in calculating the interplanar spacings gives the following values:—

"d"	I/I ₁	
7.72	0.40	
6.50	1.00	
6.12	0.40	75
5.74	0.40	
5.18	0.40	
4.71	0.08	
4.54	0.16	
4.37	0.60	80
4.23	0.02	
4.12	0.08	
3.84	0.12	
3.68	0.12	
3.63	0.12	85
3.47	0.12	
3.31	0.08	
3.19	0.12	
3.05	0.02	
2.97	0.20	
2.84	0.02	
2.74	0.04	
2.68	0.04	
2.47	0.02	
2.39	0.04	
2.33	0.04	
2.25	0.04	
2.14	0.02	
2.032	0.02	
1.908	0.02	100

Chemical tests made on the crystalline free base show the presence of methoxy, hydroxy, N-methyl, and C-methyl groups. The free base gives positive Molisch and anthrone tests for the presence of carbohydrate. It decolorizes permanganate solution. The crystalline base gives negative results in the ninhydrin, biuret, and Sakaguchi protein tests, gives a negative ferric chloride test for phenolic groups, and gives a negative maltol test.

Chromatography of crystalline Tylosin base on Whatman No. 1 paper gives the following R_f values: $R_f=0.36$ in a solvent of water containing 7 per cent magnesium sulfate (weight-volume) and 2.5 per cent methyl ethyl ketone (volume-volume); $R_f=0.86$ in a solvent of *n*-butanol saturated with water; $R_f=0.09$ in a solvent of methyl isobutyl ketone saturated with water and containing 2 per cent *p*-toluenesulfonic acid mono-hydrate (weight-volume); and $R_f=0.83$ in a solvent of methyl ethyl ketone saturated with water. In determining the



Chemical

foregoing values, the Tylosin base was applied to the paper as an acetone solution.

The free base of Tylosin has an inhibitory action against growth of microbial organisms including both gram-positive and gram-negative bacteria, some of which are plant pathogens. However, the preponderance of organisms against which it has inhibitory action are gram-positive. The levels at which Tylosin shows inhibition against the growth of illustrative organisms are numerically set forth in Table I. The inhibitory levels were determined by the agar-dilution test or by the broth-dilution test (identified in the table by the letters "ad" and "bd" respectively).

In the agar-dilution test, the test organism is streaked or implanted on a series of agar plates containing various concentrations of Tylosin to determine the minimum con-

centration of Tylosin base in mcg./ml. (micrograms per milli-liter) in an agar substrate which inhibits the growth of the organism over a period of forty-eight hours (or seventy-two hours in the case of the plant pathogen organisms).

In the broth-dilution test, a series of tubes containing nutrient broth having various concentrations of Tylosin are inoculated with the test organism to determine the minimum concentration of Tylosin base in mcg./ml. in the broth substrate which inhibits organism growth for a period of about twenty hours.

The acyl derivatives of Tylosin, and the acid addition salts of both the free base and the acyl derivatives possess activities of about the same value as those given in Table I, making allowances for the increased molecular weights of the acyl derivatives and salts.

TABLE I.

Test Organism	Inhibitory Concentration (mcg./ml.)
BACTERIA	
<i>Staphylococcus aureus</i>	1.56 ad
<i>Staphylococcus albus</i>	3.13 ad
<i>Bacillus subtilis</i>	1.56 ad
<i>Mycobacterium phlei</i>	.78 ad
<i>Mycobacterium tuberculosis</i> (607)	.78 ad
<i>Mycobacterium avium</i>	.78 ad
<i>Escherichia coli</i>	>100. ad
<i>Proteus vulgaris</i>	50. ad
<i>Pseudomonas aeruginosa</i>	>100. ad
<i>Aerobacter aerogenes</i>	>100. ad
<i>Klebsiella pneumoniae</i>	12.5 ad
<i>Salmonella enteritidis</i>	>100. ad
<i>Shigella paradysenteriae</i>	100. ad
<i>Brucella bronchiseptica</i>	25. ad
<i>Vibrio metschnikovii</i>	50. ad
<i>Streptococcus pyogenes</i>	0.195 bd
<i>Corynebacterium diphtheriae</i>	0.0975 bd
<i>Diplococcus pneumoniae</i>	0.195 bd
BACTERIAL PLANT PATHOGENS	
<i>Erwinia amylovora</i>	100. ad
<i>Agrobacterium tumefaciens</i>	>100. ad
<i>Xanthomonas campestris</i>	25. ad
<i>Xanthomonas malvacearum</i>	12.5 ad
<i>Xanthomonas phaseoli</i>	6.25 ad
<i>Pseudomonas syringae</i>	25. ad
<i>Corynebacterium insidiosum</i>	100. ad
<i>Corynebacterium sepdonicum</i>	—

It will be noted from the foregoing table that Tylosin has a relatively broad antimicrobial activity. This activity can be demonstrated not only *in vitro*, but also *in vivo*. Thus, administration of Tylosin to mice infested with various pathogenic organisms results in the prompt elimination of the

infection, whether the drug is administered subcutaneously, intraperitoneally, or orally. The ED_{50} of Tylosin (effective dosage to protect 50 per cent of the mice used as test animals) in illustrative infections is set forth in Table II.

TABLE II.

5	Infecting Organism	Route of Administering Tylosin	ED ₅₀ mg. of Tylosin/kg. body weight
10	<i>Staphylococcus aureus</i> <i>Staphylococcus aureus</i> <i>Diplococcus pneumoniae</i> <i>Meningopneumonitis virus</i> <i>Streptococcus Pyogenes</i> C203 <i>Streptococcus haemolyticus</i>	Subcutaneous Oral Subcutaneous Intraperitoneal Subcutaneous Oral	3.8 (× 2) 42 (× 2) 4.8 (× 2) 41.5 (× 5) 2.6 (× 2) 65 (× 2)

antimicrobial

15 In mice, the administration of Tylosin is effective in overcoming infections of *Spirochaeta novyi*, the orally effective dose being about 31 mg./kg. of body weight, and the intraperitoneally effective dose being about 2 mg./kg. of body weight.

20 Desmycosin, its acyl derivatives, and its acid addition salts, to be described hereinafter, have antimicrobial activities substantially the same as those set forth above for Tylosin.

25 Tylosin can be produced by culturing newly found and hitherto undescribed organism strains isolated from soil samples obtained from Nongkhai, Thailand. The organisms were grown using submerged culture fermentation under aerobic conditions in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts. The organisms were isolated from the above soil samples by suspending portions of the soil samples in sterile distilled water, and by streaking the suspensions on nutrient agar. The seeded nutrient agar plates were incubated at about 25–30° C. for several days. At the end of the incubation time, colonies of the Tylosin-producing organisms were transferred with a sterile platinum loop to inoculate agar slants. The inoculated agar slants were incubated to provide larger amounts of inoculum for the production of Tylosin.

45 The novel organisms capable of producing Tylosin have been placed on permanent deposit with the Culture Collection of the Northern Utilization Research and Development Branch of the U.S. Department of Agriculture, Peoria, Illinois, and have been assigned the culture numbers NRRL 2702 and NRRL 2703.

50 The newly discovered organisms are in many respects quite similar to the species *Streptomyces fradiae*, a number of the order Actinomycetales, as defined in Bergey's Manual of Determinative Bacteriology, 7th Edition, page 799. The novel organisms NRRL 2702 and NRRL 2703 have been classified as new strains of the species *S. fradiae*, an organism first identified by Waksman and now obtainable from the American Type Culture Collection as ATCC

10745 (Waksman 3435). One notable difference between our novel strains and the Waksman strain of *S. fradiae* lies in the failure of the Waksman strain to produce Tylosin or Desmycosin. The Waksman strain has been cultured in the preferred media herein described, but no detectable amount of Tylosin or Desmycosin has ever been observed.

This invention will be described in detail with particular reference to the newly found organism, *S. Fradiae* NRRL 2702. However, it is to be understood that the production of Tylosin by the cultivation of other Tylosin producing strains of *Streptomyces fradiae* such as *S. Fradiae* NRRL 2703 or any Tylosin producing mutants of *S. fradiae* NRRL 2702 and *S. fradiae* NRRL 2703 are within the scope of this invention. Such mutants can be produced by known procedures, as by subjecting a Tylosin-producing *Streptomyces fradiae* to X-ray or ultraviolet irradiation, or to chemical agents, for example, the nitrogen mustards.

In the following paragraphs, the results of a detailed taxonomic study of the above Tylosin-producing strain of *S. fradiae*, NRRL 2702, are set forth. The colors used in the description are in accordance with the definitions used in Ridgway: Culture Standards and Nomenclature (1912).

MICROSCOPIC MORPHOLOGY.

Tomato paste-oatmeal agar (14 days at 30° C.). Spore chains are in hooks, loops, and irregular coils. Typical spirals are rarely found. Spores are sub-globose in shape and range from about 0.8μ to about 1.5μ.

Inorganic salts-starch agar (14 days at 30° C.). Microscopic morphology like that observed on tomato paste-oatmeal agar.

Inorganic salts-ribose agar (14 days at 30° C.). Microscopic morphology like that observed on tomato paste oatmeal agar.

CULTURAL CHARACTERISTICS.

Czapek's agar (14 days at 30° C.). Growth fair. No aerial mycelium. Reverse near Cream Color.

Calcium malate agar (14 days at 30° C.).

65

70

75

cap

cap

80

85

90

95

100

110

Nomenclature procedures

Growth derate. Cream Buff in areas. Reverse Naples Yellow.

Glucose-Asparagine agar (14 days at 30° C.) Growth very scant.

5 *Inorganic salts-starch agar* (14 days at 30° C.). Growth abundant. Aerial mycelium moderate, white to Pale Ochraceous Salmon. Reverse near Cream Buff.

10 *Tomato paste-oatmeal agar* (14 days at 30° C.). Growth abundant. Aerial mycelium moderate, from white to Vinaceous Buff.

15 *Emerson's agar* (14 days at 30° C.). Growth moderate. Aerial mycelium fair, white. Reverse near Ochraceous Buff.

Yeast Extract agar (14 days at 30° C.). Growth moderate. Aerial mycelium fair, white. Reverse Antimony Yellow.

Potato Plug agar (14 days at 30° C.). No visible growth.

PHYSIOLOGY.

Gelatin liquefaction—slow.

Nitrate reduction—reduces nitrates.

H₂S production—none.

25 Starch hydrolysis—fair.

Temperature—26° C.—30° C. growth optimum.

37° Temperature—32° C.—37° C. sporulation optimum.

30 No aerial mycelium production at 26° C. In Table III are set forth the results of carbon utilization tests carried out on organism NRRL 2702. In the table, the following symbols are employed:

35 + = growth and utilization

- = no growth, no utilization.

± = limited growth probably poor utilization.

TABLE III.

CARBON UTILIZATION PATTERN FOR NRRL 2702.

Compound	Growth Response
L (+) Arabinos	+
L (+) Rhamnose	±
D (+) Ribose	-
D (+) Xylose	+
D (-) Fructose	+
D (+) Glucose	+
50 D (+) Melibiose	-
Sucrose	+
D (+) Trehalose	+
D (+) Melizitose	-
D (+) Raffinose	-
55 Cellulose	-
Inulin	-
Adonitol	-
i-Inositol	-
Mannitol	±
60 Sodium acetate	+
Salicin	-

As noted, Tylosin can be produced by cultivation of *Streptomyces fradiae* NRRL 2702. The culture medium can be any one of a number of media, since, as is apparent from the above-described utilization tests, the organism is capable of utilizing many energy sources. However, for economy of production, maximum yields of antibiotic, and ease of isolation of the antibiotic certain culture media are preferable. The media which are useful in the production of Tylosin include an assimilable source of carbon such as glucose, sucrose, fructose, starch, glycerine, molasses, dextrin, brown agar, or corn steep solids. The preferred sources of carbon are glucose and starch. Additionally, employable media include a source of assimilable nitrogen such as linseed meal, tankage, fish meal, cotton seed meal, oatmeal, ground wheat, soybean meal, beef extract, peptones (meat or soy), casein, amino acid mixtures, and the like. Preferred sources of nitrogen are soybean meal, casein, and corn steep solids.

Inorganic salts, for example, those providing sodium, potassium, ammonium, calcium, magnesium, cobalt, sulfate, chloride phosphate, carbonate, acetate, and nitrogen ions, and a source of growth factors such as distiller's solubles and yeast extract, can be incorporated into the media with beneficial results.

As is necessary for the growth and development of other microorganisms, essential trace elements should also be included in the culture medium for growing the actinomycete employed in this invention. Such trace elements are commonly supplied as impurities incidental to the addition of the other constituents of the medium.

The initial pH of the culture medium can be varied widely. However, it has been found desirable that the initial pH of the medium be between about pH 5.5 and about pH 8.0, and preferably about pH 6.5 to about pH 7.0. As has been observed with other actinomycetes, the pH of the medium gradually increases throughout the growth period of the organism during which time Tylosin is produced, and may attain a pH from about pH 7.2 to about pH 8.0 or above, the final pH being dependent at least in part on the initial pH of the medium, the buffers present in the medium, and the period of time the organism is permitted to grow.

Submerged, aerobic cultural conditions are the conditions of choice for the production of large amounts of Tylosin. For preparation of relatively small amounts, shake flasks and surface culture in bottles can be employed, but for the preparation of large amounts, submerged aerobic culture in sterile tanks is preferred. The medium in the sterile tank can be inoculated with a sporulated suspension, but because of the

growth lag experienced when a sporulated suspension is used as the inoculum, the vegetative form of the culture is preferred. By thus avoiding the growth lag, more efficient use of the fermentation equipment is realized. Accordingly, it is desirable first to produce a vegetative inoculum of the organisms by inoculating a relatively small quantity of culture medium with the spore form of the organism, and when a young, active, vegetative inoculum has been obtained, to transfer the vegetative inoculum aseptically to the large tank. The medium in which the vegetative inoculum is produced can be the same or a different medium than that utilized for the large scale production of Tylosin.

The organisms grow best at temperatures in a range of 25° C. to 32° C. Optimal Tylosin production appears to occur at a temperature of about 26–30° C.

As is customary in submerged culture processes, sterile air is blown through the culture medium. For efficient growth of the organism and Tylosin production, the volume of air employed in the tank production of Tylosin preferably is upwards of 0.1 volume of air per minute per volume of culture medium. Efficient growth and optimal yields of Tylosin are obtained when the volume of air used is at least one volume of air per minute per volume of culture medium.

The concentration of Tylosin activity in the culture medium can readily be followed during the fermentation period by testing samples of the culture medium for their inhibitory activity against the growth of an organism known to be inhibited in the presence of Tylosin. The use of the organism *Staphylococcus aureus* has been found to be very satisfactory for this purpose. The testing can be carried out by the known turbidimetric or cup-plate methods.

In general, maximum production occurs after inoculation of the culture medium within about 2 to 5 days when submerged aerobic culture or shake flask culture is employed, and within about 5 to 10 days when surface culture is used.

The mycelium undissolved solids are removed from the fermentation broth by conventional means such as by filtration or centrifugation. The antibiotic is removed from the filtered or centrifuged broth by employing adsorption or extraction techniques. It is preferred to employ an extraction procedure in recovering the antibiotic from the filtered broth since such extraction procedures are usually more rapid, efficient, and economical in antibiotic recovery. For this extraction of the antibiotic substance from the filtered broth, water-immiscible, polar, organic solvents are preferred, such including esters of fatty acids, for example,

ethyl acetate and amyl acetate; chlorinated hydrocarbons, for example, chloroform, ethylene dichloride, and trichloroethylene; water-immiscible alcohols, for example, butyl and amyl alcohols; water-immiscible ketones, for example, methyl isobutyl ketone and methyl amyl ketone; and ethers, for example, diethyl ether and methyl propyl ether. Chloroform and amyl acetate are the presently preferred extraction solvents.

In the use of adsorption techniques, various adsorbants and ion exchange resins can be used, for example, carbon, silica gel, alumina, and ion exchange resins of acidic character such as "XE" 64 and "IRC" 50 (weakly acidic cation exchange resins of the carboxylic acid type sold by Rohm and Haas Company), carboxymethyl cellulose resin, and "Dowex" 50 (a strongly acidic cation exchange resin sold by The Dow Chemical Company). "Dowex" is a Registered Trade Mark. The compound Tylosin can be adsorbed on one of the above-named adsorbants from chloroform, acetone, or benzene solution. The adsorbed Tylosin can then be eluted from the adsorbant as by washing the adsorbant on which the Tylosin is adsorbed, with a lower alcohol, having from 1 to 4 carbon atoms, such as methanol or ethanol, or with a lower alcohol containing up to about 50 per cent of a lower ketone having from 3 to 6 carbon atoms such as acetone.

The organic solvent extracts obtained by the preferred extraction method can be directly evaporated to dryness to provide crude Tylosin preparations. However, the organic solvent extracts preferably are used in obtaining purified preparations of Tylosin. For example, purified Tylosin can be obtained by concentrating *in vacuo* an organic solvent extract of Tylosin to a small volume, by decolorizing the Tylosin concentrate with carbon, and precipitating the Tylosin by the addition of a nonpolar solvent such as, for example, a 2- to 10-fold excess of petroleum ether. The precipitate thus obtained is a solid, purified Tylosin which is usually amorphous. The amorphous precipitate can be crystallized by employing one of the crystallizing solvents hereinabove mentioned.

As disclosed above, Tylosin is converted into Desmycosin by hydrolyzing Tylosin with dilute acid at about room temperature or above. Severe hydrolytic conditions should be avoided to prevent the destruction of the Desmycosin. Desmycosin can be obtained very readily simply by permitting an aqueous, acidic solution of Tylosin to stand at room temperature for a period of time sufficient to provide substantial quantities of Desmycosin as a hydrolytic product. It has been found that an excellent yield of Desmycosin is provided by dissolving Tylosin to a concentration of 4 per cent in

"Dowex"

an acidic, aqueous solution such as in dilute aqueous hydrochloric acid which is however, not to be so concentrated as to substantially destroy the Desmycosin, permitting the mixture to stand at room temperature for about three days, and then recovering the Desmycosin from the hydrolysis mixture by neutralizing the mixture, and extracting the Desmycosin with a water-immiscible solvent.

Desmycosin has substantially the same solubility properties and microbiological inhibitory properties as Tylosin. Desmycosin is characterized by the following specific physical and chemical properties: It is a white solid which is stable in solution in the range of pH 1 to pH 9. It can be obtained in crystalline form by crystallization from crystallizing solvents such as chloroform and acetonitrile. When crystallized from chloroform, the crystals of Desmycosin are blade-like in form, and are solvated by about an equimolar amount of chloroform. The chloroform solvate crystals of the free base of Desmycosin after air drying at room temperature melt at 95–115° C. Nonsolvated, crystalline Desmycosin can be obtained by heating the solvated crystals *in vacuo* at 75° C. for a few hours.

An electrometric titration of Desmycosin in diethyl formamide-water solution (2:1 parts by volume) reveals the presence of one titratable group of $pK'_a=8.0$.

The molecular weight as determined by the titration data is 785 ± 25 .

An average of several elemental analyses of the crystalline base dried *in vacuo* at 80° C. over phosphorus pentoxide, gave the following values:

Carbon	59.73 per cent
Hydrogen	8.34 per cent
Nitrogen	1.70 per cent
Oxygen (by difference)	30.23 per cent

The above data indicate for Desmycosin as empirical formula of about $C_{33}H_{47}NO_{14}$, which formula like that set forth for Tylosin is subject to possible slight modification.

The infra red absorption curve of the crystalline free base in chloroform solution is shown in Figure 2 of the accompanying drawings. The distinguishable bands in the infra red spectrum over the range of 2.0μ to 15.0μ are as follows: 2.76, 2.86, 3.38, 3.43, 3.48, 3.54, 3.69, 5.80, 5.95, 6.14, 6.26, 6.87, 7.11, 7.16, 7.25, 7.37, 7.60, 7.92, 8.48, 8.59, 8.77, 8.91, 9.26, 9.45, 9.92, 10.13, 10.40, 10.75, 11.11, 11.51, and 11.95.

An ultraviolet absorption spectrum of Desmycosin in water shows an intense absorption maximum of $288 m\mu$ having a molar extinction of $E_{1\%}^{1\text{cm}} = 275$.

The specific optical rotation of the crystalline free base of Desmycosin which was

dried at room temperature *in vacuo* over anhydrous calcium chloride for about 15 hours, is as follows: $[\alpha]_D^{25} = -15.75^\circ$; $C=2$ per cent in methanol (weight per volume).

A powder X-ray diffraction pattern using unfiltered chromium radiation and a wavelength value of $2.2896\text{\AA}$. in calculating the interplanar spacings gives the following values:

"d"	I/I ₁	
13.1	0.20	
11.6	0.20	75
10.1	0.20	
6.88	0.20	
6.16	0.50	
5.62	0.33	
5.27	1.00	80
5.03	0.13	
4.76	0.13	
4.39	0.33	
4.17	0.13	
4.01	0.20	85
3.77	0.20	
3.64	0.13	
3.51	0.13	
3.35	0.27	
3.09	0.13	90
2.91	0.13	
2.76	0.13	
2.61	0.13	
2.37	0.13	
2.25	0.43	95 0.03

Chemical tests made on the crystalline free base show the presence of methoxy, hydroxy, N-methyl, and C-methyl groups. The free base decolorizes permanganate solution, and gives a positive Molisch test for the presence of a carbohydrate. The crystalline base gives negative results in the ninhydrin, biuret, and Sakaguchi protein tests, gives a negative ferric chloride test for phenolic groups, and gives a negative maltol test.

Chromatography of Desmycosin on Whatman No. 1 paper gives the following R_f values: — $R_f = 0.65$ in a solvent of water containing 7 per cent magnesium sulfate (by weight) and 2.5 per cent methyl ethyl ketone (by volume); $R_f = 0.82$ in a solvent of *n*-butanol saturated with water; and $R_f = 0.64$ in a solvent of methyl ethyl ketone saturated with water. In determining the foregoing values, the Desmycosin was applied to the paper as an acetone solution.

Both Tylosin and Desmycosin readily form acyl derivatives, or esters, on acylation with, for example, and acid halide or the anhydride of an organic acid such as acetic, chloroacetic, propionic, or acrylic acid. Additionally, an organic acid of the foregoing character itself can be used as the



spectrum

absorption

acylating agent when a reagent such as N,N'-dicyclohexylcarbodiimide is also employed in the reaction mixture. It has been found desirable to employ lower alkylcarbacyl acylating agents, the resulting acylated compounds being lower alkylcarbacyl derivatives having from 2 to 6 carbonations in the carbacyl group or groups. The separation and purification of the novel acyl derivatives are effected by any of the commonly employed crystallisation techniques. The acyl derivatives have different melting points dependent upon the nature and number of acyl substituents and customarily have $pK'a$ values substantially lower than the $pK'a$ values of Tylosin and Desmycosin.

The acid addition salts of Tylosin and Desmycosin can be formed by employing an inorganic acid such as sulfuric, hydrochloric, or nitric acid, or an organic acid such as tartaric, gluconic, oxalic, or acetic acid. The acid addition salts can be prepared by dissolving the free base of Tylosin or Desmycosin in a solvent in which it is soluble, such as acetone or ether, and acidifying the solution with about an equimolar amount of the appropriate acid whereupon the salt usually precipitates out of solution. In the event the salt does not readily precipitate, it can be precipitated as by evaporating the solution to a smaller volume, or by adding a miscible solvent in which the salt is not soluble. Acid addition salts of the acyl derivatives of Tylosin and Desmycosin can also be prepared following the above procedures using acids such as those set forth above.

Because of their broad antibacterial spectrum and their highly effective antimicrobial action, the novel compounds of this invention are useful in many ways as sterilizing agents, both for the sterilization of laboratory and home utensils, and food products.

Additionally, the compounds are useful in the veterinary field for the treatment of infections such as turkey sinusitis. Effective control of sinusitis is obtained by direct injection into each infraorbital sinus of the afflicted bird of a solution containing about 5 to about 20 milligrams of Tylosin, Desmycosin, or an ester or acid addition salt thereof.

This invention is further illustrated by the following specific examples:—

EXAMPLE 1.

Preparation of Tylosin.

A sporulated culture of *Streptomyces fradiae* NRRL 2702 is produced by growing the organism on a nutrient agar slant having the following composition:—

Yeast extract	...	...	1.0 g.	
Beef extract	...	...	1.0 g.	
Hydrolyzed casein ("N-Z-AMINE-Type A" sold by the Sheffield Chemical Co.)	2	g.		65
Dextrin	...	...	10	g.
Cobaltous chloride heptahydrate	20	mg.		
Agar	...	...	20	g.
Water	...	...	1	L 70

The pH of the medium is adjusted to pH 7.3 by the addition of sodium hydroxide.

The slant is inoculated with spores of *Streptomyces fradiae* NRRL 2707 and is incubated for five days at about 30° C. The sporulated culture growth on the slant is covered with water, and the slant is scraped gently to remove the spores to provide an aqueous spore suspension.

1 ml. of the spore suspension is used for inoculating under aseptic conditions a 100 ml. portion of a sterile vegetative culture medium having the following composition:—

Glucose	...	...	15 g.	85
Soybean meal	...	...	15 g.	
Corn steep solids	...	...	15 g.	
Sodium chloride	...	...	5 g.	
Calcium carbonate	...	...	2 g.	
Tap water, added to make a total volume of	...	...	1 l.	90

The inoculated vegetative medium is incubated at about 30° C. for 48 hours, during which time the incubate is shaken at the rate of 114 cycles per minute on a reciprocal shaker having a 2-inch stroke.

5 ml. of the vegetative inoculum are used to inoculate aseptically 100 ml. portions of the following sterilized production medium contained in 500 ml. Erlenmeyer flasks:—

Soybean meal	...	...	15 g.	
Casein	...	...	1 g.	
Crude glucose syrup	...	...	20 ml.	
Calcium carbonate	...	...	2.5 g.	
Sodium nitrate	...	...	3 g.	105
Water, added to make a total volume of	...	...	1 l.	

The inoculated culture is incubated for 100 hours at about 26—28° C. During the incubation period, the incubate is shaken at 114 revolutions per minute on a reciprocal shaker having a 2-inch stroke. The pH of the starting medium is about pH 6.5. At the end of the incubation period, the pH of the medium increases to about pH 7.5.

The culture broth obtained is filtered to remove the mycelium and other undissolved solids. The filtered broth contains Tylosin in the amount of about 250 mcg. per ml. of broth.

EXAMPLE 2. Preparation of Tylosin.

A sporulated culture of the *Streptomyces fradiae* NRRL 2702 is produced by growing the organism on a nutrient agar slant having the following composition:—

Tomato Paste-Oatmeal Agar

Tomato paste	20 g.
Pre-cooked oatmeal	20 g.
Agar	15 g.
Water, added to make a total volume of	1 l.

The slant is inoculated with spores of *Streptomyces fradiae* NRRL 2702 and the inoculated slant is incubated for 9 days at a temperature of about 30° C. After incubation, the sporulated culture on the slant is covered with water, and the surface of the slant is scraped gently to remove the spores to obtain an aqueous spore suspension.

Employing aseptic techniques, one-half of the inoculum obtained from one agar slant is used for inoculating a 500 ml. portion of a sterilized vegetative culture medium having the following composition contained in a 2 l. Erlenmeyer flask:—

Corn-Steep Yeast I

Glucose	15 g.
Corn steep solids	5 g.
Yeast	5 g.
Calcium carbonate	3 g.
Water, added to make a total volume of	1 l.

The incubation is carried on at 28° C. for 48 hours with shaking at 110 cycles per minute on a reciprocal shaker having a 2-inch stroke.

0.25 gal. of the vegetative inoculum from the flask is added aseptically as an inoculum to 250 gal. of the sterile corn steep yeast I medium described above contained in an iron 350 gal. fermenter tank. 0.025 gal. of Antifoam A (an antifoam product sold by The Dow Corning Company) is added to the culture medium to prevent excessive foaming, and additional quantities are added as needed during the fermentation. The inoculated medium is fermented for 24 hours at a temperature of 28° C. During fermentation, the medium is aerated with sterile air at a rate of 27 cubic feet per minute and is agitated with two 16-inch impellers operated at 160 revolutions per minute.

To a 1700 gal. iron fermenter are added 1200 gal. of a medium having the following composition:—

Corn Steep Soy XII

Glucose	30 kg.	
Soybean oil meal	15 kg.	60
Corn steep solids	5 kg.	
Crude soybean oil	10 kg.	
Calcium carbonate	2 kg.	
Sodium chloride	5 kg.	
Water, added to make a total volume of	1000 l.	65

The medium is inoculated with 96 gal. of the inoculum grown in the fermentation tank. The fermentation is carried on at 28° C. for four days. The foam is controlled by the addition of "Larex" No. 1 (an antifoam product sold by Swift and Company) as needed. The fermentation medium is aerated by the addition of sterile air at the rate of 128 cubic feet per minute and is agitated with two 24-inch impellers operated at 130 revolutions per minute.

600 pounds of "Silflo" (Registered Trade Mark), a diatomaceous earth filter aid sold by The Silflo Company) are added to 1,000 gal. of the broth, and the mixture is filtered. The filtrate is adjusted to pH 8.5 by the addition of 20 per cent sodium hydroxide, 500 gal. of chloroform are added, the mixture is stirred for 30 minutes, and the chloroform layer which is in the form of an emulsion is decanted. The chloroform extraction is repeated twice with 500-gal. portions of chloroform. The chloroform emulsions which contain the Tylosin are combined and are passed through a DeLaval separator to break the emulsion. The separated chloroform solution obtained is concentrated *in vacuo* to a volume of 25 l., and the impurities are largely removed by passing the concentrate over a column 6 inches in diameter containing 10 kg. of activated carbon such as that sold by the Pittsburgh Coke and Chemical Co. The carbon column is washed with 16 l. of chloroform. The combined chloroform effluents containing the Tylosin are concentrated *in vacuo* to 2 l., and the chloroform concentrate is added slowly with stirring to 20 l. of petroleum ether, causing Tylosin to precipitate. After the mixture is stirred for 15 minutes, it is filtered to remove the white, amorphous precipitate of Tylosin which after drying *in vacuo* weighs about 1070 g.

The amorphous Tylosin is crystallized by dissolving it in 355 ml. of acetone, filtering the acetone mixture to remove a slight haze, and slowly adding the filtered acetone mixture with gentle stirring to 20 l. of water at 5° C. The resulting aqueous, acetone solution of Tylosin is permitted to stand at room temperature with gentle stirring to permit the acetone to evaporate slowly, whereupon Tylosin crystallizes out. The Tylosin crystals are removed by filtration and are

dried *in vacuo* at room temperature. They melt at about 127—132° C.

EXAMPLE 3.

Preparation of the tartrate salt of Tylosin.

- 5 5 g. of crystalline Tylosin are dissolved in 100 ml. of acetone, and 1.5 g. of d-tartaric acid dissolved in 20 ml. of acetone are added with stirring. The solution is permitted to stand at room temperature whereupon the tartrate salt of Tylosin crystallizes out of the solution. The crystals of the tartrate salt of Tylosin are removed by filtration, are washed with acetone, and are air-dried. The yield of the salt is 5.5 g. The crystalline salt melts at about 140—146° C.

EXAMPLE 4.

Preparation of gluconate salt of Tylosin.

- 20 1.03 g. glucono-delta lactone are dissolved in 10 ml. of water, and the aqueous solution is warmed to 85° C. for two hours to cause hydrolysis of the lactone to gluconic acid. 15 ml. of warm methanol are added to the aqueous solution. 5 g. of the crystals of Tylosin dissolved in 10 ml. of methanol are added to the aqueous methanol-mixture with stirring. The Tylosin methanol mixture is permitted to stand overnight at room temperature. Methanol is removed from the mixture by evaporation *in vacuo*. After the methanol is removed, 40 ml. of water is added to the aqueous Tylosin mixture. The diluted mixture is filtered, and the filtrate containing the Tylosin activity is freeze-dried, yielding 5.3 g. of a white solid consisting of the gluconate salt of Tylosin. The gluconate salt of Tylosin melts at about 114—117° C.

EXAMPLE 5.

Preparation of the hydrochloride salt of Tylosin.

- 40 890 mg. of Tylosin are dissolved in 200 ml. of ether. The ether mixture is acidified by the addition of 0.082 ml. of 12 N hydrochloric acid. The precipitate of the hydrochloride salt of Tylosin which forms is filtered off, is washed with ether, and is dried *in vacuo*. The hydrochloride salt of Tylosin is recrystallized from an ethanol-ether mixture. The hydrochloride salt of Tylosin melts at about 141—145° C.

EXAMPLE 6.

Preparation of the acetic acid ester of Tylosin.

- 55 14.3 g. of Tylosin are dissolved in 97 ml. of acetone containing 15.3 g. of potassium bicarbonate. 4.4 ml. of acetyl chloride in 5 ml. of acetone are added slowly with stirring to the Tylosin solution during a 25-minute period. The reaction mixture, while being slowly stirred, is permitted to stand at room temperature for three hours after which time the stirred mixture is poured

over cracked ice. The mixture containing acetylated Tylosin is extracted two times with 75 ml. volumes of benzene. The combined benzene extracts are slowly concentrated by evaporation causing the acetic acid ester of Tylosin to crystallize out. The crystalline ester is recrystallized from benzene.

Analyses of the recrystallized ester of Tylosin show that it has an acetyl content of about 8.22 per cent by weight and a pK^1a of about 5.1. (This and the other pK^1a values of compounds of this invention herein set forth have been determined by electro-metric titration in a dimethylformamide-water solution, 2:1 by volume).

EXAMPLE 7.

Preparation of the acetic acid ester of Tylosin.

- 200 g. of Tylosin are added to a combination of 467 ml. of acetic anhydride and 200 g. of fused anhydrous sodium acetate. The mixture is permitted to stand at room temperature for three days. The reaction mixture is poured over cracked ice, and the mixture is neutralized by the addition of potassium bicarbonate. The neutralized mixture is extracted with 2 l. of benzene. The benzene extract containing acetyl ester of Tylosin is washed with 5 per cent potassium bicarbonate solution followed by washing three times with water. The washed benzene extract containing the acetic acid ester of Tylosin is concentrated *in vacuo* to about 100 ml. The benzene concentrate is added to about 3 l. of petroleum ether causing the ester of Tylosin to precipitate. The precipitate consisting of the acetic acid ester of Tylosin is removed by filtration and is dissolved in 210 l. of chloroform. The chloroform solution is decolorized with carbon, and 10 volumes of petroleum ether are added to precipitate the acylated Tylosin. The precipitate consisting of the acetic acid ester of Tylosin is removed from the mixture by filtration, is dissolved in 62 l. of ethyl acetate, and the ethyl acetate solution is applied to a column of acid-washed alumina having a diameter of 13 cm. and a height of 110 cm. The alumina, on which the acetic acid ester of Tylosin is adsorbed, is washed with ethyl acetate. The adsorbed acetyl ester of Tylosin is eluted with a methanol-ethyl acetate combination (1:9 v/v), and the eluate containing the ester of Tylosin is evaporated to dryness *in vacuo*. The dry residue consisting of amorphous acetic acid ester of Tylosin is purified by dissolving it in hot isopropyl ether, and cooling the solution to precipitate the ester. Analyses of the purified ester of Tylosin show that it has an acetyl content of about 8.91 per cent by weight, and a pK^1a of about 5.2.

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EXAMPLE 8.

Preparation of Desmycosin.

20 g. of crystalline Tylosin are suspended in 500 ml. of distilled water, and the pH of the suspension is adjusted to pH 2.8 by the dropwise addition of 12 N hydrochloric acid. The mixture is permitted to stand at room temperature for three days after which the pH of the mixture is adjusted to pH 8.5 by the addition of solid sodium carbonate. The alkaline mixture is extracted four times with 100 ml. portions of chloroform. The combined chloroform extracts are washed two times with 250 ml. of distilled water. The washes are discarded, and the washed extract of Desmycosin is evaporated to dryness *in vacuo*. The dry residue consisting of Desmycosin is dissolved in 75 ml. of chloroform, and the chloroform solution is filtered to remove a small amount of insoluble material which is discarded. The filtrate containing Desmycosin is concentrated *in vacuo* to about 40 ml. and is cooled to a temperature of about 5° C. whereupon Desmycosin crystallizes out. The crystals of Desmycosin are removed by filtration and are dried providing a yield of 18.3 g. of crystalline Desmycosin. The crystalline preparation of Desmycosin is a solvate of chloroform containing about equimolar amounts of chloroform and Desmycosin.

The chloroform solvate crystals of Desmycosin thus obtained can be recrystallized from chloroform or, alternatively, from acetonitrile. The chloroform solvate crystals melt at about 95—115° C.

The solvated crystals of Desmycosin when heated at 75° C. *in vacuo* for two hours, lose the chloroform leaving nonsolvated, crystalline Desmycosin. The nonsolvated crystals melt at about 105—115° C. Electrometric titration shows the molecular weight of the Desmycosin to be 785 ± 30.

EXAMPLE 9.

Preparation of Desmycosin.

To 5 g. of Tylosin are added 100 ml. of 0.1 N hydrochloric acid, and the mixture is heated to 40° C. for one hour during which time the Tylosin is converted to Desmycosin. The mixture is made slightly alkaline by the slow addition of 10 per cent sodium hydroxide solution, and the Desmycosin is isolated and purified by the procedure described in Example 8.

EXAMPLE 10.

Preparation of the propionic acid ester of Tylosin.

2.5 g. of crystalline Tylosin and 0.55 g. of N,N'-dicyclohexylcarbodiimide are dissolved in 40 ml. of dichloromethane. 0.4 g. of propionic acid are added with stirring to the Tylosin solution, and the reaction mixture is permitted to stand for 20 hours.

After the reaction period, the mixture is filtered to remove a precipitate which appears. The precipitate is discarded. The filtrate is washed ~~sequentially~~ twice with 20 ml. of 5 per cent aqueous sodium bicarbonate solution and twice with 20 ml. portions of water. The washed filtrate is dried with anhydrous magnesium sulfate, is filtered, and is evaporated to dryness. The dried residue consisting of the propionic acid ester of Tylosin is dissolved in 20 ml. of hot acetone, the solution is cooled to about 5° C., and is filtered. The filtered acetone solution containing the ester of Tylosin is evaporated to dryness *in vacuo* yielding the propionic acid ester of Tylosin, which melts at about 101—111° C. and has a pK'a of about 5.2.

EXAMPLE 11.

Preparation of the acetic acid ester of Desmycosin.

1.9 g. of Desmycosin and 0.55 g. of N,N'-dicyclohexylcarbodiimide are dissolved in 40 ml. of dichloromethane. 0.3 g. of acetic acid are added with stirring to the Desmycosin solution, and the reaction mixture is permitted to stand for six hours. The reaction mixture is worked up following the procedure as described in Example 10. The product consisting of acetyl Desmycosin has a melting point of about 97—127° C. and has a pK'a of about 6.3.

EXAMPLE 12.

Preparation of the Propionic acid ester of Desmycosin.

The propionic acid ester of Desmycosin is prepared by the reaction of propionic acid with Desmycosin following the procedure described in Example 11. The ester of Desmycosin melts at about 115—127° C. and has a pK'a of about 6.3.

EXAMPLE 13.

Preparation of the hydrochloride salt of Desmycosin.

1 g. of Desmycosin is dissolved in 200 ml. of ether. To the ether solution, 0.11 ml. of 12 N hydrochloric acid are added slowly with stirring. A white precipitate of the hydrochloride salt of Desmycosin appears in the acidified mixture, is removed by filtration, is washed two times with ether, and is dried *in vacuo*. The hydrochloride salt of Desmycosin melts at about 140—153° C. and has a pK'a of about 8.

EXAMPLE 14.

Preparation of the bisulfate salt of Desmycosin.

2 g. of Desmycosin are dissolved in 200 ml. of ether, and 0.08 ml. of 36 N sulfuric acid are added slowly with stirring to the ether mixture. A white precipitate of the bisulfate salt of Desmycosin appears, is re-

sequentially



and

hexylcarbodiimide

moved by filtration, is washed two times with ether, and is dried *in vacuo*. The bisulfate salt of Desmycosin melts at about 142–159° C. and has $pK'a$ values of about 5.4 and about 8.0.

EXAMPLE 15.

Preparation of the tartrate salt of Desmycosin.

1 g. of Desmycosin is dissolved in 50 ml. of ether, and 0.2 g. of d-tartaric acid in 100 ml. of ether are added slowly with stirring. A white precipitate of the tartrate salt of Desmycosin appears, removed by filtration, is washed two times with ether, and is dried *in vacuo*. The tartrate salt melts at about 122–136° C. and has $pK'a$ values of about 5.35, 6.85, and 8.0.

EXAMPLE 16.

Preparation of the hydrochloride salt of the acetic acid ester of Tylosin.

900 mg. of the acetic acid ester of Tylosin are dissolved in 200 ml. of ether. 0.075 ml. of 12 N hydrochloric acid are added to the ether solution slowly with stirring. A precipitate of the hydrochloride salt of the acetyl ester of Tylosin appears, is removed by centrifugation, is washed with ether, and is dried *in vacuo*. The hydrochloride salt of the acetyl ester of Tylosin has a $pK'a$ of about 8.0 and melts at about 122–134° C.

EXAMPLE 17.

Preparation of the hydrochloride salt of the acetic acid ester of Desmycosin.

100 mg. of acetyl Desmycosin are dissolved in 20 ml. of ether, and 0.1 ml. of 12 N hydrochloric acid is added with stirring to the ether mixture. A white precipitate consisting of the hydrochloric salt of acetyl Desmycosin which forms in the acidified ether mixture, is removed by centrifugation, is washed two times with ether, and is dried *in vacuo*. The hydrochloride salt of the acetyl ester of Desmycosin has a melting point of about 146–150° C., has a $pK'a$ of about 6.2, is soluble in water, and is relatively insoluble in ether.

EXAMPLE 18.

Preparation of the hydrochloric salt of the propionic acid ester of Desmycosin.

The hydrochloric salt of the propionic acid ester of Desmycosin is prepared following the procedure outlined in Example 17 with the exception, that the employed starting material is the propionic acid ester of Desmycosin rather than the acetic acid ester of Desmycosin. The resulting hydrochloride salt of the propionic acid ester of Desmycosin has a melting point of about 146–150° C., has a $pK'a$ of about 6.2, is soluble in water, and is substantially insoluble in ether.

WHAT WE CLAIM IS:—

1. A method of producing the organic compound Tylosin which comprises cultivating a Tylosin producing strain of *Streptomyces fradiae* in a aqueous culture medium containing assimilable sources of carbon, nitrogen and inorganic salts, preferably under submerged aerobic conditions until a substantial amount of Tylosin is produced by said organism in said aqueous culture medium.
2. A method according to Claim 1 in which the organism is *Streptomyces fradiae* NRRL 2702.
3. A method according to Claim 1 in which the organism is *Streptomyces fradiae* NRRL 2703.
4. A method according to Claim 1 in which the culture medium is maintained at a temperature of 25–32° C., and the growth of the organism is carried out for a period of two to five days.
5. A method according to any one of Claims 1 to 4, wherein the Tylosin is recovered from said culture medium.
6. The method of preparing the organic compound Desmycosin which comprises hydrolyzing Tylosin produced in accordance with Claim 5 with dilute acid to cause hydrolysis of Tylosin and the production of Desmycosin.
7. The method according to Claim 6 in which the acid is hydrochloric acid.
8. The organic compound Tylosin which is characterized as a white, solid substance, soluble in slightly acidic aqueous solution and in most polar organic solvents, relatively insoluble in most nonpolar solvents of the aliphatic hydrocarbon class and in alkaline aqueous solutions, stable in aqueous solution at a pH in the range of pH 4 to pH 9, having a molecular weight in the range of 900 to 960 as determined by titration, containing about 59.68 per cent of carbon, about 8.53 per cent of hydrogen, about 1.55 per cent of nitrogen, and about 30.24 per cent of oxygen (by difference), having an ionizable group with $pK'a = 7$ as determined by electrometric titration in dimethyl formamide-water solution (2:1, parts by volume), having in chloroform solution spectral absorption maxima in the infra red region in the range of 2 to 5 μ at about the following wave lengths expressed in microns: 2.85, 3.36, 3.41 (shoulders at 3.47, 3.52, and 3.58), 3.68, 5.80, 5.94, 6.27, 6.88, 7.09, 7.27, 7.36, 7.61, 7.90, 8.45, 8.62, 8.75, 8.95, 9.28, 9.53, 9.85, 10.04, 10.13, 10.40, 11.10, 11.53, and 11.91, and having a spectral absorption maximum in water solution in the ultraviolet region at about 288 $m\mu$ having a molar extinction of $E_{1\%}^{1cm} = 241$, and an optical ro-



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moved by filtration, is washed two times with ether, and is dried *in vacuo*. The bisulfate salt of Desmycosin melts at about 142–159° C. and has $pK'a$ values of about

3. 5.4 and about 8.0.

EXAMPLE 15.

Preparation of the tartrate salt of Desmycosin.

1 g. of Desmycosin is dissolved in 50 ml. of ether, and 0.2 g. of d-tartaric acid in 100 ml. of ether are added slowly with stirring. A white precipitate of the tartrate salt of Desmycosin appears, removed by filtration, is washed two times with ether, and is dried *in vacuo*. The tartrate salt melts at about 122–136° C. and has $pK'a$ values of about 5.35, 6.85, and 8.0.

EXAMPLE 16.

Preparation of the hydrochloride salt of the acetic acid ester of Tylosin.

900 mg. of the acetic acid ester of Tylosin are dissolved in 200 ml. of ether. 0.075 ml. of 12 N hydrochloric acid are added to the ether solution slowly with stirring. A precipitate of the hydrochloride salt of the acetyl ester of Tylosin appears, is removed by centrifugation, is washed with ether, and is dried *in vacuo*. The hydrochloride salt of the acetyl ester of Tylosin has a $pK'a$ of about 8.0 and melts at about 122–134° C.

EXAMPLE 17.

Preparation of the hydrochloride salt of the acetic acid ester of Desmycosin.

100 mg. of acetyl Desmycosin are dissolved in 20 ml. of ether, and 0.1 ml. of 12 N hydrochloric acid is added with stirring to the ether mixture. A white precipitate consisting of the hydrochloric salt of acetyl Desmycosin which forms in the acidified ether mixture, is removed by centrifugation, is washed two times with ether, and is dried *in vacuo*. The hydrochloride salt of the acetyl ester of Desmycosin has a melting point of about 146–150° C., has a $pK'a$ of about 6.2, is soluble in water, and is relatively insoluble in ether.

EXAMPLE 18.

Preparation of the hydrochloric salt of the propionic acid ester of Desmycosin.

The hydrochloric salt of the propionic acid ester of Desmycosin is prepared following the procedure outlined in Example 17 with the exception, that the employed starting material is the propionic acid ester of Desmycosin rather than the acetic acid ester of Desmycosin. The resulting hydrochloride salt of the propionic acid ester of Desmycosin has a melting point of about 146–150° C., has a $pK'a$ of about 6.2, is soluble in water, and is substantially insoluble in ether.

WHAT WE CLAIM IS:—

1. A method of producing the organic compound Tylosin which comprises cultivating a Tylosin producing train of *Streptomyces fradiae* in a aqueous culture medium containing assimilable sources of carbon, nitrogen and inorganic salts, preferably under submerged aerobic conditions until a substantial amount of Tylosin is produced by said organism in said aqueous culture medium.

2. A method according to Claim 1 in which the organism is *Streptomyces fradiae* NRRL 2702.

3. A method according to Claim 1 in which the organism is *Streptomyces fradiae* NRRL 2703.

4. A method according to Claim 1 in which the culture medium is maintained at a temperature of 25–32° C., and the growth of the organism is carried out for a period of two to five days.

5. A method according to any one of Claims 1 to 4, wherein the Tylosin is recovered from said culture medium.

6. The method of preparing the organic compound Desmycosin which comprises hydrolyzing Tylosin produced in accordance with Claim 5 with dilute acid to cause hydrolysis of Tylosin and the production of Desmycosin.

7. The method according to Claim 6 in which the acid is hydrochloric acid.

8. The organic compound Tylosin which is characterized as a white, solid substance, soluble in slightly acidic aqueous solution and in most polar organic solvents, relatively insoluble in most nonpolar solvents of the aliphatic hydrocarbon class and in alkaline aqueous solutions, stable in aqueous solution at a pH in the range of pH 4 to pH 9, having a molecular weight in the range of 900 to 960 as determined by titration, containing about 59.68 per cent of carbon, about 8.53 per cent of hydrogen, about 1.55 per cent of nitrogen, and about 30.24 per cent of oxygen (by difference), having an ionizable group with $pK'a$ as determined by electrometric titration in dimethyl formamide-water solution (2:1, parts by volume), having in chloroform solution spectral absorption maxima in the infra red region in the range of 2 to 5 μ at about the following wave lengths expressed in microns: 2.85, 3.36, 3.41 (shoulders at 3.47, 3.52, and 3.58), 3.68, 5.80, 5.94, 6.27, 6.88, 7.09, 7.27, 7.36, 7.61, 7.90, 8.45, 8.62, 8.75, 8.95, 9.28, 9.53, 9.85, 10.04, 10.13, 10.40, 11.10, 11.53, and 11.91, and having a spectral absorption maximum in water solution in the ultraviolet region at about 288 $m\mu$ having a molar extinction of $E_{1\%}^{1cm} = 241$, and an optical ro-



tation in methanol of $[\alpha]_D^{25} = -46.3^\circ$ (C=2 per cent in methanol).

9. The organic compound Desmycosin which is characterized as a white, solid substance, soluble in slightly acidic aqueous solutions and in most polar solvents, relatively insoluble in most nonpolar solvents of the aliphatic hydrocarbon class and in alkaline aqueous solutions, stable in aqueous solution at a pH in the range of pH 1 to pH 9, having a molecular weight in the range of 760 to 810 as determined by titration, containing 59.73 per cent of carbon, 8.34 per cent of hydrogen, 1.7 per cent of nitrogen, and 30.23 per cent of oxygen (by difference), having an ionizable group with $pK'a=8.0$ as determined by electrometric titration in demethyl formamide-water solution (2:1, parts by volume), having spectral absorption maxima in chloroform solution in the infra red region in the range of 2 to 15 μ at about the following wave lengths expressed in microns: 2.76, 2.86, 3.38, 3.43, 3.48, 3.54, 3.69, 5.80, 5.95, 6.14, 6.26, 6.87, 7.11, 7.16, 7.25, 7.37, 7.60, 7.92, 8.48, 8.59, 8.77, 8.91, 9.26, 9.45, 9.92, 10.13, 10.40, 10.75, 11.11, 11.51, and 11.95, and having a spectral absorption maximum in water solution in the ultraviolet region at about 288 m. μ having a molar extinction of $E_{1\%}^{1\text{cm}} = 275$, and an optical rotation in methanol solution of $[\alpha]_D^{25} = -15.75^\circ$ (C=2 per cent in methanol, weight per volume).

10. An acid addition salt of Tylosin, the organic compound set forth in Claim 8.

11. An acid addition salt of Desmycosin, the organic compound set forth in Claim 9.

12. An alkyl carbacyl derivative of Tylosin, the organic compound set forth in Claim 8, having from 2 to 6 carbon atoms in the carbacyl group or groups.

13. An alkyl carbacyl derivative of Desmycosin, the organic compound set forth in Claim 9, having from 2 to 6 carbon atoms in the carbacyl group or groups.

14. An acid addition salt of an alkyl

carbacyl derivative of Tylosin as claimed in Claim 12.

15. An acid addition salt of an alkyl carbacyl derivative of Desmycosin as claimed in Claim 13.

16. The organic compound Tylosin substantially as hereinbefore described with reference to Example 1 or Example 2.

17. An acid addition salt of Tylosin substantially as hereinbefore described with reference to any one of Examples 3, 4 or 5.

18. An alkyl carbacyl derivative of Tylosin substantially as hereinbefore described with reference to any one of Examples 6, 7 or 10.

19. An acid addition salt of an alkyl carbacyl derivative of Tylosin substantially as hereinbefore described with reference to Example 16.

20. The organic compound Desmycosin substantially as hereinbefore described with reference to Example 3 or Example 9.

21. An alkyl carbacyl derivative of Desmycosin substantially as hereinbefore described with reference to Example 11 or Example 12.

22. An acid addition salt of Desmycosin substantially as hereinbefore described with reference to any one of Examples 13, 14 and 15.

23. An acid addition salt of an alkyl carbacyl derivative of Desmycosin substantially as hereinbefore described with reference to Example 17 or Example 18.

24. A method of producing the organic compound Tylosin substantially as hereinbefore described with reference to Example 1 or Example 2.

25. A method of producing the organic compound Desmycosin substantially as hereinbefore described with reference to Example 8 or Example 9.

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