

PATENT SPECIFICATION



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COMPLETE SPECIFICATION

Certain Organic Salts of Tetracycline and Processes for Preparing Tetracycline Hydrochloride and Acid and Metal Salts of Tetracycline

We, BRISTOL LABORATORIES LIMITED, formerly known as THE ANGLIER CHEMICAL COMPANY LIMITED, a British Company, of 211 Blackfriars Road, London, S.E.1, 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:—

10 This invention relates to certain organic acid salts of tetracycline and processes for preparing tetracycline hydrochloride and acid and metal salts of tetracycline.

Our co-pending Application No. 30108/54 (Serial No. 770,065) claims a process for producing tetracycline which comprises cultivating *Streptomyces viridifaciens* ATCC-11939 or one of its mutants in an aqueous, nitrogenous nutrient medium containing, carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to the solution and then recovering the resulting tetracycline from the fermentation broth.

25 Application No. 30108/54 (Serial No. 770,065) also claims an antibiotic substance which is a non-toxic, therapeutically effective salt of tetracycline comprising an organic acid addition salt of tetracycline, and tetracycline citrate and tetracycline tartrate.

30 Tetracycline may also be prepared from chlorotetracycline by the method described in the Journal of the American Chemical Society, Volume 75, pages 4621-4623 (1953).

35 The base tetracycline is a potent antibacterial agent exhibiting a broad spectrum of activity. Tetracycline base is not well suited for commercial manufacture and for therapeutic use for several reasons, however. Thus, tetracycline base is very hygroscopic and upon exposure to air picks up varying amounts of moisture rapidly (e.g. 12.2 per cent); such exposure to air cannot be readily avoided in practical use and storage

and the amount of active ingredient in the hydrated material thus formed is therefore unknown. As a result, accurate dosage is difficult, if not impossible.

In addition, tetracycline base has a very 50 low solubility in water, i.e. about 0.43 mgm./ml. at room temperature. Oral or parenteral aqueous solutions prepared with this material are therefore too voluminous for practical use; a dose of 500 mgms. 55 would thus require a volume of about 1000 cc. In addition, such a highly water insoluble product is slowly absorbed upon injection.

It is an object of the present invention 60 to overcome these obstacles to the commercial production and therapeutic use of tetracycline base.

We have now discovered that this object may be attained by the manufacture and 65 use of the following stable, therapeutically effective, non-toxic, organic acid addition salts of tetracycline; the formate, the acetate, the benzoate, the maleate, the ascorbate, the succinate and the nicotinate. 70

The organic acid addition salts of tetracycline of the present invention are found to be markedly superior to tetracycline base by virtue of their greater solubility in water. In addition, some of them are more stable 75 to heat in the dry state than is tetracycline base and exhibit lesser hygroscopicity.

The salts of the present invention may be prepared in various ways. For example, stoichiometric amounts of acid may be 80 added to a solution of tetracycline base in water or an organic solvent, e.g. butanol, acetone, or methanol, to form the acid addition salt; when desired the solid salt is then isolated by lyophilising the resulting 85 mixture (as in water), by collecting the precipitated salt by filtration (as from ethyl acetate), by precipitating the salt by the addition of another organic solvent (as in the addition of a hydrocarbon, e.g. Skelly- 90

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solve, to butanol or cyclohexane to ethyl acetate), or by reducing the volume of solvent by distillation *in vacuo* until the salt precipitates upon cooling (e.g. from butanol).

- 5 According to the present invention there is provided a process for producing a salt of tetracycline which comprises cultivating a strain of *Streptomyces viridifaciens* ATCC-11989 in an aqueous, nitrogenous nutrient
- 10 containing, carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to the solution, then recovering the resulting tetracycline by extracting the tetracycline from
- 15 the broth into butanol at pH 8.5, separating the butanol extract, precipitating tetracycline base by the addition of a petroleum ether to the butanol extract, separating the tetracycline base, reacting the tetracycline base
- 20 with an acid or metal hydroxide to produce a salt of tetracycline and separating the salt of tetracycline.

- The invention also provides a process for producing tetracycline hydrochloride which
- 25 comprises cultivating a strain of *Streptomyces viridifaciens* ATCC-11989 in an aqueous nitrogenous nutrient-containing, carbohydrate solution under submerged aerobic conditions until substantial anti-
 - 30 bacterial activity is imparted to the solution, then extracting the tetracycline from the broth at approximately pH 8.5 with butanol, separating the butanol extract, reducing the butanol in volume by azeotropic distillation,
 - 35 passing hydrogen chloride gas into the concentrated butanol extract to form and precipitate tetracycline hydrochloride and collecting the tetracycline hydrochloride so formed.
 - 40 In a preferred method tetracycline base in a fermentation broth having a pH about 8-9, preferably adjusted to pH 8.5 is extracted into butanol. The butanol extract is separated and the tetracycline base is
 - 45 precipitated by addition of lower hydrocarbons to the extract. Then the resulting tetracycline base is after separation converted into a salt of tetracycline by reacting it with an acid of metal hydroxide. A preferred
 - 50 method is to react the tetracycline in the butanol extract after reduction of its volume by azeotropic distillation with hydrogen chloride gas in order to form and precipitate tetracycline hydrochloride and
 - 55 collect it. In particular, the process for producing a tetracycline salt will involve the preparation of a tetracycline base in accord with the above outlined procedure using the organism *S. viridifaciens* ATCC-
 - 60 11989 and using an extraction process into butanol at pH 8 to 9 and preferably pH 8.5, separating the butanol extract, precipitating tetracycline base by the addition of a petroleum ether to the extract separating the
 - 65 base and reacting it with an acid or metal

hydroxide to produce a salt of tetracycline. The salt may be separated by crystallization and filtration. This particular method can be used to produce tetracycline hydrochloride following the steps of reducing the 70 volume of the butanol extract by azeotropic distillation, passing hydrogen chloride gas into the concentrated butanol extract, to form and precipitate tetracycline hydrochloride. 75

Salts of tetracycline are active *in vivo* as well as *in vitro* and display marked chemotherapeutic activity in animals. The salts are administered in aqueous solution. The results of testing tetracycline hydrochloride 80 for chemotherapeutic activity and toxicity are given in the following table.

Acute Toxicity		CD ₅₀ , mgm./kg.
LD ₅₀ , mgm./kg.		mouse
mouse, intravenous		intraperitoneal
186 to 230		about 6.2

The CD₅₀ (Curative Dose — 50) is the minimum dose of tetracycline hydrochloride which will cure 50 percent of a group of mice injected intraperitoneally with 100 to 90 1000 LD₅₀ doses of *Diplococcus pneumoniae* each LD₅₀ dose being sufficient if given alone to kill 50 percent of a group of mice. The infection is given at the same time as 95 the injection of test drug. The animals are observed for four days and deaths for each group expressed as the percentage of the total animals per group. The percentage death is transformed to probit values and these plotted against the log of the dose in 100 mgms. per kg. of mouse weight. The point of intersection of the probit 5 line and the best line constructed through the experimental points describes the concentration 105 of drug which should protect half of the animals under the conditions of the experiment. The antilog of this term is called the CD₅₀ value.

Following is a description by way of example of methods of carrying the invention 110 into effect:—

EXAMPLE I

In preparing tetracycline on a laboratory scale, the fermentation is conducted in shaker bottles open to the air but protected 115 from contamination with cotton or gauze covers. *Streptomyces BL 567201* (*Streptomyces viridifaciens*) is grown in a suitable aqueous nitrogenous nutrient containing carbohydrate solution by the submerged 120 culture method as disclosed in co-pending application No. 30108/54 (Serial No. 770,065), agitation and aeration of the culture being effected by placing the bottles on a reciprocating type shaker which provides 125 spraying, splashing or spilling of the mash through an oxygen-containing atmosphere. As a typical case, 500 ml. of a culture medium composed of

1% sucrose

130

1% soy bean meal
1% soy peptone
1.5% KH_2PO_4
0.5% $(\text{NH}_4)_2\text{HPO}_4$
95% Water

- 5 are introduced into 4 litre bottles and sterilized. After autoclaving, the medium is inoculated with about 1%, by volume, of a turbid aqueous spore suspension of the
10 *Streptomyces* from an agar slant. The pH is 6.0-6.2 at the start of the fermentation. The contents of the bottle are then incubated at 26°-28°C. for 48 hours while shaking at 130 strokes per minute with a 1 1/4" stroke.
15 At the end of the incubation period the fermentation liquor was a bluish-green and when assayed by the method described above, gave inhibition zones of approximately 27 mm. when broth was diluted 30-fold. This broth contains tetracycline, which can be isolated as described below.

- About 5 liters of the tetracycline broth is extracted at pH 8.5 with about 3 liters of butanol. The butanol extract is then separated and reduced in volume by azeotropic distillation to about 30 ml. When this concentrate is added to about 350 ml. of a hydrocarbon, a tetracycline base is precipitated.
30 The tetracycline free base (one gram) is dissolved in a minimum volume of anhydrous butanol and the stoichiometric amount of hydrogen chloride required to form the salt is passed in as a gas. Solid,
35 crystalline yellow tetracycline hydrochloride precipitates and is collected by filtration and found to be non-hygroscopic and to be soluble in water at room temperature to the extent of about 110 to 153 mg./ml. and to
40 melt at about 214°C. A dry sample of tetracycline hydrochloride showed an average loss of potency by bio-assay (ten replicates) of only 15% when heated 17 hours at 126°C. as compared to a loss of 48% for
45 tetracycline base under the same conditions.

- If desired, the concentrated butanol extract can be treated directly by passing in hydrogen chloride gas to form a precipitate of tetracycline hydrochloride which can be
50 recovered by filtration.

EXAMPLE II

- Tetracycline base (1.1 g.) suspended in 3 ml. ethyl acetate is mixed with a solution of benzoic acid (0.35 g.) and the resultant
55 mixture is stirred until clear. The addition of 20 ml. cyclohexane precipitates light tan tetracycline benzoate, which melts at about 159° to 162°C. after recrystallization from cyclohexane/ethyl acetate, and is found to
60 be less hygroscopic than tetracycline base and to be soluble in water to the extent of about 0.81 to 1.06 mgm./ml. at room temperature as determined by shaking excess solid in water for at least 16 hours and
65 measuring the tetracycline in solution by

ultra-violet absorption. By this method, the solubility in water at room temperature of tetracycline base was found to be about 0.42 to 0.43 mgm./ml.

A second preparation of tetracycline 70 benzoate darkened at 154°C. and melted at about 163° to 166°C. •

EXAMPLE III

To a solution of 10.0 g. hydrated tetracycline (18.75% water) in 100 ml. *n*-propanol at 60°C. was added 4 ml. of glacial acetic acid. To the resulting clear solution was added 300 ml. cyclohexane, precipitating solid yellowish tan tetracycline acetate which was collected by filtration, dried in
80 air and then *in vacuo* over P_2O_5 at room temperature and found to shrink at about 128°C. and to melt with decomposition at about 140° to 145°C.

Tetracycline acetate is found to be soluble 85 in water to the extent of about 3.15 to 3.50 mgm./ml. at room temperature.

EXAMPLE IV

Tetracycline hydrate (81.3% free base; 9.98 g.) was mixed with 3.52 g. of ascorbic 90 acid in 200 ml. of hot (50°C.) *n*-propanol. The solution became clear; upon cooling solid yellowish tan tetracycline ascorbate precipitated and was collected by filtration (7.9 g.) and found to melt at about 145° to
95 150°C. with decomposition and to be soluble in water at room temperature to the extent of about 8.3 mgm./ml.

EXAMPLE V

Tetracycline hydrate (81.3% free base; 100 9.98 g.) and 2.36 g. succinic acid were dissolved with heating in 200 ml. *n*-propanol. Upon cooling, solid yellow tetracycline succinate precipitated and was collected by filtration, recrystallized from 100 mls. hot
105 *n*-propanol, yielding 7 g., shrinking at 155°C. and melting with decomposition at about 160° to 164°C. and being soluble in water at room temperature to the extent of about 3.5 mgm./ml. 110

EXAMPLE VI

Tetracycline hydrate (10 g.) was dissolved in 90 ml. *n*-propanol at about 60°C. and added with stirring to a warm solution of 2.71 g. nicotinic acid in 50 ml. *n*-propanol. 115 The solution became clear; on cooling solid yellowish tan tetracycline nicotinate (5.7 g.) precipitated and was collected by filtration and found to melt at about 141° to 145°C. with decomposition and to be less hygroscopic than tetracycline base and to be
120 soluble in water to the extent of about 4.6 mgm./ml.

EXAMPLE VII

Tetracycline (10.0 g.; 18.75% water) was 125 suspended in 50 ml. *n*-propanol to which was added 1 g. of 98% formic acid and then 50 ml. more *n*-propanol. A clear solution was obtained upon heating to 50° to 55°C. for a few minutes. Crystalline pale 130

yellow tetracycline formate (7.5 g.) precipitated upon cooling and was collected by filtration, washed with *n*-propanol, dried in air and found to melt at about 184° to 185°C. with decomposition.

EXAMPLE VIII

Maleic acid (2.55 g.) was dissolved in 10 ml. acetone. To this solution was added 10 g. tetracycline trihydrate as a solid. The resulting solution was filtered and washed through the filter with 10 ml. acetone. Ethyl acetate was added, precipitating tetracycline maleate as a gum. The solvent was decanted and ethyl ether was added. Upon stirring, the tetracycline maleate solidified and was collected by filtration and found to melt at about 80°C. to 100°C. and to be yellow.

The hygroscopicity of tetracycline base was compared with that of the acid addition salts of tetracycline described above by drying a sample at 60°C. *in vacuo* over P_2O_5 , exposing the sample for thirty minutes at room temperature to an atmosphere of 70% humidity and then measuring the gain in weight of the sample. Using this method the following amounts of water were absorbed by the samples in terms of percent of original weight: base (crystalline) 12.2%; formate (crystalline) 0.45%; hydrochloride (crystalline) 0.48%; maleate 2.42%; benzoate 2.36%; ascorbate 3.05%; acetate 3.26%; nicotinate 3.02%; succinate 3.82%.

WHAT WE CLAIM IS:—

1. Tetracycline ascorbate.
2. Tetracycline acetate.
3. Tetracycline succinate.
4. Tetracycline benzoate.
5. Tetracycline nicotinate.
6. Tetracycline formate.
7. Tetracycline maleate.

8. A process for producing a salt of tetracycline which comprises cultivating a strain of *Streptomyces viridifaciens* ATCC-11989 in an aqueous, nitrogenous nutrient

containing, carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to the solution, then recovering the resulting tetracycline by extracting the tetracycline from the broth into butanol at pH 8.5, separating the butanol extract, precipitating tetracycline base by the addition of a petroleum ether to the butanol extract, separating the tetracycline base, reacting the tetracycline base with an acid or metal hydroxide to produce a salt of tetracycline and separating the salt of tetracycline.

9. A process for producing tetracycline hydrochloride which comprises cultivating a strain of *Streptomyces viridifaciens* ATCC-11989 in an aqueous nitrogenous nutrient-containing, carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to the solution, then extracting the tetracycline from the broth at approximately pH 8.5 with butanol, separating the butanol extract, reducing the butanol in volume by azeotropic distillation, passing hydrogen chloride gas into the concentrated butanol extract to form and precipitate tetracycline hydrochloride and collecting the tetracycline hydrochloride so formed.

10. A process for the preparation of a non-toxic therapeutically effective salt of tetracycline substantially as described with reference to any one of the specific examples hereinbefore set forth.

11. A non-toxic therapeutically effective organic acid addition salt of tetracycline when prepared by the process particularly described herein.

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Reference has been directed in pursuance of Section 9, subsection (1) of the Patents Act 1949 to Patent No. 747,779.

yellow tetracycline formate (7.5 g.) precipitated upon cooling and was collected by filtration, washed with *n*-propanol, dried in air and found to melt at about 184° to 185°C. with decomposition.

EXAMPLE VIII

Maleic acid (2.55 g.) was dissolved in 10 ml. acetone. To this solution was added 10 g. tetracycline trihydrate as a solid. The resulting solution was filtered and washed through the filter with 10 ml. acetone. Ethyl acetate was added, precipitating tetracycline maleate as a gum. The solvent was decanted and ethyl ether was added. Upon stirring, the tetracycline maleate solidified and was collected by filtration and found to melt at about 80°C. to 100°C. and to be yellow.

The hygroscopicity of tetracycline base was compared with that of the acid addition salts of tetracycline described above by drying a sample at 60°C. *in vacuo* over P₂O₅, exposing the sample for thirty minutes at room temperature to an atmosphere of 70% humidity and then measuring the gain in weight of the sample. Using this method the following amounts of water were absorbed by the samples in terms of percent of original weight: base (crystalline) 12.2%; formate (crystalline) 0.45%; hydrochloride (crystalline) 0.48%; maleate 2.42%; benzoate 2.36%; ascorbate 3.05%; acetate 3.26%; nicotinate 3.02%; succinate 3.82%.

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1. Tetracycline ascorbate.
2. Tetracycline acetate.
3. Tetracycline succinate.
4. Tetracycline benzoate.
5. Tetracycline nicotinate.
6. Tetracycline formate.
7. Tetracycline maleate.
8. A process for producing a salt of tetracycline which comprises cultivating a strain of *Streptomyces viridifaciens* ATCC-11989 in an aqueous, nitrogenous nutrient

containing, carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to the solution, then recovering the resulting tetracycline by extracting the tetracycline from the broth into butanol at pH 8.5, separating the butanol extract, precipitating tetracycline base by the addition of a petroleum ether to the butanol extract, separating the tetracycline base, reacting the tetracycline base with an acid or metal hydroxide to produce a salt of tetracycline and separating the salt of tetracycline.

9. A process for producing tetracycline hydrochloride which comprises cultivating a strain of *Streptomyces viridifaciens* ATCC-11989 in an aqueous nitrogenous nutrient-containing, carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to the solution, then extracting the tetracycline from the broth at approximately pH 8.5 with butanol, separating the butanol extract, reducing the butanol in volume by azeotropic distillation, passing hydrogen chloride gas into the concentrated butanol extract to form and precipitate tetracycline hydrochloride and collecting the tetracycline hydrochloride so formed.

10. A process for the preparation of a non-toxic therapeutically effective salt of tetracycline substantially as described with reference to any one of the specific examples hereinbefore set forth.

11. A non-toxic therapeutically effective organic acid addition salt of tetracycline when prepared by the process particularly described herein.

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