

PATENT SPECIFICATION

DRAWINGS ATTACHED

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COMPLETE SPECIFICATION

Preparation of New Antibiotics Designated Demethyltetracyclines

We, AMERICAN CYANAMID COMPANY, a corporation organised under the laws of the State of Maine, United States of America, of 30, Rockefeller Plaza, New York, State of New York, 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 therapeutic substances which are defined as demethyltetracycline antibiotics and are designated demethyltetracycline, chlorodemethyltetracycline and bromodemethyltetracycline, and epimers thereof.

The new antibiotics of the present invention are produced in a fermentation process in which mutant strains of *Streptomyces aureofaciens* are employed. The new antibiotics appear to be closely related to but are distinctly different from previously described members of the tetracycline family. They are characterized by marked stability under both alkaline and acid conditions and are superior to the tetracyclines in this respect. They have a similar antibiotic activity and may be used for the same purposes and in the same general manner as are the presently known tetracyclines.

In accordance with the invention, a method is provided of preparing demethyltetracycline, chlorodemethyltetracycline and/or bromodemethyltetracycline, which comprises aerobically fermenting an aqueous nutrient medium containing an assimilable source of carbon, nitrogen and mineral substances and having a controlled chloride and bromide ion content with a demethyltetracycline-producing strain of *S. aureofaciens*, if desired recovering the resulting demethyltetracycline antibiotics from the fermentation medium.

The invention also provides a method of preparing an epimer of demethyltetracycline, chlorodemethyltetracycline, or bromodemethyltetracycline which comprises adjust-

ing the pH value of a concentrated solution of the corresponding demethyltetracycline to a value within the range 3.0 to 5.0 and allowing the solution to stand until isomerization has come to an equilibrium.

Each of the previously known tetracycline antibiotics which are produced by fermentation with *S. aureofaciens* under particular conditions, namely tetracycline, chlorotetracycline and bromotetracycline, has a counterpart in the new series of antibiotics of the present invention. In other words, there is produced in accordance with the process of the present invention when using certain mutant strains of *S. aureofaciens* which will be more particularly described hereinafter an antibiotic substance similar to tetracycline, another similar to chlorotetracycline and another analogous to bromotetracycline. The particular new antibiotic that is formed as a result of the fermentation process will depend upon the nature of the nutrient medium and other conditions of the process. In a chloride-free medium the antibiotic that predominates will be the chloride-free analog of tetracycline. In a bromine-rich medium, the bromine-containing antibiotic will be formed; and in a chlorine-rich medium, the predominating antibiotic will be a chlorine-containing antibiotic analogous to chlorotetracycline. Ordinarily, some tetracycline, chlorotetracycline and/or bromotetracycline will also be formed during the fermentation process, the relative proportions of which will depend upon the chloride and/or bromide ion content of the medium and the strain of microorganism used. With careful selections of the strain of *S. aureofaciens*, it is possible to almost completely eliminate the formation of these known tetracycline antibiotics.

The new antibiotics of the present invention can be converted into epimers as herein-after described, similarly to the manner in which the tetracyclines are converted into the quatrimecins.

The chlorine- and bromine-containing anti-

[Price 3s. 6d.]



biotics of the present invention may also be dehalogenated by hydrogenation by using palladium on charcoal as the catalyst, just as are chlorotetracycline or bromotetracycline converted to tetracycline itself.

5 Chemical analyses and a consideration of the chemical and physical properties of the new compounds indicate that they differ essentially from the corresponding tetracyclines in having one less methyl group. The methyl group involved is most likely the one occupying the six position on the naphthacene ring of the tetracyclines. Accordingly, the tetracycline analog of the present invention would be called 4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-1,11-dioxo-2-naphthacenecarboxamide. In the case of the chlorine and bromine analogs, the halogen substituent would be on the seven position and would be named accordingly. Appropriate common names for these new antibiotics would be demethyltetracycline, chlorodemethyltetracycline and bromodemethyltetracycline and this nomenclature will be used herein. It will be understood, however, that the postulated structure has not been proven by an unequivocal synthesis and may subsequently be found to be in error. The present invention is concerned with new products and not a particular system of nomenclature or probable structure.

The new antibiotics of the present invention are produced by certain mutant strains of *S. aureofaciens*. These strains are unquestionably of the species *Streptomyces aureofaciens*, inasmuch as they were derived from the original A-377 isolated by Dr. B. M. Duggar and deposited at the Northern Regional Research Laboratories at Peoria, Illinois, and indexed there as NRRL 2209. Mutations involving treatment of A-377 with mutagenic agents including successively ultraviolet irradiation, nicotine, and nitrogen mustard were employed to develop one of the strains. Various other strains have been obtained by spontaneous mutation and by treatment with mutagenic agents. These several strains which produce the new antibiotics in differing proportions and effectiveness have the general characteristics of the species *S. aureofaciens*. They differ somewhat among themselves and from previously described

strains of *S. aureofaciens* primarily in pigmentation and their ability to produce the new antibiotics. In most cases a reddish-brown pigment is noted when the microorganisms are grown on conventional culture media. The shades of pigment range from peach-tan or copper-brown to deep brown-mahogany or burgundy depending upon the particular strain and the nutrient medium used. Although the property of producing the new antibiotics of the present invention is generally associated with strains having this type of pigmentation, yet this pigment production and production of the new antibiotic is not necessarily a common property. All of the strains studied produce some chlorotetracycline and generally some tetracycline, although the relative proportions of these antibiotics may vary considerably depending upon the nature of the fermentation medium, the fermentation conditions and the particular strain chosen. In some cases the production of chlorotetracycline and tetracycline is very low, of the order of a few per cent of total antibiotic produced by the fermentation.

The mutant strains which produce the new antibiotics of the present invention possess the same general characteristics as do the strains which produce the tetracyclines and differ among themselves in the same general manner that the tetracycline strains differ from each other, as has been described in a number of scientific papers which have been published. Although many strains of *S. aureofaciens* are available to the public from culture collections and many have been described in patents and scientific literature, the following data will serve to illustrate the type of variation of the new strains from the original A-377 strain available as NRRL 2209.

Comparison of *S. aureofaciens* Strains S-604 (A.T.C.C. 12551) and A-377 on Various Media

Streptomyces aureofaciens Strain S-604 (A.T.C.C. 12551), which produces chlorodemethyltetracycline and demethyltetracycline was compared with *Streptomyces aureofaciens* Strain A-377 (NRRL 2209) by observation of growth characteristics on various media incubated at 26—27° C.

Observations were as follows:—

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1. GLYCEROL ASPARAGINE BEEF EXTRACT AGAR

Glycerol	1.0 per cent
L-Asparagine	0.05 per cent
Beef Extract	0.2 per cent
KH_2PO_4	0.05 per cent
Bacto Agar	1.5 per cent
Distilled Water q.s.	100.0 per cent
pH Adjustment with 50% KOH	7.0
Post Sterilization pH	7.1

Streptomyces aureofaciens

	Strain S—604	Strain A—377
Growth	Abundant, Venetian Red*	Fair
Aerial Hyphae	Abundant, White to Rose Grey*	White, Uniform
Sporulation	Slight becoming Abundant	None
Diffusible Pigment	Reddish-brown	Yellow
Reverse	Brown-Mahogany*	Yellow to Light Orange-Yellow

* Color Harmony Manual, Third Edition, Container Corporation of America.

2. DEXTRIN CZAPEK-DOX AGAR

Dextrin	1.0 per cent
NaNO_3	0.2 per cent
K_2HPO_4	0.1 per cent
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.05 per cent
KCl	0.05 per cent
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.001 per cent
Bacto Agar	1.5 per cent
Distilled water q.s.	100.0 per cent
Post Sterilization pH	7.0

Streptomyces aureofaciens		
	Strain S—604	Strain A—377
Growth	Sparse, Hyaline	Profuse
Aerial Hyphae	None	Abundant, Lead Grey* Water-White Surface Globules
Sporulation	None	Abundant
Diffusible Pigment	None	Slight: Pale yellow
Reverse	Apigmentous	Apigmentous

* Color Harmony Manual, Third Edition, Container Corporation of America.

3. CORN STEEP AGAR

Corn Steep	0.4 per cent
Sucrose	1.0 per cent
MgSO ₄ ·7H ₂ O	0.025 per cent
KH ₂ PO ₄	0.2 per cent
(NH ₄) ₂ HPO ₄	0.2 per cent
Bacto Agar	2.0 per cent
Tap water q.s.	100 per cent
Post sterilization pH	6.3

Streptomyces aureofaciens		
	Strain S—604	Strain A—377
Growth	Profuse	Profuse
Aerial Hyphae	Abundant, Dk. Rose Taupe*	Abundant, Beaver*
Sporulation	Very Abundant, Uniform	Very Abundant, Uniform
Diffusible Pigment	Very concentrated; Deep Brown* to Deep Brown Mahogany*	Light Greenish- Yellow
Reverse	Dk. Brown Mahogany*	Covert Brown*

* Color Harmony Manual, Third Edition, Container Corporation of America.

4. OTHER MEDIA

Streptomyces aureofaciens		
	Strain S—604	Strain A—377
Nutrient Agar	Poor growth. Taupe brown* to dark brown*. No aerial hyphae. Reverse: Taupe brown*. Reddish brown soluble pigment.	Fair growth. No aerial hyphae. Reverse: pale yellow. Yellow to light brownish-yellow soluble pigment.
Glucose Asparagine meat extract Agar	Abundant growth. Heavy aerial hyphae mottled: Rose taupe* to Faun* to Camel*. Sporulation: abundant. Reverse: Taupe brown*. Reddish brown soluble pigment.	Fair growth. Aerial hyphae white becoming increasingly grey with increase in spore formation. Reverse: Light yellow. Light yellow soluble pigment.
Potato Slants	Profuse moist smooth modulated growth: Dark brown mahogany* with peach tan* trace. Aerial hyphae: none to abundant becoming white to camel*. Sporulation abundant in areas of heavy aerial hyphae formation. Chocolate* to chocolate brown* soluble pigment.	Profuse moist smooth nodulated growth: Light melon yellow* to Antique rose*. Aerial hyphae: Trace. No soluble pigment.
Purple Milk	Slight deep red mahogany* growth* collar. Little significant pH change nor apparent peptonization. Slight false alkaline color reaction due to diffusion of soluble pigment.	Slight white to pale yellow growth collar. Little significant pH change nor apparent peptonization in 15 days.

* Color Harmony Manual, Third Edition, Container Corporation of America.

On all agars, except Dextrin Czapek-Dox, but including potato slant growth, *S. aureofaciens* Strain S-604 characteristically produces deep pigmentation generally appearing

with growth. Similarly, readily observable is the characteristic soluble reddish-brown pigment.

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5. MICROSCOPIC OBSERVATIONS

Streptomyces aureofaciens				
Medium	Strain S—604		Strain A—377	
	Mycelium	Spores	Mycelium	Spores
Glycerol Asparagine Beef Extract Agar	Flexuous, continuous, branched. Diam. 0.8μ to $>1.0\mu$	Spheroidal to ovoidal. Diam. $1.5-2.0\mu$	Flexuous, continuous, branched. Diam. 0.7μ to $>1.0\mu$	Spheroidal to ovoidal. Diam. $1.5-2.0\mu$
Corn Steep Agar	Flexuous, continuous, branched. Diam. 0.8μ to >1.0	Spheroidal to ovoidal. Diam. $1.5-2.0\mu$	Flexuous, continuous, branched. Diam. 0.8μ to $>1.0\mu$	Spheroidal to ovoidal. Diam. $1.5-2.0\mu$

Mycelial and spore morphology for Strain S—604 is apparently similar to that observed for Strain A—377. Both strains demonstrate continuous, flexuous, branched mycelium with an occasional tendency of aerial hyphae towards spiraling. Characteristic spores are avoidal to spheroidal in shape.

To illustrate the color variations among the several strains of *S. aureofaciens* which produce the new demethyltetracyclines of the

present invention, four selected strains were grown on corn steep agar and the following observations were made:

Color Observations*: *Streptomyces aureofaciens*: Corn Steep Agar (AP₄):
Four-Day Incubation at 27° C.

Strain	Single Colonies	Mass Growth
S-604M1 (ATCC 12551)	Deep red mahogany	Deep red mahogany
S1071 (ATCC 12552)	Copper Brown	Deep brown mahogany to red mahogany
V-62 (ATCC 12553)	Wide marginal area peach tan becoming light copper brown at the colony center	Light copper brown
B-740 (ATCC 12554)	Dark Wine	Burgundy

* Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

A relatively simple procedure of selecting a strain of *S. aureofaciens* that will produce the new antibiotics of the present invention is to select a strain of *S. aureofaciens* which has dark maroon colonies when grown on corn steep-agar medium. Inocula are prepared from these colonies and a fermentation is carried out as described in Examples 1 and 2 which follow. At the end of the fermentation period, a quantity of the fermentation liquor is acidified to a pH of about 1.5 to solubilize the antibiotic and filtered to obtain a clear aqueous solution. One or two drops of the filtrate is placed on a paper strip and chromatographed by standard chromatographic development procedures. Butanol equilibrated with 0.3 molar aqueous phosphate buffer at pH 3 is used as the solvent. The paper strips are developed by descending chromatography with the solvent phase of the equilibrated mixture. The location of the spots corresponding to the antibiotics of the present invention is readily determined by inspection under ultraviolet light. An alternative and more sensitive method consists of placing the strip on an agar plate seeded with antibiotic-sensitive microorganisms such as *Bacillus cereus* or *Bacillus subtilis* and incubating the plates. Zones of inhibition become apparent in the regions corresponding to the antibiotic spots. Comparison of the spots with control samples of the pure antibiotics and the Rf values which are set forth hereinafter provides a basis for detecting the presence of the new antibiotics in the fermented liquor

and a semi-quantitative basis for determining the amount present.

An illustration of this technique may be given with the assistance of Figure 1 of the accompanying drawing which shows how paper-strip chromatography can separate and detect the various tetracyclines and the products of the present invention from each other. The drawing represents a paper strip, 1, on which a small amount of an antibiotic solution containing tetracycline, chlorotetracycline, demethyltetracycline and chlorodemethyltetracycline was placed at point 2. The total antibiotic activity of the solution placed at point 2 was 30 gammas expressed as tetracycline. The paper strips were then developed as described above. After a period of time, the strips were inspected by ultraviolet light with the results shown in the drawing. The solvent front had advanced to the point indicated by the solid line, 3. The tetracycline component of the mixed antibiotic solution had advanced to point 5, and the chlorotetracycline had advanced to point 7. The new antibiotic demethyltetracycline had advanced to point 4, and antibiotic chlorodemethyltetracycline was found at point 6. The Rf values of these antibiotics are also shown on the drawing. The Rf value is defined as the distance of the spot from the point of origin, 2, divided by the distance of the solvent front, 3, from the origin, 2, and are, of course, always less than 1. Various solvent systems may be used to obtain a series of Rf values for any particular antibiotic, and

these values are considered very valuable in identifying antibiotics and distinguishing them from others.

The Rf values of a number of the tetra-

cyclines, quatrimycins and the new products of the present invention in two specific solvent systems are shown in the following table.

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Antibiotic	Ethyl acetate/pH 4.7 Citrate phosphate Buffer (McElvain)	90—10 chloroform- Butanol/aqueous 0.3 M H ₃ PO ₄ + 0.1% trichloroacetic acid pH 1.9
Chlorotetracycline	0.76 — 0.80	0.61
Bromotetracycline	0.76 — 0.80	0.61
Tetracycline	0.46 — 0.47	0.20
Quatrimycin	0.14 — 0.16	0.12
Chlorquatrimycin	0.27 — 0.28	0.33
Bromquatrimycin	0.27 — 0.28	0.33
Oxyquatrimycin	0.00 — 0.06	0.11
Chlorodemethyltetracycline	0.68 — 0.72	0.39
Demethyltetracycline	0.27	0.22
Epichlorodemethyltetracycline	0.25 — 0.27	0.20
Epidemethyltetracycline	0.09	0.10

- 10 Several strains of *S. aureofaciens* which produce the new antibiotic have been deposited at the American Type Culture Collection, Washington, D.C. These are cataloged at the Collection as ATCC 12551, 12552, 12553 and 12554. ATCC 12551 is strain S-604. It will be understood, of course, that mutants which will also produce the new antibiotics, may be derived from these strains by conventional procedures. These mutants may vary somewhat in general morphological characteristics as do the various strains of the *S. aureofaciens* species.

- 20 One of the most important advantages of the demethyltetracycline over the previously described tetracyclines is their increased stability in acids and alkalis. The acid instability of tetracycline and the alkali instability of chlorotetracycline are well known. Chlorotetracycline in an aqueous solution with a sodium carbonate buffer at pH 9.85 has a half life of 29.2 minutes at 23° C. By contrast, chlorodemethyltetracycline loses no more than 6 per cent of its activity in 24 hours under the same conditons. Tetracycline is completely destroyed in less than 5 minutes in 1 N sulfuric acid at 100° C. On the other hand, chlorodemethyltetracycline loses only about 2 per cent of its activity in 1 N sulfuric acid at 100° C. after 15 minutes. Similarly,

the new antibiotic demethyltetracycline loses only about 33 per cent of its activity in 1 N hydrochloric acid at 100° C. in 6 minutes. In 0.1 N sodium hydroxide at 50° C., demethyltetracycline has a half life of about 20 hours as compared to 3.3 hours for tetracycline. These unexpected properties are very valuable inasmuch as the acid instability of tetracycline and the alkali instability of chlorotetracycline has limited or completely precluded the use of these valuable substances in many applications. By virtue of the much better stability of the new antibiotics, it is possible to prepare many pharmaceutical products which could not be satisfactorily compounded with the tetracyclines. The increased stability also makes it possible to improve the recovery and refining processes since more drastic pH and temperature conditions can be employed and the efficiency of various steps in the process can therefore be improved.

Chemical analyses of highly purified samples of chlorodemethyltetracycline have checked within experimental error, with the calculated empirical formula C₂₁H₂₁ClO₈. The Kuhn-Roth method for determining methyl groups resulted in an extremely low value, indicating that there was no methyl group on the six position of the naphthacene ring. Accordingly, the name chlorodemethyltetra-

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cycline appears to be appropriate. This is particularly true in view of a comparison of the ultraviolet and infrared absorption spectra of the products with the several tetracyclines.

- 5 The melting point of chlorodemethyltetracycline as the free base when measured on a hot stage was 170—175° C. with decomposition. The melting point of chlorotetracycline is 168—169° C. Demethyltetracycline, free
10 base, melts at 170—177° C. with decomposition whereas tetracycline melts on the hot stage at 160—168° C.

- Other comparisons between chlorotetracycline and chlorodemethyltetracycline are as follows. Chlorodemethyltetracycline has a
15 pK_a value of 4.45 in 50/50 dimethyl formamide-water. Chlorotetracycline has a value of 4.50 in the same system. The optical rotation of chlorodemethyltetracycline in 0.03
20 N hydrochloric acid at a concentration of 0.5 per cent was $[\alpha]_D^{25} = -261^\circ$; for chlorotetra-

cycline it is -243° . The demethyltetracyclines appear to be more water-soluble than the corresponding tetracyclines. For example, chlorodemethyltetracycline hydrochloride is
25 soluble in water to the extent of about 45 milligrams per milliliter as compared to about 14 milligrams per milliliter for chlorotetracycline.

The antibacterial activity of the tetracyclines and demethyltetracyclines is generally similar but with distinct differences as will
30 be seen from the following table.

This table compares the concentrations of chlorotetracycline and chlorodemethyltetracycline necessary to effect half maximum inhibition of growth in terms of micrograms of
35 antibiotic per milliliter of solution at periods of time ranging from 4 to 48 hours with varying microorganisms. The low values indicate more effective inhibition of growth.
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TABLE I

Microorganism	Antibiotic									
	Chlorotetracycline					Chlorodemethyltetracycline				
	Time in Hours					Time in Hours				
	4	8	24	48	4	8	24	48	4	8
<i>Staphylococcus aureus</i> ATCC 6538P	0.041	0.072	0.57	4.1	0.065	0.094	0.41	0.68		
<i>Staphylococcus albus</i>	7.5	20.5	>50.0	>50.0	16.5	50.0	>50.0	>50.0		
<i>Escherichia coli</i>	0.12	0.27	1.5	10.2	0.16	0.3	0.66	<1.56 >0.8		
<i>Salmonella gallinarum</i>	0.4	0.74	4.65	37.0	0.4	0.56	1.06	1.1		
<i>Bacillus cereus</i>	<0.025	0.035	0.15	1.1	<0.015	0.039	0.077	0.19		
<i>Pseudomonas aeruginosa</i>	0.31	0.56	6.3	35.0	0.94	1.3	4.3	6.7		
<i>Streptococcus pyogenes</i> β hemolytic	11.5	43.0	>50.0	>50.0	14.4	41.0	>50.0	>50.0		
<i>Proteus vulgaris</i>	0.28	0.56	9.2	50.0	0.61	1.12	1.38	4.4		
<i>Escherichia coli</i> ATCC 9637	0.54	1.18	6.6	34.0	0.43	0.67	0.97	1.8		

The demethyltetracyclines have also been

The ultraviolet absorption spectra of the tetracyclines and the demethyltetracyclines are very similar. Figure 2 of the drawings shows a comparison between the ultraviolet absorption spectrum of chlorotetracycline and chlorodemethyltetracycline at the same concentration. As will be noted, the form of the

A further comparison of the ultraviolet absorption spectra of the tetracyclines is shown in Table II wherein the extinction coefficient values $E_{1\%}^{1\text{ cm}}$ (spectrophotometric absorption of a 1 per cent solution measured in a 1 cm. cell) measured at the maxima and minima of the several antibiotics is shown. The values are computed from the ultraviolet absorption curves by correcting the concentration at which the samples were run to 1 per cent. All samples were measured in 0.1N H_2SO_4 solution.

TABLE II

Antibiotic	Max.		Min.		Max.		Min.		Max.	
	E ₁ % 1cm	mμ	E ₁ % 1cm	m	E ₁ % 1cm	mμ	E ₁ % 1cm	mμ	E ₁ % 1cm	mμ
Chlorotetracycline hydrochloride	209	368	135	302	356	265	288	242	343	228
Epichlorotetracycline—NH ₄	170	368	100	302	332	254	290	242	342	228
Tetracycline hydrochloride	305	355	182	299	393	268	191	232	289	217
Epitetracycline-NH ₄	290	355	140	299	326	254	200	232	275	216
Demethyltetracycline neutral	292	353	169	298	395	268	202	232	299	217
Epidememthytetracycline hydrochloride	310	355	162	299	367	255	228	232	306	217
Chlorodememthytetracycline hydrochloride	249	368	160	299	362	268	270	238	357	227
Epichlorodememthytetracycline hydrochloride	242	368	130	300	348	254	289	328	364	227

The infrared absorption spectra of several of the new products of the present invention are shown in the remaining Figures of the accompanying drawings. Figure 3 is a representation of the infrared absorption spectrum of chlorodemethyltetracycline hydrochloride. Figure 4 represents a comparison of the infrared absorption spectra of tetracycline, free base, and demethyltetracycline, free base. As will be noted, there are a number of distinct differences as well as similarities. Figure 5 shows the infrared absorption spectrum of epichlorodemethyltetracycline hydrochloride, and Figure 6 is the absorption spectrum of epidemethyltetracycline hydrochloride. These curves were run on a Perkin-Elmer Model 21 Automatic Recording Infrared Spectrophotometer with the antibiotics in a solid phase compressed in a disc with KBr. Additional data is as follows: Prism—NaCl; Resolution—2; Response—1—1; Gain—5; Speed 1/2 min./ μ ; Suppression—2; and Scale 2"/ μ .

Various methods of assaying the tetracyclines have been developed; and some of these can be used in assaying the demethyltetracyclines with, of course, suitable modifications. The fluorometric assay for chlorotetracycline is based on an increase in fluorescence due to the formation of an isochlorotetracycline under alkaline conditions. The sample to be assayed is dissolved in an alkaline phosphate buffer, and the fluorescence of the solution is determined in a fluorophotometer at 0 time (immediately on preparation of the solution) and again after standing to permit the isomerization to occur. The increase in fluorescence corresponds to the amount of chlorotetracycline present. The presence of chlorodemethyltetracycline in the solution may interfere, however, with the chlorotetracycline response tending to result in a spuriously low chlorotetracycline. A more reliable method for determining chlorotetracycline in mixtures with chlorodemethyltetracycline is based on the fact that the alkaline degradation product of chlorotetracycline does not absorb at 380m μ . The sample is prepared at pH 10 using carbonate buffer and the change in absorption at 380 m μ is measured after standing for 30 minutes (approximately three half lives of chlorotetracycline). Both chlorotetracycline and chlorodemethyltetracycline absorb before alkaline degradation but only chlorodemethyltetracycline absorbs after the degradation treatment; and the decreased absorption is a measure of the chlorotetracycline originally present in the sample.

A specific assay for chlorodemethyltetracycline in the presence of other tetracycline antibiotics is based on the so-called Hiscox method. A mild acid treatment of 15 minutes at 100° C. with 1 N HCl destroys all tetracyclines except chlorodemethyltetracycline. A portion of the sample so treated is then sub-

jected to a strong acid treatment of 15 minutes at 100° C. with 6 N HCl to destroy the chlorodemethyltetracycline. The chlorodemethyltetracycline content is calculated by measuring the loss in spectrophotometric absorption at 368 m μ or the increase at 430 m μ which is effected by the strong acid treatment and comparison with a standard.

A mixture containing only two components, such as those containing the two members of an epimeric pair, may be assayed spectrophotometrically. The spectrophotometric absorptions are determined at two wavelengths and the ratio of these absorptions is interpolated linearly between the known values for the pure components. The per cent composition of the mixture can thus be determined.

Bromodemethyltetracycline may be assayed most simply by analysis for bromine content by standard microanalytical procedures.

As indicated above, bromodemethyltetracycline may be obtained as the principal antibiotic of the fermentation by controlling the halogen content of the fermentation. Generally the aqueous nutrient medium should contain at least 50 parts per million of bromide ions and the chloride ion content should be kept as low as possible, less than about 50 parts per million of chloride ions. Preferably the medium should contain from about 100 to 1500 parts per million of bromide ions and less than about 10 parts per million of chloride ions. The bromide ion may be supplied by any water-soluble bromide salt, such as potassium bromide, which will release bromide ions for use in the biological synthesis of the antibiotic.

Except for the control of the halogen ion content of the fermentation, the process of producing bromodemethyltetracycline is similar to that of producing demethyltetracycline and chlorodemethyltetracycline as described herein. Strains of *S. aureofaciens* which produce demethyltetracycline may also be used to produce the bromo analog.

Bromodemethyltetracycline has substantially the same antibacterial activity as does chlorodemethyltetracycline and can be used for the same purposes and in the same manner.

It is not necessary to separate bromodemethyltetracycline from the other antibiotics which may be concurrently produced during the fermentation since a mixture of the various demethyltetracyclines is useful for many purposes; for example, the fermented nutrient medium may be concentrated and used directly for its antibiotic content. Another manner of such use is to acidify the fermented liquor, filter to remove mycelia and insoluble matter, neutralize and concentrate to obtain a dry powder which can be mixed with animal feeds. If the medium contains alkaline earth metal ions or they are added thereto, and the pH of the medium after fermentation is adjusted to a value of 6 to 10, alkaline earth

metal salts of the antibiotics are precipitated. The mixed antibiotics are useful in stimulating the growth of many animals.

5 A crude form of bromodemethyltetracycline and mixtures of this with other antibiotics may be used in preserving meats, poultry and fish in the same manner that the tetracyclines are now used. In these compositions very little attention need be given to the proportions of the various tetracyclines that may be present therein.

10 If it is desired to separate bromodemethyltetracycline from the other antibiotics contained in the fermented liquor, one possible method is as follows. Treatment with acid will destroy the tetracycline; and treatment with an alkali will destroy the chloro and bromotetracyclines in the fermented liquor. Bromodemethyltetracycline can be recovered from the resulting solutions by partition chromatography using diatomaceous earth in the column. A mixture of chloroform and butanol at a pH of about 2.0 will elute the antibiotics from the diatomaceous earth. Bromodemethyltetracycline comes off the column ahead of demethyltetracycline and may be recovered from the eluant. Bromodemethyltetracycline may also be separated into a single antibiotic component by use of the Craig countercurrent distribution technique. In the event that it is desired to separate chlorodemethyltetracycline from other antibiotics contained in the fermented liquor, the above-described methods for isolating bromodemethyltetracycline are applicable.

35 The epidemethyltetracyclines of the present invention are considered to be isomers of the demethyltetracyclines. The structural differences appear to be based upon a rearrangement of the dimethylamino group on the C₄ carbon atom. Apparently under certain conditions which will be described in more detail hereinafter, inversion occurs and an equilibrium mixture of the demethyltetracycline and its epimer occurs. The two may be separated to obtain substantially pure products. For each of the demethyltetracycline, chlorodemethyltetracycline and bromodemethyltetracycline, there is a corresponding isomer which we designate as epidemethyltetracycline, epichlorodemethyltetracycline and epibromodemethyltetracycline.

50 The demethyltetracyclines can be converted into their isomeric forms by simply adjusting the hydrogen ion concentration of a concentrated solution of the antibiotic within the range of pH 3.0 to 5.0 and allowing the solution to stand until the isomerization has come to an equilibrium. The most important conditions which need to be controlled for the conversion of the demethyltetracyclines to their isomers are concentration, hydrogen ion concentration, time and temperature.

65 The isomerization is most conveniently carried out at room temperature, although a

higher rate of conversion takes place at higher temperatures. The pH should be within the range of about 3.0 to 5.0, preferably between 3.5 and 4.5. Some epimerization will take place at hydrogen ion concentrations outside these ranges and even in distilled water; but the rate is very slow. The concentration of the antibiotic in the aqueous solution should be as high as possible in order to obtain the faster rates of epimerization. Complete equilibration may require the period of time of about 24 hours at 25° C., but satisfactory equilibration may require a considerably shorter time under specific conditions. Ordinarily, however, best results are obtained by allowing the solutions to stand for periods of time of one week or more. An equilibrium appears to be reached in most cases at about 50 per cent; this is, about half of the demethyltetracycline is converted to epimer at equilibrium.

Inasmuch as concentration is an important factor in obtaining high yields in short periods of time, a solvent system should be selected which will give the highest concentration of the demethyltetracycline. These solvent systems should be buffered to obtain a pH within the preferred range. Various solvents include methanol, ethanol, butanol, acetone, 2-ethoxy ethanol, 2-methoxy propanol, tetrahydrofuran, dimethylformamide and mixtures of these solvents. Still other solvents may be used. A preferred buffering agent is sodium dihydrogen phosphate, although other buffers and buffer pairs may be used which will maintain the hydrogen ion concentration within the desired range.

The epimers of the present invention may be recovered from aqueous solution in the same general manner that the tetracyclines may be recovered. Although they have slightly less antibacterial activity *in vitro* and *in vivo* as compared to the demethyltetracyclines, they are still highly useful in the treatment of disease caused by bacteria by virtue of their substantial antibacterial activity.

As would be expected, the demethyltetracyclines form salts and complexes of the same type and in the same general manner as do the tetracyclines. Treatment with acids of pH of less than about 4 will result in the formation of acid salts. The free base may be obtained at a pH within the range of about 4 to 6; and at pH's higher than 6, salts with the bases, such as the calcium salt, are obtained. Similarly, salts of the epimers are formed.

As previously indicated the process of producing the new antibiotics of the present invention will be generally the same as those for the production of chlorotetracycline, tetracycline, and bromotetracycline, with principal differences being in the selection of a mutant strain of *S. aureofaciens* which will produce the desired demethyltetracycline instead of

one of the tetracyclines. The composition of the fermentation medium, the rate of aeration, time, temperature, hydrogen ion control, method of inoculation and the like, used in the production of chlortetracycline, bromtetracycline and tetracycline, are suitable for the production of the new demethyltetracyclines.

- 5 In order that the fermentation process of
10 the invention may be fully understood, it will now be described with reference to the following specific examples:

EXAMPLE 1

- 15 A suitable medium for the preparation of inocula for these fermentations may be prepared with the following substances.

Sucrose	30 g./l.
(NH ₄) ₂ SO ₄	2 g./l.
CaCO ₃	7 g./l.
20 Corn Steep Liquor	16.5 ml./l.

- The pH of the medium thus prepared is about 6.8. An 8 milliliter portion is measured into an 8-inch Brewer tube and sterilized at 120° C. for 20 minutes. The sterilized medium is then inoculated with 0.5 ml. of an aqueous spore suspension of strain S-604 of *S. aureofaciens* containing approximately 40—60 million spores per milliliter. The inoculated medium is incubated for 24 hours at 28° C. on a reciprocating shaker operated at 110 cycles per minute.

- 30 A suitable fermentation medium contains water and one suitable for production of chlorodemethyltetracycline also the following:

35 Corn Starch	55 g./l.
CACO ₃	7 g./l.
(NH ₄) ₂ SO ₄	5 g./l.
NH ₄ Cl	1.5 g./l.
FeSO ₄ · 7H ₂ O	40 mg./l.
40 MnSO ₄ · 4H ₂ O	50 mg./l.
ZnSO ₄ · 7H ₂ O	100 mg./l.
CoCl ₂ · 6H ₂ O	5 mg./l.
Corn Steep Liquor	30 g./l.
Cottonseed Meal	2 g./l.
45 Lard Oil	2.0% v/v

- Restricting the chloride ion content of this medium tends to increase the amount of demethyltetracycline at the expense of chlorodemethyltetracycline production.

50 EXAMPLE 2

- A shaker flask fermentation which resulted in the production of some of each of demethyltetracycline and chlorodemethyltetracycline was conducted with a medium prepared as follows:

55 Corn Steep Liquor	30 g./l.
Lard Oil	2% v/v
Corn Starch	55 g./l.
Cottonseed Meal	2 g./l.
60 CaCO ₃	7 g./l.
(NH ₄) ₂ SO ₄	5 g./l.
NH ₄ Cl	1.5 g./l.
FeSO ₄ · 7H ₂ O	40 mg./l.

ZnSO ₄ · 7H ₂ O	100 mg./l.
MnSO ₄ · 4H ₂ O	50 mg./l.
CoCl ₂ · 6H ₂ O	5 mg./l.

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A 25-milliliter portion was measured into a 250 milliliter Erlenmeyer Flask, sterilized for 20 minutes at 120° C. and inoculated with 1 milliliter of mycelial growth prepared as in Example 1. After incubation for 120 hours at 26° C. on a rotary shaker at 186 r.p.m. the mash was assayed and found to contain not more than about 70 gammas per milliliter of chlorotetracycline, about 450 gammas per milliliter of demethyltetracycline and 900 gammas per milliliter of chlorodemethyltetracycline.

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EXAMPLE 3

Tank Fermentation

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A 40-liter fermentation is carried out in a pilot tank, essentially according to the method described in Example 2. The medium, after preparation, is sterilized for 25 minutes at 125° C., and inoculated with strain S-604 of *S. aureofaciens* inoculum. The fermentation is carried out with continuous agitation, at a temperature of 28° C. for the first 24 hours and 25° C. until completion at 135 hours. Sterile air is introduced at a rate of 0.3 l./l./min. for the first sixteen hours, followed by 0.5 l./l./min. to harvest.

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The following examples illustrate the recovery, refining and separation of the antibiotics produced in Example 3:

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EXAMPLE A

Refining of Chlorodemethyltetracycline and Demethyltetracycline

25.8 liters of mash prepared as in Example 3 are treated with 200 milliliters of concentrated hydrochloric acid to adjust the pH to 1.5. The treated mash is filtered, after mixing with 2.8 kilograms of a filter aid to obtain 16.3 liters of filtrate. The filter cake is reslurried with 25 liters of water at a temperature of about 50° C. and adjusted to pH 1.5. After stirring for 30 minutes, the reslurried cake is filtered and the filtrate is combined with the previous filtrate to form a pool of 42.3 liters. The pooled filtrate is treated with 6.76 kilograms of sodium chloride and extracted four times with 15 per cent of the filtrate volume of butanol. The extracts are combined, clarified by filtration, and concentrated *in vacuo* at 25 to 30° C. to a final volume of 6 liters.

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EXAMPLE B

Chromatographic Separation of Chlorodemethyltetracycline and Demethyltetracycline

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The butanol concentrate from Example A is further processed in two equal aliquots, one of which is concentrated to 900 milliliters, the other of which is concentrated to 610 milliliters by vacuum concentration. The concentrates are filtered and adjusted with 50 per cent sodium hydroxide to a pH of 2.

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The solvent system used for the chromatographic separation is prepared by equilibrating an 80 per cent butanol—20 per cent chloroform mixture with water adjusted to pH 2 with hydrochloric acid. The columns are glass columns of six-inch diameter packed with 2.8 kilograms of acid washed "Celite" No. 545 (diatomaceous earth). The word "Celite" is a registered Trade Mark). The columns are equilibrated with the aqueous phase of the solvent system prior to use.

The two butanol concentrates are treated on separate columns. The concentrates are poured gently onto the tops of the columns. The columns are then eluted with the solvent phase, which is run through at a rate of approximately 10 liters per hour. In each case, forty 500-milliliter cuts are taken, and the antibiotic content of each is determined by paper strip chromatography. In the 900 milliliter run, cuts 6 to 17 contain the chlorodemethyltetracycline and chlorotetracycline, and cuts 24 to 40 contain the demethyltetracycline and tetracycline. In the 610 milliliter run, cuts 7 to 18 contain the chlorodemethyltetracycline and chlorotetracycline, and cuts 19 to 31 contain the demethyltetracycline and tetracycline.

EXAMPLE C

Recovery of Chlorodemethyltetracycline from Column Fractions.

The chlorodemethyltetracycline-containing fractions from Example B are combined to give a volume of about 12 liters. An equal amount of water is added, and the mixture is vacuum concentrated to 1810 milliliters of an aqueous solution. The pH of the concentrate is adjusted from 2.3 to 1.9 by the addition of hydrochloric acid. The acidified concentrate is washed twice with 180 milliliters of chloroform, filtered, and concentrated in vacuum to a volume of 360 milliliters. The pH of the concentrate is adjusted from 1.2 to 2.8 with 50 per cent sodium hydroxide. The solution is freeze-dried to yield 18.56 grams of amorphous chlorodemethyltetracycline hydrochloride assaying 550 gammas per milligram.

EXAMPLE D

Recovery of Demethyltetracycline from Column Fractions

The demethyltetracycline-containing cuts from Example B were pooled to give a volume of 15 liters. An equal volume of water was added and the mixture was vacuum concentrated at 24° C. to a volume of 565 milliliters of aqueous solution. The pH of the concentrate was adjusted to 1.65 by the addition of concentrated hydrochloric acid, and the solution is washed twice with 55 milliliters of chloroform. The washed solution is filtered and adjusted with 50 per cent sodium hydroxide to a pH of 5.8. After aging for three hours at room temperature and 15 hours at 4° C., the resulting crystal slurry is filtered

and the product is washed with water and dried *in vacuo* at 40° C. A yield of 4.7 grams of crude neutral demethyltetracycline assaying 880 gammas per milligram as tetracycline hydrochloride is obtained, representing an overall yield of 73 per cent from the aqueous concentrate.

EXAMPLE E

Crystallization of Chlorodemethyltetracycline Hydrochloride from Acetone-Ether.

18.56 grams of freeze-dried chlorodemethyltetracycline as in Example C, is stirred in a mixture of 55 milliliters of acetone, 7.4 milliliters of water and 3.7 milliliters of concentrated hydrochloric acid. The solution is filtered, and the solids are rinsed twice with a total of 55 milliliters of acetone containing 1.85 milliliter of water. The filtrate and washings are combined, the pH being 0.8, and treated with 55 milliliters of ether. The mixture is seeded, aged for 18 hours at room temperature with constant agitation, and the crystals which form are removed by filtration. The product is washed with a mixture of acetone, water and ether, then with acetone, and finally with ether and vacuum dried at 40° C. A yield of 4.74 grams of crystalline chlorodemethyltetracycline hydrochloride assaying 1090 gammas per milligram is obtained, representing a 50.6 per cent step yield. The product contains 11 per cent chlorotetracycline.

EXAMPLE F

Crystallization of Chlorodemethyltetracycline Hydrochloride from Butanol "Cellosolve"

A 50.4 gram portion of freeze-dried chlorodemethyltetracycline prepared as in Example C, and assaying 700 gammas per milligram, is slurried in 22.5 milliliters of "Cellosolve", and to the resulting solution is added a total of 205 milliliters of butanol. The pH is adjusted to 0.8 by the addition of 11 milliliters of concentrated hydrochloric acid and the mixture is aged 51 hours with stirring. The resulting crystals are removed by filtration, washed with a 90—10 mixture of butanol—"Cellosolve", then with chloroform, and vacuum dried. A yield of 30.6 grams of chlorodemethyltetracycline hydrochloride assaying 1053 gammas per milligram is obtained, a yield of 84 per cent. The word "Cellosolve" is a registered Trade Mark.

EXAMPLE G

Recrystallization of Chlorodemethyltetracycline to Destroy Chlorotetracycline

One gram of the chlorodemethyltetracycline prepared in Example E, assaying 1090 gammas per milligram, is slurried in 3 milliliters of 90 per cent butanol-10 per cent "Cellosolve", and 3 milliliters of triethylamine is added thereto to adjust the pH to 10.4. The incomplete solution is allowed to stand at room temperature for 24 hours to destroy the chlorotetracycline which may be present, after which time concentrated hydrochloric

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acid is added to lower the pH to 0.8 and the mixture is aged for 24 hours with agitation. The crystalline product is filtered, washed with 90 per cent butanol-10 per cent cellosolve, then with chloroform, and vacuum dried at 40° C. A yield of 0.36 grams of chlorodemethyltetracycline hydrochloride, assaying 1228 gammas per milligram and containing about 2.5 per cent chlorotetracycline, is obtained, representing a yield of 40 per cent.

The following examples illustrate the conversion of the antibiotics into various forms:

EXAMPLE H

Chlorodemethyltetracycline Hydrobromide

1.0 gram of ammonium chlorodemethyltetracycline is dissolved in 20 milliliters of water and the solution is treated with 0.6 milliliter of 48 per cent hydrobromic acid to adjust the pH to 1.6. The mixture is aged with stirring at room temperature for 18 hours, at which time long needle-shaped crystals of chlorodemethyltetracycline hydrobromide are present. The product is filtered, washed with 6 milliliters of water adjusted to pH 2.2 with hydrobromic acid, and vacuum dried for 5 hours at 44° C. A yield of 750 milligrams of chlorodemethyltetracycline hydrobromide is obtained.

Analysis—Calculated for $C_{21}H_{22}N_2ClBrO_8$: C=46.2; H=4.1; N=5.1; Cl=6.5; Br=14.6; O=23.4. Found: C=45.94; H=4.44; N=4.71; Cl=6.78; Br=13.74; O (difference)=24.39, 24.70.

EXAMPLE I

Preparation of Chlorodemethyltetracycline Ammonium Salt

A 100-milligram portion of the chlorodemethyltetracycline prepared in Example E is dissolved in 0.5 milliliters of 90 per cent butanol-10 per cent "Cellosolve", and ammonia gas is bubbled into the solution until a thick solid appears. The slurry is thinned with a small amount of concentrated aqueous ammonia, allowed to stand at room temperature for 5 hours, and filtered. The product is washed with 90 per cent butanol-10 per cent "Cellosolve", then with acetone, and vacuum dried. A yield of 41.4 milligrams of chlorodemethyltetracycline ammonium salt assaying 1148 gammas per milligram as chlorotetracycline hydrochloride is obtained. (44 per cent yield).

EXAMPLE J

Preparation of Chlorodemethyltetracycline Neutral

A 50 milligram portion of the chlorodemethyltetracycline obtained in Example E, assaying 1090 gammas per milligram, is dissolved in 1.3 milliliter of water, and the solution is treated with dry sodium carbonate to a pH of 7-8. The resulting amorphous precipitate is centrifuged off, and the clear decantate is adjusted to pH 5.4 and aged for

16 hours at room temperature. The product is filtered, washed with water, and vacuum dried at 35° C., to yield 21.9 milligrams of crystalline chlorodemethyltetracycline neutral assaying 1250 gammas per milligram. (50 per cent yield).

EXAMPLE K

Reduction of Chlorodemethyltetracycline to Demethyltetracycline

A 12.5-milligram sample of crystalline chlorodemethyltetracycline hydrochloride is dissolved in water and one drop of triethylamine is added thereto. The solution is mixed with 10 milligrams of 10 per cent palladium on carbon, and shaken in a hydrogen atmosphere for 20 minutes. The resultant solution is filtered to remove the catalyst. Ultraviolet spectra and paper chromatography both indicate that dechlorination to demethyltetracycline occurs.

As indicated hereinabove, salts of the demethyltetracyclines may be formed by treatment with acids and bases. Hydrochloric acid is a preferred acid because the salts of this acid and the demethyltetracyclines are very useful products. Special properties may be imparted by the use of other acids, such as sulfuric acid, phosphoric acid, acetic, boric and other organic and inorganic acids. Salts with bases may be prepared with the alkali metal hydroxides, such as sodium hydroxide, and the alkaline earth metals as described above. The calcium salt is particularly useful and may be prepared by simply adjusting an aqueous solution of any one of the demethyltetracyclines to within the range 6-10 with at least a molar equivalent of the calcium being present in the solution. The barium, magnesium, strontium and other salts may be prepared in like manner. Complexes of the demethyltetracyclines with boron, zirconium, and other polyvalent metal ions are also formed, as in the case of the tetracyclines.

Obviously, if the fermentation contains no chlorine or bromine, neither chlorodemethyltetracycline nor bromodemethyltetracycline can be formed. In such a case, the principal antibiotic will be demethyltetracycline. As the chloride ion is raised, particularly above 50 parts per million, substantial quantities of chlorodemethyltetracycline are formed, as 1 part of chloride ion can result in the production of approximately 14 parts of chlorodemethyltetracycline. The amount of this latter antibiotic can be controlled. We prefer when producing this antibiotic that the fermentation medium contains less than 50 parts per million of bromide ion and over 50 parts per million of chloride ion and this amount may be increased up to 1000 parts per million or even more for maximum production of chlorodemethyltetracycline.

A small concentration of bromide ion in the fermentation medium from about 10

parts per million to several per cent tends to depress the formation of chlorodemethyltetracycline, and the production of demethyltetracycline is generally increased. When the fermentation medium contains less than about 50 parts per million of chloride ion and more than about 50 parts per million of bromide ion, the production of bromodemethyltetracycline is favored; and with high-antibiotic-yielding strains of *S. aureofaciens*, the resulting fermented liquor may contain bromotetracycline as the predominating antibiotic.

WHAT WE CLAIM IS:—

1. Demethyltetracycline, chlorodemethyltetracycline and bromodemethyltetracycline and epimers thereof.

2. A method of preparing demethyltetracycline, chlorodemethyltetracycline and/or bromodemethyltetracycline, which comprises aerobically fermenting an aqueous nutrient medium containing an assimilable source of carbon, nitrogen and mineral substances and having a controlled chloride and bromide ion content with a demethyltetracycline-producing strain of *S. aureofaciens*, if desired recovering the resulting demethyltetracycline antibiotics from the fermentation medium.

3. A method according to claim 2, in which a substantially chloride-free and bromide-free medium is used whereby demethyltetracycline is the predominant antibiotic produced.

4. A method according to claim 2, in which a medium containing at least 50 parts per million of chloride ions and less than 50 parts per million of bromide ions is used whereby chlorodemethyltetracycline is the predominant antibiotic produced.

5. A method according to claim 2, in which a medium containing at least 50 parts per million of bromide ions and less than 50

parts per million of chloride ions is used whereby bromodemethyltetracycline is the predominant antibiotic produced.

6. A method according to any one of claims 2 to 5, in which said medium contains or has added thereto alkaline earth metal ions, and the pH of the medium after fermentation is adjusted to a value of 6 to 10, whereby alkaline earth metal salts of the antibiotics are precipitated.

7. A method of preparing an epimer of demethyltetracycline, chlorodemethyltetracycline, or bromodemethyltetracycline which comprises adjusting the pH value of a concentrated solution of the corresponding demethyltetracycline to a value within the range 3.0 to 5.0 and allowing the solution to stand until isomerization has come to an equilibrium.

8. A method of preparing demethyltetracycline, chlorodemethyltetracycline or bromodemethyltetracycline, substantially as hereinbefore described with reference to Examples 1 to 3.

9. Demethyltetracycline, chlorodemethyltetracycline and bromodemethyltetracycline and epimers thereof, whenever produced by the methods according to any one of the preceding claims 2 to 8.

10. A method of preparing demethyltetracycline which comprises dehalogenating chlorodemethyltetracycline or bromodemethyltetracycline by catalytic hydrogenation preferably using palladium on charcoal as the catalyst.

11. Demethyltetracycline whenever prepared by the method according to claim 10.

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3. A method according to claim 2, in which a substantially chloride-free and bromide-free medium is used whereby demethyltetracycline is the predominant antibiotic produced.

4. A method according to claim 2, in which a medium containing at least 50 parts per million of chloride ions and less than 50 parts per million of bromide ions is used whereby chlorodemethyltetracycline is the predominant antibiotic produced.

5. A method according to claim 2, in which a medium containing at least 50 parts per million of bromide ions and less than 50

parts per million of chloride ions is used whereby bromodemethyltetracycline is the predominant antibiotic produced.

6. A method according to any one of claims 2 to 5, in which said medium contains or has added thereto alkaline earth metal ions, and the pH of the medium after fermentation is adjusted to a value of 6 to 10, whereby alkaline earth metal salts of the antibiotics are precipitated.

7. A method of preparing an epimer of demethyltetracycline, chlorodemethyltetracycline, or bromodemethyltetracycline which comprises adjusting the pH value of a concentrated solution of the corresponding demethyltetracycline to a value within the range 3.0 to 5.0 and allowing the solution to stand until isomerization has come to an equilibrium.

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