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PATENT SPECIFICATION

DRAWINGS ATTACHED

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COMPLETE SPECIFICATION

A new Antibiotic Enduracidin and a process for the production thereof

We, TAKEDA CHEMICAL INDUSTRIES LIMITED, a body corporate organized under the laws of Japan, of 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, 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 a new antibiotic and to its microbiological production.

This invention is based on the observations:

- 15 (1) that there exist microorganisms capable of producing the new antibiotic;
- (2) that the microorganisms capable of producing the antibiotic belong to the Genus "*Streptomyces*";
- (3) that the antibiotic is accumulated when the microorganisms are cultured;
- 20 (4) that the accumulated antibiotic can be

recovered in a desired purity from the culture broth, utilizing the physicochemical properties of the antibiotic; and

(5) that the antibiotic has strong antimicrobial activity against both Gram-positive and acid-fast bacteria. 25

The new antibiotic has been named "Enduracidin".

Enduracidin is produced by a variant of *Streptomyces fungicidicus*, *Streptomyces fungicidicus* No. B-5477 IFO-12439 (ATCC-21013) which has been isolated from the soil at Nishimomiya, Japan, and named as above by us. 30

The microbial characteristics of *Streptomyces fungicidicus* No. B-5477 (ATCC-21013) are as follows. 35

In the following characteristics, the colour names designated "Rdg." are based on Ridgway's Color Standard and Nomenclature. 40

A. Morphological characteristics:

Sporophores form a siral.

Spores show an oval, $0.9 \mu \times 1.6 - 1.1 \mu$

The surface of the spores is spiny.

B. Culture characteristics:

1. Czapek's agar:

Vegetative mycelium (hereinafter abbreviated as VM):

Abundant, spreading, penetrate into the medium, colourless.

Aerial mycelium (hereinafter, abbreviated as AM):

Abundant, powdery, Quaker Drab (Rdg. LI, 1''').

Reverse (hereinafter abbreviated as R):

Warm Buff (Rdg. XV, 17'-d).

Soluble pigment (hereinafter abbreviated as SP): None.

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2. Glucose Czapek's agar:
- VM : Abundant, spreading, colourless.
- AM : Abundant, powdery, Pale Quaker Drab (Rdg. XI, 1''''-d).
- R : Warm Buff.
- SP : None or faint yellow.
3. Glycerine Czapek's agar:
- VM : Abundant, folded, colourless.
- AM : Abundant, Deep Olive Gray (Rdg. LI, 23'''''), becoming Light mouse Gray (Rdg. LI, 15''''-G) to Light Quaker Drab (Rdg. LI, 1''''-b).
- R : Warm Buff.
- SP : None or faint yellow.
4. Glucose asparagine agar:
- VM : Abundant, spreading, colourless.
- AM : Abundant, Light Olive Gray (Rdg. LI 23''''-d) to Pale Quaker Drab (Rdg. LI, 1''''-d) or sometimes poor, white.
- R : Cream Colour (Rdg. XVI, 19'-f) or colourless.
- SP : None.
5. Bouillon broth:
- VM : Abundant, floating on the surface of the medium and a few colourless mycelia sink to the bottom.
- AM : Abundant, white.
- Transparent medium and no soluble pigment.
6. Bouillon agar:
- VM : Abundant, spreading, colourless.
- AM : Moderate, white to Pale Quaker Drab.
- R : Warm Buff (Rdg. XV, 17'-d).
- SP : None.
7. Glucose bouillon agar:
- VM : Abundant, folded, spreading, colourless.
- AM : Abundant, Deep Quaker Drab (Rdg. LI, 1''''-i).
- R : Yellow Ochre (Rdg. XV, 17').
- SP : Faint brown or none.

8. Glycerine bouillon agar:

VM : Abundant, spreading, colourless.

AM : Abundant, powdery, Quaker Drab to Deep Quaker Drab.

R : Isabella Colour (Rdg. XXX, 19''-i).

SP : None or faint brown.

9. Starch agar:

VM : Abundant, spreading, colourless.

AM : Abundant, Light Quaker Drab (Rdg. LI, 1''''-b), becoming Quaker Drab.

R : Light Buff (Rdg. XV, 17'-f).

SP : None.

10. Egg:

VM : Abundant, spreading, wrinkled.

AM : Moderate, white to white with yellowish tinge and sometimes, poor, white.

The colour of the medium changes from grayish white to brown.

11. Yeast extract agar:

VM : Abundant, wrinkled, spreading, colourless.

AM : Abundant, powdery, Light Mouse Gray (Rdg. LI, 15''''-b) to Quaker Drab.

R : Isabella Colour.

SP : None.

12. Potato plug:

VM : Abundant, spreading, raised, up, colourless.

AM : Abundant, Quaker Drab.

The colour of the medium changes to dark brown after 3 weeks.

13. Milk:

VM : Abundant, folded, floating on the surface of the medium, colourless.

AM : Abundant, white to Pearl Gray (Rdg. LII, 35''''-f).

Strong peptonization without coagulation.

14. Carrot plug:

VM : Abundant, spreading, raised up, colourless.

AM : Abundant, Quaker Drab (Rdg. LI, 1'''').

The colour of the plug changes to Buchthorn Brown (Rdg. XV, 17'-f).

15. Gelatin.
VM : Poor.
AM : Poor, white, slow liquefaction.
16. Nutrient gelatine:
VM : Abundant.
AM : Abundant, white, rapid liquefaction.
SP : None.
17. Nitrate reduction in Czapek's solution (containing NaNO_3 0.1%): Positive.
18. Cellulose:
VM : Abundant, spreading, colourless.
AM : Abundant, Quaker Drab (Rdg. LI, 1''''').
No decomposition.
19. Calcium malate agar:
VM : Abundant, spreading, thin, colourless.
AM : Moderate, Pallid Quaker Drab (Rdg. LI, 1'''''-d).
(Rdg. LI, 15'''''-f).
R : Naples Yellow (Rdg. XVI, 19'-d).
SP : None or faint yellow.
20. Tyrosine agar:
VM : Poor, thin spreading, colourless.
AM : None.
R : Colourless.
SP : None.
21. Peptone agar:
VM : Abundant, spreading, flat, colourless.
AM : Poor, Drab Gray (Rdg. XLV, 17'''''-b).
R : Faint Cream Colour (Rdg. XVI, 19'-f).
SP : None.
22. Starch casein agar:
VM : Abundant, spreading, flat, colourless.
AM : Abundant, velvety, Deep Mouse Gray (Rdg. LI, 15'''''-i).
R : Light Olive Gray (Rdg. LI, 23'''''-d).
SP : None.

23. Maltose tryptone agar:

VM : Abundant, spreading, flat, colourless.

AM : Abundant, Mouse Gray (Rdg. LI, 15'''''). to Quaker Drab (Rdg. LI, 1''''').

R : Light Buff (Rdg. XV, 17'-f).

SP : None or faint yellow.

24. Bennett's agar:

VM : Abundant, spreading, flat, thin, colourless.

AM : Abundant Quaker Drab to Deep Quaker Drab (Rdg. LI, 1'''''-i).

R : Colourless to Warm Buff (Rdg. XV, 17'-d).

SP : None.

25. Starch hydrolysis:

Hydrolysis, enzymatic zone/growth zone = 30mm/10mm or 28mm/10mm.

C. Utilization of carbon sources observed by the Pridham Gottlieb method:

Erythritol	±	Melibiose	++
Adonitol	±	Maltose	+++
S-Sorbitol	+	Sucrose	+
L-Inositol	+++	Lactose	+++
D-Mannitol	+++	Raffinose	+
Dulcitol	+	Trehalose	+++
D-Xylose	+++	Salicin	+++
L-Arabinose	+++	Aesculin	+
L-Sorbose	+	Inulin	±
D-Galactose	+++	Dextran	+++
D-Glucose	++	Sodium acetate	++
D-Fructose	+++	Sodium succinate	+
D-Mannose	++	Sodium citrate	+
Rhamnose	+++	Control	±

Remarks; +++ : Heavy growth;

++ : Good growth;

+ : Fair growth;

± : Faint growth.

Comparison of the above-mentioned morphological and culture characteristics with the description in "The Actinomyces, Volume II" written by S. A. Waksman, published by The Williams and Wilkins Company in 1961, shows that the strain usable in the present invention has morphological characteristics which are similar to those of *Streptomyces fungicidicus* in that the sporophores form a spiral, the surfaces of the spores is spiny, the aerial mycelium is gray, no produced, and the strain is of the non-chromogenic type.

However, there are some differences in culture characteristics between this strain and *Streptomyces fungicidicus*.

More specifically, *Streptomyces fungicidicus* produces a pink soluble pigment when cultured on calcium malate agar and weakly coagulates and peptonizes milk on a milk medium.

In contrast, the present strain does not produce such a soluble pigment or, if it does produce a soluble pigment, the latter is pale yellow, and it does not coagulate milk on a milk medium but peptonizes the milk so strongly that the peptonization is completed within one week. Further, the present strain utilizes fructose and salicin, which are not utilized by *Streptomyces fungicidicus*.

Additionally, *Streptomyces fungicidicus* forms Fungicide which is a fungicidal substance, while, the present strain does not form such a fungicidal substance in the culture broth nor in the mycelium.

From the above-mentioned properties of the present strain, we have classified it as a strain of *Streptomyces fungicidicus* and named it *Streptomyces fungicidicus* No. B-5477.

A mutant was obtained by UV-irradiation, γ -ray irradiation and monospore culture. The mutant forms spiral sporophores and the surface of the spores is spiny in the same way as the original strain. Culture characteristics of the mutant are shown in the following.

Culture characteristics of Streptomyces fungicidicus No. B-5477 mutant.

A specimen of *Streptomyces fungicidicus* No. B-5477 was deposited at American Type Culture Collection, Maryland, U.S.A., under the accession number ATCC-21013 and at the institute of Fermentation, Osaka, under the accession number IF012439.

The antibacterial spectrum observed by the cross streak method of *Streptomyces fungicidicus* No. B-5477 on the bouillon agar and glycerine bouillon agar is shown in Table 1. *Streptomyces fungicidicus* No. B-5477 was streaked on agar plates and incubated at 28°C. for 4 days. The plates were then cross streaked with test organisms shown in Table 1 and were further incubated at 37°C. for 20 hours when Gram-positive and Gram-negative bacteria were used and for 40 hours when acid-fast bacteria were used.

Finally, the inhibition length for each test organism was measured.

TABLE 1

Antibacterial spectrum of *Streptomyces fungicidicus* No. B-5477 by the cross streak method.

	Inhibitory zone (mm)			
	Bouillon agar		Glycerine bouillon agar	
<i>Escherichia coli</i>	0	0	0	0
<i>Proteus vulgaris</i>	0	0	0	0
<i>Staphylococcus aureus</i>	0	0	12	12
<i>Bacillus subtilis</i>	0	0	12	12
<i>Bacillus cereus</i>	0	0	0	0
<i>Bacillus brevis</i>	0	0	0	0
<i>Saccharomyces lutea</i>	0	0	0	0
<i>Micrococcus flavus</i>	0	0	0	0
<i>Mycobacterium avium</i>	0	0	0	0

Table 1 shows that *Streptomyces fungicidicus* No. B—5477 produces antibiotic substances which are mainly active against Gram-positive bacteria.

The microbial characteristics of Actinomyces, especially the genus *Streptomyces*, are not generally fixed and this applies also to the characteristics of the Enduracidin producing strains.

Therefore, there are many mutants and variants of *Streptomyces fungicidicus* No. B—5477.

Among the mutants of *Streptomyces fungicidicus* B—5477 (ATCC 21013), regardless of whether the mutation is caused naturally or artificially, for example with X-ray treatment, ultraviolet-ray treatment or by the action of chemical reagents, any mutant which can produce Enduracidin can be employed in the method of the present invention.

The microbial characteristics of the mutant (ATCC No. 21014) derived from *Streptomyces fungicidicus* No. B—5477, which may be obtained by ultraviolet-ray treatment, γ -ray treatment or monospore isolation of the wild strain are as follows.

A. Morphological characteristics:

Same as those of the wild strain.

B. Culture characteristics:

The mutant produces colourless or yellow vegetative mycelia on various cultures, and Massicot Yellow (Rdg.XVI, 21'—f) of Mustard Yellow (Rdg.XVI, 19'—b) aerial mycelia.

The present mutant does not produce a soluble pigment on most culture media, but it occasionally produces a pale yellow soluble pigment.

1. Czapek's agar: Vegetative mycelium (VM): Abundant, spreading, penetrates into the medium, pale yellow, Aerial mycelium (AM) Abundant, powdery, Cream Colour (Rdg.XVI, 19'—f) to Pale Ochraceous Buff (Rdg.XV, 15'—f), Reverse (R): Cream Colour, Soluble pigment (SP): None or faint yellow.

2. Glucose Czapek's agar: (VM) Abundant, spreading, colourless to faint yellow. (AM) Moderate, powdery, white to Naples Yellow (Rdg.XVI, 19'—d), (R) Cream Colour to Mustard Yellow (Rdg. XVI, 19'—b), (SP) None.

3. Glycerine Czapek's agar: (VM) Abundant, spreading, folded, Mustard Yellow. (AM) Abundant, powdery, Cream Colour to Naples Yellow, (R) Light Ochraceous Buff (Rdg.XV, 15'—d) to Ochraceous Buff (Rdg.XV, 15'—b) (SP) pale yellow.

4. Glucose asparagine agar: (VM) Colourless to Mustard Yellow, spreading, (AM) Moderate, velvety, Massicot Yellow (Rdg.XVI, 21'—f) to Mustard Yellow. (R) Yellow, (SP) None.

5. Bouillon agar: (VM) Moderate, spreading, colourless (AM) Moderate, powdery, white, (R) Colourless, (SP) None.

6. Glucose bouillon agar: (VM) Abundant, spreading, colourless, wrinkled, (AM) Moderate, powdery, white to Cream Colour, (R) Pale brown, (SP) None.

7. Glycerine bouillon agar: (VM) Abundant, spreading, colourless, wrinkled, (AM) Moderate, powdery, white, (R) Pale brown, (SP) None.

8. Starch agar: (VM) Abundant, spreading, Mustard Yellow, penetrates into the medium, (AM) Abundant, powdery, Pale Ochraceous Buff (Rdg.XV, 15'—f) to Cream Colour, (R) Naples Yellow to light Ochraceous-Salmon (Rdg.XV, 13'—d). (SP) None or faint yellow.

9. Whole egg medium: (VM) Moderate, spreading, Naples Yellow, (AM) None or very scant, white to Cream Colour, Colour of medium does not change.

10. Löffler's medium (VM) Abundant, colourless, wrinkled, (AM) None, (SP) None, Strong liquefaction.

11. Yeast extract agar: (VM) Abundant, spreading, wrinkled, colourless to faint brown, (AM) Abundant, powdery, Cream Colour to Naples Yellow, (R) Antimony Yellow (Rdg. XV, 17'—b) to Light Ochraceous-Salmon, (SP) None.

12. Potato plug: (VM) Abundant, spreading, wrinkled, antimony Yellow (Rdg.XV, 17'—b), (AM) Abundant, powdery, white to Light Ochraceous-Buff. The colour of the medium becomes brown.

13. Carrot plug: (VM) Abundant, wrinkled, Ochraceous-Buff, (AM). At first white, later Pale Ochraceous-Buff to Light Ochraceous-Salmon. The colour of the medium does not change.

14. Bouillon: (VM) Scant, sinks to the bottom and abundant growth floating on the surface of the medium, colourless to pale brown (AM) Moderate, powdery, white, (SP) None.

15. Glucose bouillon, Glycerine bouillon: Same as on bouillon, but growth is more abundant and wrinkled.

16. Litmus milk: (VM) Abundant growth, Mustard Yellow, floating on the surface of the medium and ring-form growth, wrinkled, (AM) Scant, white, peptonization strong without coagulation.

17. Nutrient gelatin: (VM) Abundant, ring-form on the edge of the tube, wrinkled, Ochraceous-Buff. (AM) Moderate, white to Cream Colour. No soluble pigment. Strong liquefaction.

18. Czapek's solution with 0.2% NaNO_3 : (VM) Moderate, floating on the surface of the medium, colourless, (AM) moderate, powdery, white, (SP) None. Nitrate reduction weak.

19. Cellulose: (VM) Moderate, colourless to Cream Colour, spreading, (AM) Moderate, powdery white to Pale Ochraceous-Salmon.

20. Calcium malate agar: (VM) Moderate,

- colourless to Cream colour to Ochraceous-Buff spreading (AM) Moderate, powdery, white to Pale Ochraceous-Salmon, (R) Cream colour to Light Ochraceous-Salmon, (SP) None.
21. Tyrosine agar: (VM) Scant, thin, colourless, (AM) None, (R) Colourless, (SP) None.
22. Peptone agar: (VM) Moderate, spreading, colourless, (AM) Moderate, powdery, white to Pale Ochraceous-Buff (Rdg.XV, 15'-f), (R) Colourless to Cream Colour, (SP) None, Non-chromogenic type.
23. Starch hydrolysis: Enzymatic zone/ Growth zone=22^{mm}/11^{mm} or 26^{mm}/13^{mm}.

C. Utilization of carbon sources observed by Pridham and Gottlieb's method:

Erythritol	+	D-maltose	+++
Adonitol	±	Sucrose	±
D-Sorbitol	±	Lactose	+++
i-inositol	+++	Raffinose	±
D-Mannitol	+++	Salicin	+++
Dulcitol	±	Aesculin	+
D-Xylose	+++	Inulin	±
L-Arabinose	+++	Na-acetate	++
L-Sorbose	+	Na-succinate	++
D-Galactose	+++	Na-citrate	++
D-Glucose	++	Starch	+++
D-Mannose	+++	Control	±
Glycerine	+++		
D-Fructose	+++		
Rhamnose	+++		
Melibiose	+		
±	faint growth	+	growth
++	Moderate growth	+++	Abundant growth

In the method of the present invention, the Enduracidin producing resin of *Streptomyces fungicidicus* is incubated in a medium containing assimilatable carbon sources, digestible nitrogen sources and other necessary nutrients.

Starch, glucose, lactose, maltose, galactose, sucrose, dextrin, glycerol or millet jelly may be employed as the carbon sources, for example. The nitrogen sources include, for example, peptone, soybean powder, cornsteep, liquor, meat extract, ammonium salts and organic or inorganic nitrogen compounds. Further, a small quantity of inorganic salts such as chlorides, phosphates, and salts of metals such as calcium, zinc, manganese and iron

may be added to the medium. If necessary, conventional nutrient factors or an antifoaming agent such as an animal oil, a wax, a vegetable oil or a mineral oil may be added.

For the culture of the Enduracidin producing-strain, a submerged culture or a shaking culture utilizing a liquid medium is preferred. But, as occasion demands, a surface culture may be employed. The culture conditions such as the temperature, culture period and pH of the medium may be determined so that the production of Enduracidin becomes a maximum. When a submerged culture is employed, the production of Enduracidin becomes a maximum generally under such conditions as at 25°C. to 45°C., at around a

neutral pH and for about 3 to 10 days.

Enduracidin thus produced is contained mostly in the mycelium, but also in the liquid part of the culture broth.

Enduracidin thus accumulated in the culture broth is recovered and refined to a desired purity by utilizing appropriate means with adequate consideration being given to the properties of Enduracidin, for example, those using the difference between Enduracidin and the impurities in solubility, in distribution co-efficient between two liquid phases, in adsorbability, or in ion-coherence.

As the Enduracidin exists not only in the mycelium but also in the liquid part of the culture broth, it is preferably for the recovery of Enduracidin to separate at first the mycelium from the culture broth and then to subject the mycelium and the liquid part of the culture broth, respectively, to a separation or purification process.

In practice, the separation or purification of Enduracidin is advantageously achieved by means of extraction with an organic solvent, and the best results are given when the extraction is carried out with *n*-butanol under basic conditions, followed by re-extraction with an aqueous acid solution.

The separation and purification of Enduracidin may be effected by utilizing the following characteristics: it is adsorbable on activated charcoal under alkaline conditions (pH not lower than about 8.0), extractable with an organic solvent (e.g. acidified aqueous acetone, and aqueous methanol) and not adsorbable on cation- or anion-exchange resins (e.g. Amberlite (trade mark) IR-120 and Amberlite IR-45).

A preferred example of the method for extracting Enduracidin may generally be carried out as follows.

The mycelium obtained from the culture broth is washed with water, and the mycelium is then subjected to extraction twice under neutral or acid conditions with an organic solvent, e.g. 50% to 70% aqueous acetone in a volume three to four times as large as the volume of the mycelium and 50 to 70% methanol, at room temperature for about 3 hours.

The extract is separated and contains plenty of Enduracidin showing a strong antimicrobial activity against gram-positive bacteria. After adjusting to a pH of 5 to 6, the extract is concentrated *in vacuo* to distill out the acetone followed by adjustment with sulphuric acid to pH 3, and then ethyl acetate is added in a proportion of $\frac{1}{3}$ rd part by volume relatively to the resulting, extract, and the whole mixture is shaken to transfer the soluble part into the ethyl acetate layer. An aqueous layer (containing some precipitate) separated from the ethyl acetate layer is adjusted to pH 8 with sodium hydroxide, sodium carbonate or sodium bicarbonate, followed by extraction

with $\frac{1}{3}$ rd volume *n*-butanol relatively to the whole volume.

After the above-mentioned extraction with *n*-butanol has been repeated once more, the *n*-butanol layer is washed with $\frac{1}{2}$ volume water relatively to the whole volume, and then extracted with an aqueous acid solution (e.g. pH 2, N/200 HCl or N/200 H₂SO₄) to produce an aqueous acid solution containing the active ingredient.

The aqueous solution is subjected to the above-mentioned extraction with *n*-butanol at pH 8.0. The *n*-butanol layer obtained contains Enduracidin produced in the mycelium in a high yield. The extraction with aqueous hydrochloric acid solution and that with *n*-butanol are further repeated to yield an *n*-butanol layer containing the active ingredient in a much higher concentration. The resulting *n*-butanol layer is concentrated to a certain degree, washed with water and further concentrated *in vacuo* at a low temperature to distill off *n*-butanol, until the remaining amount of *n*-butanol in the system becomes very small.

Ether is added to the condensed *n*-butanol in one-tenth volume to yield the yellowish-brown precipitates which are separated out by filtration. The precipitates are washed with ether to yield a crude active ingredient containing a greater part of the accumulated Enduracidin.

The crude substance shows antimicrobial activity of 5,000—10,000 units per milligram against *Staphylococcus aureus* and of 5,000—10,000 units per milligram against *Bacillus subtilis*.

In order to refine the crude substance, the following process is preferably employed.

The crude substance dissolved in an aqueous acid solution is passed through a column packed with activated charcoal, which is previously treated with an acid, and is then treated with a weakly basic ion-exchange resin (e.g. Amberlite (trade mark) IR-4b or IR-45, Rohm & Hass Co., U.S.A.) to remove the acid. The treated solution is concentrated or freeze-dried to yield the pure active ingredient.

The active ingredient obtained in this way may be further subjected to purification or may be changed into its hydrochloride by, for example, the following process. The active ingredient dissolved in a methanolic hydrochloric acid solution is passed through a column packed with activated charcoal, which has previously been treated with an acid, and the treated solution is concentrated at room temperature. Acetone-ether is added to the concentrate which is then kept standing to yield a colourless crystalline powder of Enduracidin hydrochloride.

This powder is soluble in water and methanol, and shows antimicrobial activity of 10,000 to 15,000 units per milligram against

Staphylococcus aureus and of 10,000 to 15,000 units per milligram against *Bacillus subtilis*.

A 20 to 50% aqueous methanol solution of this powder is passed through a weakly basic ion-exchange resin, and to the *n*-butanol is added to the effluent followed by distilling off water and methanol to give a condensate.

Alternatively, the aqueous solution of this

powder is adjusted to pH 8–8.5, and the treated solution extracted with *n*-butanol, followed by condensation to give a condensate.

Ether is added to the condensate obtained to yield Enduracidin in its free form.

The physico-chemical and biological properties of Enduracidin thus purified are as follows:

1. Elementary analysis:

Enduracidin consists of C, H, N, O, and Cl atoms.

	C (%)	H (%)	N (%)	(O%)	Cl (%)
Free form* ¹	53.2±0.5	6.54±0.3	14.45±0.5	22.45±0.5	3.36±0.5
Hydrochloride* ²	49.7±0.5	6.29±0.3	13.51±0.5	21.08±0.5	9.42±0.5

(The free form is hereinafter simply referred to as A whilst the hydrochloride form is hereinafter simply referred to B.*¹). The free form still contains $3.36 \pm 0.5\%$ chlorine atom in its molecule.

The chlorine content of about $3.36 \pm 0.5\%$ is considered to be due to strong ionic bonding or covalent bonding, though this has not yet been confirmed.

2. Molecular Weight and Structural Unit:

When Enduracidin is hydrolysed with a 6 N-hydrochloric acid solution at 110°C. for from 6 to 48 hours, a mixture of the product comprises threonine, serine, glycine, alanine and aspartic acid, some ninhydrin-positive substances and undetermined substances which give blue and violet-blue with Sakaguchi's reagent. These undetermined substances include those which show at first yellowish-brown, yellow or violet on the ninhydrin reaction but these colours change to violet at the end on keeping standing at room tem-

perature, and their amounts vary in accordance with the extent of hydrolysis.

From those characteristics, Enduracidin is regarded as a new basic peptide type antibiotic.

On the other hand, from a neutralizing titration curve obtained by titration of Enduracidin in 50% methanol with an N/10 hydrochloric acid solution, it is understood that Enduracidin contains some basic groups capable of dissociating in its molecule. From the accounted amount of chlorine, the minimum molecular weight of Enduracidin may be calculated as about 1055, and therefore a molecular weight of Enduracidin may be shown as (about 1000 to 1100)_n, wherein *n* is a real number.

3. Specific rotation:

A: $[\alpha]_D^{25} = 85^\circ \pm 10^\circ$ (C = 0.5, in dimethylformamide)

B: $[\alpha]_D^{25} = 70^\circ \pm 10^\circ$ (C = 0.5, in water), $80^\circ \pm 10^\circ$ (C = 0.5 in methanol)

4. The absorption spectra: are shown in the accompanying drawings.

Fig. 1 (free form) and Fig. 4 (hydrochloride

form).

The significant absorption maxima observed are as follows:

*²) The hydrochloride has a further 2 moles of chlorine relatively to the chlorine atom of the free form.

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22

	A	B
	$230\text{m}\mu$ (E $\frac{1\%}{1\text{cm}}$) = 220 ± 15	$230\text{m}\mu$ (E $\frac{1\%}{1\text{cm}}$) + 213 ± 10
λ 95% MeOH max	$263\text{m}\mu$ (E $\frac{1\%}{1\text{cm}}$) = 150 ± 10	$263\text{m}\mu$ (E $\frac{1\%}{1\text{cm}}$) = 140 ± 10
λ N/10 HCl max	$230\text{m}\mu$ (E $\frac{1\%}{1\text{cm}}$) = 195 ± 10	
	$272\text{m}\mu$ (E $\frac{1\%}{1\text{cm}}$) = 112 ± 10	
λ N/10 NaOH max	$250\text{m}\mu$ (E $\frac{1\%}{1\text{cm}}$) = 335 ± 10	

The infrared absorption spectrum of Enduracidin, measured by the Potassium bromide disc method, is shown in Fig. 2 (free form KBr-disc method), in Figs. 3 (free form, Nujol method), in Fig. 5 (hydrochloride form,

KBr-disc method) and in Fig. 6 (hydrochloride form, Nujol method), respectively.

The significant absorption bands in microns are as follows:

A:	B:
3.05 (strong)	3.05 (strong)
3.25 (shoulder)	3.25 (shoulder)
3.38 (shoulder)	3.38 (shoulder)
5.75 (shoulder)	5.77 (shoulder)
5.95 (shoulder)	5.95 (shoulder)
6.09 (strong)	6.09 (strong)
6.24 (shoulder)	6.23 (shoulder)
6.47 (shoulder)	6.47 (shoulder)
6.55 (shoulder)	6.55 (shoulder)
6.61 (strong)	6.61 (strong)
6.90 (middle)	6.95 (middle)
7.23 (middle)	7.25 (middle)
7.63 (middle)	7.48 (shoulder)
8.10 (broad, strong)	8.16 (broad, strong)
8.51 (middle)	8.51 (middle)
9.90 (middle)	9.89 (middle)
10.40 (weak)	10.40 (weak)
11.92 (middle)	11.93 (middle)
12.30 (weak)	12.30 (weak)
12.75 (very weak)	

5. Colour:

A: Colourless powder

B: Colourless crystalline powder

6. Colour reaction:

Reagent	Enduracidin	Result
ninhydrin reagent	(A)	positive (pale-brownish violet)
	(B)	do
Barton's reagent (a mixture of equal volumes of 1% aqueous ferric chloride and 1% aqueous potassium ferricyanate)	(A)	positive (pale-blue)
	(B)	do
Dragendorff reagent	(A)	Positive (orangish-yellow)
	(B)	do
Alkaline potassium permanganate	(A)	discolouration occurs (reduction)
	(B)	do

7. Melting point:

A: 205 — 225° C. (sintering) 225 — 240° C. (decomposition)

B: 215 — 220° C. (sintering) 235 — 245° C. (decomposition)

8. Solubility:

(A) is readily soluble in pyridine, N,N-dimethylformamide, aqueous 5% NIKKOL HCO—50 solution (a 5% by weight aqueous solution of polyoxyalkylene adduct of hydrogenated castor oil made by NIKKO-SHOKAI-KABUSHIKI-KAISHA, Japan), dilute acid, aqueous alkaline solution (with decomposition), and soluble in aqueous methanol, aqueous ethanol, aqueous acetone and aqueous butanol; but insoluble or hardly

soluble in water, absolute ethanol, pure butanol and pure acetone.

(B) is readily soluble in water and methanol, and soluble in ethanol, but insoluble in acetone and ether.

9. *R_f* value (paper partition chromatography):

The paper partition chromatograph measured by using the ascending method on "Whatman filter paper No. 1" (W. and R. Balston Ltd., Great Britain) is as follows:

Solvents	<i>R_f</i> value (A = B) * ¹
Acetic acid . <i>n</i> -Butanol . Water (1:4:5)	0.45 ± 0.1 * ²
<i>n</i> -Butanol saturated with water	0.0
Pyridine . <i>n</i> -Butanol . Water (3:4:7)	0.80 ± 0.1

*¹) The same *R_f* value is observed for A and B*²) The substance is easily adsorbed on a filter paper under neutral conditions.

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12. Th again 20

10. *Stability:*

(A) and (B) are rather stable in methanol and in an acid solution, but its antibacterial activity decreases to 1/5—1/10 on storage at 40°C. for 42 hours in an alkaline solution.

11. *Toxicity:*

The medium lethal dose (LD₅₀) of (A) against acute toxicity in mice is not less than 880 milligrams per kilogram of body weight when administered intraperitoneally and not less than 66 milligrams per kilogram of body weight when administered intravenously.

The medium lethal dose (LD₅₀) of (A) against acute toxicity in rats is not less than 300 milligrams per kilogram of body weight when administered intravenously.

12. *Antibacterial spectra:*

The antimicrobial activities of Enduracidin against various microorganisms are measured

by means of the agar dilution method.

Gram-positive or -negative bacteria employed as the test organisms are incubated on bouillon agar at 37°C. for 20 hours.

For acid-fast bacteria, glycerin bouillon agar is used and incubation is carried out at 37°C. for 40 hours.

In the case in which fungi or yeast are employed, glucose bouillon agar is used as the incubation medium and the incubation is carried out for 40 hours at 28°C.

Antimicrobial spectra of Enduracidin shown by minimum inhibitory concentration are shown in Table 2.

As is seen in Table 2, Enduracidin shows strong antimicrobial activities against Gram-positive bacteria and acid-acid bacteria.

The minimum inhibitory concentration against the bacteria tested at 37°C. on TSA culture containing beef blood serum for 20 hours is shown in Table 1.

TABLE 1

Test bacteria	Minimum inhibitory concentration (mcg/ml)	
	(A)	(B)
<i>Staphylococcus aureus</i> 209 p	2.5	0.78
<i>Streptococcus pyogenes</i> E-14	1.25	0.78
<i>Streptococcus pyogenes</i> Dick	1.25	0.78
<i>Streptococcus pyogenes</i> S-8	1.25	0.78
<i>Streptococcus pyogenes</i> Ni-5	2.5	1.56
<i>Streptococcus viridans</i> sp.	1.25	1.56
<i>Diplococcus pneumoniae</i> I	1.25	0.78
<i>Diplococcus pneumoniae</i> II	0.625	0.78
<i>Diplococcus pneumoniae</i> III	1.25	0.78
<i>Corynebacterium diphtheriae</i>	0.625	0.78

TABLE 2

Test bacteria	Minimum inhibitory concentration ($\mu\text{g/ml}$)
<i>Escherichia coli</i>	>100
<i>Proteus vulgaris</i>	>100
<i>Staphylococcus aureus</i>	0.1
<i>Bacillus subtilis</i>	0.1
<i>Bacillus cereus</i>	0.5
<i>Bacillus brevis</i>	1.0
<i>Sarcina lutea</i>	0.2
<i>Micrococcus flavus</i>	0.05
<i>Mycobacterium avium</i>	5.0
<i>Mycobacterium avium</i> (Streptomycin-resist)	5.0
<i>Mycobacterium avium</i> (Neomycin-resist)	5.0
<i>Mycobacterium sp. ATCC 607</i>	5.0
<i>Mycobacterium phlei</i>	5.0
<i>Mycobacterium smegmatis</i>	2
<i>Piricularia oryzae</i>	>100
<i>Aspergillus niger</i>	>100
<i>Penicillium notatum</i>	>100
<i>Candida albicans</i>	>100
<i>Saccharomyces cerevisiae</i>	>100

Physiological acidity or basicity of Enduracidin is measured by means of the cross-streak agar dish method.

5 The results are shown in Table 3.
As seen in Table 3, Enduracidin shows

stronger activities on basic assay media than on neutral or acid media, which means that Enduracidin seems to belong to a so-called physiologically basic substance.

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TABLE 3

(Difference of antimicrobial activities by pH-changes, or Enduracidin)

Test bacteria	pH	Inhibitory zone (mm)	
<i>Bacillus subtilis</i>	6	11	11.5
	8	13	12
<i>Mycobacterium avium</i>	6	0	0
	8	0	0

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The following examples set forth presently preferred embodiments of the present invention; this is intended to be solely illustrative however, and not at all limitative of the invention.

In the present specification as well as the following examples, the abbreviations μ g, mg, g, kg, ml, l and $^{\circ}$ C. refer to microgram(s), milligram(s), gram(s), kilogram(s), milliliter(s), liter(s) and degree(s) centigrade, respectively, percentages are weight/volume percentages unless otherwise described.

EXAMPLE 1

Streptomyces fungicidicus No. B-5477 (ATCC No. 21013) of one platinum loop taken from a glucose-asparagine agar slant is inoculated on 500 ml. each of aqueous culture media which are respectively placed in several 2 l.-flasks, the respective aqueous media containing 2% of glucose, 3% of starch, 1% of corn steep liquor, 1% of soybean flour, 0.5% of peptone, 0.3% of sodium chloride and 0.5% of calcium carbonate being previously adjusted to pH 7 and sterilized at 120 $^{\circ}$ C. for 20 minutes with high-pressure

steam. The respective culture media are then incubated with shaking (120 r.p.m., 10cm) at 28 $^{\circ}$ C. for 24 hours.

One l. of the resulting culture broth optionally collected from the flasks is pre-incubated in 30 l. of the culture medium of the same composition as above, placed in a 50 l.-tank for 24 hours at 28 $^{\circ}$ C. with stirring (280 r.p.m.) and aerated with a volume of 45 l./minute.

The pre-culture obtained is further incubated for 96 hours at 28 $^{\circ}$ C. in 500 l. of an aqueous culture medium containing 1% of glucose, 2% of starch, 1% of corn steep liquor, 1% of soybean powder, 0.5% of peptone, 0.3% of sodium chloride and 0.5% of calcium carbonate placed in a 1000 l. vessel of stainless steel with stirring (160 r.p.m./minute) and aeration with a volume of 750 l./minute during the first half of the cultivation and 500 l./minute during the latter half of the cultivation, while employing about 15Kg. of silicone oil as an anti-foaming agent.

Changes of pH value and of antimicrobial activity shown with agar dilution unit as the lapse of the time of cultivation are shown in the following table.

Cultivation period	pH	Culture filtrate (unit)		Mycelia (unit)	
		St*	B**	St*	B**
Sakaguchis flask (seed)	6.15	—	—	—	—
pre-culture (30 l.)	7.05	—	—	—	—
0	7.05	—	—	—	—
18	6.28	—	—	—	—
24	6.70	—	—	—	—
main culture (500 l.)	7.05	—	—	—	—
0	7.05	—	—	—	—
18	6.75	—	—	—	—
30	7.16	10>	10>	10>	10
42	7.36	10>	10>	20	20
54	7.55	35	35	350	350
66	7.70	50	50	350	350
78	8.05	75	75	750	750
90	7.93	100	100	1000	1000

* *Staphylococcus aureus*

** *Bacillus subtilis*

EXAMPLE 2

One l. of the seed culture optionally collected from the flasks as in Example 1 is incubated in 30 l. of an aqueous culture medium containing 2% of glucose, 3% of starch, 1% of corn steep liquor, 1% of soybean powder, 0.5% of peptone, 0.3% of sodium chloride, 0.5% of calcium carbonate and 0.2% of sodium glutamate. The culture

medium is then incubated at 28°C. for 96—120 hours with aeration (45 l./minute) and agitation (280. r.p.m.), while employing a silicone oil as an anti-foaming agent.

Change of pH value and of antimicrobial activity shown with agar dilution unit as lapse of the time of cultivation are shown in the following Table.

Cultivation Period	pH	Culture filtrate (unit)		Mycelia (unit)	
		St*	B**	St*	B**
0	7.25	—	—	—	—
18	6.95	—	—	—	—
30	6.81	10>	10>	10>	10>
42	7.00	10>	10>	10>	10>
54	7.30	35	20	150	150
66	7.52	35	20	150	150
78	7.62	35	35	350	350
90	7.59	500	500	1500	1500
102	7.70	500	500	2000	2000
114	7.90	500	500	2000	2000

EXAMPLE 3

500 L. of the culture broth obtained in Example 1 are subjected to filtration to separate 100 Kg. of wet mycelia from 400 l. of culture filtrate. The wet mycelia are subjected to extraction twice with 300 l. each of a 70% aqueous acetone under agitation for three hours. The acetone layers are combined (showing 200—350U/ml. against *St. aureus*) and adjusted to pH 5—6 with 4N-sulfuric acid, from which acetone is evaporated off under reduced pressure to leave 200 l. of aqueous solution. The aqueous solution is adjusted to pH 3.0 with the addition of 4N sulfuric acid. 70 L. of ethyl acetate are then added to the adjusted acid solution, and the mixture is stirred, then left standing to form two layers. The ethyl acetate layer is removed and the aqueous layer (containing some precipitate) is taken out and adjusted to pH 8.0 with 4N- sodium hydroxide, immediately followed by extraction twice with 90 l. each of *n*-butanol. The *n*-butanol solutions, showing 150—200U/ml. against *Staphylococcus aureus*, are combined, and washed with 90 l. of distilled water. The treated butanol solu-

tion is subjected to extraction twice with 50 l. each of N/200 hydrochloric-acid. The respective hydrochloric acid solutions are combined and adjusted to pH 8.0 with 4N sodium hydroxide, immediately followed by subjecting them to extraction twice with 50 l. each of *n*-butanol. The respective *n*-butanol layers are combined and washed with 50 l. of distilled water. The combined and washed *n*-butanol layer is subjected to extraction twice with 15 l. each of N/200 hydrochloric acid. The aqueous hydrochloric layers are combined and adjusted to pH 8.0 with 4N sodium hydroxide, immediately followed by extraction twice with 15 l. each of *n*-butanol. The respective *n*-butanol layers are combined and washed twice with 10 l. each of distilled water. The treated *n*-butanol layer has added thereto $\frac{1}{4}$ as much volume of distilled water, and the mixture is then subjected to concentration under reduced pressure at an outside temperature not higher than 70°C. until the remaining volume of butanol becomes 200 ml. In the course of the concentration, the precipitates of the product of this invention are observed. About 2 l. of ether (not containing

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peroxide are added to the concentrate, whereby the resulting precipitates are collected by filtration together with the precipitates which were already formed. The collected precipitates are washed with ether to yield about 3.5g. of crude powdery substance showing an antimicrobial activity of 5,000 U/mg. against *Staphylococcus aureus*.

4N-sulphuric acid is added to 400 l. of the culture filtrate referred to in the opening paragraph of this Example to adjust its pH to 3.0, followed by the addition of 150 l. of ethyl acetate. The mixture is stirred and is then left standing for a while to form two layers. The aqueous layer is taken out and is adjusted to pH 8.0 with 4N-sodium hydroxide, immediately followed by the addition of 100 l. of *n*-butanol. The mixture is stirred, and is then left standing for a while to form two layers. The *n*-butanol layer is washed with about 50 l. of distilled water, and is then subjected to extraction twice with 15 l. of N/200 hydrochloric acid. The respective hydrochloric acid layers are combined and adjusted to pH 8.0 with 4N-sodium hydroxide, immediately followed by the addition of 10 l. of *n*-butanol. The mixture is stirred for further extraction and is then left standing to form two layers. The *n*-butanol layer taken out is washed twice with distilled water each $\frac{1}{2}$ as much volume as the *n*-butanol layer.

The washed *n*-butanol layer has added thereto distilled water of $\frac{1}{2}$ as much volume as the butanol layer. The mixture is subjected to concentration under reduced pressure at an outside temperature not higher than 70°C. The concentration are subjected to similar processes to those which are applied to the wet mycelia referred to in the first paragraph of this Example, whereby about 0.5 g. of a crude powdery substance showing an antimicrobial activity of 5,000—7,500 U/mg against *Staphylococcus aureus* is produced.

EXAMPLE 4

100 l. of an acid solution containing an active ingredient which is obtained by extracting with *n*-butanol in Example 3 is passed through a column packed with 1 kg. of activated charcoal (Takeda Chemical Industries Limited) for column chromatography which was previously treated with hydrochloric acid so as to obtain 70 l. of an almost colourless fraction containing the active ingredient. The fraction is passed through a column packed with 2 l. of Amberlite (trade mark) IR-45 at S.V.5 to yield a slightly basic aqueous solution. The aqueous solution is concentrated at a relatively low temperature under reduced pressure. *n*-Butanol is added to the concentrate and the mixture is stirred to transfer the active ingredient to the *n*-butanol layer. out, or N-hydrochloric acid is added to the *n*-butanol layer as in Example 3, whereby the

free form of the active ingredient precipitates out, or N-hydrochloric acid is added to the *n*-butanol to make its pH 4.5, followed by concentration by freeze-drying, in the respective cases, 1.8g. of a powdery substance showing antimicrobial activity of 7,500—10,000 U/mg against *Staphylococcus aureus* and 1.9 g. of a powdery substance showing antimicrobial activity of 7,500—10,000 U/mg are obtained.

EXAMPLE 5

1 g. of crude powder substance prepared in the same manner as in Example 3 or Example 4 is dissolved in a mixture of 500 ml. of methanol and 1 ml. of N-hydrochloric acid.

After the solution is passed through a column packed with 10 g. of the activated charcoal (Takeda Chemical Industries, Ltd.) for column chromatography, previously treated with the mixture of 4 ml. of N/10-hydrochloric acid and 100 ml. of methanol, the column is washed with methanol to yield 1.5 l. of effluent containing active ingredient.

The effluent is concentrated in vacuo, followed by the addition of acetone-ether at a relatively low temperature whereby white precipitates are obtained.

The precipitates are collected by filtration and washed with ether to yield 500 mg. of precipitates (hydrochloride form).

The precipitates show antimicrobial activity of 15,000 units per mg. against *Staphylococcus aureus*.

On the other hand, 100mg. of precipitates (hydrochloride form) are dissolved in 100 ml. of 20% aqueous methanol, followed by passage through a column of 5 ml. of Amberlite (trade mark) IR-45 at S.V.3.

The column is washed with methanol, and the effluent is concentrated. The condensates are lyophilised, followed by washing with acetone-ether to yield 80 mg. of white powder (free from).

The precipitate shows antimicrobial activity of 15,000 units per mg. against *Staphylococcus aureus*.

EXAMPLE 6

16 Litres of the culture broth obtained in a similar manner to Example 7 below are subjected to filtration to separate out about 4 kg of wet mycelia. The mycelia are subjected to extraction at pH of about 2 with four times as much volume of 70% aqueous sulphuric acid acetone solution as the mycelia under agitation for four hours. After 16 l. of aqueous acetone extract (3500 U/me against *Staphylococcus aureus*) are adjusted to pH 8—8.4 with sodium hydroxide, 160 g of activated charcoal (SHIRASAGI-A, Takeda Chemical Industries, Ltd., Japan) and 160 g of Hyflo Super Cel (Johns Manville Sales Corp. U.S.A.) are added to the aqueous acetone layer, and the mixture is agitated

for one hour. After filtration, the activated charcoal is washed with ten times as much volume of acetone as the filtrate and then with ten times as much volume of methanol.

5 The treated activated charcoal is subjected to extraction twice with 20 times as much as volume of an acid aqueous acetone solution as the activated charcoal under agitation for three hours.

10 The extracts are combined and adjusted to pH 5, from which the solvent is evaporated off to leave 1.5 l. of an aqueous solution. The solution is passed through a column packed with 200 ml. of Amberlite (trade

15 mark) IR-120 (H) and then a column packed with 200 ml. of Amberlite IR-45, and subjected to distillation to remove the solvent under reduced pressure. The residue is mixed with 5 l. of water and the mixture

20 is adjusted to pH 8.5 and extracted three times with one third of its volume of *n*-butanol. The *n*-butanol layer is washed with water and condensed under reduced pressure while adding water thereto to leave 500 ml.

25 of the aqueous *n*-butanol solution. 5 L. of ether are added to the solution to yield preci-

pitates which are filtered off and dried to give 4 g. of powdery crude Enduracidin.

The crude Enduracidin obtained shows about 10,000 U/mg. against *Staphylococcus aureus*. 30

EXAMPLE 7

500 Ml. of a culture medium consisting of 2% glucose, 3.5% corn steep liquor, 1.5% calcium carbonate and water are poured into each of two 2 l. flasks. The medium is inoculated with *Streptomyces fungicidus* No. B-5477 mutant (IFO-12440) (ATCC-21014) and incubated at 28°C. for two days with shaking on a reciprocating shaker to prepare a preinoculation medium. In a 50 l. tank, 30 l. of an aqueous culture medium consisting of 2% glucose, 3% soluble starch, 1% corn steep liquor, 1% soybean flour, 0.3% sodium chloride, 0.5% peptone, 0.5% calcium carbonate and water and adjusted to pH 7.0 are inoculated with the resulting preincubation broth, and the inoculated medium is incubated at 28°C. with agitation and aeration of 30 l. per minute. Progress of the incubation during 90 hours is as shown in the following table. 35 40 45 50

10 (6)

Incubation period	pH of the broth	Potency of whole broth (dil. u./ml.)
0	7.05	<10
18	7.00	<10
30	7.10	10
42	7.35	50
54	7.82	350
66	7.82	1000
78	7.84	1500
90	7.84	1500

WHAT WE CLAIM IS:—

55 1. A method for producing Enduracidin and its hydrochloride, which comprises culturing *Streptomyces fungicidus* No. B5477 (ATCC 21013) or an enduracidin-producing mutant thereof, in a medium containing

60 assimilatable carbon sources, digestible nitrogen sources and other nutrients necessary for the growth of the microorganism under aerobic conditions until Enduracidin is substantially accumulated in the culture broth, and recovering

65 the accumulated Enduracidin therefrom, and converting the Enduracidin if desired to the hydrochloride thereof.

2. Enduracidin having the following characteristics:

acteristics:

- (1) Its elementary analysis is C $53.2 \pm 0.5\%$, H $6.54 \pm 0.3\%$, N $14.45 \pm 0.5\%$, O $22.45 \pm 0.5\%$ and Cl $3.36 \pm 0.5\%$; 70
- (2) When Enduracidin is hydrolysed with a 6 N-hydrochloric acid solution at 110°C. for from 6 to 48 hours, the product comprises a mixture of threonine, serine, glycine, alanine and aspartic acid, some ninhydrin-positive substances and undetermined substances which give blue and violet-blue with Sakaguchi's reagent. 75
- (3) Its specific rotation is $[\alpha]_D^{25} = +85^\circ \pm 10^\circ$ ($c=0.5$, in dimethylformamide); 80

(7)

(8)

(9)

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for one hour. After filtration, the activated charcoal is washed with ten times as much volume of acetone as the filtrate and then with ten times as much volume of methanol. The treated activated charcoal is subjected to extraction twice with 20 times as much as volume of an acid aqueous acetone solution as the activated charcoal under agitation for three hours.

- 10 The extracts are combined and adjusted to pH 5, from which the solvent is evaporated off to leave 1.5 l. of an aqueous solution. The solution is passed through a column packed with 200 ml. of Amberlite (trade mark) IR-120 (H) and then a column packed with 200 ml. of Amberlite IR-45, and subjected to distillation to remove the solvent under reduced pressure. The residue is mixed with 5 l. of water and the mixture is adjusted to pH 8.5 and extracted three times with one third of its volume of *n*-butanol. The *n*-butanol layer is washed with water and condensed under reduced pressure while adding water thereto to leave 500 ml. of the aqueous *n*-butanol solution. 5 L. of ether are added to the solution to yield precipitates which are filtered off and dried to give 4 g. of powdery crude Enduracidin.

The crude Enduracidin obtained shows about 10,000 U/mg. against *Staphylococcus aureus*.

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EXAMPLE 7

500 Ml. of a culture medium consisting of 2% glucose, 3.5% corn steep liquor, 1.5% calcium carbonate and water are poured into each of two 2 l. flasks. The medium is inoculated with *Streptomyces fungicidus* No. B-5477 mutant (IFO-12440) (ATCC-21014) and incubated at 28°C. for two days with shaking on a reciprocating shaker to prepare a preinoculation medium. In a 50 l. tank, 30 l. of an aqueous culture medium consisting of 2% glucose, 3% soluble starch, 1% corn steep liquor, 1% soybean flour, 0.3% sodium chloride, 0.5% peptone, 0.5% calcium carbonate and water and adjusted to pH 7.0 are inoculated with the resulting preincubation broth, and the inoculated medium is incubated at 28°C. with agitation and aeration of 30 l. per minute. Progress of the incubation during 90 hours is as shown in the following table.

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Incubation period	pH of the broth	Potency of whole broth (dil. u./ml.)
0	7.05	<10
18	7.00	<10
30	7.10	10
42	7.35	50
54	7.82	350
66	7.82	1000
78	7.84	1500
90	7.84	1500

WHAT WE CLAIM IS:—

- 55 1. A method for producing Enduracidin and its hydrochloride, which comprises culturing *Streptomyces fungicidus* No. B5477 (ATCC 21013) or an enduracidin-producing mutant thereof, in a medium containing assimilatable carbon sources, digestible nitrogen sources and other nutrients necessary for the growth of the microorganism under aerobic conditions until Enduracidin is substantially accumulated in the culture broth, and recovering the accumulated Enduracidin therefrom, and converting the Enduracidin if desired to the hydrochloride thereof.

2. Enduracidin having the following characteristics:

acteristics:

- (1) Its elementary analysis is C 53.2±0.5%, H 6.54±0.3%, N 14.45±0.5%, O 22.45±0.5% and Cl 3.36±0.5%; 70
- (2) When Enduracidin is hydrolysed with a 6 N-hydrochloric acid solution at 110°C. for from 6 to 48 hours, the product comprises a mixture of threonine, serine, glycine, alanine and aspartic acid, some ninhydrin-positive substances and undetermined substances which give blue and violet-blue with Sakaguchi's reagent. 75
- (3) Its specific rotation is $[\alpha]_D^{25} = +85^\circ \pm 10^\circ$ (c=0.5, in dimethylformamide); 80

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amide);

(4) Its melting point is 205°C.—225°C.
with sintering and decomposition at
225°C.—240°C.;

(5) Its ultraviolet absorption spectrum is

λ 95% MeOH max 230 m μ

263 m μ

λ N/10 HCl 230 m μ

272 m μ

λ N/10 NaOH max 250 m μ

as shown on Fig. 1 of the accompany-
ing drawings, and the significant
absorption maxima observed are as
follows:

$\frac{1\%}{1\text{cm}} = 220 \pm 15$

$\frac{1\%}{1\text{cm}} = 150 \pm 10$

$\frac{1\%}{1\text{cm}} = 195 \pm 10$

$\frac{1\%}{1\text{cm}} = 112 \pm 10$

$\frac{1\%}{1\text{cm}} = 335 \pm 10$

10 (6) Its infrared absorption spectrum is as
shown on Figs. 2 and 3 of the accom-
panying drawings, and the significant

absorption bands in microns are as
follows:

3.05 (strong)

3.25 (shoulder)

3.38 (shoulder)

5.75 (shoulder)

5.95 (shoulder)

6.09 (strong)

6.24 (shoulder)

6.47 (shoulder)

6.55 (shoulder)

6.61 (strong)

6.90 (middle)

7.23 (middle)

7.63 (middle)

8.10 (broad, strong)

8.51 (middle)

9.90 (middle)

10.40 (weak)

11.92 (middle)

12.30 (weak)

12.75 (very weak);

(7) It is positive to Ninhydrin reagent,
Barton's reagent and Dragendorff re-
agent;

(8) It is discoloured with alkaline potassium
permanganate;

(9) It is readily soluble in pyridine, N,N-
dimethylformamide, a 5% aqueous
NIKKOL HCO—50 solution (as here-
in defined), dilute acids, aqueous
alkaline solutions (with decomposition),

and soluble in aqueous methanol, aque-
ous ethanol, aqueous acetone and aque-
ous butanol; but insoluble or hardly
soluble in water, absolute ethanol, pure
butanol and pure acetone;

(10) Its R_f values obtained by paper par-
tition chromatography measured by
using the ascending method on "What-
man filter paper No. 1" are as follows:



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Solvent	Rf value
Acetic acid <i>n</i> -Butanol Water (1:4:5)	0.45 $\pm$ 0.1
<i>n</i> -Butanol saturated with water	0.0
Pyridine <i>n</i> -Butanol Water (3:4:7)	0.80 $\pm$ 0.1; and

- (11) It shows antimicrobial activity selectively against Gram-positive and acid-fast bacteria, and the hydrochloride of Enduracidin.
- 5 3. A method as claimed in claim 1 substantially as hereinbefore described with reference to any of the specific examples.
4. Enduracidin or its hydrochloride when prepared by a method as claimed in claim 10 1 or 3.
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