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# (54) Preparation of antitumor antibiotic

(57) An antibiotic referred to herein as antibiotic 81-484 and having the following physico-chemical properties:
(1) elementary analysis: $C_{33}H_{48}O_7$ (high resolution mass spectrum);
(2) molecular weight: 556 [Field desorption (FD) mass spectrum];
(3) no clear melting point (oily at 10 - 100°C);
(4) specific rotation: $[\alpha]_D^{20} = -151°$
(c = 0.1, methanol);
(5) ultraviolet absorption spectrum: substantially as shown in Figure 1 of the drawings (in methanol);
(6) infrared absorption spectrum: substantially as shown in Figure 2 of the drawings (KBr tablet);
(7) solubility: insoluble in hexane and water, soluble in diethyl ether, methanol, ethanol, dichloromethane, chloroform, ethyl acetate, butyl acetate, acetone and benzene;
(8) nuclear magnetic resonance spectrum: substantially as shown in Figure 3 of the drawings ($CDCl_3$, TMS);
(9) nature: acidic substance; and
(10) colour reaction: negative for ninhydrin, anthrone-$H_2SO_4$ and ferric chloride reaction, positive for iodine and antimony trichloride;
weakly positive for Zatkis reagent;
and the pharmaceutically acceptable salts thereof possess antitumor activity and activity against some fungi and are obtained from *Streptomyces* strain 81-484 FERM-P 7371.

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FIG. 1

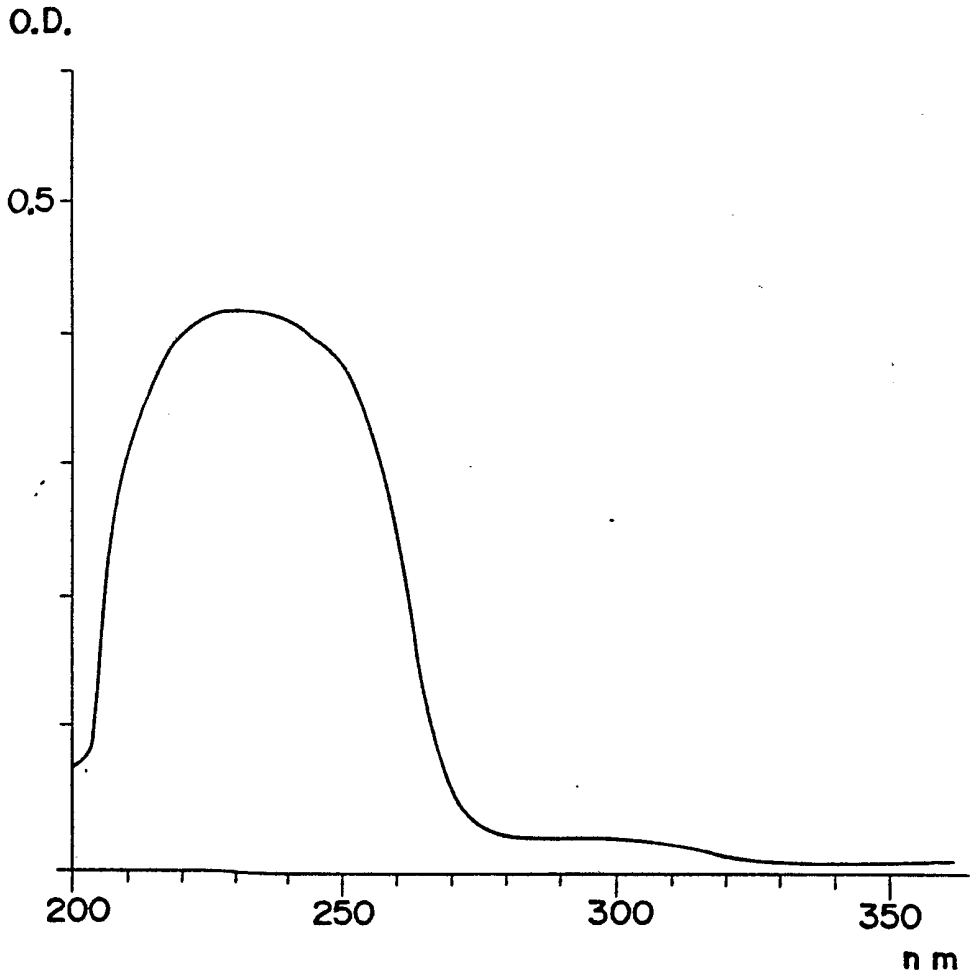
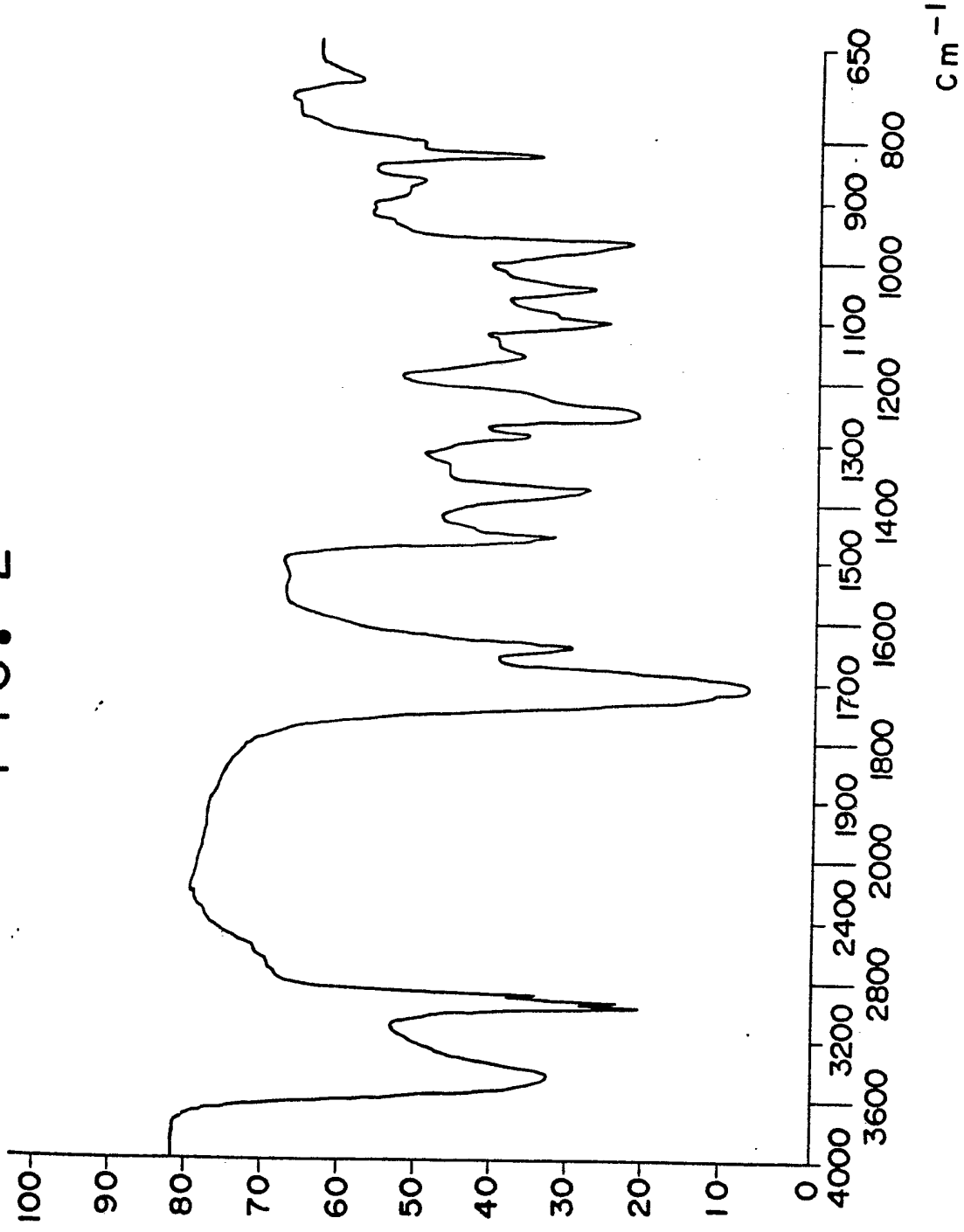


FIG. 2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cm⁻¹

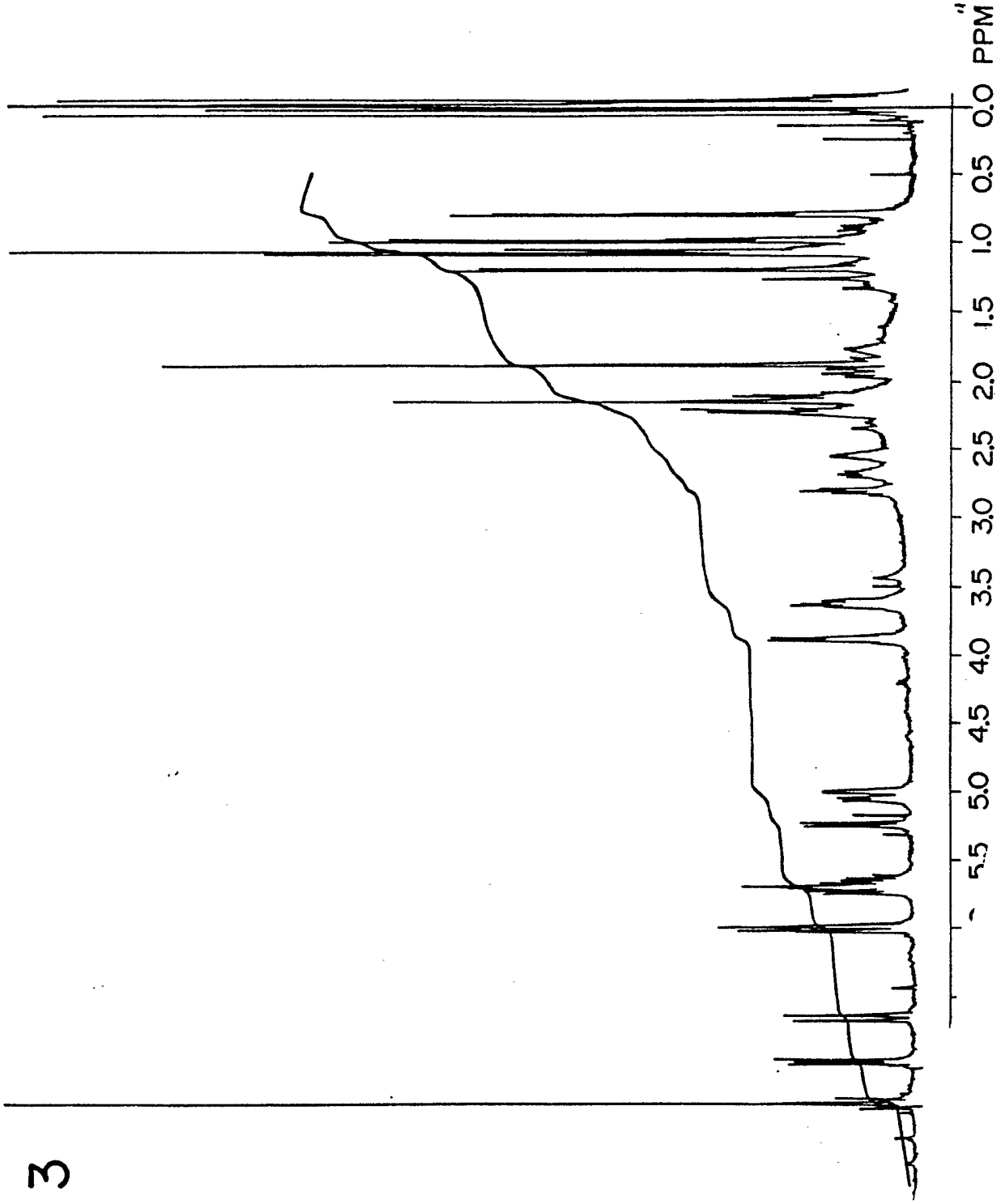


FIG. 3

SPECIFICATION

**Preparation of antitumor antibiotic**

This invention relates to an antibiotic, its production, pharmaceutical compositions containing it and to a microorganism employed in its production.

We have found that a strain of *Streptomyces*, strain 81–484, isolated from a soil sample collected in Chiba-prefecture, Japan, produces an antibiotic substance showing antimicrobial activity against some kind of fungi and having growth inhibitory activity against P388 mouse leukemia and Sarcoma 180 cells. The antibiotic has no activity against Gram-positive and negative bacteria. The antibiotic has been isolated and purified and is referred to herein as antibiotic 81–484.

Accordingly the present invention provides an antibiotic, antibiotic 81–484, having the following physico-chemical properties:

(1) elementary analysis: $C_{33}H_{48}O_7$ (high resolution mass spectrum);

(2) molecular weight: 556 [Field desorption (FD) mass spectrum];

(3) no clear melting point (oily at 10–100°C);

(4) specific rotation: $[\alpha]_D^{20} = -151°C$);

(5) ultraviolet absorption spectrum: substantially as shown in Figure 1 of the accompany drawings (in methanol);

(6) infrared absorption spectrum: substantially as shown in Figure 2 of the accompanying drawings (Kbr tablet);

(7) solubility: insoluble in hexane and water, soluble in diethyl ether, methanol, ethanol, dichloromethane, chloroform, ethyl acetate, butyl acetate, acetone and benzene;

(8) nuclear magnetic resonance spectrum: substantially as shown in Figure 3 of the accompanying drawings ($CDCl_3$, TMS);

(9) nature: acidic substance; and

(10) color reaction: negative for ninhydrin, anthrone-$H_2SO_4$ and ferric chloride reaction,
positive for iodine and antimony trichloride;
weakly positive for Zatkis reagent; and the pharmaceutically acceptable salts thereof.

The present invention also provides a process for the production of antibiotic 81–484 or a pharmaceutically acceptable salt thereof, which process comprises culturing a microorganism belonging to the genus *Streptomyces* and producing the said antibiotic, isolating the said antibiotic thus-produced from the culture, and if required converting the antibiotic into a pharmaceutically acceptable salt thereof. Preferably the antibiotic 81–484 producing microorganism is the *Streptomyces* strain 81–484.

The invention further provides a pharmaceutical composition comprising as active ingredient antibiotic 81–484 or a pharmaceutically acceptable salt thereof, together with a pharmaceutically acceptable carrier or diluent.

Taxonomical properties of strain 81–484 are as follows:

(a) Morphological properties:

Strain 81–484 grows abundantly filamentous on many agar plate media. Dissection of substrate mycelia is not observed. When aerial mycelia are formed, the sporangiophores are straight or loose incomplete spirals with spore chains of more than 20 spores at the top thereof, and no sporangia are observed. The surface of a spore is smooth and elliptical with a 0.8 μm major axis × 0.4 μm minor axis.

(b) Growth conditions on various media at 27°C for 3 weeks culture are as follows:

| *Medium* | *Growth* | *Color of reverse* | *Color of surface of aerial mycelia* | *Soluble pigment* |
|---|---|---|---|---|
| yeast-malt agar (ISP M2) | good | pastel-yellow (1½fb) | ivory (2db) | wheat (2fb) |
| oatmeal agar (ISP M3) | almost no growth | — | — | — |
| starch-inorganic salt agar (ISP M4) | restricted growth | ivory (2db) | ivory (2db) | ivory (2db) |
| glycerin-asparagine agar (ISP M5) | good | yellowish-gray (2ca) | brownish-white (3ca) | brownish white (3ba) |
| peptone-yeast-iron agar (ISP M6) | restricted growth | paleyellow-orange (3ea) | pale yellow-orange (3ea) | (2hb) |
| tyrosine agar (ISP M7) | good | light brownish gray (3ec) | light grayish-yellow brown (2ie) | light brownish-gray (2ec) |

Operation is according to the ISP (International Streptomyces Project) method. Color was determined by consulting "Color Harmony Manual" (4th Ed.) Wilhelm Ostwald.

(c) Physiological properties:

(1) Growth temperature: grown at 20–37°C, and at 27°C for optimum growth.

(2) Liquefaction of gelatin (glucose-peptone-gelatin medium): negative.

(3) Hydrolysis of starch (starch-inorganic agar medium): negative.

(4) Coagulation and peptonization of skim milk (10% skim milk medium): negative for coagulation, positive for peptonization.

(5) Formation of melanin pigment (tyrosine agar medium and peptone-yeast-iron agar medium): negative.

(6) Formation of $H_2S$ (peptone-yeast-iron agar medium): negative.

(7) Formation of sulfite (sulfate medium): positive.

(d) Assimilation of carbon sources:

(Observed on Pridham-Gottlieb agar medium at 27°C for 1 month):

(++: good utilization, +: utilization, −: non-utilization):

| | |
|---|---|
| L-arabinose | − |
| D-xylose | − |
| D-glucose | ++ |
| D-fructose | − |
| sucrose | − |
| inositol | + |
| L-rhamnose | + |
| raffinose | − |
| D-mannitol | − |

(e) Composition of cell wall:

(Method according to Becker, et al. [Appl. Microbiol., *13*, 236–243 (1965)]: LL-type diaminopimeric acid.

On the basis of these taxonomical properties, strain 81–484 belongs to the genus *Streptomyces*. The exact species of this strain has not been elucidated, and so the strain is referred to as *Streptomyces* 81–484. This strain was deposited at the Fermentation Institute, Agency of Industrial Science and Technology, M.I.T.I., Japan on 15th December 1983 assigned as FERM-P 7371.

In general, taxonomical properties of *Streptomyces* are easy to mutate, and so natural or artificial mutations derived by conventional mutation techniques, for example ultraviolet or X-ray irradiation, or treatment with a mutagen such as N-methyl-N-nitro-N-nitrosoguanidine or ethylmethane sulfonate can easily be applied. Natural and artificial mutants belonging to the genus *Streptomyces* and producing antibiotic 81–484 can therefore be used in the present invention.

In the present invention, the antibiotic 81–484 producing microorganism belonging to genus *Streptomyces*, preferably *Streptomyces* 81–484 FERM-P 7371, is cultured in a medium suitable for *Streptomyces*. Nutrient media containing assimilable carbon and nitrogen sources and, if required, inorganic salt can be used.

The present invention additionally provides a culture of the microorganism strain *Streptomyces* 81–484 FERM-P 7371 in a culture medium containing a source of assimilable carbon, a source of assimilable nitrogen, and, if desired, inorganic salts, and substantially free from other microorganisms.

The invention yet further provides a process for the propagation of the microorganism strain *Streptomyces* 81–484 FERM-P 7371, which process comprises culturing *Streptomyces* 81–484 FERM-P 7371, in a culture medium containing a source of assimilable carbon, a source of assimilable nitrogen and, if desired, inorganic salts, and substantially free from other microorganisms.

Examples of assimilable carbon souces are glucose, molasses, starch, dextrin, cellulose, glycerin or organic salts. These are used in combination or individually. Examples of assimilable nitrogen sources are organic nitrogen such as peptone, meat-extract, yeast-extract, dry yeast, soy bean powder, corn steep liquor, cotton seed oil, casein, soy bean protein hydrolysate, amino acid and urea or inorganic nitrogen such as nitrate and ammonium salt. If necessary, an inorganic salt of sodium, potassium, calcium or magnesium such as the phosphate and others can be used. Further, if required, a trace nutrient, growth stimulant or precursor of antibiotic 81–484 can optionally be added to the medium.

Cultivation is carried out, in general, by shaking culture or aeration agitation culture. Submerged aeration culture is preferable for industrial production. The pH of the medium is preferably a neutral pH. The culturing temperature is 20–37°C, generally 24–30°C, and preferably 27°C. The culture time is usually for 4–6 days for liquid culture. Cultivation is preferably stopped at the maximum antibiotic production in a medium. The above culturing conditions, temperature, agitation, aeration and other culturing conditions should naturally be controlled depending upon the nature of the individual microorganism strain used. An antifoaming agent such as silicon oil, vegetable oil and a surface active agent can be added to prevent foaming.

Antibiotic 81–484 mainly accumulates in the culture filtrate, and so the cultured mass is usually filtered with the aid of a filter-aid such as Celite or Hyflo-supercel (trade names), or centrifuged to separate the mycelia and filtrate wherefrom the antibiotic is preferably isolated.

The antibiotic 81–484 is also present in the mycelia, and can be isolated by extraction with methanol or acetone, concentrating the extract *in vacuo* and purifying in the same way as for isolation from a culture filtrate.

Since the antibiotic 81–484 is insoluble in hexane and water, and soluble in many types of organic solvent, for example alcoholic solvents such as methanol or ethanol, chloroform type solvents such as dichloromethane or chloroform or ketone type solvents such as acetone or methyl isobutyl ketone, and is acidic in nature, purification can be achieved by applying these facts.

In general, a culture filtrate can be extracted with a water-immiscible organic solvent such as chloroform methyl isobutyl ketone, ethyl acetate or butyl acetate to transfer the antibiotic into the organic solvent. For the extraction, the culture filtrate has preferably been previously adjusted to pH 3.0–5.0.

The organic solvent layer is optionally washed with an aqueous solution of ethylenediamine tetraacetate to remove metallic ions, and is dried by adding, for example, anhydrous sodium sulfate, anhydrous magnesium sulfate or beads-gel. The dehydrated organic solvent layer is concentrated *in vacuo*. Though antibiotic 81–484 is stable under heating, concentration is preferably effected at under 60°C. Hexane or petroleum ether is added to the concentrate to precipitate the antibiotic 81–484. The thus obtained precipitate is washed with hexane and purified by filtration and centrifugation. Antibiotic 81–484 is then obtained crude as a brownish coloured substance.

Further purification can be carried out by applying differences in solubility of antibiotic 81–484 and contaminants differences in distribution ratio between two immiscible liquids or differences in adsorption on adsorbents. Preferable means are chromatography, for example adsorption chromatography using an adsorption resin such as silica-gel, alumina, activated cellulose or hydroxyappatite HP-20, reverse phase partition chromatography using silanated silica-gel or octadecylsilanated silica-gel, molecular sieve gel-filtration chromatography using Sephadex (Registered Trade Mark) LH-20 or Toyopeal (Registered Trade Mark), or ion-exchange chromatography using DEAE-Sephadex or DEAE-Toyopeal (trade names).

Thus antibiotic 81–484 can be purified by chromatography, electrophoresis, counter current distribution, ultrafiltration or distillation and other means individually or in combination, optionally using a series thereof. For example, the crude substance, dissolved in a small amount of chloroform or benzene, can be adsorbed onto a column packed with silica-gel, and chromatographed with a mixture of hexane-acetone. Active fractions are collected and concentrated *in vacuo*. The concentrate, dissolved in a small amount of chloroform, is adsorbed on a silica-gel column and chromatographed with a mixed solvent of chloroform-methanol. Active fractions are collected and concentrated *in vacuo*. The concentrates, dissolved in a small amount of methanol, are again chromatographed by adsorption on a reverse phase silica-gel column and eluting with a mixture of methanol-water. In this way antibiotic 81–484 can be purified.

Antibiotic 81–484 is an acidic substance and can be prepared as a pharmaceutically acceptable salt by known processes, for example an alkaline metal salt such as of sodium or potassium, an alkaline earth metal salt such as of calcium or magnesium, or a salt with an organic amine.

The physico-chemical and biological properties of antibiotic 81–484 are as follows:

I. Physico-chemical properties:

1) Properties: colourless or pale yellowish viscous oily material;
2) Elemental formula: $C_{33}H_{48}O_7$ (High resolution mass-spectrum);
3) Molecular weight: 556 (Field desorption mass-spectrum);
4) Melting point: no clear melting point (oily at 10–100°C);
5) Specific rotation: $[\alpha]_D^{20} = -151°$ (c.=0.1, methanol);
6) Ultraviolet absorption spectrum: substantially as shown in Figure 1 of the accompanying drawings (in methanol);
7) Infrared absorption spectrum: substantially as shown in Figure 2 of the accompanying drawings (Kbr tablet);
8) Solubility: insoluble; hexane, water, soluble; diethyl ether, methanol, dichloromethane, chloroform, ethyl acetate, butyl acetate, acetone, benzene;
9) Nuclear magnetic resonance spectrum: shown in Figure 3 of the accompanying drawings (400 Hz, $CDCl_3$, TMS);
10) Nature: acidic substance;
11) Colour reaction: negative; ninhydrin, anthrone-$H_2SO_4$, ferric chloride,
positive; iodide, antimony trichloride,
weakly positive; Zatkis reagent, and
12) Silica-gel thin layer chromatography (carrier: silica-gel 60,
Merck: Rf=0.33 (ethyl acetate:methanol=40:1)
Rf=0.28 (chloroform:Methanol=10:1).

II. Biological properties:

(1) Antimicrobial spectrum:

| *Test organisms* | *MIC (g/ml)* |
|---|---|
| *Staphylococcus aureus* FDA 290P | >50 |
| *Bacillus subtilis* PCI 219 | >50 |
| *Sarcina lutea* PCI 1001 | >50 |
| *Escherichia coli* NIHJ | >50 |
| *Schigella sonnai* | >50 |
| *Saccharomyces sake* | >50* |
| *Candida albicans* | >50* |
| *Schizosaccharomyces pomb* IAM 4803 | 0.1** |
| *Trichophyton ferginium* | >50** |

MIC: minimum inhibitory concentration
Assayed by paper disc method using ToYo disc, diameter 8 mmø thick (trade name), on nutrient agar (agar: 1.0%, **0.5%), except on *potate agar.

Antibiotic 81–484 shows antimicrobial activity against some fungi but not against Gram positive bacteria.

(2) Antitumor activity:

1) Effect on P388 mouse leukemia, $1\times10^5$ cells, were inoculated intraperitoneally into groups of five mice, $CDF_1$, female, aged 5 weeks, and the antibiotic was administered as shown in Table 1:

TABLE 1

| *Administration (mg/kg/day)* | *Administ. day* | *life span days (mean value)* | *life prolongation ratio (%)* |
|---|---|---|---|
| control | — | 12 | 0 |
| 0.016 | 1–5 | 19 | 58 |
| 0.008 | 1–5 | 16 | 33 |

Date of administration was set as day 0 on the day of tumor inoculation. The life span is expressed as a mean value for the five mice in each group.

Ratio of life prolongation is calculated by the following equation:

Ratio of life prolongation (%)

$$= \frac{\text{mean value of life-span days, treated}}{\text{mean value of life-span days, control}} \times 100 - 100$$

2) Effect on sarcoma 180: Sarcoma 180, $1 \times 10^6$ cells, were inoculated intraperitoneally into mice, strain ICR, female, 5 weeks age, and treated as shown in Table 2:

TABLE 2

| *Administration (mg/kg/day)* | *Date of administ.* | *Life-span days (mean value)* | *Life-prolongation ratio (%)* |
|---|---|---|---|
| control | — | 12 | 0 |
| 0.031 | 1–5 | 20 | 67 |
| 0.016 | 1–5 | 28 | 133 |
| 0.008 | 1–5 | 22 | 83 |

Date of administration is set as day 0 on the day of tumor inoculation. The life span is expressed as a mean value for the five mice in a group and the ratio of life-prolongation is calculated by the equation above.

As shown in Tables 1 and 2, the antibiotic 81–484 has antitumor activity against P388 leukemia and sarcoma 180 ascites carcinoma.

Heretofore, antibiotics having properties resembling those of antibiotic 81–484 have been reported: antibiotic ATS–1287 (Japan. Unexam. Pat. Publ., No. 55–118499) and Leptomycin A and B [J. Antibiotics, *36*(6), 639–650 (1983)]. However antibiotic ATS-1287 has a different specific rotation and NMR spectrum. Leptomycin A and B have different molecular formula, specific rotations and NMR spectra.

The following Examples illustrate the present invention.

EXAMPLE 1

*Culture of strain 81–484:*

A liquid culture medium (pH 7) [medium A] (100 ml×30] in 500 ml Erlenmeyer flask consisting of glucose 2.0%, peptone 0.5 ml, meat extract 0.5%, dry yeast 0.3%, NaCl 0.5% and calcium carbonate 0.3% was sterilized. A loopful of *Streptomyces* 81–484 FERM-P 7371 cultured on an agar slant medium consisting of glucose 1%, peptone 0.5%, meat extract 0.5%, NaCl 0.3% and agar 1.2% was inoculated thereinto and shake cultured at 27°C for 72 hours with amplitude 17 cm, 120 reciprocations per minute to prepare a seed culture.

The seed culture (2.5 lit.) was aseptically inoculated into medium A (120 lit.) in the 200 1.-fermenter, and aerobically cultured to obtain the culture liquid (approx. 120 lit.).

EXAMPLE 2

*Extraction of antibiotic 81–484:*

The culture liquid obtained in Example 1 was filtered after adding a filter-aid. The filtrate and mycelia washed liquid (120 lit.) were passed through an Amberlite (Registered Trade Mark) XAD-7 column (5 lit.) to adsorb the active principle. The column was washed with water and 20% aqueous ethanol to elute the contaminants, and the active principle was eluted by 40% aqueous ethanol. The eluate (25 lit.) was concentrated *in vacuo* up to approx. 300 ml and the precipitate was removed by filtration. The concentrate with added ethyl acetate was agitated thoroughly. The separated ethyl acetate layer was dried by adding anhydrous sodium sulfate and concentrated *in vacuo* to obtain oily antibiotic 81–484 (20 g).

EXAMPLE 3

*Purification by Silica-gel chromatography:*

The oily material obtained in Example 2 was charged onto a column (46×600 mm) of silica-gel 60 (Merck: Registered Trade Mark) previously washed with hexane, and eluted with gradiently changed hexane to acetone. Active fractions were concentrated *in vacuo*, and again adsorbed on a column of silica-gel which had previously been washed with hexane, and eluted with gradiently changed hexane to ethyl acetate. Active fractions were concentrated *in vacuo* to obtain crude antibiotic 81–484 (100 mg, purity 50%).

EXAMPLE 4

*Isolation by HPLC:*

High performance liquid chromatography (HPLC) [Japan. Spectrophot. TRIROTAR-V, UVIDE-100-V, VL-613, GP-A40] was used for further purification. Octadecylsilane silica-gel (Showa Denko Co. Fine SIL $C_{18}$–10) was packed in a stainless steel column (10×250 mm, Showa Denko Co.).

The crude antibiotic 81–484 (1 mg), obtained in Example 3, was dissolved in methanol (100 µl) and injected into the column, developed with a mixture of water:methanol (30:70, medium for HPLC) and chromatographed. The peak corresponding to antibiotic 81–484 was collected by detecting at 220 nm uv absorption.

Methanol was distilled off *in vacuo* and ethyl acetate was added to the residue. The mixture was stirred at an acidic pH to transfer the antibiotic into the ethyl acetate layer. The ethyl acetate layer was washed with purified water and dried *in vacuo* to obtain purified antibiotic 81–484 (400 μg). The same operations were repeated scores of times to obtain antibiotic 81–484 (approx. 200 mg).

4. Brief explanation of drawings:

*Figure 1*: uv spectrum of antibiotic 81–484

*Figure 2*: IR spectrum of antibiotic 81–484

*Figure 3*: NMR spectrum of antibiotic 81–484.

CLAIMS

1. A process for the production of an antibiotic having the following physicochemical properties:

(1) elementary analysis: $C_{33}H_{48}O_7$ (high resolution mass spectrum);

(2) molecular weight: 556 [Field desorption (FD) mass spectrum];

(3) no clear melting point (oily at 10–100°C);

(4) specific rotation: $[\alpha]_D^{20} = -151°$ c=0.1, methanol);

(5) ultraviolet absorption spectrum: substantially as shown in Figure 1 of the accompanying drawings (in methanol);

(6) infrared absorption spectrum: substantially as shown in Figure 2 of the accompanying drawings (Kbr tablet);

(7) solubility: insoluble in hexane and water, soluble in diethyl ether, methanol, ethanol, dichloromethane, chloroform, ethyl acetate, butyl acetate, acetone and benzene;

(8) nuclear magnetic resonance spectrum: substantially as shown in Figure 3 of the accompanying drawings ($CDCl_3$, TMS);

(9) nature: acidic substance; and

(10) colour reaction: negative for ninhydrin, anthrone $H_2SO_4$ and ferric chloride reaction, positive for iodine and antimony trichloride;

weakly positive for Zatkis reagent; or a pharmaceutically acceptable salt thereof; which process comprises culturing a microorganism belonging to the genus *Streptomyces* and producing the said antibiotic, isolating the said antibiotic thus-produced from the culture, and if required converting the antibiotic into a pharmaceutically acceptable salt thereof.

2. A process according to claim 1 wherein the microorganism belonging to the genus *Streptomyces* is *Streptomyces* 81–484 FERM-P 7371.

3. A process for the preparation of an antibiotic as defined in claim 1, said process being substantially as hereinbefore described in Examples 1,2,3 and 4 together.

4. A pharmaceutical composition comprising as active ingredient an antibiotic, or a pharmaceutically acceptable salt thereof, which has been produced by a process as claimed in any one of the preceding claims together with a pharmaceutically acceptable carrier or diluent.

5. A culture of the microorganism strain *Streptomyces* 81–484 FERM-P 7371 in a culture medium containing a source of assimilable carbon, a source of assimilable nitrogen and, if desired, inorganic salts, and substantially free from other microorganisms.

6. A process for the propagation of the microorganism strain *Streptomyces* 81–484 FERM-P 7371, which process comprises culturing *Streptomyces* 81–484 FERM-P 7371 in a culture medium containing a source of assimilable carbon, a source of assimilable nitrogen and, if desired, inorganic salts, and substantially free from other microorganisms.

7. A process for the propagation of the microorganism strain *Streptomyces* 81–484 FERM-P 7371, said process being substantially as hereinbefore described in Example 1.

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