INTRODUCTION

The present invention provides a new antimicrobial compound, FR231956 substance and a process for producing FR231956 substance by culturing FR231956 substance-producing microorganism in a nutrient medium and recovering the FR231956 substance from the resultant cultured broth. Also provided are an antimicrobial agent comprising the FR231956 substance and carrier(s), a pharmaceutical composition comprising an effective amount of FR231956 substance and pharmaceutically acceptable carrier(s), a method for killing microorganisms by applying the FR231956 substance to the microorganisms, and use of the FR231956 substance for the treatment of infectious diseases caused by pathogenic microorganisms.

The novel Sordarin derivative as a therapeutic antimicrobial agent is described in detail.
DESCRIPTION

NOVEL SORDARIN DERIVATIVE AS A THERAPEUTIC ANTIMICROBIAL AGENT

TECHNICAL FIJLD

The present invention relates to a new antimicrobial compound, hereinafter entitled FR231956 or its salt which is useful as a medicament.

DISCLOSURE OF INVENTION

The present invention relates to a new antimicrobial compound, FR231956 or its salt

More particularly it relates to a new antimicrobial compound, FR231956 or its salt which has an antimicrobial activity against pathogenic microorganisms, especially pathogenic fungi, to a process for the preparation thereof, to a pharmaceutical composition comprising the same, which is useful as an antimicrobial agent, and to a use thereof as a medicament.

Accordingly, one object of this invention is to provide the novel compound, FR231956 or its salt which is of use for treating infectious diseases caused by pathogenic microorganisms.

Another object of this invention is to provide a process for production of the FR231956 substance or its salt by fermentation of the FR231956-producing strain such as Sordaria araneosa ATCC36386 in a nutrient medium.

A further object of this invention is to provide a pharmaceutical composition containing, as an active ingredient, the FR231956 substance or its salt.

Still further object of this invention is to provide a use of the FR231956 substance or its salt for treating infectious diseases caused by pathogenic microorganisms.
The structure of the new compound, FR231956 substance consists of a skeleton of sordarin, which is an antifungal antibiotic (see Helvetica Chimica Acta, 1971, 54, 1178-1190). The FR231956 substance is produced by fermentation of the strain ATCC36386 of the fungus species Sordaria araneosa which is known as a sordarin producing strain.

British Patent Specification No. 1,162,027 describes the preparation of an antibiotic, SL2266, later named sordarin, by the cultivation of the strain Sordaria araneosa NRRL3196 (ATCC36386). Japanese Kokai J62040292 discloses the compound zofimarin having the sordarin skeleton, which is reported to have antifungal activity. Semi-synthetic sordarin derivatives are reported in PCT Applications WO96/14326, WO96/14327 and WO98/15178.

We now describe hereinafter a novel compound, FR231956 which exhibits an excellent antifungal activity and a broad spectrum of action.

The new compound, FR231956 substance is represented by the following formula:
The FR231956 substance has the following physico-chemical properties:

Molecular formula:

\[ \text{C}_{36} \text{H}_{50} \text{O}_{11} \]

Elemental Analysis:

Calcd for \[ \text{C}_{36} \text{H}_{50} \text{O}_{11} \cdot \frac{1}{2} \text{H}_2\text{O} \]

\[ \text{C} \ 64.75, \ \text{H} \ 7.70 \]

Found:

\[ \text{C} \ 64.64, \ \text{H} \ 7.93 \]

Molecular weight:

ESI-MS (+) m/z 681 (M+Na), ESI-MS (-) m/z 657 (M-H)

Melting point:

98 - 110°C (dec.)

Optical rotation:

\[ [\alpha]_D (23^\circ\text{C}) \ +21^\circ \ (c=0.5, \text{methanol}) \]

Ultraviolet absorption spectrum:

\[ \lambda_{\text{max}} \ (\text{methanol}) : 222 \text{ nm} \ (\varepsilon=13000) \]

Solubility:

Soluble: methanol, ethyl acetate, chloroform, dimethylsulfoxide

Insoluble: water, n-hexane

Color reaction:

Positive: iodine vapor reaction, cerium sulfate reaction

Negative: Molisch reaction, ninhydrin reaction, Ehrlich's reaction, Dragendorff reaction, ferric chloride reaction

Thin layer chromatography (TLC):

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<th>Stationary phase</th>
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<td>Silica Gel 60</td>
<td>dichloromethane : methanol (20 : 1, v/v)</td>
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* made by E. Merck
High performance liquid chromatography (HPLC):

Condition:
Column: YMC-Pack ODS-AM-303** (4.6 mm φ x 250 mmL)
Mobile phase: 60% aqueous acetonitrile containing 0.5%
NaH₂PO₄·2H₂O)
Flow rate: 1 ml/minute
Detection: UV at 210 nm
Retention time: 12.8 minutes

**: YMC Co., Ltd.

Infrared absorption spectrum:

vmax (KBr): 3420, 2960, 2870, 1720, 1460, 1380, 1220, 1160,
1100, 1070, 1000, 900 cm⁻¹

¹H Nuclear magnetic resonance spectrum:

(500 MHz, CD₃OD) δ (ppm):

9.78 (1H, s), 6.02 (1H, dd, J=3 and 1 Hz), 5.65 (1H, m), 5.47
(1H, dd, J=4 and 3 Hz), 4.50 (1H, d, J=1 Hz), 4.24 (1H, dd,
J=6 and 2 Hz), 3.97 (1H, d, J=10 Hz), 3.78 (1H, dd, J=4 and
1 Hz), 3.75 (1H, d, J=10 Hz), 3.70 (1H, m), 3.34 (3H, s), 3.31
(1H, dd, J=9 and 3 Hz), 3.15 (1H, m), 2.93 (1H, dd, J=6 and
2 Hz), 2.75-2.70 (2H, m), 2.34 (1H, m), 2.16 (1H, m), 2.08-2.02
(3H, m), 1.98 (3H, d, J=2 Hz), 1.88-1.69 (4H, m), 1.36 (3H,
d, J=6 Hz), 1.27 (3H, d, J=6 Hz), 1.26-1.20 (2H, m), 1.00 (3H,
d, J=7 Hz), 0.96 (3H, d, J=7 Hz), 0.96 (1H, m), 0.82 (3H, d,
J=7 Hz)

¹³C Nuclear magnetic resonance spectrum:

(125 MHz, CD₃OD) δ (ppm):

207.1 (d), 176.0 (s), 167.3 (s), 150.5 (s), 140.2 (d), 133.9
(s), 131.3 (d), 100.6 (d), 79.6 (d), 76.5 (t), 74.8 (s), 71.1
(d), 70.4 (d), 69.9 (d), 66.9 (s), 59.6 (s), 57.9 (q), 57.8
(d), 57.3 (d), 53.4 (d), 53.0 (d), 47.3 (d), 42.8 (d), 42.6
(d), 33.2 (t), 32.5 (d), 30.6 (t), 29.9 (t), 28.8 (d), 27.3
(t), 23.2 (q), 21.6 (q), 20.5 (q), 18.4 (q), 17.9 (q), 14.2
(q)
Nature of substance:
acidic substance

The FR231956 substance can be produced by fermentation of the FR231956-producing strain such as *Sordaria araneosa* ATCC36386 in a nutrient medium.

*Sordaria araneosa* ATCC36386 is preserved and can be distributed by the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA.

It is to be understood that the production of the FR231956 substance is not limited to the use of the particular organism described herein, which is given for the illustrative purpose only. This invention also includes the use of any mutants which are capable of producing the FR231956 substance including natural mutants as well as artificial mutants which can be produced from the described organism by conventional means such as irradiation of X-ray, ultra-violet radiation, treatment with N-methyl-N'-nitro-N-nitrosoguanidine, 2-aminopurine, and the like.

Production of the FR231956 substance

The FR231956 substance is produced when the FR231956 producing strain such as *Sordaria araneosa* ATCC36386 is grown in a nutrient medium containing sources of assimilable carbon and nitrogen under aerobic conditions (e.g. shaking culture, submerged culture, etc.).

The preferred sources of carbon in the nutrient medium are carbohydrates such as glucose, sucrose, starch, fructose or glycerin, or the like.

The preferred sources of nitrogen are peanut powder, yeast extract, peptone, gluten meal, cotton seed flour, soybean powder, soybean meal, corn steep liquor, dried yeast, wheat germ, etc., as well as inorganic and organic nitrogen
compounds such as ammonium salts (e.g. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea or amino acid, or the like.

The carbon and nitrogen sources, though advantageously employed in combination, need not to be used in their pure form because less pure materials, which contain traces of growth factors and considerable quantities of mineral nutrients, are also suitable for use.

When desired, there may be added to the medium mineral salts such as sodium or calcium carbonate, sodium or potassium phosphate, sodium or potassium chloride, sodium or potassium iodide, magnesium salts, copper salts, zinc salts, iron salts, or cobalt salts, or the like.

If necessary, especially when the culture medium foams seriously, a defoaming agent such as liquid paraffin, fatty oil, plant oil, mineral oil, silicone or the like may be added.

Agitation and aeration of the culture mixture may be accomplished in a variety of ways, such as agitation by a propeller or similar mechanical agitation equipment, by revolving or shaking the fermenter, and the like.

The fermentation is usually conducted at a temperature between about 10°C and 40°C, preferably 20°C to 35°C, for a period of about 24 hours to 120 hours, which may be varied according to fermentation conditions and scales.

When the fermentation is completed, the culture broth is then subjected for recovery of the FR231956 substance to various procedures conventionally used for recovery and purification of biological active substance, for instance, solvent extraction with an appropriate solvent or a mixture of some solvents, chromatography or recrystallization from an appropriate solvent or a mixture thereof.

The FR231956 substance in its free form may also be converted to its salts by treating with an inorganic or organic
base such as sodium or potassium hydroxide, ammonium hydroxide, ethanolamine and the like and with an amino acid such as glycine, lysine, glutamic acid and the like.

5 Biological properties of the FR231956 substance

The FR231956 substance has a strong antimicrobial activity against pathogenic microorganisms, especially pathogenic fungi such as pathogenic yeast (e.g. Candida albicans etc.) and the like. Accordingly, the FR231956 substance or its pharmaceutically acceptable salt is useful as an antimicrobial agent, especially antifungal agent which is used for the treatment of infectious diseases in human beings and animals. Such infections include superficial, cutaneous, subcutaneous and systemic mycotic infections such as respiratory tract infections, gastrointestinal tract infections, cardiovascular infections, urinary tract infections, candidiasis and chronic mucocandidiasis (e.g. thrush and vaginal candidiasis) and skin infections caused by fungi, cutaneous and mucocutaneous candidiasis, dermatophytoses (e.g. ringworm and tinea infections, athletes foot, paronychia, pityriasis, intertrigo, Candida vulvitis, Candida balanitis and otitis externa). They may also be used as prophylactic agents to prevent systemic and topical fungal infections for immunocompromised patients (e.g. AIDS patients, patients receiving cancer therapy or transplant patients).

As examples for showing such pharmacological effects of the FR231956, some biological data are explained in the following.

Test 1 (Antimicrobial activity)

Antimicrobial activities of the FR231956 substance were determined by a serial broth dilution method using 96-well
microtiter plate in 100 μl of yeast nitrogen base dextrose medium. The inoculum was adjusted to 1 x 10^5 colony forming units/ml. *Candida albicans* was cultured at 37°C for 24 hours and *Cryptococcus neoformans* was cultured at 37°C for 48 hours in 5% CO₂ incubator. After incubation, the growth inhibition of microorganism in each well was determined by microscopic observation. The results were shown as MEC (minimum effective concentration: μg/ml) value (Table 1).

Table 1. Antimicrobial activities of the FR231956 substance.

<table>
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<tr>
<th>Microorganisms</th>
<th>MEC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> FP629</td>
<td>0.05</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em> YC203</td>
<td>0.03</td>
</tr>
</tbody>
</table>

From the test result, it is realized that the FR231956 substance of the present invention has an antimicrobial activity (especially, antifungal activity).

The pharmaceutical composition of this invention can be used in the form of pharmaceutical preparation, for example, in solid, semisolid or liquid form, which contains the FR231956 substance or a pharmaceutically acceptable salt thereof, as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral or parenteral applications.

The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, injections, ointments, liniments, eye drops, lotion, gel, cream, and any other form suitable for use.
The carriers which can be used are water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form, and in addition auxiliary, stabilizing, thickening, solubilizing and coloring agents and perfumes.

The object compound or a pharmaceutically acceptable salt thereof is included in the pharmaceutical composition in an amount sufficient to produce the desired antimicrobial effect upon the process or condition of diseases.

For applying the composition to a human being, it is preferable to apply it by intravenous, intramuscular, topical, percutaneous, pulmonary or oral administration, or insufflation. While the dosage or therapeutically effective amount of the FR231956 substance or a pharmaceutically acceptable salt thereof varies depending on the age and conditions of each individual patient to be treated, in the case of intravenous administration, a daily dose of 0.01 - 20 mg of the FR231956 substance per kg weight of human being, in the case of intramuscular administration, a daily dose of 0.1 - 20 mg of the FR231956 substance per kg weight of human being, in the case of oral administration, a daily dose of 0.5 - 50 mg of the FR231956 substance per kg weight of human being is generally given for treating or preventing infectious diseases.

The following examples are given for the purpose of illustrating the present invention, but not limited thereto.

Example

(1) Fermentation of Sordaria araneosa ATCC36386 for the production of the FR231956 substance

An aqueous seed medium (160 ml) containing corn starch
2%, sucrose 1%, glycerol 1%, pharmamedia 1%, gluten meal 1%, and Tween 80 0.2% was placed in each of three 500-ml Erlenmeyer flasks and was sterilized at 120°C for 30 minutes. A loopful of the slant culture of Sordaria araneosa ATCC36386 was inoculated in each of the seed flasks. The inoculated flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) at 25°C for 4 days, and 480ml (three flasks) of the seed culture were inoculated to 20 liters of sterile production medium consisting of modified starch 3%, pharmamedia 2%, oat meal 0.5%, (NH₄)₂SO₄ 0.3%, KH₂PO₄ 2 %, Na₂HPO₄·12H₂O 1.5%, Adekanol LG-109 0.05%, and Silicone KM-70 0.05% in a 30-liter jar fermentor. Fermentation was carried out at 25°C for 4 days under aeration of 20 liters/minute and agitation of 200 rpm.

The production of the FR231956 substance in the fermentation broth was monitored by HPLC analysis indicated below.

# Analytical HPLC condition
Column: YMC Pack ODS-AM 303, S-5 120A (250 x 4.6 mm I.D., YMC Co., Ltd.)
Mobile phase: 60% aqueous acetonitrile containing 0.1% trifluoroacetic acid
Flow rate: 1 ml/min.
Detection: UV at 210 nm
Retention time: FR231956 11.8 min.

(2) Isolation and purification of the FR231956 substance
The culture broth (100 liters) was extracted with an equal volume of acetone by stirring for 2 hours at room temperature. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (7 liters) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with 25% aqueous acetone. The column was washed with 50% aqueous methanol (20 liters) and
then eluted with methanol (40 liters). The active fraction (0-30 liters) was diluted to 75 liters with water and passed through a column (2 liters) of YMC-GEL (ODS-AM 120-S50, YMC Co., Ltd.) packed with 40% aqueous methanol. The column was eluted with 50% aqueous methanol (9.3 liters). The active fraction (1.3-5.3 liters) was diluted with an equal volume of water and passed through a column (2 liters) of YMC-GEL (ODS-AM 120-S50, YMC Co., Ltd.) packed with 25% aqueous methanol. The column was washed with 40% aqueous acetonitrile containing 0.5% NaH₂PO₄·2H₂O (9 liters) and eluted with 50% aqueous acetonitrile containing 0.5% NaH₂PO₄·2H₂O (9.95 liters). The active fraction (6.15-9.45 liters) was diluted with an equal volume of water and passed through a column (2 liters) of YMC-GEL (ODS-AM 120-S50, YMC Co., Ltd.) packed with 25% aqueous acetonitrile containing 0.25% NaH₂PO₄·2H₂O. The column was washed with 50% aqueous methanol (4 liters) and then eluted with 97% aqueous methanol (2.6 liters). The active fraction (1.3-2.1 liters) was concentrated in vacuo to give yellow residue. This residue was dissolved in a small volume of ethyl acetate and added with a large amount of n-hexane, and then was dried up to give 1044 mg of FR231956 as pale yellow powder.
CLAIMS

1. The FR231956 substance of the following formula:

2. A process for production of the FR231956 substance of claim 1 or its salt, which comprises culturing a FR231956 substance-producing microorganism in a nutrient medium and recovering the FR231956 substance of claim 1 or its salt from the resultant cultured broth.

3. An antimicrobial agent comprising the FR231956 substance of claim 1 or its salt and carriers.

4. The FR231956 substance of claim 1 or a pharmaceutically acceptable salt thereof for use as a medicament.

5. Use of the FR231956 substance of claim 1 or a pharmaceutically acceptable salt thereof for the preparation of a medicament.
6. A pharmaceutical composition which comprises, as an active ingredient, the FR231956 substance of claim 1 or a pharmaceutically acceptable salt thereof in admixture with pharmaceutically acceptable carriers or excipients.

7. A method of killing microorganisms by applying the FR231956 substance of claim 1 or its salt to the microorganism.

8. A method of killing fungi by applying the FR231956 substance of claim 1 or its salt to the fungi.

9. A method for the prophylactic and/or therapeutic treatment of infectious diseases caused by pathogenic microorganisms, which comprises administering the FR231956 substance of claim 1 or a pharmaceutically acceptable salt thereof to a human being or an animal.

10. Use of the FR231956 substance of claim 1 or a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of infectious diseases caused by pathogenic microorganisms.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H15/24 A61K31/70 C12P19/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07H A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>A</td>
<td>WO 96 14327 A (GLAXO WELLCOME SA ; MARTIN JOSE JULIO (ES); CHICHARRO GONZALO JESUS) 17 May 1996 (1996-05-17) cited in the application</td>
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<td>WO 98 11891 A (DOMBROWSKI ANNE W ; JANSSON RICHARD K (US); SCHWARTZ ROBERT E (US)); 26 March 1998 (1998-03-26)</td>
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<td>WO 98 15178 A (TSE BRUNO ; MERCK &amp; CO INC (US); BALKOVEC JAMES M (US)) 16 April 1998 (1998-04-16) cited in the application</td>
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<td>A</td>
<td>WO 99 09974 A (NIELSEN KAHN JENNIFER ; TSE BRUNO (US); MERCK &amp; CO INC (US)) 4 March 1999 (1999-03-04)</td>
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Date of the actual completion of the international search
22 September 2000

Date of mailing of the international search report
29/09/2000

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax. 31 651 epc nl, (+31-70) 340-3016

Authorized officer
Bardili, W
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