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PATENT SPECIFICATION

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(54) METABOLITES S7481/F-1 AND /F-2

(71) We, SANDOZ LTD., of 35 Lichtstrasse, 4002 Basle, Switzerland, a Swiss Body Corporate, 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:—

The invention provides a process for the production of compound S7481/F-1, S7481/F-2, or S7481/F-1 combined with S7481/F-2, which comprises cultivating a S7481/F-1; S7481/F-2, or S7481/F-1 and S7481/F-2 producing strain of the fungus species, *Cylindrocarpon lucidum* Booth or *Tolypocladium inflatum* Gams (earlier classed as *Trichoderma polysporum* (Link ex Pers.) Rifai) in contact with a nutrient medium and isolating S7481/F-1; S7481/F-2 or S7481/F-1 combined with S7481/F-2, compound S7481/F-1 having the following characteristics in solid form:—

Amorphous, colourless powder; M.P. 137—140°C (decomp.); $[\alpha]_D^{20} = -236^\circ$ (CHCl_3 , $c=0.5$);

U.V. Spectrum $[\text{CH}_3\text{OH}$; $c=1.063$ g/l; $d=0.01$ cm] as shown in Figure 1 of the accompanying drawings;

I.R. Spectrum $[\text{CH}_2\text{Cl}_2]$ as shown in Figure 2 of the accompanying drawings;

Proton N.M.R. Spectrum [90 MHz; CDCl_3] as shown in Figure 3 of the accompanying drawings;

Carbon-13 N.M.R. Spectrum [250 mg in 1.5 ml CDCl_3 ; sweep width 6000 MHz] as shown in accompanying Table;

High resolution mass spectrum [ion acceleration 4 kv; temperature ion source 290°C; pressure 5.10^{-6} mm Hg], peak of highest mass $m/e=1183.831$ [± 0.004] corresponding to $\text{C}_{62}\text{H}_{109}\text{N}_{11}\text{O}_{11}$ recognisable as product of elimination of water from $\text{C}_{62}\text{H}_{111}\text{N}_{11}\text{O}_{12}$;

Elementary analysis C 61.8, H 9.4, N 13.0, O 15.7% in agreement with empirical formula $\text{C}_{62}\text{H}_{111}\text{N}_{11}\text{O}_{12}$;

and release of amino acids alanine, α -aminobutyric acid, N-methylleucine, N-methylglycine and valine on reaction with 6N aqueous hydrochloric acid at reflux; and

compound S7481/F-2 having the following characteristics in solid form:—

Amorphous colourless powder; M.P. 127—130°C (decomp.); $[\alpha]_D^{20} = -243^\circ$ (CHCl_3 , $c=0.5$);

U.V. Spectrum $[\text{CH}_3\text{OH}$; $c=1.062$ g/l; $d=0.01$ cm] as shown in Figure 4 of the accompanying drawings;

I.R. Spectrum $[\text{CH}_2\text{Cl}_2]$ as shown in Figure 5 of the accompanying drawings;

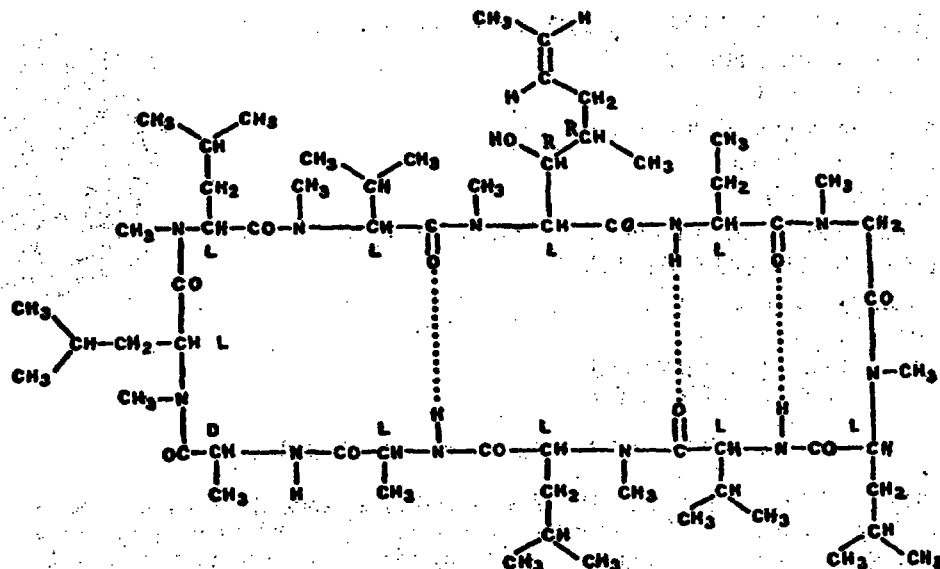
Proton N.M.R. Spectrum [90 MHz; CDCl_3] as shown in Figure 6 of the accompanying drawings;

High resolution mass Spectrum [ion acceleration 4 kv; ion source temperature 290°C; pressure 5.10^{-6} mm Hg], peak of highest mass $m/e=1169.815$ (± 0.006) corresponding to $\text{C}_{61}\text{H}_{107}\text{N}_{11}\text{O}_{12}$ the product of water elimination from $\text{C}_{61}\text{H}_{109}\text{N}_{11}\text{O}_{12}$; and

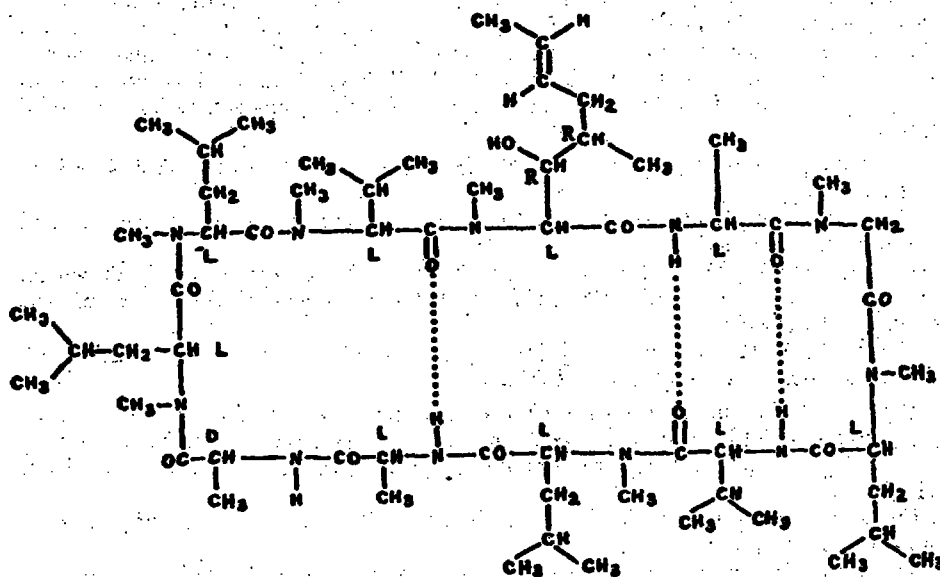
Elementary analysis found C 61.7; H 9.1, N 13.1, O 16.5% in agreement with empirical formula $\text{C}_{61}\text{H}_{109}\text{N}_{11}\text{O}_{12}$.

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The structure of S7481/F-1 is as follows:—



The structure of S7481/F-2 is as follows:—



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When the strain is a *Cylindrocarpon lucidum* Booth strain, this is preferably the strain NRRL 5760 described hereinafter, or an obvious taxonomical equivalent thereof, or a mutant or variant thereof.

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When the strain is a *Tolypocladium inflatum* Gams strain (earlier classed as *Trichoderma polysporum* (Link ex Pers.) Rifai), this is preferably the strain NRRL 8044 described hereinafter, or an obvious taxonomical equivalent thereof, or a mutant or variant thereof.

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The invention also provides the compounds S7481/F-1 and S7481/F-2 as defined above and described herein.

The invention also provides fermentation liquors produced by cultivating either strain NRRL 5760 or strain NRRL 8044, or an obvious taxonomical equivalent thereof on a synthetically produced nutrient medium.

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The new strain of the fungus species *Cylindrocarpon lucidum* Booth employed in the process of the invention was isolated from a soil sample found in the State Wisconsin, USA, and a specimen of said strain has been deposited with the United

States Department of Agriculture (Northern Research and Development Division), Peoria, Ill., USA, under the reference NRRL 5760.

In its essential characteristics, the new strain NRRL 5760 of *Cylindrocarpum lucidum* Booth conforms to the original description (C. Booth 1966, The Genus *Cylindrocarpum*, Mycological Papers No. 104, 21). On potato dextrose agar the strain NRRL 5760 forms neither chlamydospores nor microconidia. However, chlamydospore-like cells of the macroconidia are also lacking. The banana-shaped macroconidia, which are usually partitioned by 3 transverse walls, measure $28-45 \times 4.8-5.7 \mu$ and are, therefore, considerably smaller than indicated in the above mentioned description ($45-65 \times 6-6.5 \mu$). A further important difference lies in the growth rate; while the type should form colonies measuring 1-1.5 cm within 7 days, the strain NRRL 5760 grows to colonies measuring 7-8 cm within the same time, whereby colourless or brown substrate mycelium is formed almost exclusively on potato dextrose agar. The conidiophores with the phialides are formed already after a few days in tight groups and bundles, are branched in a penicillium-like or verticillium-like manner and form macroconidia over a longer period, these macroconidia accumulating as slimy, beige-coloured mass.

The new strain of the fungus species *Tolypocladium inflatum* Gams (earlier classed as *Trichoderma polysporum*) employed in the process of the invention was isolated from a soil sample found in Norway, and a specimen of said strain has been deposited with the United States Department of Agriculture (Northern Research and Development Division), Peoria, Ill., USA, under the reference NRRL 8044.

The new strain NRRL 8044 grows on a 2% malt extract agar at between 6° and 33°C, the optimum growth is around 24°C, when pure white, superficially velvet-like and unwrinkled colonies, having a diameter of 40-45 mm, are formed within 12 days. However, at the highest temperature permitting a good growth (33°C), no aerial mycelium, but a very strongly wrinkled colony is formed. With increasing culture temperatures a yellow pigment is increasingly formed on the under side of the colony, this pigment diffusing into the agar at 27°C and still more pronouncedly at 30-33°C.

In order to form conidiophores, standing scattered or in tight groups, especially however at the border of the culture vessel, the new strain NRRL 8044 requires 15 to 20 days on malt extract agar at 24°C. The morphology of the conidiophore structures slightly differ in some of their characteristics from the description of species of *Trichoderma polysporum* (Link ex Pers) Rifai (M. A. Rifai 1969, A Revision of the Genus *Trichoderma*, Mycological Papers No. 116). Thus, the main branch of the conidiophores reaches a diameter of 2.0-3.8 μ only, whereas 4-6.3 μ are indicated for the species. The phialides of the new strain are elongated, especially at the end of their development, this being untypical for this species. Finally, the conidia are somewhat smaller than what is indicated in the description of the species, i.e. $2.0-3.1 \times 1.5-2.0 \mu$, as compared with $2.4-3.8 \times 1.8-2.2 \mu$. The present strain NRRL 8044 does not form true chlamydospores under any of the indicated culture conditions.

Following the description of W. Gams *Persoonia* 6 Part 2 pp 185-191 (1971) the characteristics of the above strain fit the definition of a *Tolypocladium inflatum* strain, rather than a *Trichoderma polysporum* strain.

The new fungus strains NRRL 5760 and NRRL 8044 may be cultivated on various nutrient media containing the usual nutrients for fungi. For example, such strains make use of nutrients usually employed for carbon-heterotrophic organisms, e.g. sucrose, glucose, maltose, lactose, starch, malt extract as the carbon source, organic and inorganic nitrogen-containing compounds such as cornsteep, soy peptone, yeast or meat extracts, sodium nitrate, ammonium sulphate, ammonium nitrate, and amino acids as the nitrogen source, as well as the usual mineral salts and trace elements.

The new compounds S7481/F-1 and S7481/F-2 may be produced by

1. inoculating a liquid medium with a conidia or mycelium suspension of the strain NRRL 5760, and incubating for 9 to 13, preferably 11 days, at 24-30°C, preferably 27°C, and at a pH value of 4.8-8.8, preferably 8.1. This cultivation is effected in penicillin flasks under aerobic conditions by surface culture fermentation,

2. inoculating a liquid medium with a conidia or mycelium suspension of the strain NRRL 8044, and incubating the culture for 11 to 18, preferably 13 days, at 25-30°, preferably 27°C, and at a pH value of 4.3-6.2, preferably 5.6, in a steel fermenter, while stirring (170 r.p.m.) and aerating (1 liter of air/minute/liter of nutrient solution).

As soon as the maximum amount of antibiotics has been produced, these are

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obtained from the culture liquor in known manner by extractive and/or adsorptive methods.

One convenient method comprises the extraction of the mycelium obtained from cultivation of the fungus strain NRRL 5760, optionally after previous mechanical comminution, with 90% methanol, subsequent removal of the methanol by evaporation, and repeated extraction of the resulting aqueous mixture with ethylene chloride. The ethylene chloride extract obtained from the culture filtrate may be added to this extract. However, other organic, water-immiscible solvents, e.g. chloroform, ethyl acetate, methylene chloride or butyl acetate, may alternatively be used.

The organic solution is evaporated to dryness and the residue is defatted, either by chromatography, e.g. on silica gel with chloroform containing a small amount of methanol, e.g. at a ratio of 98:2, or by distribution between petroleum ether and methanol which has been mixed with an amount of water sufficing for a phase separation. In the first case the fractions which are antibioticly active against *Aspergillus niger* are combined, evaporated to dryness, and the residue is chromatographed in methanolic solution on Sephadex LH 20. In the second case the methanolic aqueous phase is concentrated, optionally after the addition of a further amount of water, to such an extent that the major portion of the methanol is distilled off, whereupon the aqueous mixture is extracted with ethylene chloride or another one of the above mentioned water-immiscible solvents, the resulting organic solution is evaporated to dryness, and the residue is chromatographed in methanolic solution on Sephadex LH 20. The further separation and purification of the two antibiotics is effected by repeated chromatography on aluminium oxide or silica gel or by the successive use of both adsorbents.

A further convenient method comprises the extraction of the culture liquor obtained from cultivation of the fungus strain NRRL 8044 with a water-immiscible solvent, optionally after previous mechanical comminution. Ethylene chloride is preferably used as solvent, but chloroform, ethyl acetate, methylene chloride or butyl acetate may alternatively be used. The organic phase is separated, dried and concentrated by evaporation. The residue is chromatographed, preferably with a methanol solution on Sephadex LH 20, whereby the fractions being active against *Aspergillus niger* are combined and again chromatographed on aluminium oxide with toluene to which 10-50% of ethyl acetate have been added. The detection of the active antibiotics is again effected by testing the activity against *Aspergillus niger*. Purification of the two antibiotics is effected by further chromatography.

For the production of the new antibiotics, it is likewise possible to use other strains obtained, for example, by selection or mutation by ultraviolet or X-ray irradiation, or by other measures, for example by treatment of laboratory cultures with suitable chemicals.

The above-mentioned strains NRRL 5760 and 8044 are already available to the public from the above-mentioned United States Department of Agriculture depository and also from the patentees and will continue to be available as working strains throughout the effective life of any patent issuing hereon. Any restrictions on the availability of the above-mentioned strains to the public have already been irrevocably removed.

In the following non-limitative Examples all temperatures are indicated in degrees Centigrade. Unless indicated otherwise all values mentioned herein are subject to the usual experimental error.

"Polygram" is a Trade Mark for plastic foil supports for silica gel suitable for thin layer chromatography.

"Sephadex LH 20" is a Trade Mark for silica gel suitable for column chromatography.

"Turrax", "Merck" and "Ultra turrax" are registered trade marks.

All percentages refer to parts by volume unless otherwise stated. Elemental analytical percentages refer however to parts by weight.

EXAMPLE 1

10 liters of a nutrient solution (of which each liter contains 30 g of sucrose, 10 g of Corn steep, 3 g of NaNO_3 , 1 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.5 g of KCl and 0.01 g of $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$) are inoculated with 100 cc of a conidia and mycelium suspension of the strain NRRL 5760, and incubation is effected in 700 cc penicillin flasks at 27° for 11 days.

The mycelium, which has been separated from the culture liquid, is extracted in a Turrax apparatus by crushing and stirring with 3.5 liters of 90% methanol, and the crushed mycelium, which is separated from the solvent by filtering with

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suction, is again treated twice in the same manner with 90% methanol. The combined filtrates are concentrated by evaporation in a vacuum at a bath temperature of 40° to such an extent that the vapour mainly consists of water alone. The resulting mixture is extracted six times with the same volume of ethylene chloride by shaking, whereupon the combined ethylene chloride solutions are purified by extraction with water and are concentrated by evaporation in a vacuum at a bath temperature of 40°. The resulting residue is chromatographed on 250 g of silica gel (silica gel 60 "Merck", grain size 0.063—0.200 mm), using chloroform containing 2% of methanol as eluant, and is collected in 200 cc fractions. The fractions which are antibiologically active against *Aspergillus niger* in the plate diffusion test are combined, evaporated to dryness as described above, and after dissolving in methanol are chromatographed on 110 g of Sephadex LH 20 with the same solvent, whereupon those 20 cc fractions showing an antibiotic effect against *Aspergillus niger* in the test indicated above, are combined. A test in the thin layer chromatogram, e.g. with silica gel on "Polygram" foils and hexane/acetone (1:1) as eluant, indicates that the residue of the methanol solution evaporated as described above mainly consists of the two new compounds S7481/F-1 and S7481/F-2. These are separated and simultaneously purified by a further chromatography of the mixture thereof, using a 1000-fold amount of silica gel of the above indicated quality and chloroform containing 2% of methanol. A testing of the eluate fractions, having a volume in milliliters which is half as large as the weight of the silica gel in grams, in the thin layer chromatogram, indicates that the compound S7481/F-1 appears first in the eluate, followed by a mixture of the two compounds and finally by homogeneous S7481/F-2. Further amounts of the two antibiotics may be obtained from the mixture by repeating chromatography under the same conditions.

The new compound S7481/F-1 may be characterized as follows:

Amorphous, colourless powder having an M.P. of 137—140° (decomp.); $[\alpha]_D^{20} = -236^\circ$ (CHCl₃, c=0.5);

UV spectrum: see Figure 1; IR spectrum: see Figures 2; proton nuclear magnetic resonance: see Figure 3; ¹³C Nuclear magnetic resonance: see Table.

Mass spectrum (CEC mass spectrograph 21—110B, source of ions E.B., admission system: direct, electron energy: 70 eV, ion acceleration: 4 kV, temperature ion source: 290°, pressure: 5.10⁻⁸ mm Hg, peak matching process, reference substance "PCR 8" [Tris-pentadecafluoroheptyl-1,3,5-triazine, M=1184.9374]: highest mass peak m/e=1183.831 (±0.004), corresponding to the mass of the ion C₆₂H₁₀₉N₁₁O₁₂, the product of elimination of water from the molecule C₆₂H₁₁₁N₁₁O₁₂.

Solubility: readily soluble in most of the usual organic solvents, practically insoluble in petroleum ether and water. Behaviour in thin layer chromatogram (substance spots made visible with iodine vapour):

- Silica gel on glass plates; chloroform+4% of methanol as eluant: Rf=0.52.
- Silica gel on "Polygram" foils; hexane/acetone 1:1 as eluant: Rf=0.37; chloroform/acetone 2:1 as eluant: Rf=0.34.

Elementary analysis (sample dissolved in benzene, solution evaporated to dryness, residue dried in a high vacuum at 100° for 5 hours): found C 61.8, H 9.4, N 13.0, O 15.7%.

The values found are in agreement with the empirical formula C₆₂H₁₁₁N₁₁O₁₂. The amino acids alanin, α-aminobutyric acid, N-methyl-leucin, N-methylglycin and valin may be detected in the hydrolysis product obtained by boiling the compound S7481/F-1 with 6N hydrochloric acid (20 hours).

The new compound S7481/F-2 may be characterized as follows:

Amorphous, colourless powder having an M.P. of 127—130° (decomp.); $[\alpha]_D^{20} = -243^\circ$ (chloroform, c=0.5);

UV spectrum: see Figure 4; IR spectrum: see Figure 5; proton nuclear magnetic resonance: see Figure 6.

Mass spectrum (taken under the conditions as for antibiotic S7481/F-1): highest mass peak m/e=1169.815 (±0.006), corresponding to the mass of the ion C₆₁H₁₀₇N₁₁O₁₁, the product of elimination of water from the molecule C₆₁H₁₀₉N₁₁O₁₂.

Solubility: readily soluble in most of the usual organic solvents, practically insoluble in petroleum ether and water. Behaviour in the thin layer chromatogram (substance spots made visible with iodine vapour):

- silica gel on glass plates; chloroform+4% of methanol as eluant: Rf=0.46.
- Silica gel on "Polygram" foils; hexane/acetone 1:1 as eluant: Rf=0.28; chloroform/acetone 2:1 as eluant: Rf=0.26.

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Elementary analysis (sample dried in a high vacuum at 100° for 5 hours): found C 61.7, H 9.1, N 13.1, O 16.5%

The values found are in agreement with the empirical formula $C_{61.7}H_{9.1}N_{13.1}O_{16.5}$.

The conidia and mycelium suspension used as starting material for the inoculation is produced from a culture of the originally isolated strain NRRL 5760, which is cultivated for 10 days on an agar medium, of which each liter contains 20 g of malt extract, 20 g of agar, 4 g of yeast extract, and demineralized water. The conidia and the mycelium are taken up in a physiological common salt solution. This suspension is used for the inoculation of the above nutrient solution.

EXAMPLE 2

250 liters of a nutrient solution (of which each liter contains 30 g of sucrose, 10 g of Corn steep, 3 g of $NaNO_3$, 1 g of K_2HPO_4 , 0.5 g of $MgSO_4 \cdot 7 H_2O$, 0.5 g of KCl and 0.01 g of $FeSO_4 \cdot 7 H_2O$) are inoculated with 2.5 liters of a conidia and mycelium suspension of the strain NRRL 5760, and are incubated in 700 cc penicillin flasks at 27° for 11 days.

The mycelium is separated from the culture liquid by centrifuging and is homogenized in an Ultraturrax apparatus with 80 liters of 90% methanol, whereupon the methanolic extract solution is separated from the solid material by centrifuging, and the solids are again extracted twice in the same manner with 90% methanol. The combined extract solutions are concentrated to 15 liters in a vacuum at 30–40°. The resulting aqueous phase is extracted 8 times with the same volume of ethylene chloride. Each of the ethylene chloride extracts is washed with 5 liters of water. The ethylene chloride solutions are combined and are then concentrated by evaporation in a vacuum at 30–40°. Defatting is effected by dividing the extract residue between petroleum ether and 90% methanol in such a manner that after dissolution in 2 liters of 90% methanol it is successively shaken 3 times with 2 liter amounts of petroleum ether (B.P. 30–35°) in three separatory funnels, whereupon the three petroleum ether phases are again successively extracted twice with 2 liter amounts of 90% methanol. 3 liters of water are added to the combined methanolic solutions, and the mixture is concentrated at 30–40° to a volume of 2 liters. The aqueous concentrate is extracted by shaking 5 times with 2 liters of ethylene chloride; the ethylene chloride solutions are washed with 0.5 liter amounts of water, are combined and evaporated to dryness in a vacuum at 30–40°. The residue is subjected to chromatography on a 25-fold volume of Sephadex LH 20, whereby methanol is used as eluant. The eluate is collected in 250 cc fractions; those fractions exhibiting an antibiotic effect against *Aspergillus niger* in the plate diffusion test are combined and evaporated to dryness in a vacuum at 30–40°. The residue is subsequently chromatographed in a 70-fold quantity of aluminium oxide (neutral) of activity 1, whereby mixtures of toluene and ethyl acetate are used for elution. The first ten fractions (volume of the fractions: half as much liters as adsorbent in kg), eluted with toluene containing 15% of ethyl acetate, first show the major amount of the antibiotic S7481/F-1, followed by a mixture of S7481/F-1 and S7481/F-2. A further amount of the same mixture, containing a small amount of foreign matter, may be obtained by subsequent elution with toluene/ethyl acetate (1:1). The detection of the antibiotics in the eluate fractions is effected by thin layer chromatography, e.g. with silica gel on "Polygram" foils and hexane/acetone (1:1) as eluant. The antibiotic S7481/F-1 is obtained from the combined fractions containing the antibiotic S7481/F-1 in homogeneous form, by evaporating in a vacuum at 30–40° and subsequent drying of the residue in a high vacuum at 50°. The combined eluate fractions, containing the mixture, are also evaporated under the same conditions. For the obtaining of a further amount of the compound S7481/F-1 and of homogeneous S7481/F-2, the residue is subjected to chromatography on a 1000-fold amount of silica gel, whereby chloroform containing 2% of methanol is used as eluant, proceeding in a manner analogous to that described in Example 1.

EXAMPLE 3

500 liters of nutrient solution (of which each liter contains 40 g of glucose, 5 g of casein peptone, 5 g of $MgSO_4 \cdot 7 H_2O$, 2 g of KH_2PO_4 , 3 g of $NaNO_3$, 0.5 g of KCl, 0.01 g of $FeSO_4$, and demineralized water) are inoculated with 50 liters of a preculture of the strain NRRL 8044, and incubated in a steel fermenter at 27° for 13 days while stirring (170 revolutions/minute) and aerating (1 liter of air/minute/liter of nutrient solution).

500 liters of culture liquid are crushed with an Ultraturrax apparatus and extracted several times with 500 liter amounts of 1,2-dichloroethane. The organic phase

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is separated, dried over sodium sulphate and concentrated by evaporation in a vacuum. The evaporation residue is chromatographed on a 100-fold quantity of Sephadex LH 20 with methanol. The fractions which are active against *Aspergillus niger* are combined, and the mixture is chromatographed on a 100- to 200-fold quantity of neutral aluminium oxide (activity 1). Elution is effected with toluene to which 10–50% of ethyl acetate have been added. By-products are chiefly eluted first, then S7481/F-1, and finally S7481/F-2. Detection of the active antibiotics in the fractions is again effected by testing the activity against *Aspergillus niger*; purity is tested by thin layer chromatography on silica gel plates with chloroform/methanol (96:4), whereby the antibiotics are made visible with iodine vapour. The pure fractions are combined and triturated with hexane, whereby the compounds S7481/F-1 and S7481/F-2 are obtained in the form of a white amorphous powder, which is dried in a high vacuum at 50°.

The preculture used as starting material is obtained as follows:

The spore and mycelium suspension used for inoculation is produced from a culture of the originally isolated strain NRRL 8044, which is cultivated for 21 days at 27° on an agar medium (of which each liter contains 20 g of malt extract, 20 g of agar, 4 g of yeast extract, and demineralized water). The spores and the mycelium of this culture are taken up in a physiological common salt solution.

This suspension is used for inoculation of 50 liters of a nutrient solution having the same composition as the contents of the 500 liter steel fermenter, and incubation is effected in a steel fermenter at 27° for 3 days while stirring (200 revolutions/minute) and aerating (1 liter of air/minute/liter of nutrient solution). This fermentation solution is used as inoculating material for the 500 liter steel fermenter.

TABLE

¹³C spectra data of S 7481/F-1; 250 mg in 1.5 cc of CDCl₃;

Bruker HX-90E; Sweep-width 6000 Hz.

Address	Frequency [Hz]	δ [ppm]	Intensity
1260	3915.5273	173.0237	184
1264	3909.6679	172.7648	192
1293	3867.1875	170.8876	91
1299	3858.3984	170.4992	215
1312	3839.3554	169.6577	207
1317	3832.0312	169.3341	268
1937	2923.8281	129.2014	220
1992	2843.2617	125.6412	225
2745	1740.2343	76.8994	20
2785	1681.6406	74.3102	165
3027	1327.1484	58.6455	187
3042	1305.1757	57.6745	223
3047	1297.8515	57.3509	253
3051	1291.9921	57.0920	40*
3081	1248.0468	55.1501	844
3159	1133.7890	50.1011	172
3184	1097.1679	48.4828	566
3189	1089.8437	48.1592	276
3239	1016.6015	44.9227	206
3305	919.9218	40.6505	158
3327	837.6953	39.2264	192
3332	880.3710	38.9028	222
3357	843.7500	37.2845	167
3363	834.9609	36.8961	30*
3380	810.0585	35.7957	567
3387	799.8046	35.3426	304
3411	764.6484	33.7891	182
3449	703.9843	31.3294	167
3454	701.6601	31.0057	518
3475	670.8984	29.6464	544
3478	666.5039	29.4522	179

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TABLE (Cont.)

¹³C spectra data of S 7481/F-1; 250 mg in 1.5 cc of CDCl₃;

Bruker HX-90E; Sweep-width 6000 Hz.

	Address	Frequency [Hz]	δ [ppm]	Intensity	
5	3486	654.7851	28.9343	310	5
	3543	571.2890	25.2447	287	
	3549	562.5000	24.8563	631	
	3553	556.6406	24.5974	843	
	3566	537.5976	23.7559	1000	
10	3574	525.8789	23.2381	762	10
	3582	514.1601	22.7202	58*	
	3596	493.6523	21.8140	606	
	3607	477.5390	21.1020	231	
	3622	455.5664	20.1310	165	
15	3628	446.7773	19.7427	254	15
	3645	421.8750	18.6422	285	
	3650	414.5507	18.3186	314	
	3654	408.6914	18.0597	278	
	3658	402.8320	17.8007	277	
20	3674	379.3945	16.7651	224	20
	3688	358.8867	15.8588	202	
	3781	222.6562	9.8389	139	
	3933	0.0000	0.0000	111 = tetramethylsilane	

* Registered signals of CDCl₃ and resonances

25 The compounds S7481/F-1 and S7481/F-2 exhibit pharmacological properties, for example they inhibit the growth of *Aspergillus niger*. 25

Furthermore the compounds exhibit an arthritis inhibiting effect as indicated in standard tests e.g. by an inflammation inhibiting effect in the Freund's adjuvant arthritis latent period test in rats and in the Freund's adjuvant arthritis therapy test in rats.

30 The antibiotics are therefore indicated for use as arthritis-inhibiting agents. 30

The antibiotics exhibit an immunosuppressive effect, with little cytostatic activity as indicated by the following tests using S7481/F-1:—

35 a) Local hemolysis in gel *in vitro*, Jerne test: 35

The inhibition of hemolysis areas in % as compared with the controls is 99% at a dose of 2×60 mg/kg i.p., 90% at a dose of 1×150 mg/kg p.o., and 99.5% at a dose of 3×200 mg/kg p.o. The formation of immunoglobulin M antibodies and immunoglobulin G antibodies is inhibited.

40 b) Hemagglutination test (HAT) in mice: 40

The antibodies formed against sheep erythrocytes are detected. The inhibition is expressed in the suppressive index (SI), whereby the untreated controls are indicated by 1.00; SI=0.3 after 5×200 mg/kg p.o.

45 c) Skin transplantation test in mice: 45

The prolongation of the period of survival of the skin graft in H-2 histoincompatible mice is significantly prolonged after a dose of 10×200 mg/kg p.o., as compared with the untreated control animals: about 16 days longer than the controls.

50 d) Experimental allergic encephalomyelitis (EAE) in rats: 50

The experimentally induced nerve tissue damages paralyze 8 of 10 animals in the untreated control animals, but in the case of the same number of test animals treated with 13×25 mg/kg i.p., a 100% protection is ascertained.

e) Oxazolone test in mice: 55

The decrease of ear swelling is expressed as suppressive index (SI): SI=0.6 after 6×75 mg/kg p.o.

f) Cytopenias in the bone marrow and blood: 55

The high oral administration of 6×500 mg/kg does not show any significant depression of the blood-forming cells in the bone marrow nor a hypoleukocytosis in the blood on the 1st, 3rd and 7th day after the last administration of the compound.

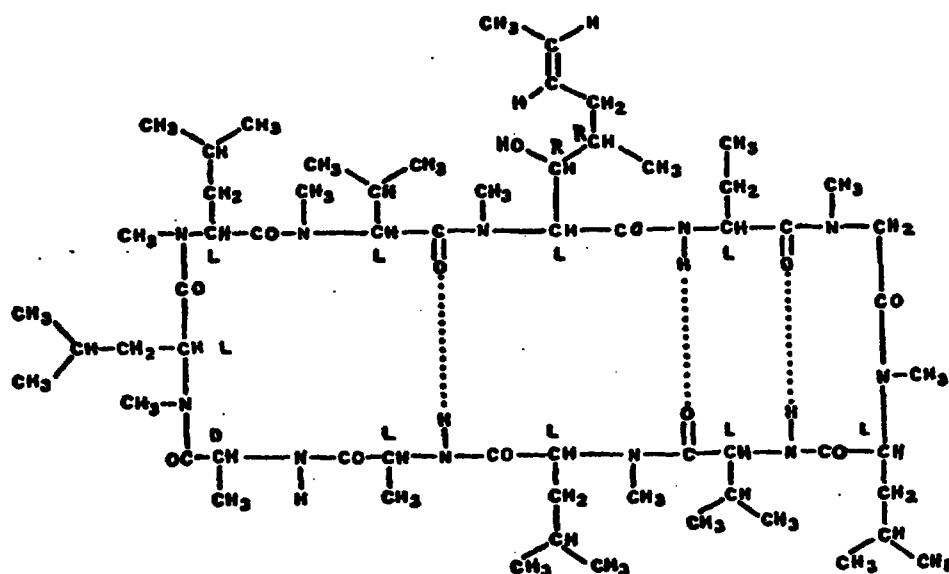
The compounds are therefore further indicated for use in the treatment of autoimmune diseases, e.g. in the treatment of rejection provoked by skin transplant.

For all the above uses, the indicated daily dose is from about 50 to about 900 mg, conveniently administered in divided doses 2 to 4 times a day in unit dosage form containing about 12 to about 450 mg, or in sustained release form.

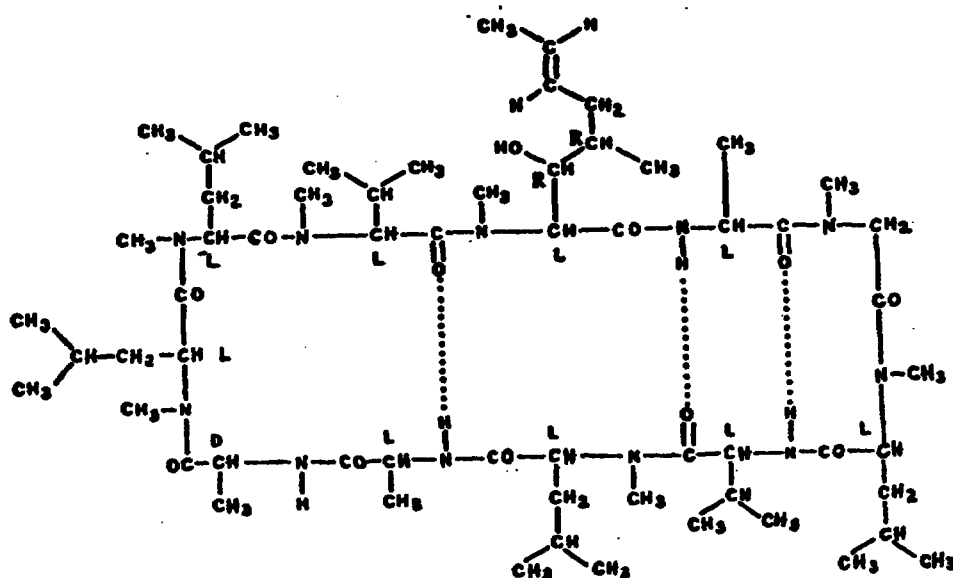
The present invention also provides a pharmaceutical composition comprising compound S7481/F-1 or S7481F-2 in pure form or as a pharmaceutically acceptable crude concentrate in association with a pharmaceutical carrier or diluent. Such compositions may be made using conventional techniques to be in the form of a solution or tablet.

WHAT WE CLAIM IS:—

1. A process for the production of the compound S7481/F-1 for formula I,



or the compound S7481/F-2 of formula II,



or the compound S7481/F-1 combined with the compound S7481/F-2, which comprises cultivating a S7481/F-1; S7481/F-2, or S7481/F-1 and S7481/F-2 producing

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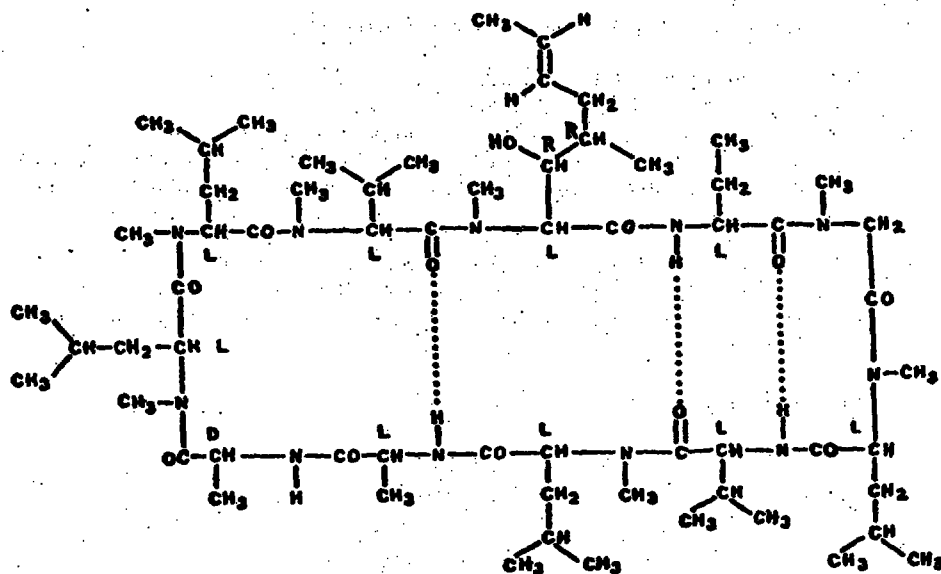
The compounds are therefore further indicated for use in the treatment of auto-immune diseases, e.g. in the treatment of rejection provoked by skin transplant.

For all the above uses, the indicated daily dose is from about 50 to about 900 mg, conveniently administered in divided doses 2 to 4 times a day in unit dosage form containing about 12 to about 450 mg, or in sustained release form.

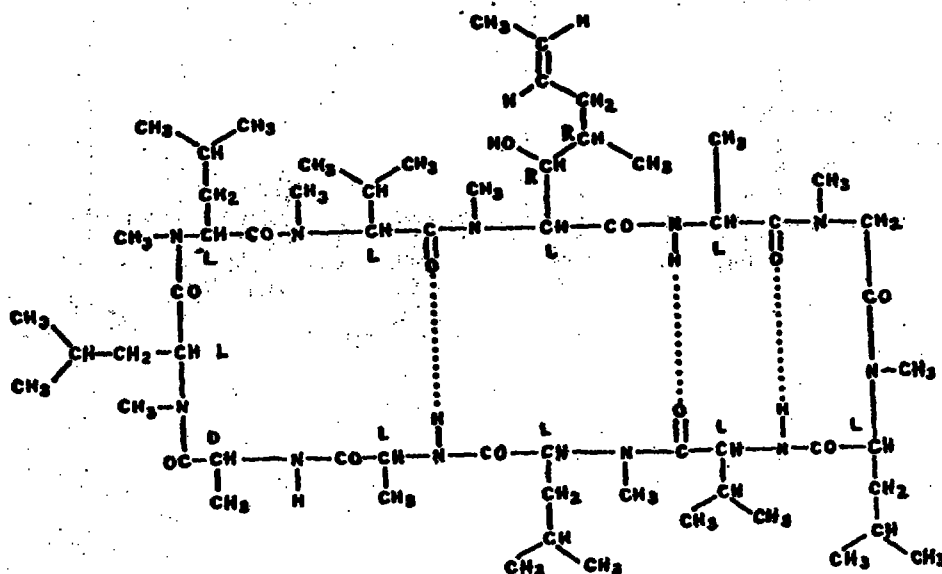
The present invention also provides a pharmaceutical composition comprising compound S7481/F-1 or S7481F-2 in pure form or as a pharmaceutically acceptable crude concentrate in association with a pharmaceutical carrier or diluent. Such compositions may be made using conventional techniques to be in the form of a solution or tablet.

WHAT WE CLAIM IS:—

1. A process for the production of the compound S7481/F-1 for formula I,



or the compound S7481/F-2 of formula II,



or the compound S7481/F-1 combined with the compound S7481/F-2, which comprises cultivating a S7481/F-1; S7481/F-2, or S7481/F-1 and S7481/F-2 producing



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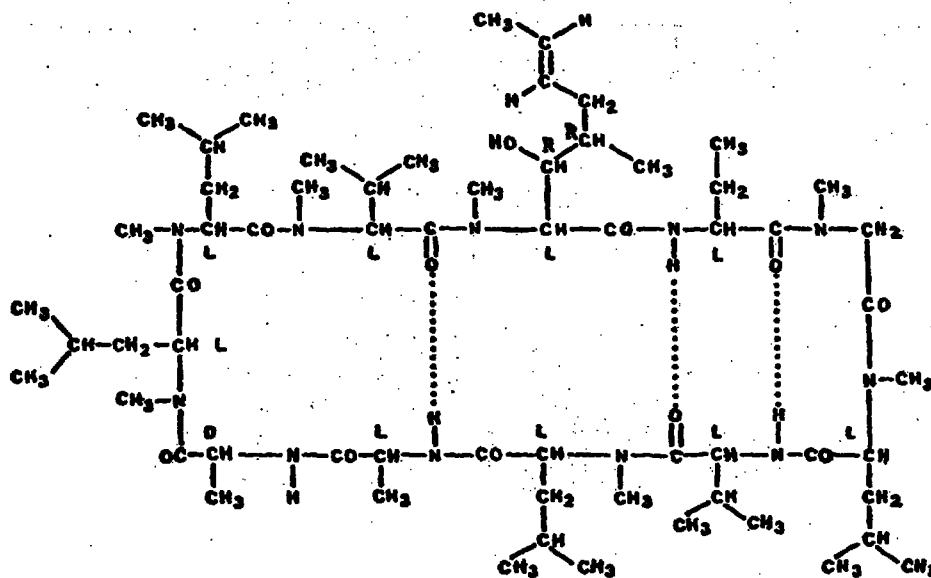
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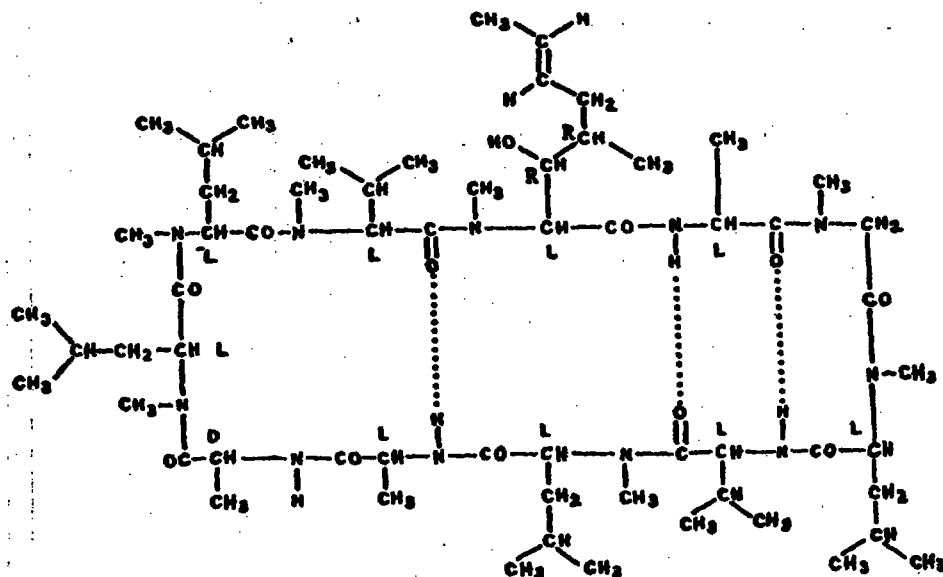
strain of the fungus species, *Cylindrocarpon lucidum* Booth or *Tolypocladium inflatum* Gams in contact with a nutrient medium and isolating compound S7481/F-1; compound S7481/F-2 or compound S7481/F-1 combined with compound S7481/F-2.

- 5 2. A process according to claim 1, wherein the strain is a *Cylindrocarpon lucidum* Booth strain.
3. A process according to claim 2, wherein the strain is NRRL 5760.
4. A process according to claim 1, wherein the strain is a *Tolypocladium inflatum* strain.
- 10 5. A process according to claim 4, wherein the strain is NRRL 8044.
6. A process for the production of the compound S7481/F-1 or /F-2 substantially as hereinbefore described with reference to any one of the Examples.
7. The compound S7481/F-1 or /F-2 whenever obtained by the process of any one of claims 1 to 6.
- 15 8. The compound S7481/F-1 having the following characteristics:—
- (a) When in amorphous form a M.Pt. of 137°—140° (decomp.)
- (b) $[\alpha]_D^{20} = -236^\circ$ (CHCl_3 ; $c=0.5$)
- (c) I.R. (CH_2Cl_2) see Figure 2 of the accompanying drawings,
- 20 (d) U.V. spectrum [CH_3OOH ; $c=1.063$ g/l; $d=0.01$ cm] as shown in Figure 1 of the accompanying drawings or S7481/F-2 having the following characteristics:—
- (a) When in amorphous form a M.Pt. of 127°—130° (decomp.)
- (b) I.R. (CH_2Cl_2) see Figure 5 of the accompanying drawings,
- 25 (c) U.V. spectrum [CH_3OH ; $c=1.062$ g/l; $d=0.01$ cm] as shown in Figure 4 of the accompanying drawings, obtainable by cultivating the strain NRRL 5760.

9. S7481/F-1 of structure



10. S7481/F-2 of structure



11. The compound S7481/F-1 having the following characteristics, when in amorphous form:—

U.V. spectrum [CH_3OH ; $c=1.063$ g/l; $d=0.01$ cm] as shown in Figure 1 of the accompanying drawings,

I.R. spectrum [CH_2Cl_2] as shown in Figure 2 of the accompanying drawings,

Proton N.M.R. spectrum [90 MHz; CDCl_3] as shown in Figure 3 of the accompanying drawings,

High resolution mass spectrum [ion acceleration 4 kv; temperature ion source 290°C ; pressure 5.10^{-6} mm Hg] peak of mass $m/e=1183.831 [\pm 0.004]$ corresponding to $\text{C}_{62}\text{H}_{108}\text{N}_{11}\text{O}_{11}$ recognisable as product of elimination of water from $\text{C}_{62}\text{H}_{111}\text{N}_{11}\text{O}_{12}$; and empirical formula $\text{C}_{62}\text{H}_{111}\text{N}_{11}\text{O}_{12}$;

or S7481/F-2 having the following characteristics, when in amorphous form:—

U.V. spectrum [CH_3OH ; $c=1.062$ g/l; $d=0.01$ cm] as shown in Figure 4 of the accompanying drawings;

I.R. spectrum [CH_2Cl_2] as shown in Figure 5 of the accompanying drawings;

Proton N.M.R. spectrum [90 MHz; CDCl_3] as shown in Figure 6 of the accompanying drawings;

High resolution mass spectrum [ion acceleration 4 kv; ion source temperature 290°C ; pressure 5.10^{-6} mm Hg] peak of mass $m/e=1169.815 (\pm 0.006)$ corresponding to $\text{C}_{61}\text{H}_{107}\text{N}_{11}\text{O}_{11}$, the product of water elimination from $\text{C}_{61}\text{H}_{109}\text{N}_{11}\text{O}_{12}$; and empirical formula $\text{C}_{61}\text{H}_{109}\text{N}_{11}\text{O}_{12}$.

12. A pharmaceutical composition comprising a compound as claimed in any one of claims 7 to 11 in association with a pharmaceutical carrier or diluent.

13. Fermentation liquor comprising a compound as claimed in any one of claims 7 to 12.

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