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**(54) Title:** NOVEL PRODUCTS

**(57) Abstract**

Compounds related to paraherquamide are prepared by culturing Penicillium sp. IMI 332995. A synthetic method of preparing N-oxides of paraherquamide derivatives is also disclosed.

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NOVEL PRODUCTS

The present invention relates to a novel microorganism and to parasiticidal compounds obtainable therefrom and
5 synthetically.

Compounds known as marcfortines are disclosed by Polonsky et al in J.Chem.Soc.Chem.Comm (1980) pp 601-602 (marcfortine A) and Tet. Letters (1981) Vol 22 pp 1977-1980 (marcfortine B
10 and C). The compounds are metabolites of the fungus Penicillium roqueforti.

Structurally related to the marcfortines is a compound known as paraherquamide, disclosed by Yamazaki et al in Tet.
15 Letters (1981) Vol 22 pp 135-136. Paraherquamide is a metabolite of the fungus Penicillium paraherquei. Also disclosed is its dihydro derivative, obtained by catalytic hydrogenation.

20 US-A-4866060 describes the use of marcofortines A, B, C as anti-parasitic agents. US-A-4923867 discloses derivatives of the marcfortines and their use as antiparasitic agents.

EP-A-0301742 describes the use of paraherquamide and
25 dihydroparaherquamide as antiparasitic agents. EP-A-0322937, EP-A-0354615 and EP-A-0390532 disclose derivatives of paraherquamide and their use as antiparasitic agents.

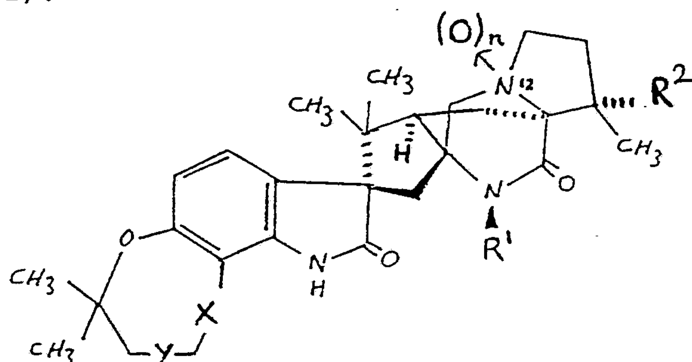
We have now discovered a novel paraherquamide producing
30 fungus. Compounds produced as metabolites in addition to paraherquamide also have parasiticidal properties, and therefore are of use in the treatment of parasitic infestations in humans and animals.

35 We have also discovered a novel modification to the basic

marcfortine/paraherquamide structure which provides compounds with parasiticidal properties.

A first aspect of the invention provides Penicillium sp. IMI
5 332995, or a mutant thereof, more particularly in biologically pure form.

A second aspect of the invention provides compounds of formula (I):
10

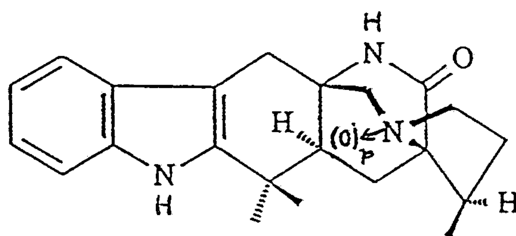



15

(I)

wherein: X is oxygen or a bond, Y is a single bond or a double bond, $R^1$ is hydrogen or methyl, $R^2$ is hydrogen or
20 hydroxy; and n is 0 or 1; but excluding paraherquamide and dihydroparaherquamide.

A third aspect of the invention provides a compound of formula (II):
25




30

(II)

wherein: p is 0 or 1.

International Application No: PCT/GB90 / 01997

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 2, line 4 of the description [1]

**A. IDENTIFICATION OF DEPOSIT** [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

C.A.B. International Mycological Institute

Address of depositary institution (including postal code and country) [4]

Ferry Lane, Kew, Surrey TW9 3AF, United Kingdom.

| Date of deposit [5] | Accession Number [6] |
|---|---|
| 31st May 1989 | IMI 332995 |

**B. ADDITIONAL INDICATIONS** [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4)EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** [8] (if the indications are not for all designated States)

**D. SEPARATE FURNISHING OF INDICATIONS** [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

J.L. Lloyd-Thomas

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

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Form PCT/RO/134 (January 1981)

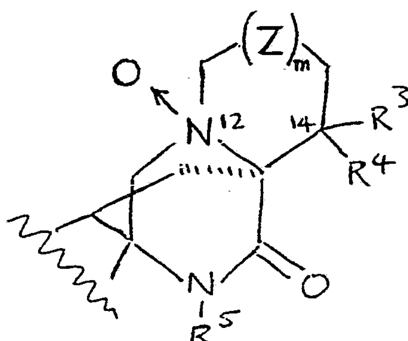
SUBSTITUTE SHEET

This compound is believed to be a bio-precursor of paraherquamide and may be used as an intermediate in the synthesis of paraherquamide or derivatives thereof.

5

A fourth aspect of the invention provides a marcfortine or paraherquamide derivative of partial formula (III):

10




(III)

15

wherein Z is $CH_2$ and m is 0 or 1, $R^3$ and $R^4$ are selected from hydrogen, hydroxy, $C_{1-6}$ alkyl, $C_{1-6}$ alkoxy, $C_{1-6}$ alkenyl, $C_{1-6}$ alkenyl-$C_{1-6}$ alkoxy, $C_{1-6}$ alkynyl-$C_{1-6}$ alkoxy, $C_{1-6}$ alkanoyloxy, poly $C_{1-6}$ alkoxy-$C_{1-6}$ alkoxy, phenyl,
20 phenyl-$C_{1-6}$ alkyl, tri-$C_{1-6}$ alkylsilyloxy, diphenylphosphoryloxy and halogen, or $R^3$ and $R^4$ together form (epoxide) or = $CH_2$, and $R^5$ is hydrogen or $C_{1-6}$ alkyl.

In effect, this aspect of the invention provides an N(12)
25 oxide of a marcfortine or parherquamide or a derivative thereof (when the usual numbering for these compounds is used). Substitution at other positions is optional, but in the partial formula preferably occurs on the carbon atom at position 14, or the amide nitrogen atom.

30

The marcfortine derivative may be any of those disclosed in US-A-4923867, the disclosure of which is incorporated herein by reference. The paraherquamide derivative may be any of those disclosed in EP-A-0301742, EP-A-0322937, EP-A-0354615
35 or EP-A-0390532, the disclosures of which are incorporated herein by reference.

The preferred N(12)oxide is VM55596, the N(12)oxide of paraherquamide itself.

Compounds of formula (I) in which Y is a double bond 5 obtainable as metabolites of IMI 332995 are set out in Table I below:

Table I

| Compound | MASS | X | $R^1$ | $R^2$ | N→O |
|---|---|---|---|---|---|
| VM 29919 (Paraherquamide) | 493 | -O- | $CH_3$ | OH | absent |
| VM 55596 (Paraherquamide N(12)-oxide) | 509 | -O- | $CH_3$ | OH | present |
| VM 54159 | 477 | -O- | $CH_3$ | H | absent |
| VM 54158 | 477 | - | $CH_3$ | OH | absent |
| VM 55594 | 461 | - | $CH_3$ | H | absent |
| VM 55595 | 447 | - | H | H | absent |

The compound of formula (II) in which P is 0 (VM55599) is 30 also obtainable as a metabolite of IMI 332995.

A further aspect of the invention provides a process for the production of compounds of formula (I) or formula (II), or a derivative thereof, which comprises cultivating Penicillium 35 sp. IMI 332995, or a mutant thereof, and subsequently isolating the compound or derivative thereof from the culture.

The present invention also provides a process for the production of a compound of the invention or a derivative thereof, which comprises cultivating a producing microorganism, and subsequently isolating the compound or 5 derivative thereof from the culture.

The present invention furthermore provides a process for the preparation of a compound of the invention or derivative thereof, which comprises chromatographically separating the 10 compound or derivative thereof from a solution thereof in admixture with other substances into a fraction comprising the compound or derivative thereof and other fractions.

The term 'cultivation' (and derivatives of that term) as 15 used herein means the deliberate aerobic growth of an organism in the presence of assimilable sources of carbon, nitrogen, sulphur and mineral salts. Such aerobic growth may take place in a solid or semi-solid nutritive medium, or in a liquid medium in which the nutrients are dissolved or 20 suspended. The cultivation may take place on an aerobic surface or by submerged culture. The nutritive medium may be composed of complex nutrients or may be chemically defined.

25 It has been found that suitable microorganisms for use in the cultivation process according to the invention for the production of VM 55595 and/or VM 55596 and/or VM 55599 include strains of fungi belonging to the genus <u>Penicillium</u> that are capable of elaborating compounds according to the 30 invention. It has further been found that <u>Penicillium sp.</u> IMI 332995, which has been isolated from soil, and also mutants and natural variants thereof, is particularly suitable for this purpose.

35 The term 'mutant' as used herein includes any mutant strain which arises spontaneously or through the effect of an external agent whether that agent is applied deliberately or otherwise. Suitable methods of producing mutant strains

include those outlined by H.I.Adler in 'Techniques for the Development of Micro- organisms' in ''Radiation and Radioisotopes for Industrial Microorganisms'', Proceedings of a Symposium, Vienna, 1973, page 241, International Atomic 5 Energy Authority, and these include:

(i) Ionizing radiation (e.g. X-rays and γ-rays), u.v. light, u.v. light plus a photosensitizing agent (e.g. 8-methoxypsoralen), nitrous acid, hydroxylamine, 10 pyrimidine base analogues (e.g. 5-bromouracil), acridines, alkylating agents (e.g. mustard gas, ethyl-methane sulphonate), hydrogen peroxide, phenols, formaldehyde, nitrosoguanidine, heat, and

15 (ii) Genetic techniques, including, for example, recombination, transformation, transduction, lysogenisation, lysogenic conversion, protoplast fusion, and selective techniques for spontaneous mutants.

20

Penicillium sp. IMI 332995 is believed to comprise a previously unreported strain in the genus Penicillium. It has been deposited in the C.A.B. International Mycological Institute, Ferry Lane, Kew, Surrey, TW9 3AF, England, the 25 deposit (IMI 332995; filing date 31st May, 1989) being made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure.

30 The characteristics of Penicillium sp. IMI 332995 were as follows:

After being grown for seven days on Czapek Dox agar medium at 28°C, Penicillium sp. IMI 332995 had produced colonies 35 which were 20-30mm diameter. Margins of the colonies were low and velutinous, central areas were floccose; mycelium

was mostly white in peripheral areas, at the centres yellow green to grey green. A yellow exudate was produced over the colony; reverse colour of colony was yellow brown.

5 Conidiophores were borne from subsurface or surface hyphae, stipes 100-300 x 2.2-3.0μm, smooth walled, characteristically terminating in well defined verticils of 3-5 divergent metulae. Some subterminal or intercalary metulae were produced also. Metulae were usually of uniform 10 length (12-18 x 2.2-2.5μm), phialides were in compact verticils of 8-12, ampulliform usually with short collula; conidia were spheroidal to subspheroidal, 2.2-3.0μm diam, with walls smooth or finely roughened, typically borne in long well defined columns, one per metula.

15

The fermentation medium for cultivating the producing organism may be solid, semisolid or liquid and suitably contains sources of assimilable carbon and assimilable nitrogen together with inorganic salts. Suitable sources of 20 nitrogen include yeast extract, soybean flour, meat extract, cottonseed flour, malt, distillers dried solubles, amino acids, protein hydrolysates and ammonium and nitrate nitrogen. Suitable carbon sources include glucose, sucrose, lactose, maltose, starch and glycerol. Suitably the culture 25 medium also includes alkali metal ions (for example, sodium), alkaline earth metal ions (for example, magnesium), halogen ions (for example, chloride), and trace elements (for example,iron).

30 The cultivation may suitably be effected at a temperature of about 20 to 35°C, advantageously 27 to 28°C, and the culture may suitably be harvested after 2 to 35 days, advantageously about 5 to 20 days, after the initiation of fermentation in order to give an optimum yield of the desired compound.

35

The desired compound or derivative thereof may then be isolated from the culture medium and worked up and purified using conventional techniques.

5 The desired product may be obtained from either the mycelial growth or from the culture medium. It may therefore be convenient for the first isolation step to involve the separation of the mycelium from the culture medium by, for example, filtration or centrifugation of fermentation broth, 10 to give a clarified culture filtrate and solid material. Alternatively, the whole medium including the mycelium can be extracted directly.

It may be convenient to include an organic solvent 15 extraction step in the isolation or purification procedure, suitably using a solvent such as acetone or chloroform.

Further isolation of the desired compound may conveniently be effected by chromatographic techniques. The extract may 20 contain additional substances, and therefore chromatographic separation may result in a plurality of fractions, of which the desired fraction or fractions is or are the fraction(s) comprising the desired compound or a derivative thereof.

25 The desired fraction(s) may readily be identified in a routine manner by testing for anthelmintic activity and/or by monitoring each fraction chromatographically. The desired fraction(s) is/are that/those identified by such procedures as containing the desired compound or a 30 derivative thereof.

If necessary, repeated chromatographic separation may be carried out in a routine manner. At each stage of the separation procedure, the fractions containing the desired 35 compound or a derivative thereof may be combined and then

subjected to further purification steps. In the initial separation steps, it may be convenient to identify the desired fractions merely as those having anthelmintic activity and to combine all such fractions. In later stages of the separation, it may be necessary to identify the desired fraction or fractions more precisely in order to separate the desired compound or a derivative thereof from any other substances that may be present. Separation may advantageously be continued in order to give one or more fractions consisting essentially of the desired compound or a derivative thereof.

The expression 'fraction consisting essentially of the desired compound or a derivative thereof' means a fraction containing the desired compound or a derivative thereof as the sole component present in that fraction, or as the major component (whether other components are active or are inactive impurities) present in that fraction. The expression 'major component' means the component that is present in the greatest amount relative to other individual components (exclusive of solvent). Suitably, the major component is present in an amount greater than the sum of the amounts of all other components (excluding solvent). More suitably, the major component is present in an amount of at least 60%, advantageously at least 70%, preferably at least 80%, especially from 90% to 100%, by weight, relative to the total amount of active material, or relative to the total amount of material whether active or inactive (exclusive of solvent), as the case may be, present in the fraction. Typically, the compounds of formula (I) are produced in admixture with one another, so that fractions may be obtained which consist essentially of a mixture of two or more compounds of formula (I).

It has been found convenient to carry out chromatographic separation using HPLC. Two or more chromatographic separation steps may be carried out successively.

In a further aspect of the invention VM 55596 may be prepared by the N-oxidation of paraherquamide. A suitable procedure is described in J. Org. Chem. [1970] 35, 1721 et seq. The same procedure may be used to prepare the compound 5 of formula (II) in which p is 0 by N-oxidation of VM 55599. Similarly compounds of partial formula (III) can be obtained by N-oxidation of the derivatives disclosed in the patents incorporated herein by reference.

10 Accordingly the present invention provides a method for the production of a compound of partial formula (III) which comprises reacting a marcfortine or paraherquamide on derivative thereof of partial formula (IV)

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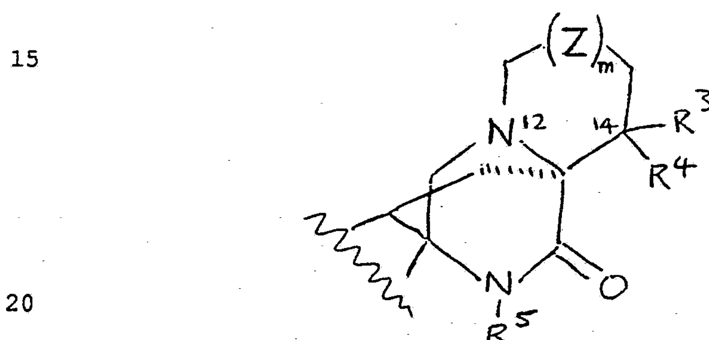



20 (IV)

in which Z, m, $R^3$, $R^4$ and $R^5$ are as defined for formula (III), with a N-oxidising agent.

25 General procedures for the preparation of heteroaromatic N-oxides can be found in Chapter II of "Chemistry of the Heterocyclic N-Oxides", A.R.Katritzky and J.M.Lagowski, published 1971 Academic Press (Vol.19 of ORGANIC CHEMISTRY - A Series of Monographs). Typically the N-oxide is formed by 30 reaction with a percarboxylic acid in an appropriate solvent. Most suitably an aromatic peracid in a non-polar solvent is used, since the reaction may usually be carried out at room temperature. Suitable aromatic peracids include perbenzoic acid, chloroperbenzoic acid perphthalic acid.

Preferably the reaction is carried out in chloroform using m-chloroperbenzoic acid. Of the non-aromatic peracids, peracetic acid is the most common reagent, but is less suitable in view of the more adverse conditions, such as 5 heating the heterocyclic compound with glacial acetic acid and hydrogen peroxide at temperatures from 20 to 90°C.

Compounds of formula (I) that are not obtainable as metabolites of IMI 332995, or by N-oxidation as described 10 above, may be prepared by chemical modification of compounds that are obtainable as metabolites, using substitution techniques that are well known in organic chemistry, for example for a alkylation of amide nitrogens.

15 The dihydro derivatives of the compounds of formula (I) (where Y is a single bond) may be prepared from the compounds of formula (I) in which Y is a double bond using the procedure described in Example 11 of EP-A-0 301742, referred to above.

20

The compound or mixture of compounds according to the invention is suitably provided in substantially pure form, for example at least 50% pure, suitably at least 60% pure, advantageously at least 75% pure, preferably at least 85%

25

pure, more preferably at least 95% pure, especially at least 98% pure, all percentages being calculated as weight/weight. An impure or less pure form of a compound according to the invention may, for example, be used in the preparation of a 30 more pure form of the same compound or of a related compound (for example a corresponding derivative) suitable for pharmaceutical use.

The compounds of the invention have parasiticidal 35 properties, for example against nematodes such as

Trichostrongylus colubriformis, and are useful for the treatment of helminthiasis in animals such as mammals, including humans and domesticated animals (including farm animals).

5

Accordingly the present invention also provides a compound according to the invention, for use in the treatment of the human or animal body, especially for treating endo- and ectoparasitic infestations and particularly for treating 10 helminthiasis of domestic and farm animals.

The term helminthiasis encompasses those diseases of man and animals caused by infestation with parasitic worms such as Strongyles, Ascarids, hookworms lungworms, filarial worms, 15 whipworms, and liver flukes. The compound may also be used against nematodes occurring in the soil or parasitic to plants.

The compounds of the invention are also active against 20 Arthropods. The phylum Arthropoda comprises insects - such as biting flies, lice, bugs, beetles and fleas - and arachnids - such as mites and ticks.

Thus, a broad aspect of the invention provides a method of 25 eradicating arthropod or nematode infestations, which method comprises applying a compound according to the invention or a derivative thereof to the arthropods or nematodes or to their environment.

30 The present invention thus provides a pesticidal composition comprising a compound according to the invention or a derivative thereof together with a suitable carrier or excipient, such as an aerosol formulation.

35 The present invention also provides a pharmaceutical or veterinary composition comprising a compound according to the invention or a pharmaceutically acceptable derivative

thereof together with a pharmaceutically or veterinarily acceptable carrier or excipient.

The present invention also provides a method of treatment or prophylaxis of endo- and ectoparasitic infestations, especially helminthiasis, of animals, especially humans and domesticated mammals, which comprises administering an effective non-toxic amount of a compound according to the invention or a pharmaceutically acceptable derivative thereof, or a composition according to the invention, to a patient in need thereof.

The composition according to the invention may be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other anthelmintics.

In suitable formulations the drug may be administered to animals orally (as a paste, drench, bolus, capsule or tablet), parenterally, percutaneously, as a food additive (eg granules, pellets or powder), or may be prepared as an aerosol spray formulation.

The compounds of the invention may be formulated as a mixture with each other and/or with other anthelmintics, insecticides, acaricides or other pharmacologically active substances.

Suitably the composition consists of sufficient material to provide a dose of from 0.01 to 50mg of active ingredient per kg of animal body weight per dose, more suitably 0.1 to 10mg/kg per dose.

A composition according to the invention may suitably contain from 0.001 to 99% by weight of the compound according to the invention (based on the total weight of the composition), depending on the method of administration.

In certain circumstances the crude fermentation broth may be administered, for example by incorporating the freeze-dried fermentation broth into the feed of the animal.

5 It will be appreciated that, in some cases, it will be advisable to repeat the dosing of the infected or potentially infected human or animal with the compound of the invention according to conventional dosage regimes used with anthelmintics.

10

The following Examples illustrate the invention.

Example 1

A conidial suspension of Penicillium sp. IMI 332995 was prepared in phosphate buffered saline containing 10% glycerol and 1% Tween 80 (PBSGT) from a well grown potato dextrose agar* plate culture.

2-3mls of suspension were spread onto Czapek Dox agar in large bioassay dishes ($245mm^2$) and incubated at 28°C. The culture and agar was harvested after 20 days growth into large glass beakers.

Czapek Dox Agar

| | g/L |
|---|---|
| Sucrose (BDH Analar) | 30 |
| $NaNO_3$ | 3 |
| $MgSO_4.7H_2O$ | 0.5 |
| KCl | 0.5 |
| $FeSO_4.7H_2O$ | 0.01 |
| $K_2HPO_4$ | 1.0 |
| Technical Agar (Oxoid) | 18g |
| Deionised water | 1000ml |

* Potato Dextrose Agar (Oxoid Ltd. Basingstoke, UK)

Extraction and Purification of VM 29919, VM 54148, VM 54159, VM 55594 and VM 55595

Culture IMI 332995, grown for 20 days on Czapek Dox agar (5L) was macerated in acetone (6L) (Waring commercial blendor, Dynamics Corporation of America) centrifuged and the solids further extracted with 2 washes of acetone (6L per wash, the solids being separated by centrifugation). The acetone was evaporated under vacuum yielding an aqueous residue which was then extracted three times with 2 volumes of $CHCl_3$. The organic phase was evaporated under vacuum leaving 4.23g of solid extract.

The primary purification was by preparative reverse phase HPLC (Dynamax - 60A C-18 column 41.4 x 250mm, Rainin Instrument Co., USA) eluted with methanol- water (75:25) at 20 mls/min. The entire extract was suspended in methanol, after allowing to stand the soluble portion was injected on to the column via a 5ml sample loop (4 consecutive injections). (Subsequent weighing of the insoluble residue showed that 3.34g of extract was loaded on to the column at this stage).

The flow from the column was continually collected into a fraction collector in 0.8min. (16ml) fractions. 1ml aliquots were taken from alternate fractions, dried under vacuum, redissolved in 0.5ml $CHCl_3$ and examined by TLC (Plastic backed Silica -60 plates, E. Merck, Darmstadt, FRG (TYPE 5735) developed with $CHCl_3$: methanol:glacial acetic acid 89:10:1) Spots were visualised by spraying with Ehrlichs reagent (1% 4-Dimethylaminobenzaldehyde in 1:3 concentrated HCl:Methanol) followed by heating. TLC of the starting material showed three major spots giving a pink colour with Ehrlichs reagent. The largest at Rf 0.55 and two smaller spots at Rf 0.6 and 0.5.

The TLC of column fractions showed that the Rf 0.55 spot eluted from the column first (fractions 10-27) followed by the other two spots together in fractions 28-56. These fractions were therefore separately pooled to give bulk fractions 'A' (Fractions 10-27) and 'B' (Fractions 28-56).

The insoluble material not loaded onto the inital column was dried and weighed (0.89g). It was almost completely soluble in a larger volume (200ml) of methanol. This was filtered and re concentrated under vacuum to 20mls. This was chromatographed under essentially identical conditions to those previously described and similar bulk fractions A and B were obtained.

[Fractions A and B from each column were pooled and weighed (A:0.93g, B:0.18g).

Further purification of both fractions A and B was by preparative Silica HPLC (Dynamax - 60A Si column 21.4 x 250mm. Rainin Instrument Co USA) eluted with $CH_2Cl_2$:methanol gradients at 8ml/min. Fractions were examined directly by TLC developed with 90:10: $CHCl_3$:methanol. The metabolites were visualized as pink spots after spraying with Ehrlichs reagent.

Sample A was dissolved in $CH_2Cl_2$ and injected onto the silica column which was eluted with a linear gradient of $CH_2Cl_2$:methanol, 100:0 to 90:10 over 120 mins. Fractions were collected throughout. By reference to TLC results it was found that the material of interest (the most abundant Ehrlich positive pink spot) eluted between 60 and 71 minutes (at 97:3 $CH_2Cl_2$:methanol). Fractions containing this material were pooled and dried to give 353 mg of pale yellow solid.

This sample was divided into two and each portion chromatographed by Silica HPLC as before except that in

addition the column eluent was monitored by UV at 235 and 290nm (1000S Diode Array Detector, Applied Biosystems Ltd. Warrington, UK). It was found that, in each run, the material eluted as before except that two distinct peaks
5 were discernable by UV monitoring. Each was collected separately and corresponding material from each column run was pooled and dried down on a rotary evaporator. The faster eluting peak yielded 117mg of VM 29919.

10 Characterisation Data for VM 29919

UV λmax 225nm (in MeOH)
Mass (FAB, NOBA/Na) $[MH^+]$ = 494
δ13C ($CDCL_3$) 183.1, 171.6, 146.2, 139.1, 135.4,
15 132.7, 125.1, 120.3, 117.3, 115.0, 79.8, 78.1, 71.4, 65.3, 63.2, 59.2, 51.8, 51.6, 46.4, 38.2, 37.1, 29.94, 29.85, 25.9, 23.7, 22.2, 20.4, 19.2

This was found to be identical to Paraherquamide
20 (Yamazaki and Okuyama 1981, Tetrahedron Letters 22 135-136)

Fractions containing the slower peak yielded 34mg of VM 54158.

25 Characterisation Data for VM 54158

UV λmax 244mm. (in MeOH)
Mass (FAB, NOBA/Na) $[MH^+]$ = 478
δ13C ($CDCl_3$ 184.3, 153.1, 137.1, 131.3, 125.6,
30 121.3, 116.1, 109.6, 105.3, 78.0, 76.4, 71.4, 65.3, 62.6, 59.2, 51.9, 51.7, 46.4, 38.1, 37.1, 27.9, 27.8, 25.9, 22.2, 22.2, 20.4, 19.1

Sample B (from the reverse phase separation) was dissolved in 4mls $CH_2Cl_2$ and injected onto a silica column eluted with a $CH_2Cl_2$:methanol gradient from 100:0 to 97:3 over 30 min (linear) followed by isocratic elution at 97:3. The column
5 was monitored by TLC and UV (235, 244 and 320nm) monitoring with spectral scanning (190-375nm) at 0.5min intervals (AB1 1000S diode array detector). Fractions were collected throughout.

10 VM 54159 and VM 55594 eluted as adjacent peaks (43-44 mins and 45-49 mins respectively) being distinguished on the basis of UV λmax of the spectral scans (VM 54159 λmax 226nm, VM 55594 λmax 244nm).

15 VM55595 eluted as a discrete peak at 59-65 mins (λmax 244nm) obtained after evaporation of solvent.

The yields of essentially pure material from the appropriate fractions were: VM 54159, 10.7mg; VM 55594, 16.3mg;
20 VM 55595, 9.6mg.

<u>Characterisation Data</u>

<u>VM 54159</u>

25

UVλ max 225nm (in MeOH)
Mass (FAB, NOBA/Na) $[MH^+]$: 478
δ13C ($CDCl_3$) 182.5, 172.2, 146.0, 139.0, 135.2, 132.3, 125.4, 120.5, 117.3, 115.1, 79.8, 67.7,
30 65.2, 62.9, 60.1, 53.4, 52.1, 46.3, 40.3, 37.5, 30.2, 30.0, 29.8, 27.7, 25.5, 24.0, 20.8, 13.1

VM 55594

UV λmax 245nm (in MeOH)
Mass (FAB, NOBA/Na) $[MH^+]$ = 462
5 δ13C ($CDCl_3$) 185.7, 172.4, 153.0, 137.5, 131.2, 125.5, 121.6, 116.3, 109.5, 105.6, 76.4, 67.7, 65.2, 62.8, 60.0, 53.3, 52.4, 46.2, 40.3, 37.3, 30.3, 27.9, 27.8, 27.7, 25.5, 23.9, 20.6, 13.2.

10 VM 55595

UV λmax 245nm (in MeOH)
Mass (FAB, NOBA/Na) $[MH^+]$ = 448
δ13C ($CDCl_3$) 184.9, 174.3, 153.0, 137.5, 131.2,
15 125.6, 121.8, 116.3, 109.4, 105.4, 76.3, 68.2, 62.5, 61.8, 60.2, 53.4, 52.8, 46.2, 40.0, 39.9, 30.3, 28.0, 27.8, 27.6, 24.1, 20.6, 13.1

EXAMPLE 2

20

EXTRACTION AND PURIFICATION OF VM 55596

Culture IMI 332995, grown for 24 days on 10L Czapek Dox agar (Example 1), was harvested into acetone and extracted into
25 chloroform, as described in Example 1, to produce 5.1 g of extract.

This extract was divided into 2 aliquots, I and II(containing approximately 4 and 1 g respectively).
30 Each was chromatographed by preparative reverse phase HPLC (Dynamax - 60A C-18 41.4 x 250 mm, Rainin Instrument Co., USA), eluted with methanol:water (75:25) at 15 ml/min. 15 ml fractions were collected throughout and an aliquot of each was examined by TLC (Merck glass-backed silica gel

60 plates, 20 x 20 cm, developed with 90:10 chloroform:methanol, visualised by spraying with Ehrlichs reagent, and heating). Fractions containing the major Ehrlichs positive component in each run (corresponding to fraction A in Example 1) were combined (fractions 9-24 in run I and 7-14 in run II) and concentrated to dryness under vacuum to yield 1.93 g.

This material was divided into 2 equal aliquots and each was chromatographed by Silica HPLC (Dynamax-60A Si 21.4 x 250 mm, Rainin Instrument Co. USA) with $CH_2Cl_2$:methanol gradient elution (8 ml/min). In one run, the gradient was as follows:

| Time | $CH_2Cl_2$:MeOH |
|---|---|
| 0-20 minutes | 100:0 to 97:3 linear gradient |
| 20-40 minutes | 97:3 isocratic |
| 40-80 minutes | 97:3 to 90:10 linear gradient |
| 80 minutes onwards | 90:10 isocratic |

8 ml fractions were collected throughout.

The column was monitored by UV absorbance (235 and 244 nm, 1,000S Diode Array Detector, Applied Biosystems Limited, Warrington, UK), and each fraction examined by TLC as before. Paraherquamide-rich material eluted in fractions 34-37. Fractions 110-115, corresponding to a peak in UV absorbance and an Ehrlichs positive spot on TLC (Rf 0.19) were pooled and combined with the corresponding fractions(by TLC) from the other column run (which was identical except that the 97:3 isocratic elution was continued for 60 minutes). After evaporating the solvent 19 mg of material was recovered and was purified by a final silica column (Dynamax - 60A, 10 x 250 mm) eluted with a linear gradient of $CH_2Cl_3$:methanol, 98:2 to 88:12 over

90 minutes at 4 ml/min. Monitoring was by UV absorbance at 235 and 244 nm with spectral scanning every 0.5 minutes (AB1 1000S Diode Array Detector). 4 ml fractions were collected throughout. A major peak of UV absorbance occurred between fractions 28-47. UV spectra indicated that the early fractions were impure. Therefore fractions 37-47 were pooled and dried under vacuum. This yielded 10.0 mg of VM 55596.

Characterisation of VM 55596

UV max. 225 nm (in MeOH) Mass (FAB, Thioglycerol) $[MH]^+$ = 510 with a major ion at 494 =$[MH-16]^+$.

$\delta$13C ($CDCl_3$) 182.2, 167.7, 146.4, 138.9, 135.4, 132.4, 124.1, 120.5, 117.7, 115.2, 83.8, 79.9, 77.9, 76.2, 69.4, 62.9, 62.8, 51.0, 46.8, 39.1, 36.0, 29.9, 29.7, 27.1, 23.3, 22.2, 21.1, 14.5

Further evidence that VM 55596 was the N-oxide derivative of paraherquamide came from the demonstration that de-oxygenation with triphenylphosphene (TPP) produced paraherquamide (0.5 mg VM 55596 in 0.5 ml dioxane was mixed with 3 mg TPP in 0.5 ml dioxane. After heating to 60°C for one hour, the product was examined by TLC (as above) and HPLC (Microsorb C-18 4.2 x 250 mm column, 90:10 methanol:water at 1 ml/min, Waters 990 Diode Array Detector) and found to be indistinguishable from paraherquamide).

Example 3

A conidial suspension of Penicillium sp. IMI 332995 was prepared in phosphate buffered saline containing 10% glycerol and 1% Tween 80 (PBSGT) from a well grown culture on a potato dextrose agar (Oxoid Ltd. Basingstoke, UK) plate.

2-3mls of suspension were spread onto Czapek Dox agar plates and incubated at 28°C.

5

| Czapek Dox Agar | g/L |
|---|---|
| Sucrose (BDH Analar) | 30 |
| $NaNO_3$ | 3 |
| $MgSO_4.7H_2O$ | 0.5 |
| KCl | 0.5 |
| $FeSO_4.7H_2O$ | 0.01 |
| $K_2HPO_4$ | 1.0 |
| Technical Agar (Oxoid) | 18g |
| Deionised water | 1000ml |

10

15

20

Two batches were grown on 10 and 20 L of agar for 27 and 26 days respectively. Each batch was separately extracted into acetone by macerating the agar with 1-2 volumes of solvent and removing the solids by centrifugation and filtration
25 after which the agar was twice more extracted with acetone.

The acetone was evaporated under vacuum yielding an aqueous residue which was then extracted, four times, each with 2.5 volumes of chloroform. The organic phase was separated and
30 evaporated under vacuum to leave 3.64 g and 11.15 g of yellow oil from the two batches, which were then combined.

The combined extract was suspended in approximately 200 ml of methanol which caused a white precipitate to form. This was filtered off and rinsed with 50 ml of methanol. The precipitated material was resuspended in approximately 5 200 ml of warm (30°C) methanol and again filtered. The precipitate was discarded and the methanolic solutions pooled and the solvent evaporated to leave 9.44 g of material.

10 A column 10 cm diameter x 25 cm was packed with HP20-SS (Mitsubishi Chemicals, Japan), well washed with acetone and equilibrated in 7:3 methanol/water.

The sample was suspended in 50 ml of 7:3 methanol/water and 15 applied to the top of the column which was then eluted as follows, 500 ml fractions were collected throughout.

| | | | | | |
|---|---|---|---|---|---|
| 6.5 L | 70:30 | methanol:water, | | Fractions | 1-13 |
| 3.5 L | 75:25 | '' | '' | '' | 14-20 |
| 3.5 L | 80:20 | '' | '' | '' | 21-27 |
| 6.5 L | 85:15 | '' | '' | '' | 28-40 |
| 8.0 L | 90:10 | '' | '' | '' | 41-56 |

A 10 ml aliquot of each fraction was dried under vacuum and 25 redissolved in 0.5 ml of chloroform and examined by TLC (10 x 10 cm HPTLC plates, Silica gel 60 $F_{254}$ (E. Merck) developed with 9:1 $CHCl_3$/MeOH, spots were visualized under UV light (254 nm) and by spraying with Ehrlichs reagent (1% 4-Dimethylaminobenzaldehyde in 1:3 concentrated 30 HCl:methanol) followed by heating).

On the basis of this data the individual fractions showing similar spots on TLC were pooled. Thus fractions 40-46 were combined and designated as group 'G'

35

When pooled and the solvent evaporated under vacuum group 'G' yielded 0.297 g of material. TLC showed this to contain a number of Ehrlichs positive components. This was further purified by normal phase HPLC using a Dynamax 60A Si column 41.4 x 250 mm (Rainin Instrument Co. USA) eluted with $CH_2Cl_2$/Methanol, initially as a linear gradient (100% $CH_2Cl_2$ to 96:4 $CH_2CL_2$/MeOH over 60 mins) and then isocratically at 96:4 ($CH_2CL_2$/MeOH), all at 20 ml/min. 18 ml fractions were collected from the start of the isocratic phase. Each fraction was examined by TLC (20 x 20 cm TLC plates, Silica gel 60 $F_{254}$ (E. Merck) developed and visualized as above). Tubes 29-31 were found to show a single Ehrlichs positive spot of Rf 0.7 so were combined. After evaporation of the solvent this sample (designated 'G1') weighed 15.1 mg.

Sample 'G1' was further chromatographed by normal phase HPLC using a Dynaxax 60A Si column 10 x 250 mm eluted with hexane/propan-2-ol pumped at 4 ml/min. The solvent composition was as follows: 0-18 min Isocratic, 98:2 hexane/propan-2-ol; 18-88 min linear gradient 98:2 to 90:10hexane/propan-2-ol; over 60 mins but held at 95:5 for 10 mins as the main peak eluted. 4 ml fractions were collected throughout. The column was monitored by UV detection at 226 and 205 nm, and by TLC. The main peak detected by UV eluted in fractions 42-53. TLC indicated that each of these fractions contained the same, pure, material corresponding to the Ehrlichs positive spot seen in sample 'G1'. Thus fractions 42-53 were pooled and the solvent evaporated to yield 5.0 mg of product (VM 55599).

The product VM 55599 was characterized as follows:-

UV max (MeOH) 226, 282 nm

Mass spectrometry (E1 and FAB) indicated a molecular weight of 349.

$^{13}C$ ($CDCl_3$) 174.8, 141.2, 136.5, 126.8, 121.3, 119.0, 117.7, 110.6, 104.0, 66.3, 58.8, 55.6, 53.5, 46.7, 34.2, 33.0,

30.5, 30.2, 30.0, 26.8, 23.9, 17.4.

Example 4

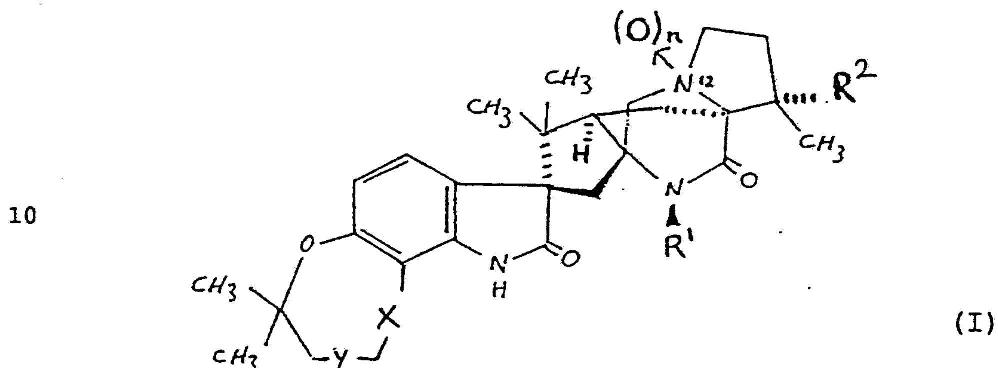
5 Synthesis of Paraherquamide N-Oxide (VM 55596)

To a solution of paraherquamide (25 mg) in chloroform (1.5 ml) at room temperature was added a solution of m-chloroperbenzoic acid (10.4 mg 85%) in chloroform (1 ml) and 10 the mixture stirred for 2h. The solvent was evaporated and the product purified by preparative TLC (silica eluted with 5% methanol in dichloromethane).

Claims

1. Penicillium sp IMI 332995 or a mutant thereof.

5 2. A compound of formula (I):




10

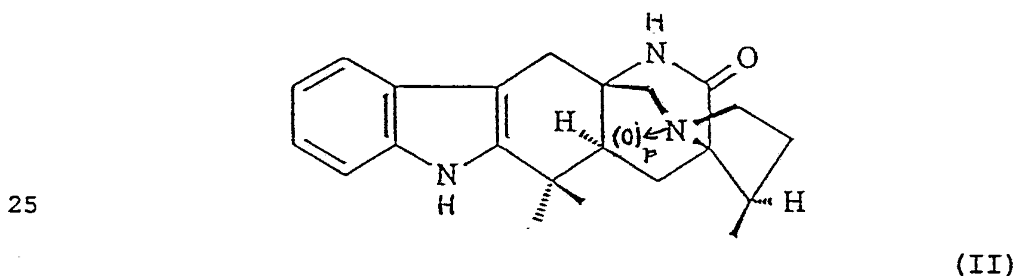
(I)

wherein X is oxygen or a bond, Y is a single bond or a

15 double bond, $R^1$ is methyl or hydrogen, $R^2$ is hydrogen or hydroxy, and n is 0 or 1; but excluding paraherquamide and dihydroparaherquamide.

3. A compound of formula (II):

20

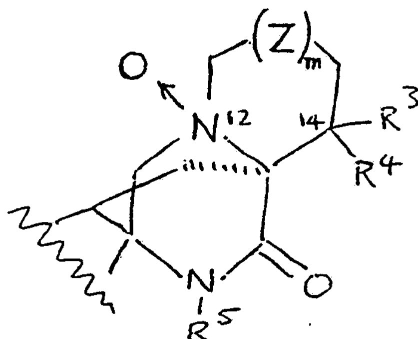



25

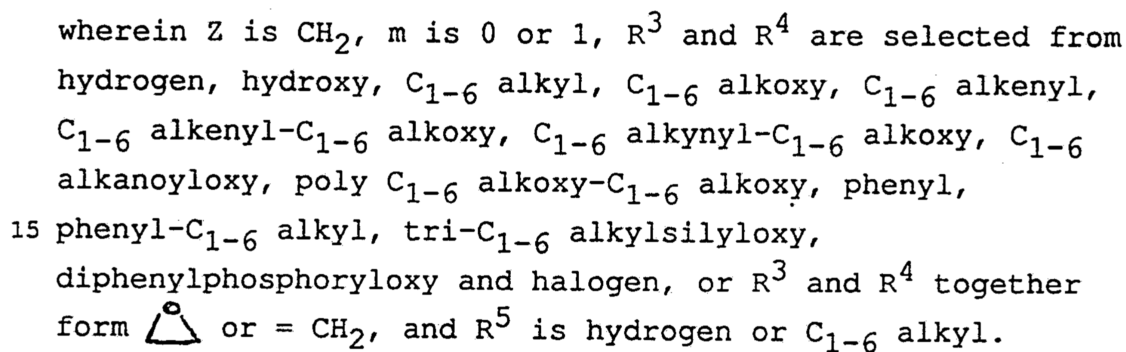
(II)

wherein p is 0 or 1.

4. A marcfortine or paraherquamide derivative of partial formula (III):




(III)



wherein Z is $CH_2$, m is 0 or 1, $R^3$ and $R^4$ are selected from hydrogen, hydroxy, $C_{1-6}$ alkyl, $C_{1-6}$ alkoxy, $C_{1-6}$ alkenyl, $C_{1-6}$ alkenyl-$C_{1-6}$ alkoxy, $C_{1-6}$ alkynyl-$C_{1-6}$ alkoxy, $C_{1-6}$ alkanoyloxy, poly $C_{1-6}$ alkoxy-$C_{1-6}$ alkoxy, phenyl, phenyl-$C_{1-6}$ alkyl, tri-$C_{1-6}$ alkylsilyloxy, diphenylphosphoryloxy and halogen, or $R^3$ and $R^4$ together form [epoxide] or = $CH_2$, and $R^5$ is hydrogen or $C_{1-6}$ alkyl.

5. A compound according to claim 2 in which Y is a double bond selected from the group consisting of:

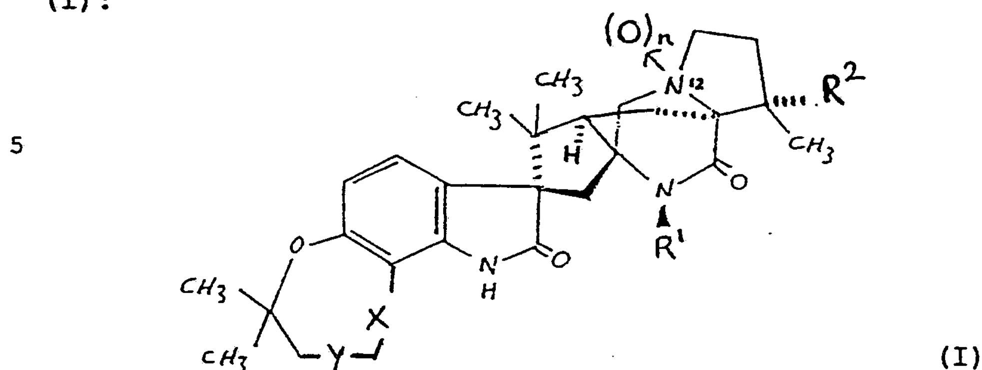
VM 55596 (X = O, $R^1$ = $CH_3$, $R^2$ = OH, n = 1)
VM 54159 (X=O, $R^1$ = $CH_3$, $R^2$ = H, n = 0)
VM 54158 (X is a bond, $R^1$ = $CH_3$, $R^2$ = OH, n = 0)
VM 55594 (X is a bond, $R^1$ = $CH_3$, $R^2$ = H, n = 0)
VM 55595 (X is a bond, $R^1$ = H, $R^2$ = H, n = 0).

6. A compound according to claim 3 which is:

VM 55599 (p = 0).

7. A process for the production of a compound of formula (I):

5

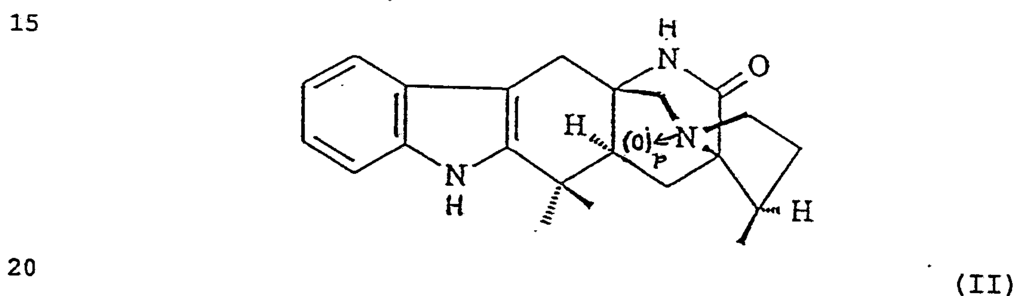



(I)

10

wherein X is oxygen or a bond, Y is a single bond or double bond, $R^1$ is methyl or hydrogen, $R^2$ is hydrogen or hydroxy, and n is 0 or 1; or a compound of formula (II):

15




20

(II)

wherein p is 0 or 1;

or a derivative thereof which comprises cultivating a
25 producing organism, isolating the compound or derivative from the culture, and where necessary converting one compound of formula (I) or (II) into another compound of formula (I) or (II).

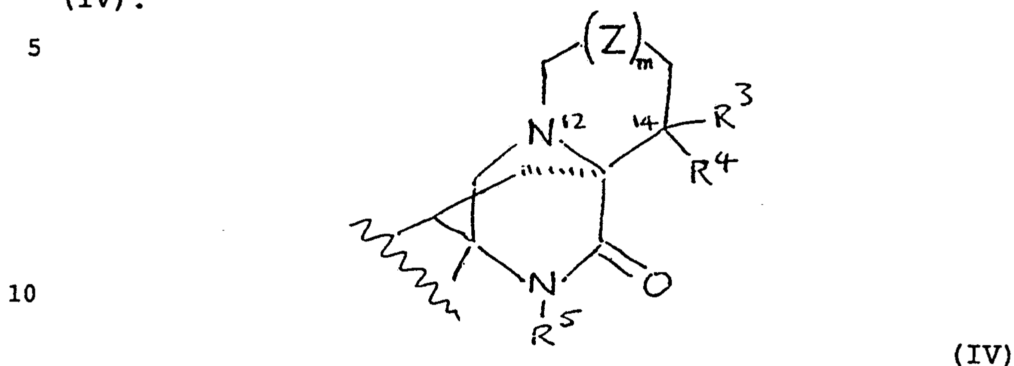
30

8. A process according to claim 7 which comprises cultivating Penicillium sp. IMI 332995 or a mutant thereof capable of producing the desired compound or a compound convertible to the desired compound.

35

9. A process for the production of a compound of partial formula (III) which comprises reacting a marcfortine or paraherquamide or a derivative thereof of partial formula (IV):

5




10

(IV)

where Z, m, $R^3$, $R^4$, $R^5$ are as defined for formula (III), with a N-oxidising agent.

15

10. A compound according to claim 2, 3, 4, 5 or 6, for use in therapeutic treatment of the human or animal body.

11. A compound according to claim 2, 3, 4, 5 or 6, for use

20 in combatting of infestations of endo-and ecto-parasites.

12. The use of a compound according to claim 2, 3, 4, 5 or 6, for the manufacture of a medicament for combatting infestations of ecto- and endo-parasites.

25

13. A pesticidal composition comprising a compound according to claim 2, 3, 4, 5 or 6, and a carrier or excipient.

30 14. A pharmaceutical or veterinary composition comprising a compound according to claim 2, 3, 4, 5 or 6, and a pharmaceutically or veterinarially acceptable carrier or excipient.