MM 46115, A NEW ANTIVIRAL ANTIBIOTIC FROM
ACTINOMADURA PELLETIERI
CHARACTERISTICS OF THE PRODUCING CULTURES, FERMENTATION,
ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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Six strains of Actinomadura pelletieri were shown to produce a novel macrolide antibiotic,
designated MM 46115. MM 46115 was active against parainfluenza virus 1 and 2 and Gram-
46115 by strain IP 729.63,
positive bacteria. Methods are described for the production of MM 46115 by strain IP 729.63,
and for the isolation of the compound from a butanol extract of the culture filtrate.

During the course of a screening programme for microbial products with antiviral activity, a new
antibiotic, designated MM 46115, was found to be produced by several strains of Actinomadura pelletieri.
Spectroscopic data† (will be reported separately) has revealed MM 46115 to be a novel tetronic acid
containing macrolide (Fig. 1).

In this paper we describe the cultural characteristics of the producing strains, and the fermentation,
isoLation, physico-chemical and biological properties of the compound. The structure elucidation will be
published elsewhere.

Materials and Methods

Fermentation Conditions
In order to study the fermentation of A. pelletieri in shake flasks, sterile water containing 0.025%
Tween 80 was added to glucose-yeast extract agar slope cultures to produce a vegetative mycelium
fragment suspension (10 ml per slope). Portions of this suspension (2.5 ml) were used to inoculate 50 ml
of the seed stage medium (Arkasoy 0.9%, glucose
2.0%, pH 6.5) contained in 250-ml Erlenmeyer flasks
closed with foam plastic bungs. The flasks were
incubated for periods of between 144 and 216 hours
at 26°C on a New Brunswick G53 shaker at 240 rpm.
The seed culture was added as a 3% inoculum to
further 250-ml Erlenmeyer flasks each containing
50 ml of International Streptomyces Project 2 (ISP2)
medium (yeast extract 0.4%, malt extract 1%, and
glucose 0.4% prepared in deionised water and the
pH adjusted to 7.3). The cultures were then grown
(26°C, 240 rpm) for periods of up to 192 hours.
For the production of MM 46115 by strain IP 729.63 in fermenter vessels, the contents of 12 flasks
containing the seed culture were combined and
added as a 3% inoculum to a 15-liter Biolafite in

Fig. 1. Structure of MM 46115.
**Fermentation Analyses**

Mycelial growth was measured as packed cell volume (PCV) by centrifuging the fermentation broth at 5,000 rpm for 35 minutes. Production of MM 46115 was determined by HPLC analysis. A μBondapak C18 cartridge, 8 x 100 mm (Millipore, Watford, England) was used with a mobile phase of methanol - 50 mM aqueous NH4OAc (3:1), pH 6.5. The flow rate was 2 ml/minute. Duplicate shake flasks were harvested at 24 hour intervals. Samples of culture broth were removed, and 100 μl aliquots of culture filtrate (sterile filtered) were injected. The eluate was monitored at 280 nm. The size of the absorbance peak due to MM 46115, produced by the samples of culture filtrate and by a standard reference solution, was measured.

**Antiviral and Cytotoxicity Assays**

Antiviral activity was determined against herpes simplex virus 1 in MRC-5 cells (human diploid lung), influenza A virus and parainfluenza virus 1 (Sendai strain) in MDCK cells (Madin and Darby canine kidney), human parainfluenza viruses 2 and 3 and mumps virus in Vero cells (African Green Monkey kidney), respiratory syncytial virus strain A2 and 8/60 in HEp-2 cells (human epidermoid carcinoma of the larynx), visna virus strain K184 in SCP cells (sheep choroid plexus) and human immunodeficiency virus-1 in human peripheral blood lymphocytes.

Eight three-fold dilutions of MM 46115 (100 to 0.05 μg/ml) were tested for antiviral activity against virus infected cells, and/or for cytotoxicity both by visual assessment of uninfected cells and by inhibition of [3H]thymidine incorporation. Cytotoxicity was also determined against mouse L1210 tumour cells.

**Antifungal Assays**

Solutions of MM 46115 at 500 and 100 μg/ml were added to wells cut in agar plates seeded with Candida albicans, Cryptococcus neoformans, Saccharomyces cerevisiae, Aspergillus niger, Hendersonula toruloidea, Paecilomyces varioti, Rhizopus oryzae and Trichophyton mentagrophytes. Following overnight incubation, at 37°C for the yeasts and 30°C for the filamentous fungi, activity was assessed by measuring zones of inhibition. Additionally a 50 μg/ml solution was tested for its ability to prevent morphological transformation (i.e. germination and hyphal elongation) of C. albicans after 6 or 24 hours.

**Antibacterial Assay**

MICs were determined by a 2-fold microplate method. Todd-Hewitt broth (Oxoid) was used for Streptococcus sp. and Nutrient broth (Oxoid No. 2) was used for all other organisms. Final inoculation level was approximately 10^5 cfu per ml.

**Anthelmintic Assay**

A solution of MM 46115 in methanol was serially diluted in microwell plates to give replicate concentrations from 200 to 0.2 μg/ml. The solvent was removed by evaporation, then 100 μl suspensions of nematodes added and the plates incubated overnight. The effect of MM 46115 on mortality of Haemonchus contortus L3 larvae and Turbatrix aceti or the prevention of Haemonchus egg hatch was determined by examination under a low power microscope.

**Results**

**Cultural Characteristics**

A metabolite, designated MM 46115, with activity against the Sendai strain of parainfluenza virus type 1, was detected in culture extracts from the six strains of A. pelletieri listed in Table 1. The highest levels of MM 46115 were observed with strain IP 729.63. This culture was used to produce the compound for
Table 1. Cultural characteristics of six *Actinomadura pelletieri* strains producing MM 46115.

<table>
<thead>
<tr>
<th>Culture numbers</th>
<th>Glucose - yeast extract agar&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Oatmeal agar&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Reverse</td>
</tr>
<tr>
<td>IP 729.63,</td>
<td>Good (+ +)</td>
<td>Pale pink/red</td>
</tr>
<tr>
<td>CCM 2774 (red variant)</td>
<td>Brownish/red (10C7)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IP 374,</td>
<td>Good (+ +)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Pink/brown</td>
</tr>
<tr>
<td>NCPF 1171</td>
<td>Post office red (10B6)</td>
<td></td>
</tr>
<tr>
<td>NCPF 1211</td>
<td>Good (+ +)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Brown/pink</td>
</tr>
<tr>
<td></td>
<td>Auroa (10B4)</td>
<td></td>
</tr>
<tr>
<td>IP 308.52,</td>
<td>Fair (+)</td>
<td>Orange/pink</td>
</tr>
<tr>
<td>NCPF 1066,</td>
<td>Greyish red (9B6)</td>
<td></td>
</tr>
<tr>
<td>LSH A291</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP 84,</td>
<td>Fair (+)</td>
<td>Pale pink/brown</td>
</tr>
<tr>
<td>RV 27823</td>
<td>Greyish red (7B3)</td>
<td></td>
</tr>
<tr>
<td>IP 729,</td>
<td>Good (+ +)</td>
<td>White</td>
</tr>
<tr>
<td>CCM 2775 (white variant)</td>
<td>Yellowish white (4A2)</td>
<td></td>
</tr>
</tbody>
</table>

Incubation for 21 days at 26°C.

<sup>a</sup> Colonial morphology of all cultures raised and crenated.

<sup>b</sup> Colonial morphology of all cultures smooth and convex.

<sup>c</sup> No aerial mycelium except where noted.

<sup>d</sup> Colour code (ref 3).

<sup>e</sup> Sparse white aerial mycelium.

<sup>f</sup> Good grey/white aerial mycelium.

Fig. 2. Time course of MM 46115 production by strain IP 729.63 in shake flasks.

--- pH, —— PCV ml/10 ml, ○ μg/ml MM 46115.

Cultivation conditions: ISP2 medium, 26°C, 240 rpm.

an investigation of its physico-chemical and biological properties, and for structure elucidation studies.

Strain IP 729.63 was originally isolated from a case of mycetoma in Cameroon, Africa. It was deposited with the Institut Pasteur and recorded as IP 729.63 (red variant). In 1976, it was also deposited with the
Czechoslovak Collection of Microorganisms with the designation CCM 2774 (red variant). We obtained the culture from the latter collection in 1987.

The classification of strain IP 729.63 as *A. pelletieri* has been supported by analyses of polar lipids and cell wall diamino acids carried out in our own laboratories. The results were in accordance with those recorded for reference strains of *A. pelletieri* (Lechevalier et al.1) and Mordarska et al.2). The cultural characteristics of all six MM 46115-producing *A. pelletieri* strains, after growth on glucose-yeast extract and oatmeal agars, are compared in Table 1.

**Fermentation**

A typical time course of the fermentation of strain IP 729.63 in a 250-ml Erlenmeyer shake flask is shown in Fig. 2. A maximum titre of 4.7~4.9 μg/ml of MM 46115 was achieved after 168 hours. In order to produce sufficient quantities of MM 46115 to determine the properties and structure of the compound,

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**Fig. 3. Isolation procedure for MM 46115.**

- BuOH extract at pH 2
  - evaporated to dryness (22.0 g)
  - dissolved in MeOH (50 ml)
  - diluted with MDC (300 ml)

- Filtrate
  - evaporated to dryness (15.0 g)
  - dissolved in MDC (0.2 liter)
  - filtered through SiO₂ (20 g)

- MDC eluate
  - (no MM 46115)

- Precipitate (0.77 g)
  - dissolved in MeOH (30 ml)
  - add pH 7 buffer (5 ml)
  - preparative HPLC
    - 2.0 x 25 cm S5 ODS2

- Crude MM 46115 (0.06 g)
  - preparative HPLC
    - 1.0 x 25 cm S5NH2

- MM 46115 (0.04 g)
  - crystallized from MeOH

- MM 46115
  - (mp 196~201°C)
strain IP 729.63 was grown in two 75-liter fermenters at 26°C for 168 hours.

Isolation

Fermentation broth (75 liters) was mixed with 25 liters of water. The diluted culture broth was clarified in an Westfalia CSA19 disc-stack centrifuge and then passed through a 0.2 micron nylon membrane filter. The diluted culture filtrate (60 liters) was adjusted to pH 2.0, and then extracted with 40 liters butanol. The butanol layer (33 liters) was recovered. Fig. 3 outlines the procedures used to isolate MM 46115, from the butanol extract to the crystalline product.

Physico-chemical Properties

The physico-chemical properties of MM 46115 are summarised in Table 2.

Biological Properties

MM 46115 was active against influenza A virus, parainfluenza virus 1 and 2 and respiratory syncytial virus strains A2 and 8/60 (Table 3). However, significant antiviral activity in the absence of cytotoxicity was only seen against parainfluenza virus 1 and 2 at 0.046 and 0.1 μg/ml. The ratio of antiviral activity to cytotoxicity was 9- and 10-fold, respectively. By comparison in these assays the positive control, ribavirin (1β-D-ribofuranosyl-1,2,4 triazole-3-carboxamide) was active at 5.05 μg/ml against parainfluenza virus 1 and at 13.5 μg/ml against parainfluenza virus 2. Cytotoxicity, as assessed by inhibition of [3H]thymidine incorporation ranged from 0.33 μg/ml in MRC-5 cells to 0.78 μg/ml in SCP cells (Table 3). The concentration of MM 46115 giving 50% inhibition of mouse L1210 tumour cells was 0.65 μg/ml compared to 0.043 μg/ml with the positive control 5-fluorouracil. By comparing this figure with those given in Table 3 MM 46115 showed no selectivity towards these tumour cells.

### Table 2. Physico-chemical properties of MM 46115.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline appearance</td>
<td>Elongated pentagonal plates</td>
</tr>
<tr>
<td>MP</td>
<td>196~201°C</td>
</tr>
<tr>
<td>$[\alpha]_D^2$ (c 0.84, CHCl₃)</td>
<td>$-122.1^\circ$</td>
</tr>
<tr>
<td>FAB-MS (m/z)</td>
<td>891 (M + H)$^+$</td>
</tr>
<tr>
<td>UV $\lambda_{max}$ nm</td>
<td>235 (sh), 280 (6 11,000)</td>
</tr>
<tr>
<td>HPLC$^*$ Rt (minutes)</td>
<td>10.5</td>
</tr>
</tbody>
</table>

* Waters $\mu$Bondapak C₁₈, 8 × 100 mm; MeOH - 50 mm aq NH₄OAc (3 : 1), pH 7.0; flow rate 2 ml/minute.

### Table 3. Antiviral activity and cytotoxicity of MM 46115.

<table>
<thead>
<tr>
<th>Test</th>
<th>Antiviral activity</th>
<th>Cytotoxicity (μg/ml)</th>
<th>Inhibition of $[^3]$H]thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀$^*$ (μg/ml)</td>
<td>MTC</td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus-1 + 100 U/ml Interferon β</td>
<td>None</td>
<td>0.41</td>
<td>0.33</td>
</tr>
<tr>
<td>Influenza A virus</td>
<td>0.12</td>
<td>0.41</td>
<td>0.70</td>
</tr>
<tr>
<td>Parainfluenza virus-1 (Sendai strain)</td>
<td>0.046</td>
<td>0.41</td>
<td>0.70</td>
</tr>
<tr>
<td>Human parainfluenza virus-2</td>
<td>0.1</td>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td>Human parainfluenza virus-3</td>
<td>None</td>
<td>0.74</td>
<td>ND</td>
</tr>
<tr>
<td>Mumps virus</td>
<td>None</td>
<td>0.74</td>
<td>ND</td>
</tr>
<tr>
<td>Respiratory syncytial virus strain A₂</td>
<td>0.08</td>
<td>0.08</td>
<td>0.56</td>
</tr>
<tr>
<td>Respiratory syncytial virus strain 8/60</td>
<td>0.08</td>
<td>0.08</td>
<td>0.56</td>
</tr>
<tr>
<td>Visna virus</td>
<td>None</td>
<td>0.1</td>
<td>0.78</td>
</tr>
<tr>
<td>Human immunodeficiency virus-1</td>
<td>None</td>
<td>&lt;0.09</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^*$ IC₅₀ concentration to inhibit virus damage by 50%.

MTC: Minimal toxic concentration.

ND: Not determined.
Table 4. Antibacterial activity of MM 46115.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (μg/ml)</th>
<th>Organism</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> ESS</td>
<td>&gt;4</td>
<td><em>S. aureus</em> V573 MR</td>
<td>0.25</td>
</tr>
<tr>
<td><em>E. coli</em> NCTC 10418</td>
<td>&gt;4</td>
<td><em>S. aureus</em> V1100 MR</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 1771P</td>
<td>&gt;4</td>
<td><em>S. saprophyticus</em> FL1</td>
<td>1</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1771M</td>
<td>&gt;4</td>
<td><em>S. saprophyticus</em> FL2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> C977</td>
<td>&gt;4</td>
<td><em>S. epidermidis</em> 60137</td>
<td>0.25</td>
</tr>
<tr>
<td><em>P. rettgeri</em> I</td>
<td>&gt;4</td>
<td><em>S. epidermidis</em> 54815</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em> N1</td>
<td>&gt;4</td>
<td><em>Streptococcus pyogenes</em> CN10</td>
<td>0.125</td>
</tr>
<tr>
<td><em>Klebsiella aerogenes</em> A</td>
<td>&gt;4</td>
<td><em>S. pyogenes</em> 1950</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> US32</td>
<td>&gt;4</td>
<td><em>S. agalactiae</em> 2798</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> BRL 2400</td>
<td>&gt;4</td>
<td><em>S. agalactiae</em> Hester</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 6633</td>
<td>0.25</td>
<td><em>S. sanguis</em> ATCC 10556</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Corynebacterium xerosis</em> NCTC 9755</td>
<td>0.06</td>
<td><em>S. viridans</em> Harding</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em> NCTC 8340</td>
<td>0.25</td>
<td><em>S. pneumoniae</em> PU7</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Smith</td>
<td>0.5</td>
<td><em>S. faecalis</em> I</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. aureus</em> Oxford</td>
<td>0.5</td>
<td><em>S. faecalis</em> T814</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. aureus</em> Russell</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MR: Multi-resistant (methicillin-, tetracycline-, erythromycin- and gentamycin-resistant).

MM 46115 showed slight inhibition of growth of both *H. toruloidea* and *T. mentagrophytes*.

MM 46115 was inactive against Gram-negative organisms but inhibited all of the Gram-positive organisms with MICs ranging from 0.06 μg/ml for *C. xerosis* NCTC 9755 to 1 μg/ml for *S. saprophyticus* FL1 (Table 4). The activity against Gram-positive organisms compared favourably with vancomycin, where MICs varied between 0.5 and 4 μg/ml for these organisms.

No activity was detected against any of the nematode species.

Discussion

All six of the *A. pelletieri* strains investigated have been shown to produce MM 46115. In contrast, antiviral activity was not detected in culture extracts of other species of *Actinomadura*, namely *A. madurae*, *A. citrea*, *A. spadix*, *A. roseoviolacea*, and *A. pusilla*, when they were grown under the same conditions. Our studies to date would suggest therefore that the production of MM 46115 is a characteristic property of *A. pelletieri*.

MM 46115 inhibited the replication of both parainfluenza virus 1 and 2 at concentrations that were not inhibitory to uninfected cells. Therapeutic ratios of between nine and ten were achieved. In these antiviral assays the activity of MM 46115 compared favourably with that of the positive control ribavirin. In contrast, no significant activity was seen against a range of other viruses. In addition, good activity has been observed against Gram-positive bacteria. However, MM 46115 failed to show any significant activity in the antifungal or anthelmintic assays.

Structural determinations have shown that MM 46115 is related to kijanimicin obtained from *Actinomadura kijaniata*4-5 and to the tetrocarcin/antlermicin family of antibiotics from *Micromonospora chalcea*.6-11 These compounds have been shown to exhibit activity against Gram-positive bacteria and to be antitumour agents in mice. In addition kijanimicin has been found to be effective against malaria in mice. Unlike MM 46115 they have not been reported to have antiviral properties. Our results are in agreement with the activity against Gram-positive bacteria but only moderate antitumour activity was detected in the L1210 assay. Further work is planned to determine the activity of MM 46115 in an animal model and will be reported in the future.

Acknowledgements

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References