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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(Received for publication May 21, 1990)

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-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.

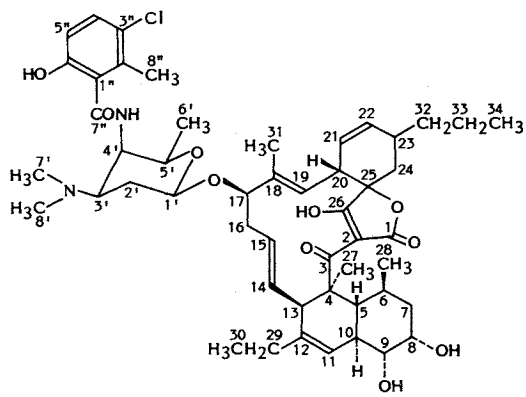
Materials and Methods

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 Biolaffite *in*

Fig. 1. Structure of MM 46115.



situ steam sterilizable stainless steel fermenter containing 10 liters of ISP2 medium. Fermentation was carried out at 26°C for 168 hours under an aeration rate of 0.5 v/v/m (5 liters/minute) and an agitation rate of 200 rpm. 1.5 liters of this secondary seed culture were used to inoculate each of two 75-liter Bioengineering fermenters containing 50 liters of ISP2 production medium. The culture was grown for 168 hours under the same incubation conditions as the secondary seed stage.

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 C_{18} cartridge, 8 × 100 mm (Millipore, Watford, England) was used with a mobile phase of methanol - 50 mM aqueous NH_4OAc (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 [^{3}H]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* L_3 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.

Culture numbers	Glucose-yeast extract agar[a]			Oatmeal agar[b]		
	Growth[c]	Reverse	Soluble pigment	Growth[c]	Reverse	Soluble pigment
IP 729.63, CCM 2774 (red variant)	Good (++) Brownish/red (10C7)[d]	Pale pink/red	Pale yellow	Good (++) Coral red (9B7)	Red/pink	Pale yellow
IP 374, NCPF 1171	Good (++)[e] Post office red (10B6)	Pink/brown	Pale brown	Fair (+) Pastel red (9A4)	Pale pink	Faint yellow
NCPF 1211	Good (++)[f] Auroa (10B4)	Brown/pink	Yellow/brown	Fair (+) Orange white (6A2)	White	Yellow
IP 308.52, NCPF 1066, LSH A291	Fair (+) Greyish red (9B6)	Orange/pink	Brown/yellow	Fair (+) Pastel red (9A5)	Pink/orange	Pale yellow
IP 84, RV 27823	Fair (+) Greyish red (7B3)	Pale pink/ brown	Pale yellow	Poor (½+) Pale red (7A3)	Uncoloured/ white	Faint yellow
IP 729, CCM 2775 (white variant)	Good (++) Yellowish white (4A2)	White	Pale yellow	Fair (+) Orange white (5A2)	Uncoloured/ white	Pale yellow

Incubation for 21 days at 26°C.

[a] Colonial morphology of all cultures raised and crenated.

[b] Colonial morphology of all cultures smooth and convex.

[c] No aerial mycelium except where noted.

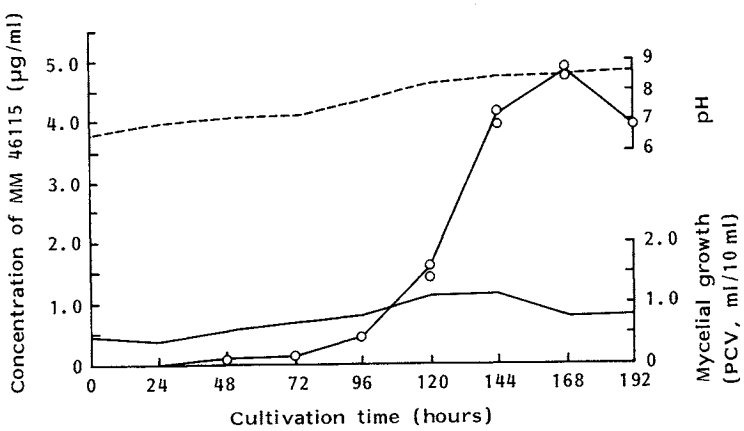
[d] Colour code (ref 3).

[e] Sparse white aerial mycelium.

[f] 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

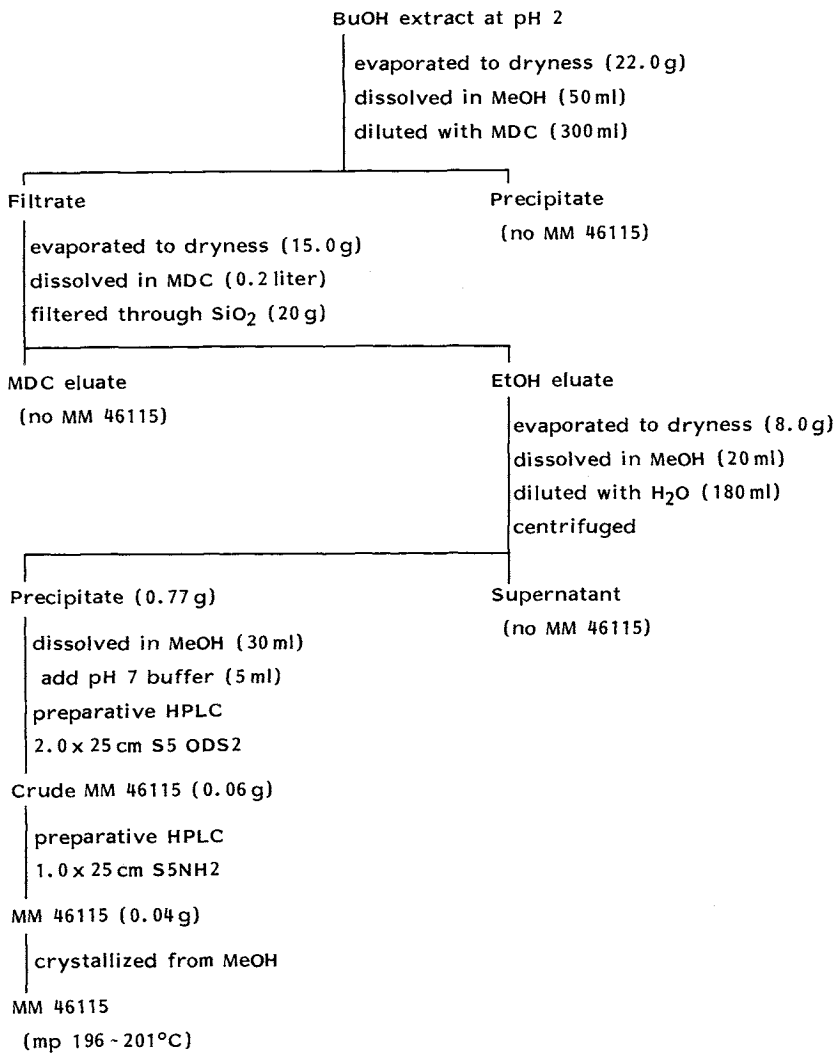
Czechoslovak Collection of Microorganisms with the designation CCM 2774 (red variant). We obtained the culture from the latter collection in 1987.

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,

Fig. 3. Isolation procedure for MM 46115.



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 A_2 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 [^{3}H]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.

Crystalline appearance	Elongated pentagonal plates
MP	196~201°C
$[\alpha]_D^{25}$ (c 0.84, $CHCl_3$)	−122.1°
FAB-MS (m/z)	891 $(M+H)^+$
UV λ_{max}^{MeOH} nm	235 (sh), 280 (ε 11,000)
HPLC[a] Rt (minutes)	10.5

[a] Waters μBondapak C_{18}, 8 × 100 mm; MeOH - 50 mM aq NH_4OAc (3 : 1), pH 7.0; flow rate 2 ml/minute.

Table 3. Antiviral activity and cytotoxicity of MM 46115.

Test	Antiviral activity	Cytotoxicity (μg/ml)	
	IC_{50}[a] (μg/ml)	MTC	Inhibition [^{3}H]thymidine incorporation
Herpes simplex virus-1 + 100 U/ml Interferon β	None	0.41	0.33
Influenza A virus	0.12	0.41	0.70
Parainfluenza virus-1 (Sendai strain)	0.046	0.41	0.70
Human parainfluenza virus-2	0.1	1.0	ND
Human parainfluenza virus-3	None	0.74	ND
Mumps virus	None	0.74	ND
Respiratory syncytial virus strain A_2	0.08	0.08	0.56
Respiratory syncytial virus strain 8/60	0.08	0.08	0.56
Visna virus	None	0.1	0.78
Human immunodeficiency virus-1	None	<0.09	ND

[a] IC_{50} concentration to inhibit virus damage by 50%.
MTC: Minimal toxic concentration.
ND: Not determined.

Table 4. Antibacterial activity of MM 46115.

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

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

We thank Dr. M. GOODFELLOW (University of Newcastle, UK), Dr. C. CAMPBELL (Curator, NCPF, UK), and Dr.

M. KOCUR (Head, CCM, Czechoslovakia) for supplying strains of *A. pelletieri*. We are also grateful to our colleagues Mr. C. GERSHATER, Mrs. H. EDWARDS, and Mr. I. DACEY for fermentation studies, Mrs. C. WINCH, Mrs. E. HAYES and Mr. B. MANGER for antifungal, antibacterial and anthelmintic assays respectively, and to Miss S. LOVE, Mr. B. POTTS, Mrs. K. REYNOLDS and Miss R. SHORTLAND for excellent technical assistance. We acknowledge Drs. A. IMMELMANN, K. HENCO (Diagen) and Dr. R. PERKINS for the HIV test.

References

1) LECHEVALIER, M. P.; C. DEBIEVRE & H. LECHEVALIER: Chemotaxonomy of aerobic actinomycetes: phospholipid composition. Biochem. Systematics Ecology 5: 249~260, 1977
2) MORDARSKA, H.; M. MORDARSKI & M. GOODFELLOW: Chemotaxonomic characters and classification of some nocardioform bacteria. J. Gen. Microbiol. 71: 77~86, 1972
3) KORNERUP, A. & J. H. WANSCHER (*Ed.*): Methuen Handbook of Colour. 3rd Ed., Eyre Methuen, 1978
4) WAITZ, J. A.; A. C. HORAN, M. KALYANPUR, B. K. LEE, D. LOEBENBERG, J. A. MARQUEZ, G. MILLER & M. G. PATEL: Kijanimicin (Sch 25663), a novel antibiotic produced by *Actinomadura kijaniata* SCC 1256. Fermentation, isolation, characterization and biological properties. J. Antibiotics 34: 1101~1106, 1981
5) MALLAMS, A. K.; M. S. PUAR, R. R. ROSSMAN, A. T. MCPHAIL, R. D. MACFARLANE & R. L. STEPHENS: Kijanimicin. Part 3. Structure and absolute stereochemistry of kijanimicin. J. Chem. Soc. Perkin Trans. I 1983: 1497~1534, 1983
6) TOMITA, F.; T. TAMAOKI, K. SHIRAHATA, M. KASAI, M. MORIMOTO, S. OHKUBO, K. MINEURA & S. ISHII: Novel antitumor antibiotics, tetrocarcins. J. Antibiotics 33: 668~670, 1980
7) TOMITA, F. & T. TAMAOKI: Tetrocarcins, novel antitumor antibiotics. I. Producing organism, fermentation and antimicrobial activity. J. Antibiotics 33: 940~945, 1980
8) TAMAOKI, T.; M. KASAI, K. SHIRAHATA, S. OHKUBO, M. MORIMOTO, K. MINEURA, S. ISHII & F. TOMITA: Tetrocarcins, novel antitumor antibiotics. II. Isolation, characterization and antitumor activity. J. Antibiotics 33: 946~950, 1980
9) TAMAOKI, T.; M. KASAI, K. SHIRAHATA & F. TOMITA: Tetrocarcins E_1, E_2, F and F-1, new antibiotics. Fermentation, isolation and characterization. J. Antibiotics 35: 979~984, 1982
10) KOBINATA, K.; M. URAMOTO, T. MIZUNO & K. ISONO: A new antibiotic, antlermicin A. J. Antibiotics 33: 244~246, 1980
11) KOBINATA, K.; M. URAMOTO, T. MIZUNO & K. ISONO: Antlermicins B and C, new members of the antlermicin family. J. Antibiotics 33: 772~775, 1980