A NOVEL SERIES OF MILBEMYCIN ANTIBIOTICS FROM *STREPTOMYCES* STRAIN E225

I. DISCOVERY, FERMENTATION AND ANTHELMINTIC ACTIVITY

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A novel series of milbemycin antibiotics were produced by soil isolate, strain E225 which was shown to be a *Streptomyces* species. The antibiotics displayed anthelmintic activity against *Trichostrongylus colubriformis* in the gerbil. Two of the compounds, VM 44857 and VM 44866 were shown to be potent anthelmintics against mixed nematode infections in sheep.

The milbemycins are a large family of closely related macrolide antibiotics produced by species of the genus *Streptomyces*. The first reported members of the family, milbemycins, α_1 to α_{10} and β_1 to β_3 were isolated from *Streptomyces hygroscopicus* subsp. *aureolacrimosus* as a result of an insecticidal screening programme[1]. Subsequent communications described further members of the series from the original strain[2], or its mutants[3,4]. Biological properties of these milbemycins have not been extensively reported but the anthelmintic activity of milbemycin D against dog parasites has been published[5]. A further series of milbemycins with anthelmintic activity was described from *Streptomyces cyaneogriseus* subsp. *noncyanogenus*[6] and *Streptomyces thermoarchaensis*[7,8]. Milbemycins were also isolated from a hybrid microorganism obtained by protoplast fusion of *Streptomyces avermitilis* and *S. hygroscopicus*[9].

This paper describes a novel series of milbemycin anthelmintics isolated from a new strain of the genus *Streptomyces*.

Materials and Methods

In Vitro Anthelmintic Test

A suspension of the infective larval stage (L_3) of *Haemonchus contortus* was prepared by culturing faeces from experimentally infected sheep. The suspension was cleaned and concentrated by the standard BAERMANN technique. Samples for anthelmintic testing, whether purified compounds or microbial culture extracts were prepared in methanol solution. Samples (0.1 ml) were evaporated to dryness in microtitre plates and L_3 suspension (0.1 ml) was added. After incubation at 4°C overnight the plates were warmed to room temperature and larval motility was assessed visually in comparison to controls. For purified compounds MIC values were defined as the minimum concentration which caused approximately 75% of the worms to show reduction in motility.

In Vivo Anthelmintic Tests

Gerbil-*Trichostrongylus colubriformis* Model: The gerbil-*Trichostrongylus* model, first described as an anthelmintic test system by KATES and THOMPSON[10], was adapted for screening purposes. Gerbils (*Meriones unguiculatus*) were infected with 500 larvae of *Trichostrongylus colubriformis* of ovine origin, and were normally suitable for dosing three weeks after infection when worms had reached egg laying maturity. Samples were dosed orally and activity was assessed either by faecal egg counts or in controlled

Table 1. Composition of seed and production media.

Seed medium		Production medium F1	
Constituent	g/litre	Constituents	g/litre
Special peptone (Oxoid)	2.5	Soluble starch (BDH)	20
Beef extract (Oxoid Lab. Lemco)	2.5	Casein (Sigma)	2
Tryptone (Oxoid)	2.5	Soybean flour (Arkasoy 50)	10
Neutralised soya peptone (Oxoid)	2.5	Glucose	20
Soluble starch (BDH)	2.5	Calcium carbonate	5
Glucose	2.5	Magnesium sulfate	1
Malt extract (Oxoid)	2.5	($MgSO_4 \cdot 7H_2O$)	
Glycerol	2.5		
Trace elements solution[a]	5 ml		

[a] Composition of trace elements solution (all concentrations in g/litre): $CaCl_2 \cdot 2H_2O$, 10; $MgCl_2 \cdot 6H_2O$, 10; NaCl, 10; $FeCl_3$, 3; $ZnCl_2$, 0.5; $CuCl_2 \cdot 2H_2O$, 0.5; $MnSO_4 \cdot 4H_2O$, 0.5; $CoCl_2 \cdot 6H_2O$, 1.

tests by post-mortem worm counts.

Egg counts, using a modified McMaster method, were performed on ten pooled faecal pellets collected 1 day before dosing and on days 4 and 5 after dosing to determine any reduction in egg output. For worm counts the animals were sacrificed and the worms removed from the small intestine. Anthelmintic activity was calculated by comparison of treated and untreated animals, and expressed as a percentage efficacy.

Sheep Tests: In preliminary tests, anthelmintic activity was manifested by depression of nematode egg output from sheep harbouring naturally acquired nematode infections. Three g samples of fresh sheep faeces were suspended in salt and nematode eggs were counted in McMaster slides.

Controlled tests were performed in sheep harbouring naturally acquired worm infestations, or in animals experimentally infected with appropriate numbers of nematode larvae. Animals were euthanased several days after treatment. Their entire gastrointestinal tracts were removed and the worms present were identified and counted. Efficacy was calculated from a comparison of worm numbers present in treated sheep with those in untreated control animals.

Culture Conditions of Strain E225

Strain E225 was maintained on starch-casein agar slopes, at room temperature or at −70°C in seed medium.

Fermentation studies were performed in 250-ml Erlenmeyer flasks containing 50 ml medium on a rotary shaker at 220 rpm and 27°C. Seed stage flasks were incubated for 2 days and used as inoculum (4%) for production flasks. The composition of seed medium and production medium F1 are given in Table 1.

HPLC Analysis of Milbemycins

Acetone (1 ml) was added to whole broth (1 ml) and stirred for 30 minutes. The cells were removed by centrifugation and clear supernatant (20 μl) was injected onto an Ultrasphere ODS 5 μm column (4.6 × 250 mm). The solvent system was methanol-water (9:1) at a flow rate of 1 ml/minute. The milbemycins were monitored using a Waters 990 diode array UV detector.

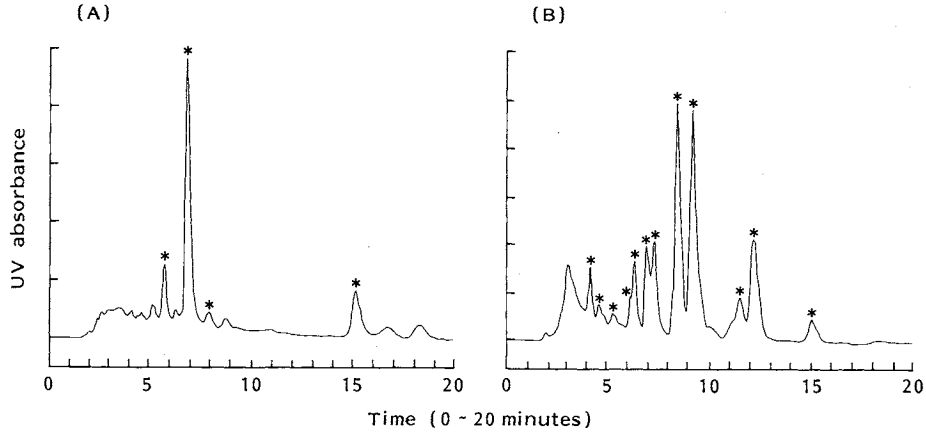
Results and Discussion

Discovery

During the screening of a wide range of microorganisms for anthelmintic activity, the strain designated E225 was selected by the *in vitro Haemonchus* L_3 test. Subsequently when a crude extract of the culture broth was dosed to the gerbil-*Trichostrongylus* model a reduction in the faecal egg count was observed.

Other culture broths previously detected by our anthelmintic screens had been shown by extraction and HPLC to be producing milbemycin antibiotics of the type described from *S. hygroscopicus* subsp.

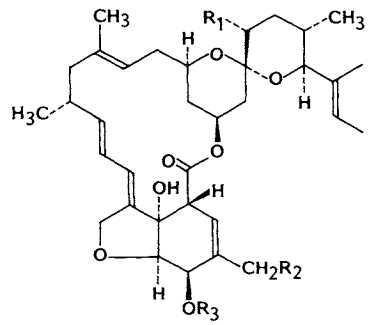
Fig. 1. HPLC of extracts from cultures E225 (A) and NRRL 5739 (B) monitored at 244 nm.



The peaks marked * displayed UV spectra consistent with a milbemycin structure.

aureolacrimosus NRRL 5739[1]. Thus an aqueous acetone extract of the culture broth of strain E225, was compared with an extract of NRRL 5739 using the same HPLC procedure. A different profile of peaks was clearly present in the novel culture, (typical chromatograms are illustrated in Fig. 1) and the spectra obtained from the diode array monitor indicated that several of the compounds belonged to the milbemycin family. The metabolites VM 44857, VM 44864, VM 44865 and VM 44866 were subsequently extracted and purified and shown to be a series of milbemycins with novel structures, as illustrated in Fig. 2. The isolation and structure determination will be published separately.

Fig. 2. Structures of four milbemycins from culture E225.



VM 44866	R_1=OH	R_2=H	R_3=H
VM 44864	R_1=OH	R_2=H	R_3=CH_3
VM 44857	R_1=H	R_2=H	R_3=H
VM 44865	R_1=OH	R_2=OCOC(=CH_3)CH_3	R_3=CH_3

Strain E225

Strain E225 was isolated from a sample of mud collected from the River Mole in Surrey, England. The morphology of the culture growing on a variety of agar media was studied under a light microscope. Vegetative hyphae showed no evidence of fragmentation and spore chains were borne in spirals. Scanning electron microscopy revealed a rugose spore coat. LL-Diaminopimelic acid was detected by analysis of the cell wall using the method of BECKER *et al.*[11]. Strain E225 was concluded to belong to the genus *Streptomyces*.

During titre improvement studies with strain E225 it was noted that three different morphological types were present in the stock culture. The different types were separated, submitted to standard streptomycete classification tests[12] and concluded to be variants of the same species. The differences between variants are shown in Table 2.

Strain E225 was compared with *S. hygroscopicus* subsp. *aureolacrimosus* NRRL 5739, in physiological tests. Carbohydrate utilization was similar; both strains grew well on glucose, adonitol, arabinose, cellobiose,

Table 2. Cultural characteristics of strains E225B, C and D on starch casein agar.

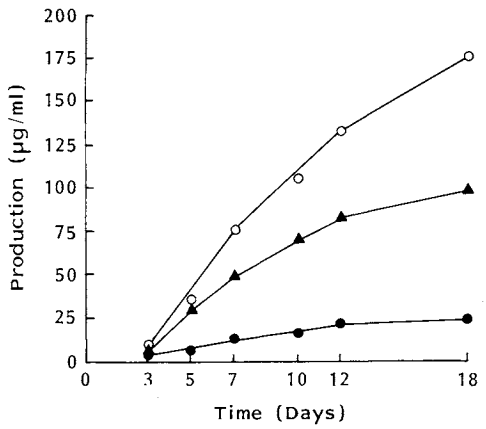
B:	White to pale grey aerial hyphae, sparsely sporing, diffusable yellow pigment
C:	Grey aerial hyphae, densely sporing, diffusable yellow pigment
D:	Yellow aerial hyphae, non-sporing, little diffusable yellow pigment

Table 3. Physiological properties of strain E225 and *Streptomyces hygroscopicus* subsp. *aureolacrimosus* NRRL 5739.

Characteristic	Strain E225	NRRL 5739	Characteristic	Strain E225	NRRL 5739
Nitrogen utilisation:			Arbutin	+	+
Asparagine	+	+	Hypoxanthine	+	−
α-Aminobutyric acid	−	−	Xanthine	−	−
Histidine	−	+	Pectin	−	−
Methionine	−	−	Lecithin	+	+
Hydroxyproline	+	+	L-Tyrosine	+	−
Melanin production	−	−	Growth on:		
Nitrate reduction	+	+	Sodium azide 0.01%	−	+
H_2S production	+	+	NaCl 7%	−	−
Degradation of:			Phenol	−	−
Allantoin	−	−	45°C	−	−

Fig. 3. Production of milbemycins by E225B in medium F1 at 27°C.

○ VM 44857, ▲ VM 44864, ● VM 44866.



Eight replicate flasks were used in the experiment. Each point is the mean of at least three samples.

Table 4. Production of milbemycins by E225 variants B, C and D.

Organism	Titre whole broth (μg/ml)		
	VM 44866	VM 44864	VM 44857
E225	5.9	10.6	30.2
B	22.7	58.1	233.8
C	Trace	17.4	39.8
D	0	0	0

Table 5. Evaluation of milbemycins produced by strain E225 in the gerbil-*Trichostrongylus colubriformis* model.

Compound	% of reduction in worm counts at dose (mg/kg)			
	1.0	0.25	0.1	0.025
VM 44866	99	98	95	32
VM 44864	99	83	38	29
VM 44857	99	100	96	65
VM 44865	99		35	

Four or five gerbils per group were used for each compound at each dose level.

fructose, inositol, inulin, mannitol, raffinose, rhamnose, sucrose and xylose. Differences were noted in other tests as itemised in Table 3.

The two organisms are clearly different strains which are also distinguished by the production of two different series of milbemycin antibiotics.

Production of Milbemycins

The three variants, designated strains E225B, C and D, were examined for milbemycin production.

Table 6. Evaluation of VM 44857 and ivermectin against naturally acquired roundworm infections in sheep: Mean group worm counts and efficacy in parentheses (%).

Treatment (single oral dose)	Abomasum			Small intestine				Large intestine		
	Haemonchus	*Ostertagia*	*T.a.*	*Trichostrongylus*	*Cooperia*	*Nematodirus*	*Strongyloides*	*Trichuris*	*Chabertia*	*Oesophagostomum*
Controls	684	1,592	6,488	12,032	3,512	536	56	12.8	9.2	14.6
Ivermectin[a]	0	0	0	0	0	0	0	0	0	0
(0.2 mg/kg)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
VM 44857	0	16	8	0	0	0	0	0.6	0	0
(0.2 mg/kg)	(100)	(99)	(99.9)	(100)	(100)	(100)	(100)	(95)	(100)	(100)
VM 44857	0	0	0	0	0	0	0	2.6	0	0
(0.4 mg/kg)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(80)	(100)	(100)

Five sheep per group.
[a] Ivermectin was used as the commercial formulation Ivomec.
T.a.: *Trichostrongylus axei.*

Table 7. Evaluation of VM 44866 and milbemycin D against experimental roundworm infections in sheep: Mean worm burdens and efficacy in parentheses (%).

Treatment (single oral dose)	Abomasum			Small intestine				Large intestine
	Haemonchus	*Ostertagia*	*T.a.*	*Trichostrongylus*	*Cooperia*	*Nematodirus*	*Immatures*[a]	*Chabertia*
Controls	1,167	5,627	12,033	13,000	2,000	6,067	6,600	388
VM 44866	0	7	0	0	773	0	240	0
(0.4 mg/kg)	(100)	(99.9)	(100)	(100)	(61)	(100)	(96)	(100)
VM 44866	0	0	0	0	280	13	33	0
(0.8 mg/kg)	(100)	(100)	(100)	(100)	(86)	(99.8)	(99.5)	(100)
Milbemycin D[b]	0	13	0	0	15	0	0	0
(0.2 mg/kg)	(100)	(99.8)	(100)	(100)	(99.3)	(100)	(100)	(100)
Milbemycin D	0	0	0	0	0	0	0	0
(0.4 mg/kg)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)

Three or four sheep per group.
[a] *Cooperia* and *Nematodirus.*
[b] Milbemycin D was obtained from the commercial veterinary product from Sankyo Co., Ltd., Japan.
T.a.: *Trichostrongylus axei.*

After 13 days fermentation in medium F1 at 27°C samples were analysed by HPLC. The results shown in Table 4 indicated variant B for further titre improvement. Strains E225 and E225B were deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland and were assigned accession Nos. NCIB 12310 and NCIB 12509, respectively.

A time course for milbemycin production by strain E225B in medium F1 is shown in Fig. 3. The long production period is typical of this strain. Only the three major metabolites are represented.

Anthelmintic Activity

In the *in vitro* test against *H. contortus* L_3 larvae MIC values for VM 44857, VM 44864 and VM 44866 were 0.05, 0.1 and 0.2 μg/ml, respectively. VM 44865 was much less active with an MIC of 25 μg/ml.

Results of controlled tests with single oral doses in the gerbil model, shown in Table 5, indicated VM 44857 and VM 44866 to be of greatest potency. In preliminary sheep tests, egg counts were reduced to zero by VM 44857 at 0.2 mg/kg, VM 44866 effected partial reduction whereas VM 44864 and VM 44865

displayed no significant activity at that dose. Subsequent evaluation of VM 44857 and VM 44866 in sheep, assessing efficacy by post-mortem worm counts is summarized in Tables 6 and 7. Ivermectin and milbemycin D were included for comparison. In naturally infected sheep VM 44857 removed all major nematode species at a dose of 0.4 mg/kg. VM 44866 at 0.8 mg/kg cleared many but not all species from experimentally infected sheep, *Cooperia* being notably refractory.

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