Further Novel Milbemycin Antibiotics from *Streptomyces* sp. E225

Fermentation, Isolation and Structure Elucidation

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Ten novel α and β class milbemycins have been isolated and characterized from the *Streptomyces* sp. E225, which has previously been shown to produce four related milbemycins. Some of the metabolites contain new structural features including, VM48641 which possesses an α-methoxyl substituent at C-27, and VM48642 which contains a furan ring at the terminus of the C-26 side chain. Several of these new compounds were shown to possess potent anthelmintic activity. An analysis of NMR chemical shift trends in this series of metabolites is presented.

The potent anthelmintic activity exhibited by the milbemycins continues to spur research to find new natural product and new semi-synthetic milbemycins with improved properties[1,2]. We have previously reported the isolation and structure elucidation of the milbemycins VM44857, VM44864, VM44865 and VM44866 (see Scheme 1) from a novel *Streptomyces* sp. designated E225.[3,4] In view of the potent *in vitro* and *in vivo* anthelmintic activity of two of these compounds[3] we investigated the fermentation broths of this organism in more detail, and have isolated 10 further, novel metabolites (compounds (**1**) to (**10**), Schemes 1 to 3[††]). In this paper, we describe the fermentation, isolation and structure elucidation studies on these metabolites, two of which possess new structural features for milbemycins. In addition, we describe NMR chemical shift trends in this series of compounds which may be useful in the structure elucidation of further metabolites.

Results and Discussion

Producing Strain

The metabolites were produced by fermentation of *Streptomyces* sp. E225 (NCIB 12310) or its morphological variant referred to as E225B (NCIB 12509)[3]. Both strains were maintained as vegetative mycelium in liquid nitrogen.

Fermentation

More than one fermentation was used for the production of the metabolites, and for minor metabolites (VM47704, VM48130, VM48633 and VM48642) the fermentations were conducted on a larger scale (3000 liter) than for the more abundant metabolites (VM44867, VM44868, 100 liter). The typical procedures used in large scale (A) and smaller scale (B) fermentations are described below.

Fermentation Process A, 3000 liter, Inoculated with Strain E225B

A (i) The first stage seed medium consisted of (all concentrations in g or ml/liter): Special peptone (Oxoid) 2.5, beef extract (Oxoid Lab. Lemco) 2.5, Tryptone (Oxoid) 2.5, neutralised soya peptone (Oxoid) 2.5, soluble starch (BDH) 2.5, malt extract (Oxoid) 2.5, glucose monohydrate 2.5, glycerol 2.5 and trace elements solution 10. The trace elements solution was as previously described[4] except that the concentration of $CoCl_2 \cdot 6H_2O$ was 0.5 g/liter. The pH of this solution was unadjusted. One flask of sterilized, first stage seed medium (100 ml in 500 ml Erlenmeyer) was inoculated with one ampoule

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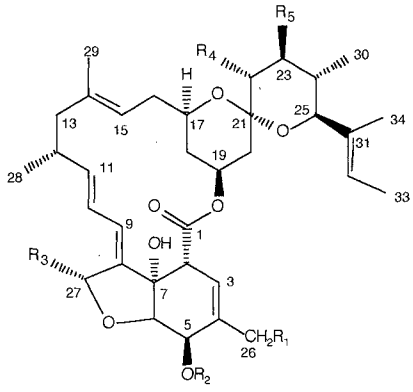
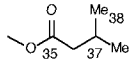
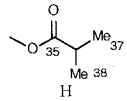
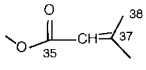
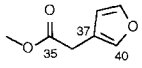
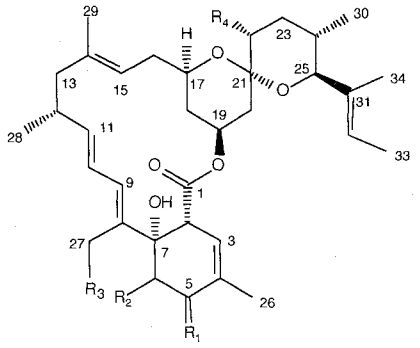
[††] See also European patents EP 254583 and EP 325462.

Scheme 1.



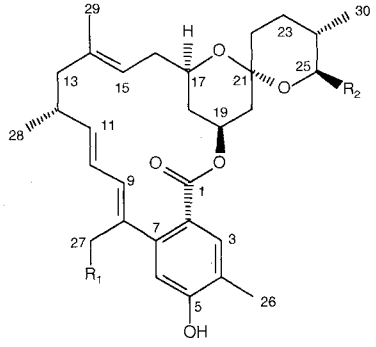
	R_1	R_2	R_3	R_4	R_5
VM47704 (**1**)		H	H	OH	H
VM48130 (**2**)	H	H	H	OH	
VM48633 (**3**)		H	H	OH	H
VM48641 (**4**)	H	Me	OMe	OH	H
VM48642 (**5**)		H	H	OH	H
VM44857	H	H	H	H	H
VM44864	H	Me	H	OH	H
VM44865	OCOC(Me)=CHMe	Me	H	OH	H
VM44866	H	H	H	OH	H

Scheme 2.



	R_1	R_2	R_3	R_4
VM44867 (**6**)	H,	OH	H	OH
VM44868 (**7**)	O	H	OH	H
VM48640 (**8**)	H,	H	OH	OH
VM54168 (**9**)	H,	H	=O	H

Scheme 3.



	R_1	R_2
VM54339 (**10**)	OH	
Milbemycin β_3	H	Me

of preserved culture (1.5 ml) and incubated at 25°C on a rotary shaker at 240 rpm for 48 hours.

A (ii) The second stage seed medium, Medium A, consisted of (all concentrations in g or ml/liter): Arkasoy 50 soybean flour (British Arkady) 10, glucose monohydrate 20, corn steep liquor (Roquette UK Ltd) 5 and NaCl 3. The pH of this medium was adjusted to 6.8 and 100 ml aliquots were sterilized in 500 ml Erlenmeyer flasks closed with cotton gauze caps. The flasks were inoculated with 4% of the first stage seed inoculum and incubated at 26°C on a rotary shaker at 240 rpm for 72 hours.

A (iii). The third stage seed medium consisted of 15 liters of Medium A together with 0.1% v/v polypropylene glycol (P2000). The medium was sterilized in a fully baffled, 20 liter fermenter (Biolafitte, France) fitted with 3 vaned-disc impellers. Sterile air was supplied (15 liter/minute) and agitation was at 200 rpm. The fermenter was inoculated with the contents of two second stage seed flasks and the fermentation was carried out at 26°C for 48 hours.

A (iv). Fourth stage seed. 100 liters of Medium A together with 0.1% antifoaming agent (10% Pluronic L81 (Blagden-Campbell, UK) in soybean oil) were sterilized at 121°C in a fully baffled, 150 liter fermenter (Braun, Germany) fitted with 3 vaned-disc impellers. Sterile air was supplied (100 liter/minute) and agitation was at 120 rpm. The fermenter was inoculated with 4 liters of the third stage seed and the fermentation was carried out at 28°C for 48 hours.

A (v). The production stage medium consisted of (all concentrations in g or ml/liter): Arkasoy 50 10, glucose monohydrate 20, Dextrin 07005 (Corn Products, UK) 20, casein (Oxoid) 2, $MgSO_4$ 1, $CaCO_3$ 5 and 10%

Pluronic L81 in soybean oil 1. The pH was adjusted to 6.0. The medium (3000 liters) was sterilized in a fully baffled, 4500 liter fermenter (Bioengineering-Wald, Switzerland) fitted with 3 vaned-disc impellers. Sterile air was supplied at 1500 liter/minute. The fermenter was inoculated with 100 liters of the fourth stage seed inoculum and the fermentation was carried out at 26°C for 17 days, adding extra aliquots of antifoaming agent as required. The agitation rates were 50, 75 and 100 rpm for the periods 0~2, 2~3 and 3~17 days respectively.

Fermentation Process B, 100 liters, Inoculated with Strain E225

B (i), First stage seed. Strain E225 was grown at 27°C on an agar plate of starch casein medium. A portion of the culture was used to inoculate six, 500 ml Erlenmeyer flasks, each containing 100 ml of sterilized seed medium. The seed medium had the following composition (all concentrations in g or ml/liter): Arkasoy 50 10, glycerol 20, maltose 2, trace element solution as in A (i) 10 and deionized water to 1 liter. The pH was adjusted to 6.5 prior to sterilization. The inoculated flasks were incubated at 27°C on a rotary shaker at 240 rpm for 48 hours.

B (ii), second stage seed. The contents of the flask from B (i) were used to inoculate 15 liters of sterilized medium in a 20 liters fermenter. The medium was the same as in B (i) except that tap water was used in place of deionized water. Prior to sterilization the pH was 6.3 without adjustment. Sterile air was supplied (15 liter/minute) and agitation was at 120 rpm. The fermentation was carried out at 28°C for 48 hours with automatic addition of polypropylene glycol P2000 to control foaming.

B (iii), Production. The production stage medium consisted of (all concentrations in g or ml/liter): potato starch 20, glucose monohydrate 20, casein hydrolysate (Oxoid) 2, casein (BDH) 2, K_2HPO_4 0.5, $MgSO_4$ 0.5, $CaCO_3$ 0.5, trace elements solution as in A (i) 10, made up to 100 liters with tap water. Prior to sterilization the pH was 7.0. Sterile air was supplied (50 liter/minute) and agitation was at 160 rpm. The fermentation was carried out at 28°C for 15 days with automatic addition of polypropylene glycol P2000 to control foaming.

Isolation and Purification

The isolation and purification procedures for VM47704, VM48633, VM48130 (large scale) and VM44867 (smaller scale) are detailed below and these are representative of the procedures used for all ten metabolites.

VM47704 (Process A, 3000 liter). The whole broth was transferred to a separate vessel, 10% v/v BuOH was added and the mixture was stirred for 16 hours at 7°C. The whole broth was then fed (5 liter/minute) together with BuOH (1.7 liter/minute) through an in-line static mixer to a liquid/liquid/solid separator (Westfalia SA7-03-076). The raffinate and mycelial solids were combined and subjected to a second, similar extraction. The combined BuOH extracts were then concentrated *in vacuo* to 20 liters prior to the addition of petroleum ether (60~80°C, 40 liters) to precipitate pigmented impurities. After centrifugation, the supernatant was concentrated *in vacuo* to 15 liters and chromatographed on a column (600 mm diameter × 200 mm) of silica gel (32~63 μm), eluting with a stepwise gradient of EtOAc in petroleum ether (60~80°C). Fractions with >8% EtOAc containing VM47704 and other active components were retained. All of the remaining milbemycin-containing fractions (see reference 4 for an HPLC procedure) were rechromatographed on a similar column to give a further quantity of VM47704 plus other active components, which was combined with the material already obtained and evaporated to give an oily concentrate (208 g). The concentrate was again chromatographed on a column (150 mm × 180 mm) of silica gel (32~63 μm), eluting with a stepwise gradient of EtOAc in petroleum ether (60~80°C). The eluant was pure petroleum ether (3.3 liters) followed by 6.6 liters each of 15, 25, 30, 35 and 40% v/v EtOAc in petroleum ether. The first 20 liters of eluate was discarded, thereafter collecting fractions of 2 liter. Fractions 3 to 8 were combined, concentrated to an oil and chromatographed on a column (75 × 500 mm) of Sephadex LH 20 (Pharmacia, UK) using MeOH as eluant. All milbemycin-containing fractions were combined. From here on, all purification was achieved using chromatography on reverse phase silica gel (Matrex C_{18} silica gel, 20~45 μm, 60 Å pore size, Amicon). The concentrate from the LH 20 column was chromatographed on a column (100 × 180 mm) of C_{18} silica gel, eluting with 85% MeOH. The first 1 liter was discarded, thereafter 20 × 100 ml fractions, all containing milbemycins, were collected, combined and rechromatographed on a similar column, eluting first with 85% MeOH (first liter discarded, next 36 × 85 ml fractions contained other active components) and then with 87.5% MeOH to give VM47704 mixed with other active components. Two more C_{18} columns were then used to further purify the VM47704 (column 1, elution with 85% MeOH, 100 × 260 mm column, 100 ml fractions, VM47704 in fractions 42~55; column 2, elution with

82.5% MeOH, similar column, 100 ml fractions, first 7.2 liters discarded, VM47704 in fractions 66~85). The impure fractions (35~41 from column 1 and 57~65 from column 2) were further purified (column 3, elution with 81% MeOH, 80 × 600 mm column, 100 ml fractions, first 9.3 liters discarded, VM47704 mainly in fractions 76~86). Fractions 76~86 from column 3 were then combined with fractions 66~85 from column 2 and given a final purification (column 4, elution with 79% MeOH, 100 ml fractions, first 20 liters discarded, VM47704 in fractions 34~55) to give 209 mg of VM47704.

VM48633 (Process A, 3000 liter). The isolation procedure was almost identical to that for VM47704 up to the stage of initial silica gel chromatography (600 mm diameter column). A 1.8 g fraction from this column containing both VM48633 and VM48130 was purified by reverse phase preparative HPLC (21.4 × 500 mm Dynamax-60A C_{18} column (Rainin, U.S.A.)) with UV monitoring at 244 nm. Gradient elution (10 ml/minute, 82:10 MeOH-H_2O rising to 100% MeOH over 140 minutes) was used to yield fractions still containing VM48633 mixed with VM48130 (97.4 mg after evaporation). This material was purified by normal phase preparative HPLC (21.4 × 500 mm Dynamax-60A silica gel column). Gradient elution (10 ml/minute, 87:13 hexane-acetone rising to 82:18 over 120 minute) was used to yield fractions containing substantially pure VM48633 (4.9 mg). Fractions were monitored by TLC (silica gel plates eluted with 60:40 hexane-acetone).

VM48130 (Process A, 3000 liter). The procedure for VM48130 was identical to that for VM48633 except that further purification of the VM48130-containing fractions from the preparative, normal phase HPLC was required. These fractions (10.6 mg) were purified by reverse phase HPLC (10 × 250 mm Hypersil ODS column (HPLC Technology Ltd)) using gradient elution (77:23 MeOH-H_2O rising to 85:15 over 60 minutes) and monitoring by UV at 244 nm to yield substantially pure VM48130

Table 1. HPLC Characteristics of metabolites (**1**) to (**10**).

Compound	Structure	UV max (nm)	HPLC retention time Rt (minutes) System*			
			(1)	(2)	(3)	(4)
VM47704	**1**	244	~15.2	—	7.0	—
VM48130	**2**	244	14.5	12.3	—	6.2
VM48633	**3**	244	~14.7	12.9	—	6.6
VM48641	**4**	244	13.6	—	—	—
VM48642	**5**	244	12.8	—	—	—
VM44867	**6**	244	—	—	9.5	7.6
VM44868	**7**	237	—	—	11.7	—
VM48640	**8**	240	13.6	—	—	—
VM54168	**9**	285	—	—	13.6	—
VM54339	**10**	250	—	—	20.6	—

* HPLC systems.
(1) Microsorb C-18 (4.6 × 250 mm) column (Rainin) eluted with 85:15 MeOH-H_2O at 1 ml/minute.
(2) Ultrasphere ODS 5 μmm (4.6 × 250 mm) column (Altex) eluted with 85:15 MeOH-H_2O at 1ml/minute.
(3) Ultrasphere ODS 5 μm (4.6 × 250 mm) column (Altex) eluted with 90:10 MeOH-H_2O at 1 ml/minute.
(4) Microsorb Phenyl (4.6 × 250 mm) column (Rainin) eluted with 88:12 MeOH-H_2O at 1 ml/minute.

Table 2. Physico-chemical properties of metabolites (**1**) to (**10**).

Compound	Molecular formula	Molecular weight	EI-MS (m/z)	FAB-MS (m/z of $(MNa)^+$)	λ_{max} (CH_3OH, nm)
VM47704 (**1**)	$C_{39}H_{56}O_{10}$	684	684	—	244
VM48130 (**2**)	$C_{38}H_{54}O_{10}$	670	—	693	244
VM48633 (**3**)	$C_{39}H_{54}O_{10}$	682	—	705	244
VM48641 (**4**)	$C_{36}H_{52}O_9$	628	—	651	244
VM48642 (**5**)	$C_{40}H_{52}O_{11}$	708	—	731	244
VM44867 (**6**)	$C_{35}H_{52}O_8$	600	600.3659	623	244
VM44868 (**7**)[a]	$C_{34}H_{48}O_7$	568	568	591	237
VM48640 (**8**)	$C_{35}H_{52}O_8$	600	—	623	240
VM54168 (**9**)	$C_{34}H_{48}O_7$	568	—	591	285
VM54339 (**10**)	$C_{34}H_{46}O_6$	550	—	573	250

[a] $[\alpha]_D^{25}$ +108° (*c* 0.29 acetone).

(4.1 mg).

VM44867 (Process B, 100 liter). The mycelial mass was extracted with acetone (2×40 liter) and then concentrated by removing the acetone. The residue (14 liter) was then extracted with CH_2Cl_2 (2×6 liter) and the combined extracts were dried over $MgSO_4$ and evaporated to an oil (40 g). The residue was chromatographed on silica gel and eluted sequentially with 0 to 60% diethyl ether in hexane. Fractions containing VM44867 (HPLC detection[4]) were combined and evaporated to an oil (66 mg). Final purification was achieved by preparative TLC using silica gel taper plates (Analtech) eluted with MeOH-CH_2Cl_2, 3:97, to give substantially pure VM44867 (4.7 mg).

Throughout the isolation and purification process the metabolites were monitored by HPLC using diode-array detection. Many of them were initially identified by their distinctive UV spectra (Table 1).

Table 3. Assignment of ^1H NMR data for metabolites **1** to **10** in $CDCl_3$/TMS, δ_H in ppm ($\delta_{TMS}=0$).

Atom	(1)	(2)	(3)	(4)[c]	(5)	(6)[e]	(7)	(8)	(9)	(10)
2	3.32	3.27	3.33	3.20	3.31	3.77	3.91	3.50	3.90	—
3	5.74	5.42	5.75	5.37	~5.73	5.30	6.41	5.30	5.26	7.40
5	4.46	4.29	4.48	4.00	4.46	4.01	—	4.03	4.38	—
5-OH	2.69	2.32	2.72	—	~2.55[d]	—	—	—	~1.85	6.49
5-OMe	—	—	—	3.528	—	3.43	—	3.36	—	—
6	3.97	3.96	3.99	4.34	3.98	3.97	2.94[f]	~2.23	2.41	6.78
							2.57	~1.87[g]	2.04	
7-OH	4.02	3.90	3.99	3.95	[a]	3.83[d]	3.79	3.63	3.90	—
9	~5.77	~5.76	~5.78	5.92	~5.77	6.20	6.37	6.38	7.34	5.78
10	~5.75	~5.75	~5.73	6.06	~5.73	6.10	6.20	6.27	6.78	6.22
11	~5.35	~5.32	5.34	5.40	5.33	5.31	5.45	5.40	5.76	5.37
12	2.42	2.42	2.42	2.48	2.43	2.44	2.48	2.47	2.56	2.51
13eq	~2.20	~2.20	~2.20	~2.22	~2.20	~2.16	~2.17	~2.2	2.20	2.21
13ax	~1.85	~1.85	~1.85	~1.88	~1.85	~1.8	1.86	~1.84	1.91	1.83
15	4.99	~4.97	4.99	5.00	4.99	4.84	4.89	4.88	4.89	4.90
16	~2.23	~2.22	~2.22	~2.22	~2.20	~2.30e	2.29e	~2.2	2.3 to	2.35 to
						~2.22a	2.21a		2.1	2.17
17	3.62	~3.59	3.62	3.63	3.63	3.67	3.59	~3.63	3.57	3.71
18eq	~1.80	~1.80	~1.80	~1.81	~1.80	~1.8	1.78	~1.86	1.63	1.99
18ax	~0.87	0.90	0.88	0.90	0.89	0.83	0.77	0.79	0.70	0.82
19	~5.35	~5.31	~5.35	5.32	~5.36	~5.38	5.38	~5.33	5.30	5.46
20eq	~1.9	~1.90	~1.90	~1.9	~1.9	~1.8	1.96	~1.80	1.89	1.98
20ax	~1.9	~1.90	~1.90	~1.9	~1.9	~1.99	1.41	~1.93	1.36	1.41
22	~3.34	3.21	3.33	3.34	3.35	~3.35	1.7 to	3.32	1.7 to	1.7 to
							1.5		1.5	1.5
22-OH	[a]	1.93	1.77	~1.77	~1.77	~1.79	—	~1.70	—	—
23eq	~1.86	—	~1.86	~1.87	~1.87	1.87	1.7 to	~1.86	1.7 to	1.7 to
							1.5		1.5	1.5
23ax	~1.40	4.93	1.41	1.41	1.41	1.41	1.7 to	1.40	1.7 to	1.7 to
							1.5		1.5	1.5
24	~1.70	~1.82	~1.69	~1.70	~1.70	~1.69	~1.53	~1.69	1.50	1.53
25	3.40	3.58	3.40	3.40	3.40	3.37	3.43	3.37	3.41	3.46
26	~4.77,	1.87	~4.82,	1.81	~4.81,	1.78	1.86	1.81	1.84	2.19
	~4.67		~4.68		~4.72					
27a	~4.69	~4.68	~4.69	5.54	4.69	1.94	4.35	4.26	10.19	4.51
						(CH_3)				
27b	~4.69	~4.65	~4.69	—	4.69	—	4.25	4.16	—	4.51
28	1.00	1.00	1.00	1.02	1.00	1.03	1.03	1.02	1.08	1.03
29	1.54	1.53	1.54	~1.56	1.54	1.62	1.60	1.60	1.54	1.63
30	0.70	0.69	0.70	0.70	0.70	0.69	0.68	0.69	0.66	0.68
32	5.41	5.45	5.41	5.41	5.41	5.36	5.37	~5.37	5.35	5.34
33	1.65	1.66	1.65	1.65	1.65	1.59	1.62	1.61	1.61	1.62
34	1.57	1.59	1.57	~1.56	1.58	1.53	1.56	1.53	1.60	1.57
36	2.23	2.61	5.72	—	3.51	—	—	—	—	—
37	2.11	1.201[b]	—	—	—	—	—	—	—	—
38	0.96	1.197[b]	1.90[b]	—	6.39	—	—	—	—	—
39	0.96	—	2.17[b]	—	7.38	—	—	—	—	—
40	—	—	—	—	~7.40	—	—	—	—	—

[a] Not clearly observed. [b] Assignment uncertain between pairs labelled b. [c] C-27-OMe $\delta_H=3.533$ ppm. [d] Assignment uncertain. [e] Broad singlet OH resonance at $\delta_H=2.68$ ppm probably due to C-6-OH. [f] $^4J_{6,7\text{-OH}}=1.6$ Hz. [g] $^4J_{6,7\text{-OH}}=1.4$ Hz.

Physico-chemical Properties and Structure Elucidation

Selected physico-chemical properties of the ten metabolites isolated from strain E225 are shown in Table 2. The structure elucidation of the new metabolites was greatly facilitated by the availability of spectral data for the four related compounds already isolated and characterized[4] and relied mainly on MS (Table 2), ^{1}H NMR (Table 3) and ^{13}C NMR data (Table 4). This section focuses on the key points of the structure elucidation of the more unusual metabolites.

VM48641 (**4**). FAB-MS indicated a molecular weight of 628. The ^{13}C NMR spectrum (Table 4) contained 36 resonances including those of 6 CH_3-C groups, 2 CH_3-O groups, 5 CH_2 groups, 10 sp^3 CH groups, 6 sp^2 CH groups, 2 sp^3 C atoms and 5 sp^2 C atoms (DEPT editing and chemical shift analysis), indicating the presence of 50 non-exchangeable protons. The complex and overlapped ^{1}H NMR spectrum (Table 3) was solved with the aid of 2D ^{1}H COSY-45 and deuterium exchange experiments, and contained the resonances of 2 hydroxyl protons. Thus VM48641 (**4**) was formulated as $C_{36}H_{52}O_9$ (CH_2O more than VM44864[4], see Scheme 1). The pattern of proton-to-proton scalar coupling connectivities in the 2D COSY spectrum was very similar to that of VM44864. However, an extra CH_3O group was present, no signals were seen for a CH_2-27 unit, and H-9 was found to have a long-range connectivity to an

Table 4. Assignment of ^{13}C NMR data for metabolites **1** to **10** in $CDCl_3$/TMS, δ_C in ppm ($\delta_{CDCl_3}=77.0$).

Atom	(1)	(2)	(3)	(4)[c]	(5)	(6)	(7)	(8)	(9)	(10)[d]
1	173.0[a1]	173.7	173.2	173.7	173.1	174.7	172.3	173.6	174.3	169.1
2	45.5	45.7	45.6	46.6	45.5	44.4	49.9	49.1	48.0	124.1[a1]
3	121.7	118.1	121.4	118.4	122.1	118.8	136.3	118.5	117.8	132.1
4	136.4	137.9	136.8	135.6	136.2	134.7[a1]	136.8	138.5	137.9[a1]	123.6[a1]
5	64.6	67.7	64.7	76.0	64.7	79.8	196.5	76.8	68.1	156.4
5-OMe	—	—	—	57.2	—	57.4	—	56.6	—	—
6	79.0	79.2	79.1	74.2	79.0	69.5	48.0	37.4	41.3	114.4
7	80.3	80.2	80.3	79.9	80.3	~77	78.0	75.8	74.1	140.7[a2]
8	139.1	139.5	139.2	139.6	139.1	136.1	137.6	139.8	139.2[a1]	135.4[a2]
9	120.47[a2]	120.3	120.5[a1]	125.4	120.57	125.5	130.4	129.6	147.5	131.8
10	123.3	123.4	123.4	123.9	123.3	124.7	123.5	124.1	121.6	123.7
11	142.9	142.8	142.9	144.3	143.08	140.4	144.8	143.7	152.5	143.1
12	35.9	36.0	36.0	35.9	36.0	35.5	36.3	36.3	36.6	36.0
13	48.4	48.5	48.5	48.6	48.5	48.6	48.3	48.4	48.0	48.6
14	137.1	137.4	137.2	137.3	137.2	137.4[a1]	136.0	136.6	135.3[a1]	135.8[a2]
15	120.54[a2]	120.4	120.6[a1]	120.6	120.59	120.6	121.0	120.5	121.5	121.7
16	34.6	34.6	34.6	34.6	34.6	34.5	34.5	34.5	34.5	33.8
17	67.9	68.1	68.0	68.0	68.0	68.0	67.4	68.1	67.2	67.5
18	36.4[a3]	36.21[a1]	36.45[a2]	36.5[a1]	36.37	36.16	36.6	36.4	36.54	36.7
19	68.8	68.5	68.8	68.7	68.9	68.1	69.5	68.7	68.4	68.9
20	36.3[a3]	36.14[a1]	36.37[a2]	36.4[a1]	36.43	36.24	41.0	36.4	40.8	41.2
21	98.8	100.2	98.9	98.9	98.9	98.8	97.6	98.9	97.6	97.8
22	71.5	75.4[a2]	71.6	71.6	71.6	71.6	35.6	71.5	35.45	35.6
23	36.8	76.0[a2]	36.9	36.9	36.8	36.9	27.6	36.9	27.5	27.6
24	32.0	37.7	32.1	32.1	32.1	32.1	31.5	32.1	31.5	31.5
25	81.9	80.2	81.9	81.9	81.9	81.8	82.4	81.9	82.4	82.3
26	64.1	19.9	63.5	19.7	64.8	19.3	15.6	19.3	19.2	15.5
27	68.4	68.5	68.5	102.3	68.5	13.8	58.0	57.9	189.3	61.4
28	22.2	22.3	22.3	22.3	22.3	21.4	21.7	21.8	21.3	20.9
29	15.5	15.6	15.5	15.6	15.5	16.1	16.0	16.0	16.0	16.0
30	17.4	13.0	17.5	17.5	17.5	17.5	17.7	17.5	17.7	17.7
31	134.0	133.1	134.1	134.1	134.1	134.0[a1]	134.8	134.1	134.9	134.9
32	123.6	124.9	123.7	123.6	123.7	123.7	123.0	123.7	122.9	123.0
33	13.1	13.2	13.1	13.1	13.1	13.1	13.0	13.1	13.0	13.0
34	10.9	10.8	10.9	10.9	10.9	10.9	11.0	10.9	10.9	11.0
35	172.9[a1]	177.8	166.4	—	170.9	57.4	—	—	—	—
36	43.2[b]	34.3	115.6	—	30.8	—	—	—	—	—
37	25.6[b]	19.2	157.8	—	117.1	—	—	—	—	—
38	22.4[b]	19.0	27.5	—	111.3	—	—	—	—	—
39	22.4[b]	—	20.3	—	143.05	—	—	—	—	—
40	—	—	—	—	140.5	—	—	—	—	—

[a] Assignment uncertain between pairs labelled ai, where "i" is a number. [b] The calculated chemical shifts for C-36, C-37, C-38 and C-39 are 43.4, 25.6, 22.4 and 22.4 ppm respectively[5]. [c] δ_C C-27-OMe = 55.3 ppm. [d] The resonances of C-2, C-4, C-7, C-8 and C-14 were assigned by reference to those of milbemycin β3[6].

sp^3 CH proton resonating at $\delta_H = 5.54$ ppm ($^4J_{9,27} \sim 1.7$ Hz). This proton was directly connected to a carbon resonating at 102.3 ppm (2D ^{1}H, ^{13}C HETCOR experiment) and exhibited a long-range connectivity to C-6 in a COLOC experiment. With the exceptions of C-2, C-5, C-6, C-9, C-11 and C-27, the ^{13}C NMR chemical shifts of (**4**) were almost identical to those of VM44864 (mean difference $= 0.12 \pm 0.12$ ppm (σ)), and it was concluded that VM48641 (**4**) was the C-27 methoxyl analogue of VM44864.[4] The observation of mutual positive NOE's between H-2 and H-27 established that the stereochemistry at C-27 was α, that is *S*, assuming the natural *R* stereochemistry exists at C-2. No significant NOE was observed between H-27 and H-6. The observation of $^3J_{2,3} \sim {}^5J_{2.5} \sim {}^5J_{2,26} \sim 2.5$, $^3J_{5,6} \sim 5.7$, $^3J_{9,10} \sim 11.3$, $^3J_{10,11} \sim 14.8$, $^3J_{11,12} \sim 10.0$, $^3J_{17,18ax} \sim {}^3J_{19,18ax} \sim 11.7$, $^3J_{22,22-OH} \sim 12.8$! (a remarkably high value for a coupling to an hydroxyl proton and indicating no rotation around the C-22–O-22 bond), $^3J_{22,23ax} \sim {}^3J_{24,23ax} \sim 11.9$, $^3J_{24,25} \sim 10.2$, $^4J_{32,34} \sim {}^5J_{33,34} \sim 1.1$ Hz, together with the close agreement of ^{13}C chemical shifts, indicates that the relative stereochemistry of VM48641 (**4**) is the same as VM44864[4] at all centres, and that the solution conformations of VM48641 (**4**) are very similar to those of VM44857 (Scheme 1)[4].

VM48642 (**5**). FAB-MS indicated a molecular weight of 708; the highest of any milbemycin produced by strain E225. The ^{13}C NMR spectrum (Table 4) contained 40 resonances including those of 5 CH_3-C groups, 8 CH_2 groups, 9 sp^3 CH groups, 9 sp^2 CH groups, 2 sp^3 C atoms and 7 sp^2 C atoms, indicating the presence of 49 non-exchangeable protons. The complex and overlapped ^{1}H NMR spectrum (Table 3) was solved as above and found to contain the resonances of 3 hydroxyl protons. Thus VM48642 (**5**) was formulated as $C_{40}H_{52}O_{11}$. The pattern of proton-to-proton scalar coupling connectivities in the 2D ^{1}H COSY-45 spectrum was identical to that of VM44866 ($C_{34}H_{48}O_8$)[4], except that the resonance of CH_3-26 was missing. Protons H-2 and H-3 exhibited long-range scalar coupling to a pair of geminal protons whose chemical shift indicated that they were connected to the oxygen of an ester function. It was concluded that the structure of VM48642 was the same as VM44866 but with a CH_2-26 methylene group substituted by an O-CO-C_5H_5O moiety: the base peak in the EI-MS was at *m/z* 81 (C_5H_5O). The structure of the C_5H_5O moiety was deduced to be -CH_2-3-furanyl (see Scheme 1) on the basis of 2D COSY-45 and COLOC conectivities including: H-38 to H-36, H-39 and H-40; H-40 to H-36, H-38 and H-39; H-36 to C-35, C-37; and C-40 and H-40 to C-37 and C-39. The following couplings were observed in this system: $^3J_{38,39} \sim 1.8$, $^4J_{38,40} \sim 0.9$ and $^4J_{39,40} \sim 1.7$ Hz. With the exceptions of C-1 to C-5 and C-26, the ^{13}C chemical shifts of VM48642 (**5**) were within experimental error of those of VM44866[4] (mean difference $= 0.09 \pm 0.13$ ppm (σ)). This result, together with the observation of $^3J_{2,3} \sim {}^5J_{2.5} \sim 2.2$, $^3J_{5,6} \sim 6.2$, $^3J_{10,11} \sim 13.5$, $^3J_{11,12} \sim 10$, $^3J_{17,18ax} \sim {}^3J_{19,18ax} \sim 11.6$, $^3J_{22,23ax} \sim {}^3J_{24,23ax} \sim 12.0$, $^3J_{24,25} \sim 10.2$, $^4J_{32,34} \sim {}^5J_{33,34} \sim 1.1$ Hz, indicates that the relative stereochemistry of VM48642 (**5**) is the same as VM44866[4] at all centres, and that the solution conformations of VM48642 (**5**) are very similar to those of VM44857 (Scheme 1)[4].

The structures of all other eight metabolites were solved in a similar fashion, mainly on the basis of the NMR data (see Schemes 1 to 3 and Tables 3 and 4). The structure elucidation of VM44868 (**7**) should be described specifically. It was difficult to establish fragments of structure in the "southern" part of the molecule, on the basis of consecutive proton-to-proton connectivities,

Fig. 1. Contour plot of an expansion of the 2D ^{1}H, ^{13}C COLOC NMR spectrum of VM44868 (**7**) in $CDCl_3$/TMS in the region of the resonance of C-7.

The corresponding 1D ^{1}H and ^{13}C NMR spectra are plotted on the left and on top. Note that the intensity of the H-6a resonance is reduced relative to that of H-6b due to an unresolved 4-bond stereospecific "W" coupling to the C-7-OH proton. The 1:1:1 triplet resonating at $\delta_C \sim 77$ ppm (lines marked ○) is due to the solvent $CDCl_3$.

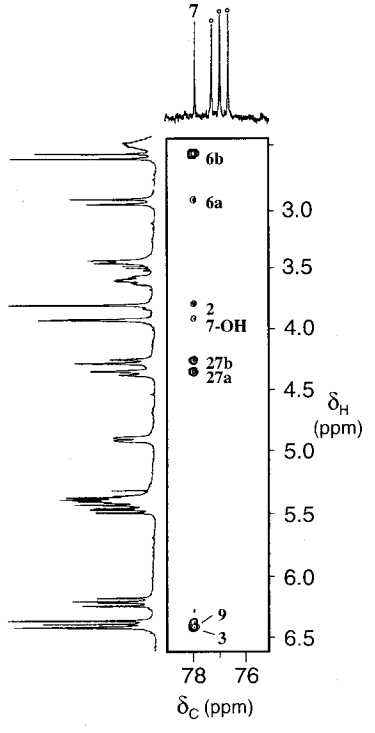


Table 5. Chemical shifts diagnostic for certain structural features in the milbemycins.[a]

Atom	α Milbemycin	α Milbemycin with C-26 ester	α Milbemycin with O at C-13[b]	β Milbemycin
C-3	117 to 119 unless extra sp^2 C in C-6 ring	121 to 122	118 to 119	118 to 119 unless extra sp^2 C in C-6 ring
C-28	22.2 to 22.4	22.2 to 22.4	*ca.* 19	20.9 to 21.8
C-29	15.5 to 15.6	15.5 to 15.6	*ca.* 11	15.9 to 16.1
H-2	3.20 to 3.40 unless extra sp^2 C in C-6 ring	3.20 to 3.40	*ca.* 3.3	3.48 to 3.90
H-3	5.32 to 5.45 unless extra sp^2 C in C-6 ring	5.75 to 5.80	*ca.* 5.4	5.25 to 5.30 unless extra sp^2 C in C-6 ring
H-9[c]	5.7 to 6.0	5.7 to 6.0	5.7 to 6.0	6.20 to 6.4 unless C-7 or C-27 are sp^2 hybridised
H-15	4.95 to 5.00	4.97 to 5.00	5.2 to 5.4	4.81 to 4.90

[a] Data for $CDCl_3$ solutions referenced to tetramethylsilane. This data applies to α milbemycins with the general structure shown in Scheme 1 and to β milbemycins with the general structure shown in Scheme 2. [b] Data from ref 8 for compounds with C-13β OH or OCOR groups. [c] The behaviour of H-10 is similar.

because of a large number of "spectroscopically silent" quaternary carbons in the region of the cyclohexenone ring (C-4, C-5, C-7 and C-8). In this case the 2D ^1H, ^{13}C COLOC experiment was particularly valuable for establishing long-range proton-to-carbon connectivities. Fig. 1 shows a contour plot of an expansion of the COLOC spectrum of (**7**) in the region of the resonance of C-7. This carbon displayed all 8 possible two-bond and three-bond connectivities, vital for the structure elucidation of this difficult region of the molecule.

Chemical shift trends in the ^1H and ^{13}C NMR data. Having assembled such a large volume of NMR data (Tables 3 and 4, and Tables 2 and 3 in ref 4) for this series of milbemycins, the data were examined for diagnostic trends. There are many large and obvious chemical shift changes at atoms close to a position of substitution and these are generally as expected. We focused on smaller, but reliable and diagnostic, changes in *characteristic and readily identifiable signals* that may be of help in solving the structures of further milbemycins in this series without recourse to a full and unambiguous structure elucidation. For instance, it is possible from a casual inspection of chemical shift data to determine whether or not a particular milbemycin belongs to the α or β class (Table 5). For α milbemycins (Scheme 1) it is also possible to determine if C-13[8] or C-26 are oxidatively substituted. For both α and β milbemycins it is also possible to determine the presence of certain oxygenation patterns in the O-21 to C-25 tetrahydropyran ring, from characteristic changes to the chemical shift of the readily identified spiroketal carbon C-21. The ^{13}C chemical shift of C-21 changes from 97.4~97.8 in an "unsubstituted" O-21 to C-25 ring to *ca.* 98.8~99.1, *ca.* 99.6~99.7[7] and *ca.* 99.8~100.2 ppm in rings with C-22-OHeq, C-23-OHax[7] and C-22-OHeq, C-23-(OCOR)eq substitution respectively. A single case of a molecule with C-23-OHeq, C-22-(OCOR)eq substitution (unpublished metabolite from a different organism) gave a shift of 99.1 ppm, indicating the occurrence of overlap of shifts. These chemical shift guides should assist in the structure elucidation of further novel milbemycins but they will not be valid for structures which differ significantly in type from those discussed here.

The further ten metabolites isolated from strain E225 are of note for their wide range of structures and the possession of unusual structural features: the C-27α-OMe group in VM48641 (**4**) and the furan sidechain in VM48642 (**5**) in particular. However, we note that compounds with C-27 hydroxylation have recently been reported as natural products and are known as metabolites of the milbemycins and avermectins[1]. Finally, we also note that since the completion of our structure elucidation work, Pfizer have reported[8] a metabolite named UK-77,021, which is identical to VM48130 (**2**).

Anthelmintic Activities

Anthelmintic activity was assessed *in vitro* against *Haemonchus contortus* L_3 larvae and *in vivo* in an adult *Trichostrongylus colubriformis*—gerbil model (at 1 mg/kg) as previously described[3]. Compounds (**4**), (**5**), (**6**), (**8**) and (**10**) were without significant activity in either test. Compounds (**7**) and (**9**) were effective at 3.1 and 0.8 μg/ml respectively in the *in vitro* test but did not show significant activity *in vivo*. VM47704 (**1**), VM48633 (**3**)

and VM48130 (**2**) were active at 1.6, 0.2 and 0.002 μg/ml respectively *in vitro* and all were >99% effective *in vivo* at the 1 mg/kg dose.

Experimental

All the NMR experiments were conducted at circa 300 K in 0.5 cm^3 $CDCl_3$/TMS solution (10 to 120 mM). ^1H and ^{13}C NMR spectra were acquired under the same conditions as previously[4] except for VM48642 for which some experiments had to be repeated with increased relaxation delays due to the unexpectedly long spin-lattice relaxation times of the protons in the furan ring. When the 2D ^1H, ^{13}C HETCOR and 1D ^{13}C DEPT-135 experiments were repeated the relaxation delays were set to 2.5 and 1.8 second respectively, and the sequences were also retuned for $^1J_{C,H}$ values of 180 rather than the normal 140 Hz, to take account of the very large values of $^1J_{C,H}$ in the furan ring. The 2D ^1H, ^{13}C COLOC experiment was repeated with relaxation delays increased from 1.0 to 2.5 seconds. HR-EI-MS and FAB-MS data were recorded on VG Analytical ZAB-1F instrument using thioglycerol and a saturated solution of sodium acetate in 3-nitro-benzyl alcohol as the FAB matrices.

UV spectra were obtained during HPLC analysis with diode-array detection (Waters 990 PDA detector). HPLC conditions were as shown in the footnote to Table 1.

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References

1) DAVIES, H. G. & R. H. GREEN: Avermectins and milbemycins part I. Chem. Soc. Rev. 20: 211~269, 1991
2) DAVIES, H. G. & R. H. GREEN: Avermectins and milbemycins part II. Chem. Soc. Rev. 20: 271~339, 1991
3) HOOD, J. D.; R. M. BANKS, M. D. BREWER, J. P. FISH, B. R. MANGER & M. E. POULTON: A novel series of milbemycin antibiotics from *Streptomyces* strain E225. I. Discovery, fermentation and anthelmintic activity. J. Antibiotics 42: 1593~1598, 1989
4) BAKER, G. H.; R. J. J. DORGAN, J. R. EVERETT, J. D. HOOD & M. E. POULTON: A novel series of milbemycin antibiotics from *Streptomyces* strain E225. II. Isolation, characterisation, structure elucidation and solution conformations. J. Antibiotics 43: 1069~1076, 1990
5) BREMSER, W. *et al.*: Chemical Shift Ranges in Carbon-13 NMR Spectroscopy, Verlag Chemie, 1982
6) KOCIENSKI, P. J.: A directed aldol approach to (+)-milbemycin β_3. J. Chem. Soc. Perkin Trans. I: 2171~2181, 1987
7) CARTER, G. T.; J. A. NIETSCHE, M. R. HERTZ, D. R. WILLIAMS, M. M. SIEGEL, G. O. MORTON, J. C. JAMES & D. B. BORDERS: LL-F28249 Antibiotic complex: A new family of antiparasitic macrocyclic lactones. Isolation, characterisation and structures of LL-F28249 α, β, γ, λ. J. Antibiotics 41: 519~529, 1988
8) HAXELL, M. A.; B. F. BISHOP, P. BRYCE, K. A. F. GRATION, H. KARA, R. A. MONDAY, M. S. PACEY, D. A. PERRY, Y. KOJIMA, H. MAEDA, S. NISHIYAMA, J. TONE & L. H. HUANG: C-13β-Acyloxymilbemycins, a new family of macrolides. Discovery, structural determination and biological properties. J. Antibiotics 45: 659~670, 1992