C-13β-ACYLOXYMILBEMYCINS, A NEW FAMILY OF MACROLIDES

DISCOVERY, STRUCTURAL DETERMINATION AND BIOLOGICAL PROPERTIES

MARK A. HAXELL*, BERNARD F. BISHOP, PATRICIA BRYCE, KENNETH A. F. GRATION, HAMIDA KARA, ROBERT A. MONDAY, MICHAEL S. PACEY and DAVID A. PERRY

Central Research Division, Pfizer Ltd.,
Ramsgate Road, Sandwich, Kent CT13 9NJ, England

YASUHIRO KOJIMA, HIROSHI MAEDA, SATOSHI NISHIYAMA and JUNSUKE TONE

Central Research Division, Pfizer Inc.,
5-Gochi, Taketoyo-cho, Chita-gun, Aichi 470-23, Japan

LIANG H. HUANG

Central Research Division, Pfizer Inc.,
Eastern Point Road, Groton, Connecticut 06340, U.S.A.

(Received for publication August 12, 1991)

A family of novel milbemycins possessing *C*-13β-acyloxy substitution was produced by *Streptomyces hygroscopicus* ATCC 53718. These compounds were detected by HPLC diode array analysis and possess anthelmintic and ectoparasiticidal activity. The origin of the oxygen atom at C-13 is discussed.

Since the discovery of milbemycins by workers at Sankyo in 1974[1,2] there have been many reports of similar related structures[3~9]. These natural products all possess anthelmintic activity but generally have weaker ectoparasiticidal activity than the avermectins[10]. The major structural difference between these two classes of metabolites is the presence of a disaccharide substitution possessing α-stereochemistry on the C-13 position of the macrolide ring in the avermectins.

There are many reports in the literature of semi-synthetic modifications of milbemycins to allow the introduction of substituents at the C-13 position[11~13]. Here we describe the discovery and structural elucidation of a novel series of milbemycins[14], the first natural compounds discovered possessing C-13 substitution since the discovery of the avermectins.

Taxonomy

Strain No. N787-182 was isolated from a soil sample collected in Kurashiki City, Okayama prefecture, Japan. Taxonomic methods similar to those employed by SHIRLING and GOTTLIEB[15] were used to study this strain. Colours were determined with colour chips[16]. Table 1 shows selected cultural characteristics of this strain. Morphology was determined by light and scanning electron microscopy of cultures grown on potato-carrot agar. The vegetative mycelium developed without signs of fragmentation, producing a grey mass of rugose spores ($1.1 \sim 1.8 \times 0.9 \sim 1.2\ \mu m$) which were arranged in spiral coiled chains. These chains occasionally coalesced into a hygroscopic mass. Sporangia, flagellae, sclerotia or other specialised structures were not observed.

Table 1. Cultural characteristics of *Streptomyces hygroscopicus* ATCC 53718.

Medium	Colony surface	Aerial mycelium	Colony reverse	Soluble pigment
Yeast extract-malt extract agar[15]	Good; pale yellow/brown	Yellowish, yellowish grey	Pale yellowish brown	Yellowish brown
	3ec, 1½ea, 1½ec, 1½ge, 1½ig	1½ea, 1½ec, 1½ge, 1½ig	3gc	3lc
Oatmeal agar[15]	Moderate; cream, pale grey, pink-gray, dark grey to black	Pale grey, grey, pink grey, dark grey to black	Cream, grey, dark grey to black	Cream to pale yellow
	2ca, 3dc, 3fe, 5fe, 3ml, 3po	3dc, 3fe, 5fe, 3ml, 3po	2ca, 3fe, 3ml, 3po	2ca, 2ea
Inorganic salts-starch agar[15]	Moderate; cream 2ca	Pale yellowish, pale grey to dark grey	Cream, pale grey, grey to dark grey	Pale yellowish
		2ea, 3dc, 3fe, 3ih, 3ml	2ca, 3fe, 3ih, 3ml, 3dc	2ia
Tyrosine agar[26]	Moderate; brown 4lg	White; in small dots	Pale yellowish brown 3gc	Dark brown 4ni
BENNETT's agar[27]	Good; cream, pale yellowish pale grey, pink grey to dark pink grey	Same as surface	Pale yellowish, pale grey to dark grey	Pale yellowish
	2ca, 2ea, 3dc, 3fe, 5fe, 3ih, 5ih, 5ml		2ea, 3dc, 3fe, 3ih, 3ml	2ga
Potato-carrot agar[18]	Moderate; cream pale grey to pink grey	Same as surface	Cream to grey	No pigment
	2ca, 3dc, 3fe, 3ih		2ca, 3fe, 3dc	

Physiological properties of strain No. N787-182 are summarised in Table 2.

Table 2. Physiological characteristics of strain *Streptomyces hygroscopicus* ATCC 53718.

Optimum temperature range for growth	21~37°C
Coagulation and peptonisation of milk	+
Melanin production in Tryptone-yeast extract broth	−
H_2S production on peptone yeast extract-iron agar	+
Liquefaction of gelatin	+
Nitrate reduction in organic nitrate or glucose nitrate broths	−
Glucose, arabinose, fructose, inositol, mannitol, raffinose, rhamnose, sucrose and xylose utilisation	+
Decomposition of cellulose	−

Cell wall analysis for amino acids and sugars was performed as described by BECKER *et al.*[17] and LECHEVALIER[18]. LL-Diaminopimelic acid was detected, thus classifying the cell wall as type I.

On the basis of the data presented above and in accordance with TRESNER and BACKUS[19], the culture was considered to be a new strain of *Streptomyces hygroscopicus* (Jensen) Waksman and Henrici, and deposited at the American Type Culture Collection under the accession number ATCC 53718.

Fermentation

Mycelial preparations of *S. hygroscopicus* ATCC 53718 (2 ml) stored at −70°C in 20% w/v aqueous glycerol were thawed and used to inoculate sterile seed medium (50 ml) containing glucose 0.1%, starch 2.4%, peptone 0.5%, yeast extract 0.5%, Lab. Lemco (Oxoid Ltd.) 0.3% and calcium carbonate 0.4% contained in 300 ml Erlenmeyer flasks. These were cultured with shaking at 28°C for 1 day after which aliquots (40 ml) were withdrawn and used to inoculate 2 × 3-litre Fernbach flasks each containing 700 ml of the same medium. These were incubated as above for one day, combined and used to inoculate a

Fig. 1. HPLC conditions for the analysis of fermentation broth extracts.

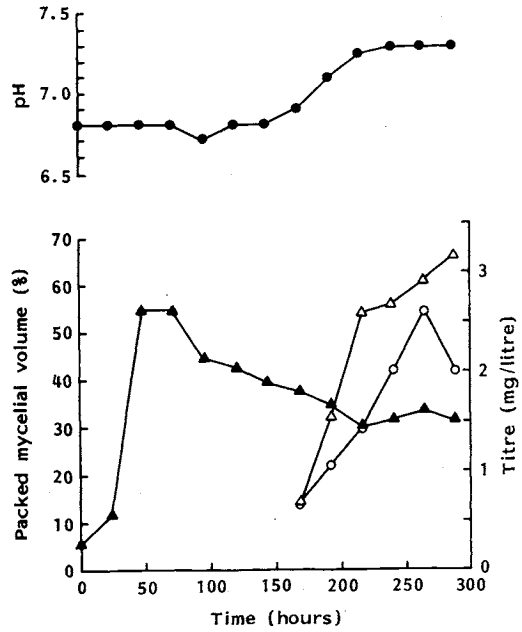
Sample preparation:	Acetone-methylene chloride extracts of mycelia were concentrated to dryness and redissolved in methanol prior to HPLC analysis
System:	Hewlett Packard 1090A with diode array detection
Column:	Beckman Ultrasphere C-18 (5 μm) (4.6 × 250 mm)
Mobile phase:	Methanol-water Linear gradient from 80:20 to 95:5 over 40 minutes
Detection:	UV 243 nm Spectra of the milbemycins were recorded on the upslope, apex and downslope of peaks over the UV range 210~300 nm
Flow:	0.85 ml/minute
Temperature:	40°C

Table 3. Effect of culture pH on production.

Initial pH	Presence of MOPS (2% w/v)	Final culture pH (10 days)	Titre of milbemycin complex (mg/litre)
6.50	−	7.65	6
6.50	+	7.00	14
6.75	+	6.90	21
7.00	+	7.20	8

Fig. 2. Fermentation profile of *Streptomyces hygroscopicus* ATCC 53718.

▲ Packed mycelial volume, ▼ UK-78,629+UK-80,694 (titre), ◇ UK-78,624 (titre), ● pH.



100-litre New Brunswick fermenter containing 70 litres of production medium consising of starch 1%, soyabean meal 1.25%, cotton seed oil 0.84%, $Na_2HPO_4 \cdot 12H_2O$ 0.3%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.02%, $FeSO_4 \cdot 7H_2O$ 0.002%, $CoCl_2 \cdot 6H_2O$ 0.001% and 3-(*N*-morpholino)propane sulfonic acid (MOPS) 2%.

The medium was adjusted before autoclaving to pH 6.5~6.7. The fermenter was run for 12 days using an agitation speed of 200 rpm and an air flow rate of 35 litres per minute. N787-182 complex formation was monitored by analysis of mycelial solvent extracts as shown in Fig. 1. The fermentation profile is shown in Fig. 2.

Early results indicated that production was significantly increased if a stable, neutral pH could be maintained. To achieve this, the inorganic non-metabolizable buffer MOPS was added to the production medium at a level of 2% (Table 3). Titre of the required compounds was low (<2 mg/litre for individual components). Medium optimisation yielded only modest improvements (3-fold), but culture improvement studies using single colony isolation and chemical mutation techniques employing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)[20] increased titres by a further 10~20-fold (Table 4). Investigations in the scale-up of a fermentation to produce larger quantities of the N787-182 complex will be reported at a later stage.

Isolation and Purification of the N787-182 Complex

The procedure for the isolation of this series of milbemycins is outlined in Fig. 3. The whole broth (70 litres) was filtered and the mycelial cake extracted with 2 × 50-litre volumes of acetone, concentrated

Table 4. Culture improvement of *Streptomyces hygroscopicus* ATCC 53718.

	Medium[b]	Potency (mg/litre)[a]	
		UK-78,624	UK-78,629+ UK-80,694
Parent culture	1	0.6	1
Parent culture	2	1.9	2.5
Single colony isolate M30	2	6	8
NTG mutant of M30	2	17	36

[a] The HPLC system shown does not resolve these two components. Results shown are for shake flask studies 10 days harvest.

[b] Medium 1 as in text, medium 2 is as follows: Starch 1%, soyabean meal 0.625%, cotton seed oil 0.84%, $Na_2HPO_4 \cdot 12H_2O$ 0.15%, KH_2PO_4 0.025%, $MgSO_4 \cdot 7H_2O$ 0.02%, $FeSO_4 \cdot 7H_2O$ 0.002%, $CoCl_2 \cdot 6H_2O$ 0.001%, MOPS 2%, pH 6.5~6.7.

Fig. 3. Isolation of milbemycin complex.

Mycelium
- (from 70 litres broth)
- 2 × 50 litres acetone
- concentrated

Aqueous suspension
- 3 × 10 litres ethyl acetate
- concentrated

Oil
- silica gel
- eluted with
  - (i) dichloromethane - ethyl acetate (4 : 1)
  - (ii) dichloromethane - ethyl acetate (1 : 1)
  - (iii) ethyl acetate

Fractions
- concentrated

Analysed by HPLC

Table 5. Chromatographic properties of individual components.

Compound	HPLC retention time[a] (minutes)	TLC[b] (Rf)
UK-78,618	6.0	0.07
UK-78,621	6.6	0.1
UK-78,614	8.5	0.095
UK-78,624	9.7	0.096
UK-78,629	12.4	0.30
UK-80,694	12.5	0.23
UK-78,622	15.5	0.38
UK-77,021	16.1	0.17
UK-79,465	18.1	0.4
UK-78,623	19.5	0.40
UK-80,695	23.5	0.48
UK-78,630	28.5	0.60

[a] See Fig. 1 for conditions.

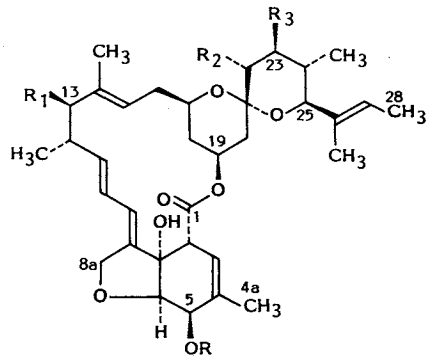
[b] Merck Kieselgel 60 F254; dichloromethane - ethyl acetate (4 : 1).

to an aqueous suspension and then further extracted with 3 × 10-litre volumes of ethyl acetate. This was concentrated to an oily residue which was chromatographed on silica gel (Kieselgel 60, 230~400 mesh, Merck). The silica was initially eluted with a 4 : 1 mixture of dichloromethane and ethyl acetate followed by a 1 : 1 mixture of the same solvents, and finally with ethyl acetate. Compounds eluted in an order consistent with the number of hydroxyl groups in the molecule. Fractions were analysed by TLC and analytical HPLC (see Table 5). Similar fractions were combined, evaporated under vacuum and the individual compounds were isolated in a pure state by semi-preparative reversed phase HPLC.

Structure Elucidation of the N787-182 Complex

The close structural relationship between the N787-182 compounds and previously described milbemycins was immediately apparent from a comparison of UV, NMR (Table 6) and mass spectroscopic data. The structure of UK-78,629, a major component of the complex, was confirmed by a combination of 2D ^1H COSY, ^1H-^{13}C COSY and DEPT NMR experiments. A particularly close correspondence was

Fig. 4. Structures of members of the N787-182 complex.



Compound	R	R_1	R_2	R_3	Mass spectroscopy	
					EI or ACE M^+	FAB $(M+Na)^+$
UK-80,695	H	$OCOCHMe_2$	H	H	654	677
UK-78,630	Me	$OCOCHMe_2$	H	H	668	
UK-78,618	Me	OH	OH	H		637
UK-78,624	H	$OCOCHMe_2$	OH	H	670	
UK-78,629	Me	$OCOCHMe_2$	OH	H	684	707
UK-77,021	H	H	OH	$OCOCHMe_2$	670	693
UK-78,623	Me	H	OH	$OCOCHMe_2$	684	707
UK-78,621	H	OH	OH	$OCOCHMe_2$		709
UK-78,614	Me	OH	OH	$OCOCHMe_2$		723
UK-80,694	H	$OCOCHMe_2$	OH	$OCOCHMe_2$		779
UK-78,622	Me	$OCOCHMe_2$	OH	$OCOCHMe_2$	770	
UK-79,465	Me	OCOCHMeEt	OH	$OCOCHMe_2$	784	

Explanation of abbreviations; EI: electron impact, ACE: alternating chemical and electron impact ionisation, FAB: fast atom bombardment.

seen between the ^{13}C NMR chemical shifts of UK-78,629 and the published ^{13}C NMR data for LL-F28249γ[6] and VM 44864[8] (Table 7). This supports the conclusion that all three compounds share the identical C-1 to C-28 carbon skeleton (Fig. 5). Unique features of the ^{13}C NMR spectrum of UK-78,629 include an additional four carbon resonances which, together with the chemical shifts of the attached protons (Table 6), was identified as an isobutyroyl group. The chemical shift of the C-13 methine carbon, 83.5 ppm, strongly supports the proposed site of attachment of this ester group. In the ^1H NMR spectrum the C-13 proton appears as a sharp doublet at 4.93 ppm ($J_{H12,13}=11$ Hz) which is consistent with the substituent being attached with β-stereochemistry[21]. In compounds with an oxygen substituent attached at C-13 with α-stereochemistry, such as the avermectins, the C-13 proton appears as a broad singlet[22].

UK-78,629 is also characterised by the presence of a hydroxyl group at C-22, a feature also found in VM 44864. The stereochemistry at C-22 in both compounds is identical as attested by the appearance of the signal of δ 1.4 which was unambiguously assigned to the C-23 axial proton by joint application of ^1H-^1H and ^1H-^{13}C correlation spectroscopy. This signal, a characteristic quartet ($^3J_{22ax\sim23ax}=^3J_{23ax\sim23eq}=^3J_{23ax\sim24ax}=12$ Hz), can only arise if the flanking substituents at C-22 and C-24 are equatorial (Fig. 6).

The geometry about the C-26, 27 double bond has not been firmly established but is assumed to

Table 6. ^{1}H NMR data for representative N787-182 compounds[a].

Assignment[b]	UK-78,614	UK-78,622	UK-78,629	UK-78,630	UK-79,465	UK-77,021
2	3.32 (q)	3.32 (q, J=2.3)	3.33 (q, J=2.5)	3.32 (q)	3.32 (q, J=2.3)	3.27 (sextet, J=2.3)
3	5.4 (br s)	5.40 (m)	5.4	5.4	5.4 (m)	5.42 (br s)
4a	1.82 (br s)	1.82 (br s)	1.85 (br s)	1.84 (br s)	1.82 (br s)	1.88 (br s)
5	3.97 (d)	3.96 (d, J=5.3)	3.98 (d, J=6),	3.98 (d)	3.96 (d, J=5.6)	4.29 (br t, J=6)
5-OCH_3	3.50 (s)	3.50 (s)	3.52 (s)	3.52 (s)	3.50 (s)	—
6	4.02 (d)	4.02 (d, J=5.5)	4.05 (d, J=6)	4.04 (d)	4.02 (d, J=5.6)	3.95 (d, J=6.2)
8a	4.64 (d)	4.63 (dd, J=14.5, 2.3)	4.65 (dd, J=14, 2)	4.65 (d)	4.63 (dd, J=14.6, 2.2)	4.68 (m, 2H)
	4.70 (d)	4.69 (dd, J=14.5, 2.3)	4.73 (dd, J=14, 2)	4.74 (d)	4.69 (dd, J=14.6, 2.2)	
9	5.70 (d)	5.72 (dt, J=11.3, 2.3)	5.75 (dt, J=11, 2)	5.75 (d)	5.73 (dt, J=11.4, 2.2)	5.76 (m)[d]
10	5.80 (dd)	5.83 (dd, J=14.6, 11.4)	5.85 (dd, J=14, 11)	5.85 (dd)	5.83 (dd, J=14.6, 11.3)	5.73 (t, J=10.3)
11	5.25 (m)	5.30 (dd, J=14.9, 10.1)	5.35 (m)	5.35 (m)	5.3 (m)	5.3 (m)
12	2.6 (m)	2.57 (m)	2.60 (m)	2.58 (m)	2.56 (m)	2.43 (m)
12a	1.15 (d)	0.99 (d, J=6.6)	1.02 (d, J=6.3)	1.02 (d)	1.00 (d, J=6.5)	1.00 (d, J=6.7)
13a	3.70 (d)	4.91 (d, J=10.5)	4.93 (d, J=11)	4.95 (d)	4.93 (d, J=10.5)	2.2 (m)
13b	—	—	—	—	—	1.9 (m)
14a	1.60 (s)	1.59 (s)[c]	1.59 (s)[c]	1.60 (s)[c]	1.59 (s)[c]	1.54 (s)
15	5.25 (m)	5.37 (m)	5.35 (m)	5.35 (m)	5.37 (m)	4.97 (m)
16a, b	2.25 (m, 2H)	2.25 (m),	2.27 (m),	2.27 (m),	2.25 (m),	2.25 (m, 2H)
		2.32 (m)	2.33 (m)	2.35 (m)	2.32 (m)	
17	3.60 (m)	3.58 (m)	3.62 (m)	3.56 (m)	3.58 (m)	3.61 (m)
18ax	0.95 (q)	0.93 (q, J=12.2)	0.93 (q, J=12)	0.90 (q)	0.93 (q, J=12.2)	0.90 (q, J=12.4)
18eq	1.85[d]	1.85[d]	1.7	1.85[d]	1.85[d]	1.85 (m)[d]
19	5.25 (m)	5.28 (m)	5.35 (m)	5.35 (m)	5.28 (m)	5.3 (m)
20ax	~1.9[d]	1.92 (m, 2H)	1.92 (m, 2H)	1.36 (t)	1.92 (m, 2H)	~1.8[d]

20eq				2.06 (dd)		~1.9[d]
22ax	3.21 (t)	3.20 (dd, J=11.6, 9.5)	3.35	~1.6[d]	3.20 (dd, J=11.5, 9.5)	3.21 (dd, J=11.2, 9.6)
22eq	—	—	—	~1.6[d]	—	—
23ax	4.94 (t)	4.91 (t, J=10.0)	1.42 (q, J=12)	~1.6[d]	4.91 (t, J=10.0)	4.93 (dd, J=10.6, 9.5)
23eq	—	—	1.87[d]	~1.6[d]	—	—
24	~1.7[d]	~1.7[d]	1.72	~1.55[d]	~1.7[d]	~1.7[d]
24a	0.69 (d)	0.68 (d, J=6.6)	0.72 (d, J=6)	0.70 (br s)	0.68 (d, J=6.6)	0.68 (d, J=6.6)
25	3.58 (d)	3.54 (d, J=10.4)	3.38 (d, J=11)	3.44 (d)	3.54 (d, J=10.5)	3.58 (d, J=10.4)
26a	1.60 (s)	1.55 (s)[c]	1.57 (s)[c]	1.57 (s)[c]	1.57 (s)[c]	1.60 (s)
27	5.45 (q)	5.43 (dq, J=1.2, 6.6)	5.4	5.4	5.43 (q, J=6.8)	5.45 (dq, J=1.2, 6.7)
28	1.67 (d)	1.66 (d, J=6.6)	1.67 (d, J=6)	1.68 (d)	1.67 (d, J=6.6)	1.66 (dd, J=6.7, 1)
2′	2.61 (heptet)	2.60 (heptet, J=7)	—	—	2.60 (heptet, J=7.0)	2.61 (heptet, J=7)
2′a	1.20 (d)	1.20 (d, J=7)	—	—	1.20 (d, J=7)	1.20 (d, J=6.95)
3′	1.20 (d)	1.19 (d, J=6.8)	—	—	1.19 (d, J=6.9)	1.20 (d, J=7)
2″	—	2.55 (heptet, J=6.8)	2.57 (heptet, J=6.8)	2.57 (heptet)	2.38 (m)	—
2″a	—	1.18 (d, J=6.5)	1.17 (d, J=6.6)	1.20 (d)	1.14 (d, J=7.0)	—
3″	—	1.16 (d, J=6.5)	1.19 (d, J=6.6)	1.18 (d)	~1.5[d]	—
4″	—				0.89 (t, J=7.2)	—
5-OH[e]	—	—	—	—	—	2.33 (br d, J=7.9)
7-OH[e]	3.9 (s)	3.88 (s)		4.05 (s)		3.89 (s)
22-OH[e]				—		1.19 (d, J=8.6)

[a] Spectra were recorded in $CDCl_3$ at either 300 or 500 MHz; chemical shifts are given in ppm relative to residual $CHCl_3$ at 7.26 ppm, multiplicities and observed coupling constants in Hz are given in parentheses.

[b] Numbers marked with single prime (2′) refer to the substituent at C-23, numbers marked with double prime (2″) refer to the C-13 substituent.

[c] Assignments may be interchanged.

[d] Approximate chemical shift of incompletely resolved or obscured signal.

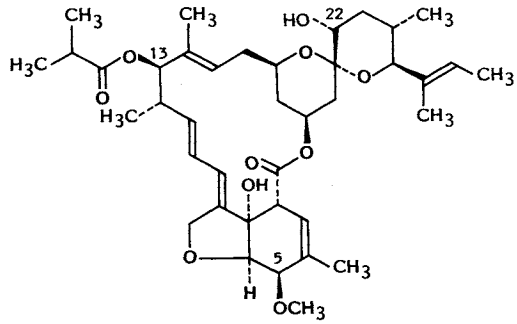
[e] Hydroxyl protons are not always apparent in the spectra.

Table 7. ^{13}C NMR assignments for UK-78,629, LL-F28249γ and VM 44864.

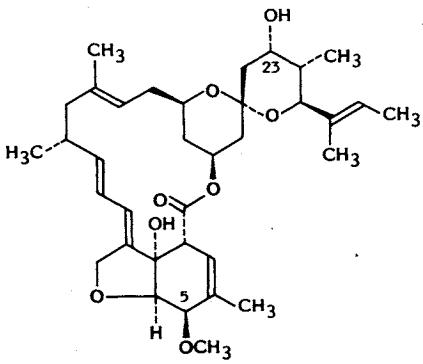
Carbon atom assignment[d]	δ (ppm)[a]			Carbon atom assignment[d]	δ (ppm)[a]		
	UK-78,629	LL-F282498γ[b]	VM 44864[c]		UK-78,629	LL-F282498γ[b]	VM 44864[c]
1	173.9	173.5	173.8	16	34.6	34.7	34.6
2	45.7	45.5	45.6	17	67.6	68.5	67.9
3	118.6	118.4	118.4	18	36.5	35.9	36.3
4	135.9	137.3	135.8	19	68.7	67.7	68.6
4a	20.1	19.8	19.8	20	37.1	40.6	36.4
5	77.0	76.8	76.9	21	99.0	99.7	98.8
5-OMe	58.0	57.6	57.7	22	71.7	40.9	71.5
6	77.8	77.5	77.5	23	36.6	69.2	36.8
7	80.6	80.3	80.3	24	32.2	35.85	32.0
8	141.3	139.6	139.7	24a	17.6	13.7	17.4
8a	68.4	68.1	68.2	25	82.2	76.6	81.8
9	119.4	119.5	119.4	26	134.1	133.9	134.0
10	124.8	123.4	123.47	26a	11.1	10.7	10.9
11	137.6	142.3	142.3	27	125.9	123.7	123.53
12	34.5	35.81	35.9	28	13.3	13.1	13.1
12a	18.8	22.2	22.3	1″	176.4	—	—
13	83.5	48.4	48.5	2″	40.1	—	—
14	136.2	135.8	137.2	3″	19.2	—	—
14a	11.1	15.4	15.5	3″a	19.2	—	—
15	124.1	120.2	120.5				

[a] Spectra recorded in $CDCl_3$ solution, δ values in ppm downfield from TMS.
[b] Data from reference 6.
[c] Data from reference 8.
[d] Numbers marked with a double prime refer to the substituent at C-13.

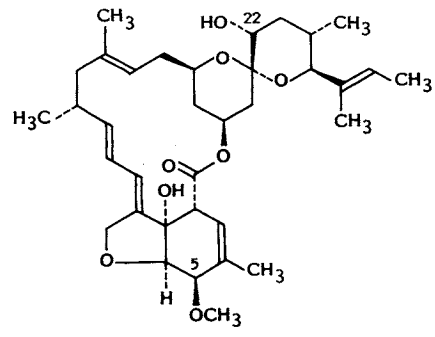
Fig. 5. Structures of UK-78,629, LL-F28249γ and VM 44864.



UK-78,629

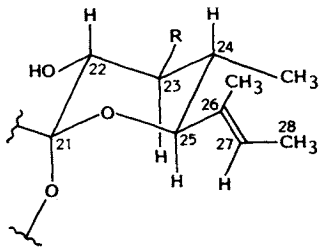


LL-F28249γ



VM 44864

Fig. 6. Stereochemistry of C-21 to C-28 region.



UK-78,629 R=H
UK-77,021 R=OCOCH(CH$_3$)$_2$

Table 8. Principal high mass fragment ions in electron-impact mass spectra.

Compound	Fragment ions m/z
UK-78,623	684[a], 666, 634, 542, 524, 151, 125
UK-78,629	684[a], 666, 596[b], 578, 524, 151, 125
UK-78,622	770[a], 682[b], 664, 540, 522, 151, 125
UK-79,465	784[a], 682[a], 664, 540, 522, 151, 125

[a] Molecular ion M^+.
[b] $(M-RCO_2H)^+$.

equate with that found in the related natural products from the closely comparable spectroscopic features.

The structure of UK-78,629 having thus been established the structures of the closely related members of the complex could be determined by spectroscopic comparison. The compounds are found to differ from one another in the nature of the substituents at C-5, C-13, C-22 and C-23. With the exception of UK-80,695 and UK-78,630 all of the compounds possess an equatorial hydroxyl substituent at C-22. Many of the compounds such as UK-77,021 are characterised by isobutyroyl group attached at C-23. In these compounds the C-23 methine proton appears downfield as a sharp triplet ($^3J_{22ax\sim23ax}=^3J_{23ax\sim24ax}=$ 10 Hz) confirming the equatorial nature of the substituents at C-22, C-23 and C-24[23]. Compounds in which the C-5 hydroxy group is replaced by a methoxy group are readily recognised by the appearance of a new singlet in the ^{1}H NMR spectrum at $\delta\sim3.5$ and concomitant shifts in the position of the C-5 and C-6 methine protons.

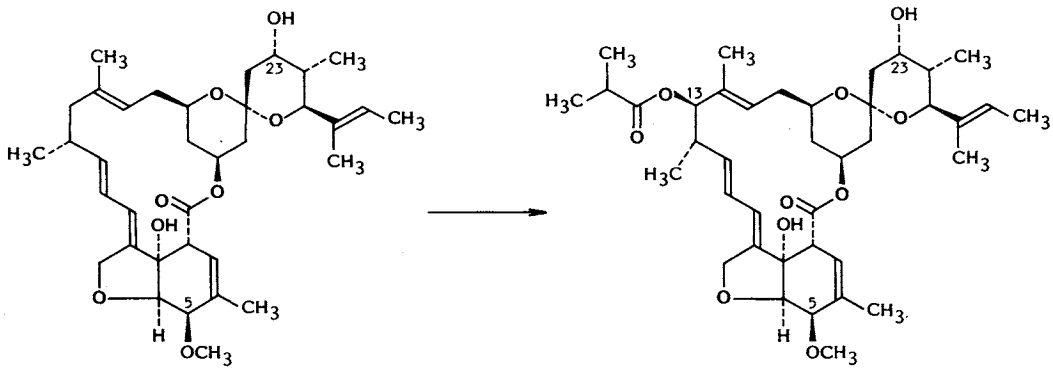
Compounds UK-78,618, UK-78,621 and UK-78,614 were characterised by a doublet at $\delta\sim3.7$ ($J=10$ Hz) in the ^{1}H NMR spectrum assigned to the C-13 methine proton. This is consistent with the presence of a hydroxyl substituent with β-stereochemistry at C-13. Compounds with this functionality prepared semisynthetically give rise to a very similar resonance in the ^{1}H NMR spectrum[21]. Coupling between the C-13 methine proton and the hydroxyl proton is not routinely observed.

Preparative HPLC purification of fractions rich in UK-78,622 (molecular weight 770) yielded a small amount of the compound UK-79,465. Mass spectroscopy gave a molecular weight of 784 for this compound, the highest yet found for a milbemycin, corresponding to the formula $C_{44}H_{64}O_{12}$. ^{1}H NMR spectroscopy clearly indicated that UK-79,465 differed from UK-78,622 at one of the two isobutyryl ester groups by the addition of a methylene unit to give a 2-methylbutyrate group. The question as to whether this ester group was attached at C-13 or C-23 was most convincingly resolved by a comparison of the EI mass spectra of the four compounds UK-78,623, UK-78,629, UK-78,622 and UK-79,465 (Table 8). Only the latter three compounds give a fragment ion corresponding to the loss of a carboxylic acid. The conclusion drawn from this experiment is that the elimination of the ester group (MacLafferty rearrangement) at C-13 is a much more facile process than at C-23. Since both UK-78,622 and UK-79,465 give rise to an ion of mass 682 resulting from the loss of isobutyric acid and 2-methylbutyric acid, respectively, their difference lies in the nature of the C-13 substituent.

Biosynthetic Origin of the C-13 Oxygenation

It has been shown by feeding experiments with labelled precursors that the oxygen atom at C-13 in

Fig. 7. Biotransformation of LL-F28249γ by *Streptomyces hygroscopicus* ATCC 53718.



the avermectins, possessing α-stereochemistry, is derived from a propionate residue inserted during the course of polyketide biosynthesis[24]. By contrast, we suspected that the C-13 oxygen atom in the N787-182 complex which exhibits β-stereochemistry was derived oxidatively from 13-unsubstituted intermediates. We addressed this hypothesis by feeding a known milbemycin, not produced by our organism, to a growing culture of *S. hygroscopicus* ATCC 53718.

The milbemycin LL-F28249γ[5] was added to a flask culture of *S. hygroscopicus* ATCC 53718 fermented under the previously described conditions. LL-F28249γ (0.3 mg) was dissolved in methanol (0.3 ml) and added aseptically to the culture 72 hours after inoculation. Fermentation was continued under standard conditions and the flask contents were harvested and analysed after a further 7 days incubation. Under these conditions this compound was cleanly converted to its 13β-isobutyryloxy derivative. This novel biotransformation product was chromatographically distinct from the compounds naturally produced by the organism ATCC 53718, and all its spectroscopic characteristics were consistent with the structure proposed (Fig. 7).

Biological Properties

Anthelmintic activity was evaluated against *Caenorhabditis elegans* using an *in vitro* screen[25]. Insecticidal activity was evaluated against larval stages of the blowfly *Lucilia cuprina* (Q strain). Filter papers were treated with the test compound which was applied as an acetone solution. Treated filter papers were placed in tubes containing 1 ml of calf serum and first instars were added. Tubes were assessed for mortality after 2 days.

Compounds UK-78,624, UK-80,694 and UK-80,695 all showed potent *in vitro* nematicidal activity with a minimum of 95% activity at 0.01 ppm against *C. elegans* and full activity at 1 mg/m^2 against *Lucilia*.

Acknowledgements

The authors wish to thank their colleagues in the Animal Health Discovery and Discovery Spectroscopy Departments for their invaluable assistance in carrying out this work. In particular thanks are due to B. E. HOILE, J. H. CACKETT and K. C. HOW (Fermentation), G. B. T. GOODWIN and J. HORSMAN (Recovery), D. V. BOWEN, T. LEE, M. J. NEWMAN, F. S. PULLEN, J. M. SUGDEN and A. G. SWANSON (Spectroscopy).

References

1) MISHIMA, H.; M. KURABAYASHI, C. TAMURA, S. SATO, H. KUWANO, A. SAITOH & A. AOKI: Structures of milbemycins

α_1, α_2, α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_{10} and β_1. Abstract Papers 18th Symp. Chem. Natural Products, p. 309, Kyoto, Oct. 17~19, 1974

2) TAKIGUCHI, Y.; H. MISHIMA, M. OKUDA, M. TERAO, A. AOKI & R. FUKUDA: Milbemycins, a new family of macrolide antibiotics: Fermentation, isolation and physico-chemical properties. J. Antibiotics 33: 1120~1127, 1980

3) TAKIGUCHI, Y.; M. ONO, S. MURAMATSU, J. IDE, H. MISHIMA & M. TERAO: Milbemycins, a new family of macrolide antibiotics. Fermentation, isolation and physico-chemical properties of milbemycins D, E, F, G and H. J. Antibiotics 36: 502~508, 1983

4) MISHIMA, H.; J. IDE, S. MURAMATSU & M. ONO: Milbemycins, a new family of macrolide antibiotics. Structure determination of milbemycins D, E, F, G, H, J and K. J. Antibiotics 36: 980~990, 1983

5) CARTER, G. T.; J. A. NIETSCHE & D. B. BORDERS: Structure determination of LL-F28249 α, β, γ and λ, potent antiparasitic macrolides from *Streptomyces cyaneogriseus* spp. *noncyanogenus*. J. Chem. Soc. Chem. Commun. 1987: 402~404, 1987

6) 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, characterization and structures of LL-F28249 α, β, γ, λ, J. Antibiotics 41: 519~529, 1988

7) 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

8) 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, characterization, structure elucidation and solution conformations. J. Antibiotics 43: 1069~1076, 1990

9) POOLE, N. J.; P. HENDLEY, M. W. SKIDMORE & R. S. I. JOSEPH (I. C. I. plc.): Pesticidal & anthelmintic milbemycins. UK Pat. Appl. 2,170,499, Aug. 6, 1986

10) DYBAS, R. A.; N. J. HILTON, M. H. FISHER, H. MROZIK, F. A. PREISER, P. ESKOLA, T. L. SHIH & G. J. DOLCE: Novel second-generation avermectin insecticides and miticides for crop protection. *In* Novel Microbial Products for Medicine and Agriculture. *Eds.*, A. L. DEMAIN *et al.*, pp. 203~212, Elsevier, 1989

11) FISHER, M. H. (Merck): Derivative of antibiotic substance milbemycin and processes therefore. U.S. 4, 093, 629, June 6, 1978

12) SATO, K.; T. YANAI, T. KINOTO & S. MIO (Sankyo): Process for preparing milbemycin derivatives and certain novel compounds employed therein. Eur. Pat. Appl. 184, 308, Jan. 11, 1986

13) RAMOS, G.; O. GHISALBA, H.-O. SCHAR, B. FREI, P. MAIENFISCH & A. C. O' SULLIVAN (Chiba-Geigy): Mikrobielles Verfahren zur Herstellung von Milbemycin-Derivaten. Eur. Pat. Appl. 277,916, Aug. 10, 1988

14) PERRY, D. A.; M. A. HAXELL, H. MAEDA & J. TONE (Pfizer): Antiparasitic macrolide antibiotics. Eur. Pat. Appl. 334, 484, Sept. 27, 1989

15) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966

16) Colour Harmony Manual. 4th Edition. Container Corporation of America, 1958

17) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964

18) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968

19) TRESNER, H. D. & E. J. BACKUS: A broadened concept of the characteristics of *Streptomyces hygroscopicus*. Appl. Microbiol. 4: 243~250, 1956

20) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic Manipulation of *Streptomyces*. A Laboratory Manual. The John Innes Foundation.

21) FREI, B. & H. B. MEREYALA (Chiba-Geigy): 13-Beta-Zuckerderivate von Milbemycinen, deren Herstellung und Verwendung gegen Ekto-und Endoparasiten am Nutztier oder ander Nutzpflanze. Eur. Pat. Appl. 235, 085, Sept. 2, 1987

22) ALBERS-SCHÖNBERG, G.; B. H. ARISON, J. C. CHABALA, A. W. DOUGLAS, P. ESKOLA, M. H. FISHER, A. LUSI, H. MROZIK, J. L. SMITH & R. L. TOLMAN: Avermectins. Structure determination. J. Am. Chem. Soc. 103: 4216~4221, 1981

23) BLANCHFLOWER, S. E.; R. J. J. DORGAN, J. R. EVERETT & S. A. READSHAW. An investigation of the stereochemistry of a 22, 23-dioxygenated milbemycin. Tetrahedron Lett. 29: 6645~6648, 1988

24) CANE, D. E.; T-C. LIANG, L. KAPLAN, M. K. NALLIN, M. D. SCHULMAN, O. D. HENSENS, A. W. DOUGLAS & G. ALBERS-SCHÖNBERG: Biosynthetic origin of the carbon skeleton and oxygen atoms of the avermectins. J. Am. Chem. Soc. 105: 4110~4112, 1983

25) SIMPKIN, K. G. & G. L. COLES: A new *in vitro* test for anthelmintics. Parasitology 79: xix, 1979
26) GORDON, R. E. & M. M. SMITH: Proposed group of characters for the separation of Strepromyces and Nocardia. J. Bacteriol. 69: 147~150, 1955
27) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Description of Genera and Species. Williams and Wilkins Co., 1961