LL-F28249 ANTIBIOTIC COMPLEX: A NEW FAMILY OF ANTIPARASITIC MACROCYCLIC LACTONES

ISOLATION, CHARACTERIZATION AND STRUCTURES OF LL-F28249 $\alpha, \beta, \gamma, \lambda$ †

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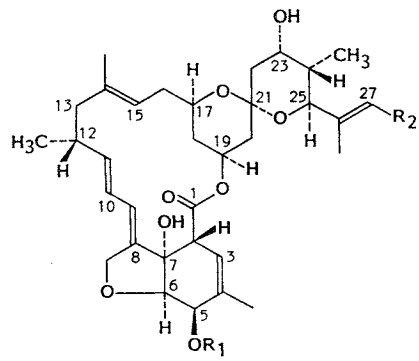
A new family of antiparasitic macrolides has been isolated from *Streptomyces cyaneogriseus* sp. *noncyanogenus*. The compounds, designated LL-F28249 α, β, γ and λ, possess potent antiparasitic activity. The isolation, purification and structure determination by spectroscopic methods are presented.

Culture LL-F28249, identified as *Streptomyces cyaneogriseus* sp. *noncyanogenus*, produces a new family of antiparasitic macrocyclic lactones. Characteristics of the producing organism and conditions for its fermentation were described in a preceding publication.[1] We previously reported the structures of the major bioactive compounds derived from the culture, LL-F28249 α, β, γ and λ (**1**~**4**) in a brief communication,[2] which included an X-ray structure determination of γ (**3**). These compounds contain the macrocycle, the fused cyclohexene - tetrahydrofuran ring system, and bicyclic 6,6-spiroketal found in milbemycins[3] and avermectins.[4]

Unique unsaturated side chains extending from position 25 are the structural features which differentiate **1**~**4** from milbemycins and the aglycone portion of avermectins. These extended unsaturated side chains are manifestations of a fundamental difference in the biosynthesis of the LL-F28249 compounds. Our experiments[5] have shown that the polyketide precursor of **1** is composed of 14 acyl units as opposed to the precursors of the milbemycins[6] and avermectins[7] which contain only 13 acyl units. The additional unit of the LL-F28249 precursor becomes the terminal portion of the characteristic side chains.

LL-F28249 α (**1**), the main component, controls experimental infections of *Trichostrongylus colubriformis* in the gerbil with single oral doses of 0.2 mg/kg. Antiparasitic efficacy will be the subject of a separate report.

In this paper are described the isolation, chromatographic purification and structure determination of the four major LL-F28249 com-



α (**1**) $R_1 = H$ $R_2 = CH(CH_3)_2$
β (**2**) $R_1 = H$ $R_2 = CH_3$
γ (**3**) $R_1 = CH_3$ $R_2 = CH_3$
λ (**4**) $R_1 = CH_3$ $R_2 = CH(CH_3)_2$

† The name nemadectin has recently been approved for LL-F28249α by the USAN Council.

ponents α (**1**), β (**2**), γ (**3**) and λ (**4**).

Materials and Methods

Fermentation

As described in a preceding publication,[1] inoculum was prepared from frozen vegetative growth maintained at −80°C. The seed medium employed (g/liter) glucose 10, dextrin 20, yeast extract 5, NZ-Amine A 5, and $CaCO_3$ 1. Three successive stages of 24 hours incubation at 28°C were used to prepare 300 liters of inoculum for the production tank. The inoculum was introduced into 3,000 liters of the sterilized production medium consisting of (g/liter) glucose 50, cottonseed flour (Pharmamedia) 25 and $CaCO_3$ 7. The tank was maintained at 30°C, agitation was provided by an impeller driven at 120 rpm, and aeration was supplied at 1,950 liters of air per minute. At 128 hours the titer of LL-F28249 α had risen to 575 μg/ml by HPLC analysis and the tank was harvested for processing.

Processing

The fermentation broth (3,000 liters) was stirred with toluene (30 liters) for 30 minutes to kill the cells. Diatomaceous earth (Celite 512, 50 kg) was added with agitation followed by filtration. The resulting cake was washed with H_2O (100 liters) and both filtrate and wash were discarded. MeOH (1,500 liters) was used to extract the washed cake. The MeOH extract was concentrated to approximately 200 liters under reduced pressure and the resulting concentrate was mixed with an equal volume of methylene chloride. The methylene chloride phase was separated and concentrated under reduced pressure to 1.5 liters.

Chromatographic Purification

A portion of the crude methylene chloride concentrate (100 ml) was chromatographed on silica gel (600 g, 55~105 μm) eluted with EtOAc - hexane (1 : 9). The flow rate was 200 ml/minute and fractions were collected at 2 minute intervals. On the basis of HPLC analysis, fractions 30~50 were combined to give 8.2 g of material (designated preparation I) upon evaporation of the solvents. Similarly fractions 58~80 were combined yielding preparation II (15.8 g).

Preparation I (8.0 g) was subjected to reversed-phase chromatography on C_{18} bonded-phase silica (800 g, 55~105 μm) eluted with MeOH - water (3 : 1). The flow rate was 150 ml/minute and fractions were collected at 2.5 minute intervals. Fractions 37~46 were combined, concentrated under reduced pressure, and the residual aqueous solution was extracted with methylene chloride. Evaporation of the methylene chloride gave a colorless residue which was redissolved in *tert*-butanol and freeze-dried yielding LL-F28249 γ (**3**, 2.2 g), as a white fluffy solid. Fractions 72~81 were combined and processed in the same manner to give LL-F28249 λ (**4**, 1.4 g).

Preparation II was chromatographed using the same reversed-phase system. Combining fractions 21~26 yielded LL-F28249 β (**2**, 0.17 g) and fractions 32~54 gave LL-F28249 α (**1**, 11.1 g).

Analytical HPLC

Reversed-phase HPLC was used to monitor fermentation titers and to guide the processing and chromatographic purification steps. The system consisted of a C_{18} bonded-phase column (5 μm, 2.1 × 100 mm) developed with 73% MeOH - water at 0.8 ml/minute. Detection was by UV absorbance at 244 nm. A typical chromatogram of the crude extract of the culture is shown in Fig. 1.

Instrumental

^1H and ^{13}C NMR spectra were recorded on either a Nicolet NT300WB or General Electric QE300 spectrometer. High resolution (HR) mass spectral data were obtained with a VG Analytical ZAB-SE instrument.

Results and Discussion

The isolation and purification scheme used to obtain LL-F28249 α, β, γ and λ (**1**~**4**) is outlined

Fig. 1. Typical HPLC chromatogram of LL-F28249 crude extract.

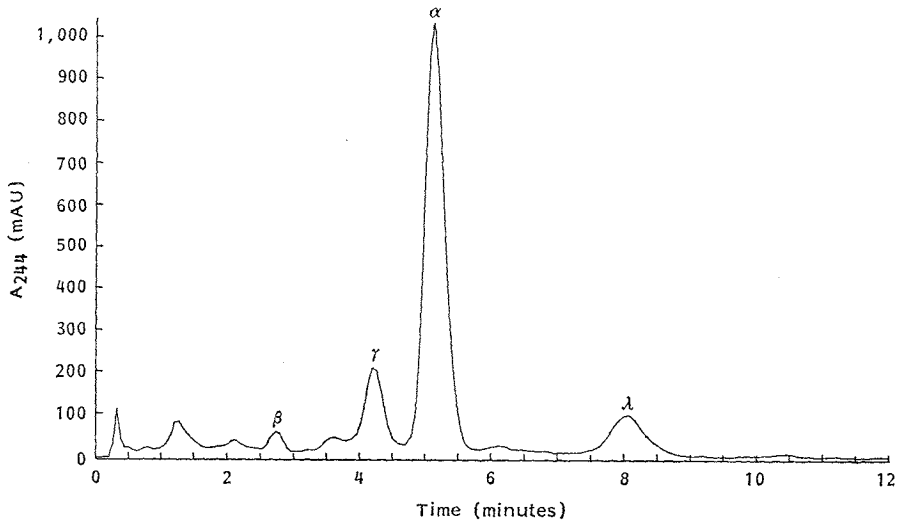
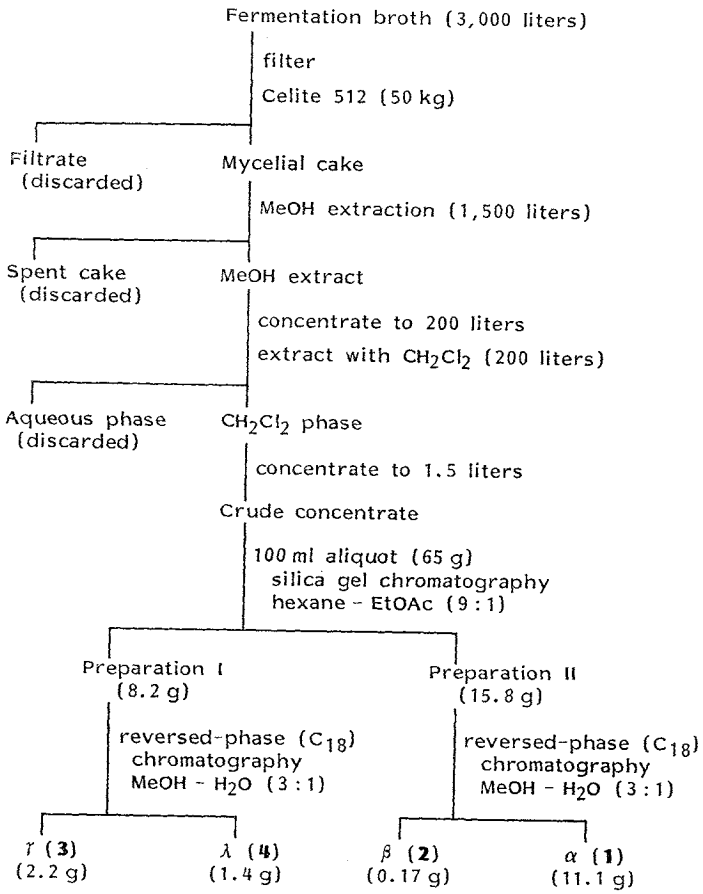


Fig. 2. Isolation and purification of LL-F28249 α, β, γ and λ (**1**~**4**).



in Fig. 2. The macrolides were recovered from the cells with methanol and were subsequently purified by chromatography on silica gel and C_{18} reversed-phase columns.

White fluffy solids were obtained for **1**~**4** by freeze-drying from *tert*-butanol. These substances dissolve readily in all common organic solvents and are practically insoluble in water. A summary of selected physico-chemical properties is presented in Table 1. The molecular formulas were determined from the measured masses of their molecular ions ($M^{\pm}$), obtained by HR electron impact mass spectrometry (EI-MS). All of the compounds are optically active and contain a maximum in their UV absorption spectra at 244 nm with shoulders at approximately 235 and 255 nm. The IR spectra of the compounds are similar, each showing a related series of strong bands.

Fig. 3. UV absorption spectrum (MeOH) of LL-F28249 α (**1**).

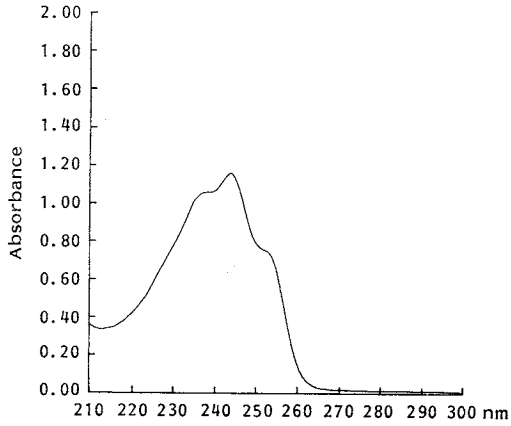


Table 1. Selected physico-chemical properties of LL-F28249 α, β, γ and λ.

	LL-F28249 α (**1**)	LL-F28249 β (**2**)	LL-F28249 γ (**3**)	LL-F28249 λ (**4**)
Formula	$C_{36}H_{52}O_8$	$C_{34}H_{48}O_8$	$C_{35}H_{50}O_8$	$C_{37}H_{54}O_8$
HREI-MS (*m/z*, $M^{\pm}$)	612.3705	584.3388	598.3543	626.3806
$[\alpha]_D^{26}$ (Me_2CO)	+136° (*c* 1.04)	+116° (*c* 1.02)	+153° (*c* 1.06)	+148° (*c* 1.18)
UV λ_{max}^{MeOH} nm (ε)	244 (30,500)	244 (26,000)	244 (28,900)	244 (28,400)
IR ν_{max} (KBr) cm^{-1}	3521, 1714, 1181, 997	3520, 1714, 1166, 995	3528, 1712, 1168, 996	3523, 1713, 1180, 997

Fig. 4. IR absorption spectrum (KBr) of LL-F28249 α (**1**).

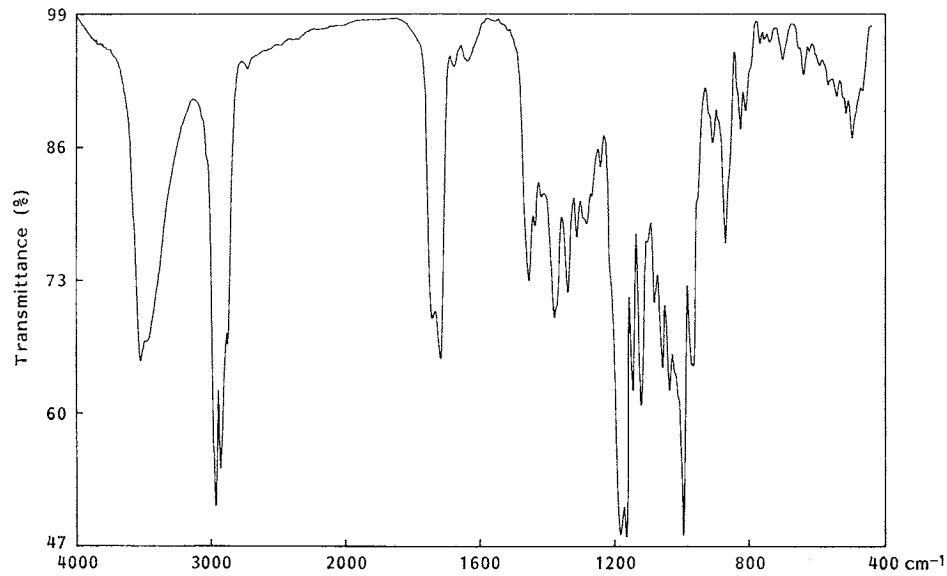


Fig. 5. ^{1}H NMR spectrum ($CDCl_3$) of LL-F28249 α (1).

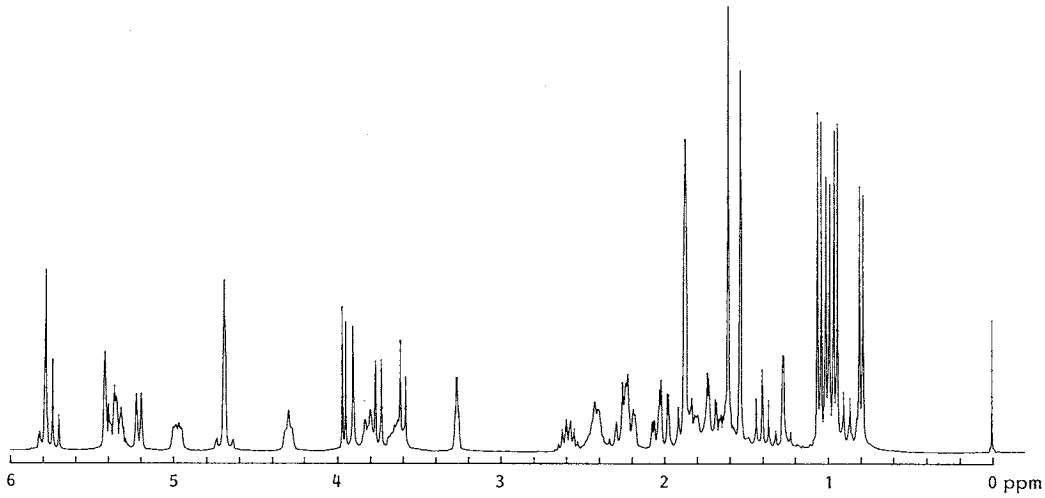
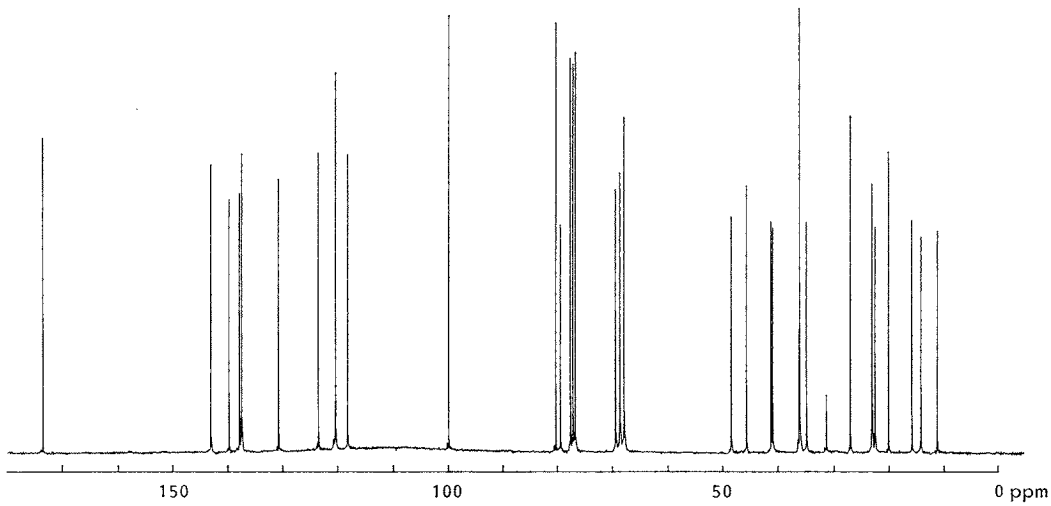


Fig. 6. ^{13}C NMR spectrum ($CDCl_3$) of LL-F28249 α (1).

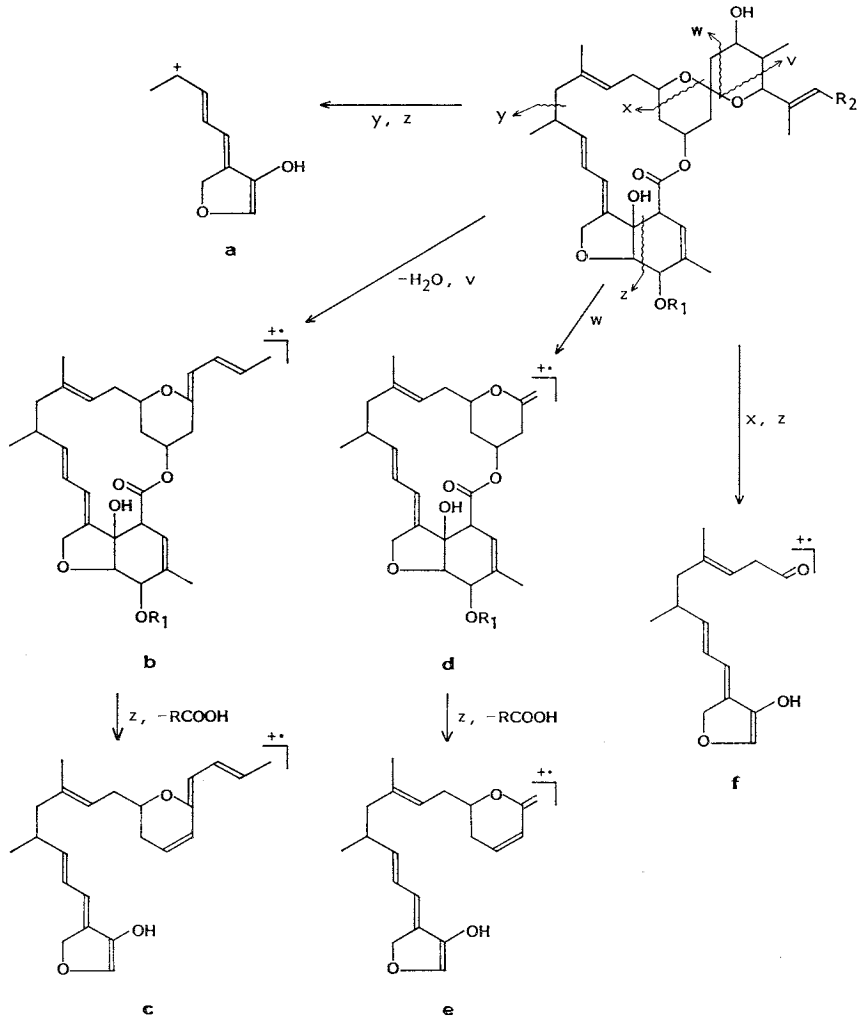


The UV, IR, ^{1}H NMR and ^{13}C NMR spectra for **1** are reproduced in Figs. 3~6, respectively.

The relationship of LL-F28249 α, β, γ and λ (**1**~**4**) to the milbemycins was initially recognized by the appearance of characteristic fragment ions in their EI-MS. Structurally significant fragment ions are represented in Fig. 7. Ions at *m/z* 151, 248 and 314 were observed in the spectra of **1**~**4** (ions **a**, **e** and **f**, Fig. 7); these have previously been described for milbemycins α_1, α_3 and D.[8] The observation of ions of type **d** provided evidence for the macrocycle and suggested the presence of the substructural unit C-1 through C-21 as shown.

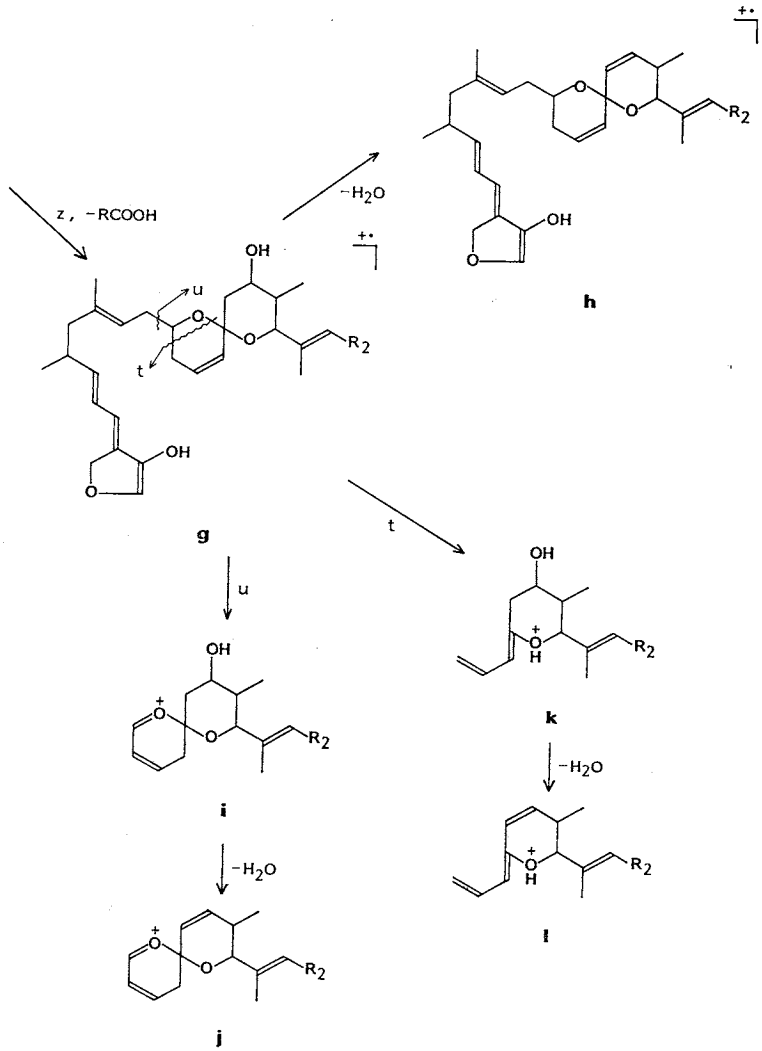
Further evidence for this partial structure was obtained by comparison of ^{13}C NMR data with

Fig. 7. Characteristic EI-MS fragmenta



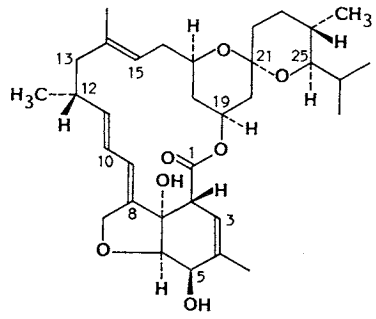
	m/z (relative	
	1	**2**
a	151.0753 (76)	151 (100)
b	482.2648 (1)	482 (1)
c	354.2818 (9)	354 (12)
d	442.2375 (1)	442 (1)
e	314.1877 (8)	314 (11)
f	248.1405 (4)	248 (7)
g	484.3211 (1)	456.2876 (1)
h	466.3097 (15)	438.2780 (18)
i	265.1786 (3)	237.1491 (8)
j	247.1705 (6)	219.1380 (11)
k	237.1838 (5)	209.1534 (10)
l	219.1740 (6)	191.1427 (13)

tions of LL-F28249 α, β, γ and λ (**1**~**4**).



abundance, %)	
3	**4**
151 (100)	151 (88)
496.2824 (1)	496 (1)
354 (27)	354 (14)
456.2512 (4)	456 (2)
314 (20)	314 (11)
248 (4)	248 (7)
456.2876 (3)	484 (2)
438 (55)	466 (26)
237 (6)	265 (4)
219 (10)	247 (7)
209 (13)	237 (6)
191 (14)	219 (8)

assignments made for milbemycin D[6] (Table 2). Excellent correlation† of the C-1 ~ C-21 chemical shifts was noted for **1** and **2** with some minor changes observed for methoxy derivatives **3** and **4**. On the basis of these data, it was concluded that the carbon skeleton from C-1 to C-21 was the same in **1** ~ **4** and that **3** and **4** represented the 5-*O*-methyl derivatives of **2** and **1**, respectively. Placement of the *O*-methyl at position 5 was established by 14 amu shifts of ions **b** and **d** (Fig. 7) and not for ions **a**, **c**, **e**, or **f**.



Milbemycin D

The structurally novel features of **1** ~ **4** are contained in the remainder of the molecules, consisting of $C_{11}H_{20}O_2$ for **1** and **4** and $C_9H_{16}O_2$ for **2** and **3**. In each case, a tetrahydropyran ring is formed by C-21 ~ C-25 and the remaining ketal oxygen. The substitution of this ring was established through spectroscopic analysis. In the EI-MS of **1** ~ **4** appearance of the ion at *m/z* 314 (Fig. 7, type **e**) was strong evidence for a methylene group at position 22. Hydroxy substitution at C-23 was indicated by EI-MS ions of type **b**. These presumably arise by initial dehydration to the Δ^{22} compounds followed by retro Diels-Alder fragmentation. Methyl substitution at C-24 is supported by the elemental compositions of the **b** ions. This methyl group (24a) gives rise to the highest-field signal in the ^{1}H NMR spectra of the molecules (Table 3), which correlates with ^{13}C NMR signals between δ 13.7 ~ 13.9 (Table 2).

The unique unsaturated side chains at position 25 were defined by analysis of NMR data. In the case of **1**, the trisubstituted olefin was recognized by ^{13}C NMR signals at δ 130.6 (s) C-26 and 137.2 (d) C-27. Placement of the isopropyl and methyl substituents about the double bond was established by ^{1}H NMR ($J_{H_{27,28}}$=9.1 Hz) and ^{1}H correlation spectroscopy (COSY) experiments. The olefinic proton 27-H was shown by COSY to be coupled to the 28-H methine proton at δ 2.58 which was in turn linked to 2 methyl groups at δ 0.953 and 1.05 (CH_3 28a and 29). Coupling was also indicated between 27-H and CH_3-26a.†† The magnitude of this coupling is quite small as no splitting of the signals was observed. The same general pattern was seen for **4** (Table 3). Allylic coupling was indicated between 27-H and CH_3-26a and in this case, both ^{1}H NMR signals show additional splitting.

For compounds **2** and **3**, EI-MS ions of types **g**, **h**, **i**, **j**, **k** and **l** (Fig. 7) clearly showed their side chains to be C_2H_4 less than that of **1** and **4**. Replacement of the isopropyl group at C-27 with a methyl group explains the nature of this homology. The terminal olefinic methyl groups give rise to doublets at δ 1.66 in the ^{1}H NMR spectra of **2** and **3** (Table 3). The signal due to the olefinic proton (27-H) appears at δ 5.47 in both spectra as a broadened quartet. Some allylic coupling to CH_3-26a appears to be responsible for the broadened nature of these signals.

† We suggest a revision of the ^{13}C NMR assignments for milbemycins α_2, α_4 and D made in ref 6. On the basis of ^{1}H COSY and two-dimensional (2D) ^{1}H, ^{13}C correlation spectroscopy, we have consistently found the signal for C-19 at higher field than that for C-17. In addition, for compounds **1** and **2** the resonance attributed to C-5 is at higher field than either that of C-17 or C-19. We believe the correct assignments for milbemycin D should be C-5 (67.4), C-17 (68.7) and C-19 (67.8). For milbemycins α_2 and α_4 the assignments of C-17 and C-19 should be reversed.

†† In our original communication (ref 2) this coupling was mistakenly attributed to a long-range interaction between 25-H and 27-H.

Table 2. ^{13}C NMR assignments for the LL-F28249 macrolides and milbemycin D.

C-Assignment[a]	LL-F28249 α (1)	LL-F28249 β (2)	LL-F28249 γ (3)	LL-F28249 λ (4)	Milbemycin D[b]
1	173.4	173.4	173.5	173.5	173.6
2	45.7	45.6	45.5	45.5	45.8
3	118.0	118.0	118.4	118.4	118.2
4	137.3	137.7	137.3	137.3	137.8
4a	19.9	19.8	19.8	19.8	19.9
5	67.4	67.5	76.8	76.8	67.8
5-OCH_3	—	—	57.6	57.6	—
6	79.3	79.3	77.5	77.5	79.3
7	80.2	80.1	80.3	80.3	80.3
8	139.4	139.4	139.6	139.5	139.6
8a	68.4	68.4	68.1	68.1	68.5
9	120.3[c]	120.21	119.5	119.5	120.4
10	123.3	123.3	123.4	123.4	123.5
11	142.8	142.7	142.3	142.4	142.8
12	36.0	35.9[c]	35.81	35.8	36.0
12a	22.2	22.2	22.2	22.2	22.3
13	48.4	48.4	48.4	48.3	48.6
14	137.3	137.3	135.8	135.7	136.9
14a	15.3	15.5	15.4	15.4	15.5
15	120.3[c]	120.25	120.2	120.1	121.0
16	34.7	34.7	34.7	34.6	34.7
17	68.5	68.5	68.5	68.4	67.4
18	36.1	36.0	35.9	35.92	36.7
19	67.8	67.7	67.7	67.7	68.7
20	40.7	40.7	40.6	40.6	41.4
21	99.7	99.7	99.7	99.6	97.5
22	41.1	41.0	40.9	41.0	35.8
23	69.3	69.2	69.2	69.2	28.1
24	35.9	35.9[c]	35.85	35.86	31.6
24a	13.9	13.8	13.7	13.8	17.4
25	76.7	76.7	76.6	76.6	78.4
26	130.6	133.9	133.9	130.5	28.4
26a	11.0	10.7	10.7	10.9	14.2
27	137.2	123.9	123.7	137.0	21.0
28	26.8	13.1	13.1	26.7	
28a	22.8[c]			22.7[d]	
29	22.8[c]			22.8[d]	

[a] Spectra obtained in $CDCl_3$, δ values in ppm downfield from TMS.
[b] Data from ref 6.
[c] Unresolved signals.
[d] Assignments may be reversed.

The relative stereochemistry shown for these molecules is that found for the γ component **3** by X-ray crystallography.[2]

Addendum in Proof

Recently RAMSAY *et al.* (RAMSAY, M. V. J.; S. M. ROBERTS, J. C. RUSSELL, A. H. SHINGLER, A. M. Z. SLAWIN, D. R. SUTHERLAND, E. P. TILEY & D. J. WILLIAMS: Novel antiparasitic agents derived by modification of a new natural product series. Tetrahedron Lett. 28: 5353~5356, 1987) reported to have independently isolated compounds identical with LL-F28249 α, β, γ and λ.

Also, a refinement of the NMR assignments has been completed by our colleague Dr. S. RAJAN. This work will be published separately in the near future.

Table 3. ^{1}H NMR data for LL-F28249 α, β, γ and λ.

H-Assignment[a]	LL-F28249 α (1)	LL-F28249 β (2)	LL-F28249 γ (3)	LL-F28249 λ (4)
2	3.27 (q, J=2.0)	3.26 (br s)	3.30 (q, J=2.4)	3.30 (q, J=2.4)
3	5.41 (br s)	5.39 (br s)	5.38 (q, J=1.6)	5.39 (d, J=1.5)
4a	1.87 (br s)	1.86 (br s)	1.81 (br s)	1.81 (br s)
5	4.29 (br d, J=6.0)	4.29 (br d)	3.97 (br m)	3.95 (br m)
5-OCH_3	—	—	3.50 (s)	3.50 (s)
6	3.95 (d, J=6.2)	3.92 (d, J=6.0)	4.01 (d, J=5.6)	4.01 (d, J=5.6)
8a	4.68 (AB)	4.68 (AB)	4.66 (AB)	4.66 (AB)
9	5.75[b]	5.76	5.73	5.77
10	5.73	5.72	5.75	5.74
11	5.35	5.33	5.30	5.35
12	2.45	2.41 (br m)	2.42 (br m)	2.43 (m)
12a	1.00 (d, J=6.6)	0.997 (d, J=6.5)	0.996 (d, J=6.6)	0.999 (d, J=6.7)
13a	2.20	2.20	2.20	2.20
13b	1.90	1.90	1.90	1.90
14a	1.53 (s)	1.53 (s)	1.53 (s)	1.53 (s)
15	4.95 (dd, J=11, 5)	4.97 (br d)	4.97 (br d, J=10)	4.96 (dd, J=9.9, 3.7)
16a,b	2.22 (m)	2.22 (m)	2.25 (m)	2.22 (m)
17	3.62 (m)	3.64 (m)	3.63 (m)	3.66 (m)
18a	0.883	0.900	0.900	0.902
18b	1.85	1.82	1.83	1.82
19	5.32	5.33	5.35	5.32
20_{ax}	1.40 (t, J=12)	1.39 (t, J=12)	1.41 (t, J=12)	1.40 (t, J=12)
20_{eq}	2.04	2.03	2.04	2.07
22_{ax}	1.70 (dd, J=14, 3.2)	1.72	1.70	1.70 (dd, J=14, 3.0)
22_{eq}	1.99 (dd, J=14, 2.4)	2.00	1.99 (dd, J=14, 2.7)	2.00 (dd, J=14, 2.5)
23	3.80 (m)	3.81	3.81	3.80 (m)
24	1.60	1.70	1.60	1.60
24a	0.802 (d, J=5.9)	0.793 (d, J=6.8)	0.792 (d, J=6.9)	0.794 (d, J=6.9)
25	3.74 (d, J=11)	3.78 (d, J=11)	3.78 (d, J=11)	3.74 (d, J=11)
26a	1.61 (s)[c]	1.60 (s)	1.60 (br s)[c]	1.60 (br s)[c]
27	5.20 (d, J=9.1)	5.47 (br q, J=6.4)	5.47 (br q, J=6.7)[c]	5.20 (d, J=9.0)[c]
28	2.58 (m)	1.66 (d, J=6.4)	1.66 (d, J=6.7)	2.57 (m)
28a	0.953 (d, J=6.6)[d]			0.952 (d, J=6.7)[d]
29	1.05 (d, J=6.6)[d]			1.05 (d, J=6.6)[d]

[a] Spectra obtained in $CDCl_3$, δ in ppm from TMS, coupling constants in Hz.
[b] When only a chemical shift value is given it represents the approximate position of an incompletely resolved signal as indicated by ^{1}H COSY experiments.
[c] Additional splitting of these signals has been observed, however no distinct pattern was recognized.
[d] Assignments may be reversed.

Acknowledgments

We thank Mr. J. D. KORSHALLA for providing the tank fermentation, and Mr. J. L. BAKER for the preparative chromatography.

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