MILBEMYCINS*, A NEW FAMILY OF MACROLIDE ANTIBIOTICS: FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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A search for novel insecticides has yielded the milbemycins, a new family of macrolide antibiotics with insecticidal and acaricidal activity. They are produced in submerged cultures of *Streptomyces hygroscopicus* subsp. *aureolacrimosus*. Fermentation studies on the strain were conducted in shaken flasks and 30-liter jar fermentors. From the culture broth 13 milbemycins were purified to homogeneity by column and thin-layer chromatography on silica gel and alumina. Physico-chemical data, such as mass spectra, UV and IR absorption spectra, optical rotations and melting points of the milbemycins are described.

In a search for novel insecticides, we discovered that the culture broth of a *Streptomyces* was active against acarus, harmful agricultural and horticultural insects such as aphids and larvae of insects of the order *Lepidoptera*.[1] The *Streptomyces* is a new subspecies and is designated *S. hygroscopicus* subsp. *aureolacrimosus*. Taxonomic studies will be reported elsewhere.

From the culture broth thirteen milbemycins were isolated and purified to homogeneity by column and thin-layer chromatography on silica gel and alumina. As reported briefly,[2,3] the milbemycins have the 16-membered macrolide structure shown in Fig. 1.

Subsequently the avermectins, isolated from the culture broth of *Streptomyces avermitilis*, were found to have structures closely related to the milbemycin αs, differing only in being substituted at the C-13 position by an oleandosyl oleandosyloxy moiety.[4,5]

In this paper we describe the fermentative production, isolation and physico-chemical properties of the milbemycins.

Materials and Methods

Fermentation studies

Cultures were grown in Erlenmeyer flasks on a rotary shaker (220 rpm, 7 cm) at 28°C. Inocula were grown for 2 days in 500-ml Erlenmeyer flasks containing 50 ml of seed medium with the following ingredients (in g/1,000 ml water): glucose 20, soybean meal 10, corn-steep liquor 5 and NaCl 3. The pH of the medium was adjusted to 6.8 before autoclaving at 120°C for 20 minutes.

Production cultures in 100-ml Erlenmeyer flasks, each containing 30 ml of the medium, were inoculated with 1 ml of the seed culture. Fermentations with the *Streptomyces* were also conducted

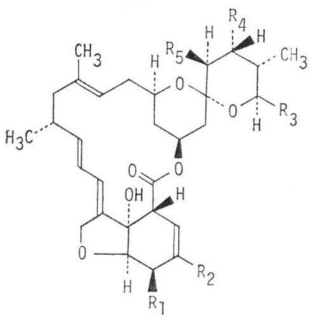
* The antibiotics were initially designated as B-41.

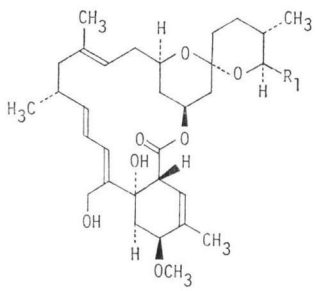
Fig. 1. Structures of milbemycins.

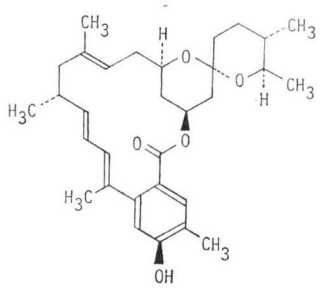
(1)

Milbemycins (B-41)	R_1	R_2	R_3	R_4	R_5
α_1 (A_3)	OH	CH_3	CH_3	H	H
α_2 (B_2)	OCH_3	CH_3	CH_3	H	H
α_3 (A_4)	OH	CH_3	C_2H_5	H	H
α_4 (B_3)	OCH_3	CH_3	C_2H_5	H	H
α_5 (A_2)	OH	CH_3	CH_3	$OCOCH(CH_3)(CH_2)_3CH_3$	OH
α_6 (B_1)	OCH_3	CH_3	CH_3	$OCOCH(CH_3)(CH_2)_3CH_3$	OH
α_7	OH	CH_3	C_2H_5	$OCOCH(CH_3)(CH_2)_3CH_3$	OH
α_8	OCH_3	CH_3	C_2H_5	$OCOCH(CH_3)(CH_2)_3CH_3$	OH
α_9 (C_1)	OH	CH_2OCO-(N H)	CH_3	H	H
α_{10}(C_2)	OH	CH_2OCO-(N H)	C_2H_5	H	H

(1) (2) (3)







	R_1
β_1(A_1)	CH_3
β_2	C_2H_5

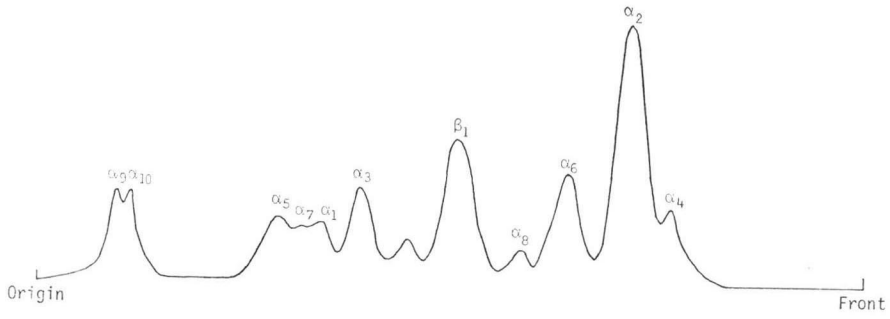
β_3

in aerated and stirred 30-liter jar fermentors. Twenty liters of medium was sterilized in the fermentor by heating to 100°C for 30 minutes and then 120°C for 20 minutes. One liter of a good preculture grown in two 2-liter Erlenmeyer flasks incubated at 28°C for 2 days was transferred to the jar-fermentors. Growth of the microorganism was estimated by measuring the packed mycelial volume from 10 ml of culture broth centrifuged in a 15-ml graduated centrifuge tube at 3,000 rpm for 10 minutes. The amount of milbemycins produced in the broth was estimated by thin-layer chromatography (TLC). Reducing sugar in the broth was estimated by the Somogyi method.

Thin-layer chromatography assay

To determine the amount of milbemycins produced in the culture broth, 3 ml of the broth was mixed with 7 ml of acetone and shaken for about 2 minutes. The mixture was centrifuged at 3,000 rpm for 5 minutes and 1 ml of the supernatant solution was evaporated to dryness *in vacuo*. The residue was dissolved in 1 ml of MeOH, and 10 μl of the MeOH solution was applied to a silica-gel 60-F_{254} plate (E. Merck, Darmstadt, Germany). The plate was developed by the ascending technique for 4 hours at room temperature, using a solvent system composed of carbon tetrachloride and dioxane (82: 18). The milbemycins on the plate were detected under short-wave UV light. For a quantitative assay, the plate

Fig. 2. Densitometer trace of a broth extract of *Streptomyces* sp. B-41.



was scanned at 240 nm with a dual-wavelength TLC Scanner (Model CS-900, Shimazu Co., Ltd., Japan). A scan of the broth extract is shown in Fig. 2. The peak heights on the scan were compared with those of a standard extract which had been calibrated from the ultraviolet absorbance of purified milbemycin components. Of the antibiotics listed in Fig. 1, milbemycins α_1 and α_3 have much the greatest acaricidal and insecticidal activity. Therefore, the quantity of milbemycins was expressed as the sum of milbemycins α_1 and α_3, unless otherwise specified.

Isolation

Fermentation broth (120 liters) containing milbemycins was filtered with the aid of infusorial earth (Celite 545 from Johns-Manville Products Corp., Calif., U.S.A.). The cake was extracted twice with 50 liters of MeOH; to the 100 liters of extract 50 liters of water was added. The resulting aqueous MeOH was extracted twice with 100 liters of *n*-hexane. The *n*-hexane was evaporated under reduced pressure and the residue was dissolved in 2 liters of MeOH. The resulting solution was allowed to stand overnight at −20°C and the precipitate was removed by filtration. The filtrate was concentrated to obtain 90 grams of a brown oily substance. This was dissolved in 150 ml of *n*-hexane and applied to a silica gel (Wakogel C-200) column. The column was eluted with a mixture of *n*-hexane and acetone (95: 5) to obtain successively, fractions I (800 ml), II (2 liters), III (1 liter) and IV (3 liters). Monitoring of the eluate was by thin-layer chromatography. The column was then eluted with *n*-hexane - acetone (90: 10) and (80: 20) to obtain fraction V (1 liter) and VI (1 liter), respectively.

Fraction I, containing a large quantity of α_4 and a small quantity of α_2, was evaporated under reduced pressure. Two grams of the crude powder thus obtained was dissolved in 5 ml of *n*-hexane and applied to a 50-ml silica-gel column, which was eluted with *n*-hexane - acetone (95: 5) to separate milbemycins α_4 from α_2 and others. Appropriate fractions were pooled on the basis of TLC analysis and the solvent was removed under reduced pressure. The solid thus obtained was recrystallized from acetone - water yielding 1.2 g of milbemycin α_4 as amorphous powder.

Fraction II, containing milbemycin α_2 as a major component and α_4 as a minor component, was evaporated and 15 g of the residue in 40 ml of *n*-hexane was chromatographed on a 500-ml silica gel column with *n*-hexane - acetone (95: 5). The fractions containing milbemycin α_2, based on TLC analysis, were pooled and evaporated under reduced pressure. The solid thus obtained was recrystallized from acetone - water to yield milbemycin α_2 (7 g) as colorless crystals.

Fraction III, containing milbemycins α_6 and α_8, was evaporated under reduced pressure and 1 g of the residue in 3 ml of *n*-hexane was chromatographed on a 30-ml silica-gel column with *n*-hexane - acetone (94: 6). Fractions selected from TLC analysis to contain milbemycins α_6 or α_8 were pooled separately and evaporated under reduced pressure. Recrystallization from acetone - water yielded 400 mg of α_6 as colorless crystals and 200 mg of α_8 as amorphous powder.

Fraction IV, containing α_1, α_3, β_1 and β_2, was evaporated under reduced pressure. Five grams of the oily residue in 13 ml of chloroform was chromatographed on a 75-ml alumina (Wako, Co., Ltd., Osaka, Japan) column with chloroform - ethyl acetate (9: 1) to separate the βs and αs components. The fractions containing β_1 as a major component and β_2 as a minor component were pooled and concentrat-

ed to dryness under reduced pressure. The yellowish syrup thus obtained in 5 ml of *n*-hexane was chromatographed on a 50-ml silica-gel column with *n*-hexane - acetone (94: 6) to separate β_1 from β_2. Appropriate fractions, based on TLC analysis, were pooled and the solvent was removed under reduced pressure. Each yellowish syrup thus obtained was dissolved in acetone, decolorized with active carbon and the solvent was removed *in vacuo*. The residues thus obtained were recrystallized from acetone - water to yield 1.2 g of β_1 and 300 mg of β_2. Two and a half grams of the yellowish syrup obtained by partial separation of fraction IV was dissolved in 10 ml of *n*-hexane and chromatographed on a 60-ml silica-gel column with *n*-hexane - acetone (94: 6) to separate milbemycins α_1 and α_3. Fractions containing α_1 and α_3, based on TLC analysis, were separately pooled and the solvent was removed *in vacuo*. The yellowish syrups thus obtained were dissolved in acetone, decolorized with active carbon and recrystallized from acetone-water to yield crystals of milbemycin α_1 (700 mg) and α_3 (1.1 g).

Fraction V, containing α_5, α_7 and β_3, was evaporated under reduced pressure. Two grams of the yellowish residue in 10 ml of MeOH was chromatographed on silica-gel 60-F_{254} plates (2-mm thickness, E. Merck, Darmstadt, Germany). The plates were developed with carbon tetrachloride - dioxane (82: 18) at room temperature for 4 hours to separate α_5, α_7 and β_3. Zones located under short-wave UV light were scraped from the plate and eluted with 400 ml of MeOH. The solvent was removed under reduced pressure. Each yellowish syrup thus obtained was dissolved in 50 ml of acetone and decolorized with 500 mg of active carbon. Milbemycin α_5 (850 mg) were obtained from the acetone-water solution by concentration *in vacuo* at 45°C. Crystals of milbemycin β_3 (100 mg) was obtained as a minor component from the α_5 fraction. Milbemycin α_7 obtained by evaporation of the acetone solution was crystallized from acetone-water to yield α_7 (150 mg) as amorphous powder.

Fraction VI, containing α_9 and α_{10}, was evaporated under reduced pressure. Eight hundred milligrams of yellowish syrup thus obtained, in 8 ml of MeOH, was applied to the origin of silica-gel 60-F_{254} plates (2-mm thickness). The plates were developed with carbon tetrachloride - dioxane (80: 20) at room temperature for 4 hours. Milbemycin α_9 and α_{10} zones, located under short-wave UV light, were scraped from the plate and eluted with 400 ml of MeOH. The solvent was removed under reduced pressure. Each yellowish substance thus obtained was dissolved in acetone and decolorized with active carbon. Milbemycins α_9 (260 mg) and α_{10} (240 mg) were obtained as amorphous powder from the acetone-water solution.

Analytical procedures

Melting points were determined using a Büchi capillary melting point apparatus and were uncorrected. UV absorption spectra were obtained in EtOH with a Hitachi 200–20 spectrophotometer. IR spectra were obtained in potassium bromide disks using a JASCO IRA-2 spectrometer. Mass spectra were recorded on a JEOL MLS-01SG at 75 eV using a direct inlet system.

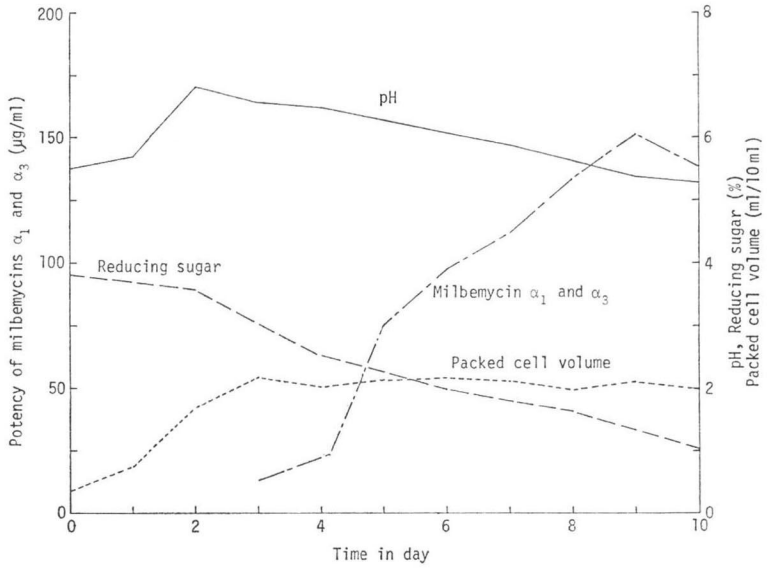
Results

Fermentation

Numerous ingredients of the culture medium were screened for their ability to support the production of milbemycins. Using 1.0% soybean meal as a nitrogen source, carbohydrates were screened as possible carbon sources. We examined glucose, sucrose, maltose, glycerol, soluble starch, corn starch, inositol and mannitol. Of these, glucose, sucrose, soluble starch and corn starch appeared to be favorable for milbemycin production. Using 2% glucose as a carbon source, various types of nitrogen compounds were tested. We found soybean meal, cotton-seed meal, corn-steep liquor and skim milk to be suitable.

On the basis of these results numerous complex media were formulated and screened for their ability to support rapid growth and subsequent antibiotic production. The best was medium BM-1, which contained the following (in g/1,000 ml water): glucose 40, corn starch 5, soybean meal 10, corn-steep liquor 2 and NaCl 3.

Fig. 3. Time course of milbemycin production on the BM-1 medium in a 30-liter-jar fermentor.



Reducing sugar and packed cell volume were determined as described in the text.

The effects of various additions to medium BM-1 on the production of milbemycins were determined by TLC. Of various vitamins tested at 10 μg/ml, folic acid, vitamins B_1, B_2 and B_6 stimulated the production of milbemycins, especially α_1 and α_3. Twelve amino acids tested at 1 and 5 mg/ml were without effect. The addition of 10 mg of $CaCO_3$ per ml or 5 mg of $MgSO_4 \cdot 7H_2O$ per ml to the medium, however, stimulated the production of milbemycins α_1 and α_3. A mixture of trace elements also stimulated the production, although this effect was not always reproducible.

The packed cell volume in the culture broth increased in the first 3 days and remained constant thereafter (Fig. 3). The antibiotics were first detected in the broth after 3 days cultivation and increased at a linear rate for the next 6 days. The yield (milbemycins α_1 and α_3, 150 μg/ml) was a maximum at 9 days (Fig. 3).

Isolation

The milbemycins were isolated from the mycelium by extraction with MeOH. The isolation procedures are outlined in Figs. 4 and 5.

Physical and Chemical Properties

All of the milbemycins are easily soluble in common organic solvents, *n*-hexane, benzene, acetone, EtOH, MeOH and chloroform. They are sparingly soluble in water.

Fig. 4. Extraction and isolation of milbemycins.

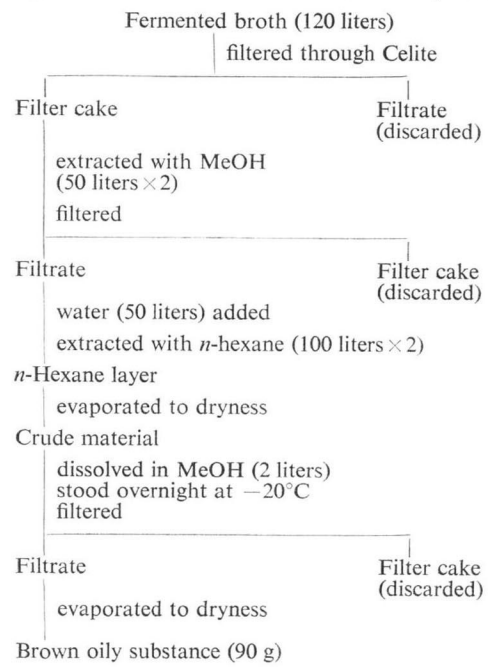
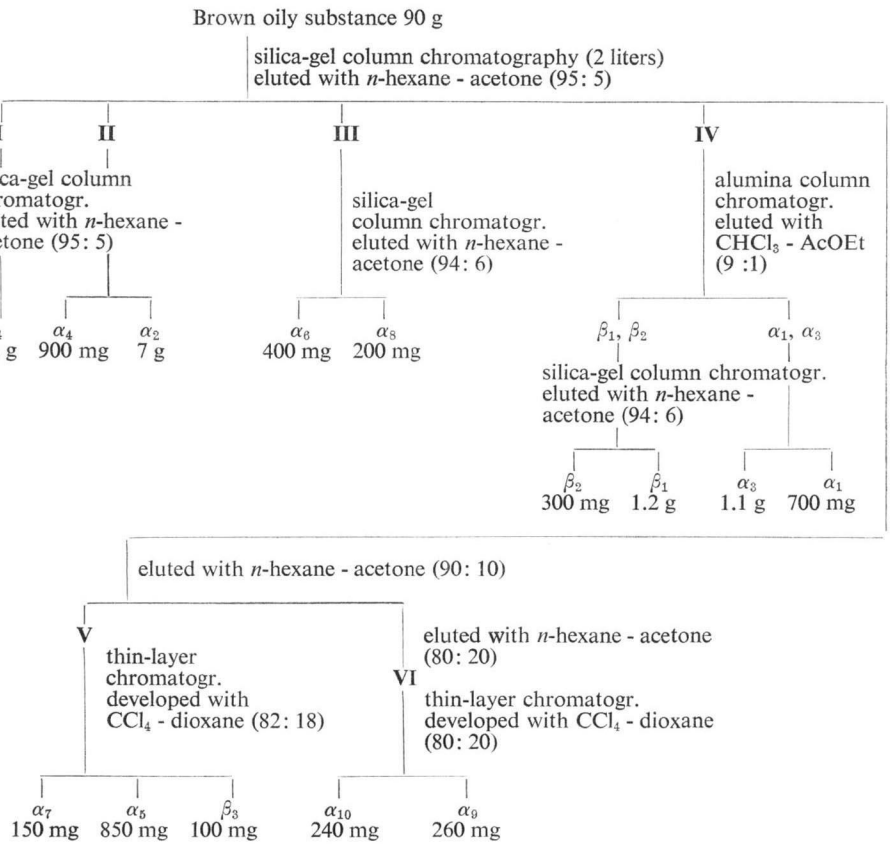


Fig. 5. Separation of milbemycins.



The mass spectrum of each milbemycin shows several prominent fragment-ion peaks and an intense molecular ion peak (Table 1). Molecular weights and formulae were determined by high-resolution mass spectrometry (Table 1).

UV spectra of the milbemycins $\alpha_{1\sim10}$ showed a maximum (244~245 nm) having two shoulders in the region of 238~254 nm. The wavelengths of the maxima and their molar extinction values are shown in Table 2. UV spectra of milbemycins α_9 and α_{10} showed a peak at 266 nm in addition to the peaks observed in the spectra of milbemycins $\alpha_{1\sim8}$. UV spectra of milbemycins $\beta_{1\sim3}$ showed only one peak at 245~247 nm.

In all IR spectra of milbemycins, absorption bands due to the hydroxyl and lactone groups were observed at 3330~3600 cm^{-1} and at 1707~1710 cm^{-1}, respectively (Table 2). The IR spectra of milbemycins α_5, α_6, α_7 and α_8 showed the absorption band due to an ester group at 1721~1722 cm^{-1}. In addition to the absorption bands due to hydroxyl and lactone groups, absorption bands due to an aromatic ring were observed in the IR spectrum of milbemycin β_3 at 1670, 1608 and 1582 cm^{-1}.

The melting points and optical rotations of milbemycins are listed in Table 2. Rf values on silica-gel thin-layer plates developed with various solvent systems are shown in Table 3.

Discussion

Of the several thousand microbial fermentation products which have been described, only a few have been reported to have insecticidal and acaricidal activity. They are aspiculamycin[6], aureothin[7], MYC

Table 1. Fragment ion peaks (m/e) in the mass spectra of milbemycins.

Milbemycin (B-41)	M^+	Fragment ions										Molecular formula
α_1 (A_3)	528				400	356	250		181	153	151	$C_{31}H_{44}O_7$
α_2 (B_2)	542				400	356	250		181	153	151	$C_{32}H_{46}O_7$
α_3 (A_4)	542				414	356	264		195	167	151	$C_{32}H_{46}O_7$
α_4 (B_3)	556				414	356	264		195	167	151	$C_{33}H_{48}O_7$
α_5 (A_2)	672			444	414		264		195	167	151	$C_{38}H_{56}O_{10}$
α_6 (B_1)	686			458	414		264		195	167	151	$C_{39}H_{58}O_{10}$
α_7	686			458	428		278	209	181	165		$C_{39}H_{58}O_{10}$
α_8	700			472	428		278	209	181	165		$C_{40}H_{60}O_{10}$
α_9* (C_1)	679	637	568		400		250		181	153	151	$C_{36}H_{47}O_9N$
α_{10}* (C_2)	693	651	582		414		264		195	167	151	$C_{37}H_{49}O_9N$
β_1 (A_1)	544		526		402	387	294		181	153		$C_{32}H_{48}O_7$
β_2	558				416				195	167		$C_{33}H_{50}O_7$
β_3	494		476	450	408		245	227				$C_{31}H_{42}O_5$

* monoacetate

Table 2. Physico-chemical properties of milbemycins.

Milbemycin (B-41)	Melting point (°C)	$[\alpha]_D^{20}$**	λ_{max}^{EtOH} nm(ε)	ν_{max} (cm^{-1})
α_1 (A_3)	212~215	+106	238 (27800), 244 (30500), 253 (sh.)	3600, 3480, 1708
α_2 (B_2)	139~142	+131	238 (sh), 244 (26500)	3600, 1708
α_3 (A_4)	212~215	+106	238 (27800), 244 (30500), 253 (sh.)	3480, 1707
α_4 (B_3)	*	+126	238 (sh.), 241 (26500), 253 (sh.)	3480, 1707
α_5 (A_2)	*	+54	238 (27800), 245 (30500), 253 (sh.)	3580, 1722, 1707
α_6 (B_1)	176~178	+75	238 (27800), 245 (30500), 253 (sh.)	3450, 1721, 1707
α_7	*	—	238 (27800), 245 (30500), 253 (sh.)	3580, 1722, 1707
α_8	*	—	238 (27800), 245 (30500), 253 (sh.)	3450, 1721, 1707
α_9 (C_1)	*	+57	238 (31300), 245 (35200), 254 (31000), 266 (sh.)	3330, 1710
α_{10} (C_2)	*	+54	238 (31300), 245 (35200), 254 (31000), 266 (sh.)	3330, 1710
β_1 (A_1)	*	+160	245 (26500)	3450, 1707
β_2	*	—	245 (26500)	3450, 1707
β_3	185~187	—	247 (29200)	3450, 1707, 1670, 1608, 1582

* amorphous, ** c=0.25, acetone
—; not measured, sh; shoulder

8005[8], orthosomycins[9], tetranactin[10] and avermectins[11]. From the results described above, as well as from the structures and characteristic biological activities, one can conclude that the milbemycins are a new family of macrolide antibiotics. The insecticidal and acaricidal activities will be reported elsewhere.

Table 3. Rf values from thin-layer chromatography of milbemycins.

Milbemycin (B-41)	Solvent systems			Milbemycin (B-41)	Solvent systems		
	I	II	III		I	II	III
α_1 (A_3)	0.35	0.42	0.39	α_7	0.32	0.35	0.37
α_2 (B_2)	0.76	0.61	0.79	α_8	0.69	0.51	0.77
α_3 (A_4)	0.40	0.44	0.41	α_9 (C_1)	0.10	0.22	0.12
α_4 (B_3)	0.81	0.63	0.81	α_{10} (C_2)	0.13	0.24	0.13
α_5 (A_2)	0.28	0.32	0.35	β_1 (A_1)	0.53	0.47	0.27
α_6 (B_1)	0.67	0.47	0.75				

Solvent system I ; Dioxane - CCl_4 (15:85)
Solvent system II ; Acetone - *n*-hexane (30:70)
Solvent system III; Ethyl acetate - $CHCl_3$ (25:75)
Pre-coated silica-gel 60 F-254 plates (Merck) were used.

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