MILBEMYCINS α_{11}, α_{12}, α_{13}, α_{14} and α_{15}: A NEW FAMILY OF MILBEMYCINS FROM *Streptomyces hygroscopicus* subsp. *aureolacrimosus*

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL PROPERTIES

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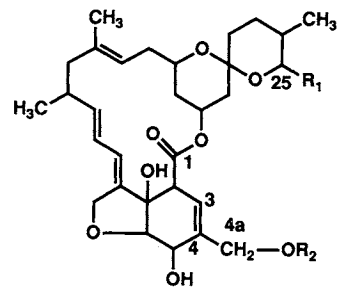
Streptomyces hygroscopicus subsp. *aureolacrimosus* SANK 60286 produces a new family of milbemycins, named milbemycins α_{11}, β_{12}, α_{13}, α_{14} and α_{15}, together with other milbemycins. Their structures are 3-methyl-2-butenoyloxy and 3-methylbutyroyloxy derivatives at C-4a of milbemycins A3 and A4, or 3-methyl-2-pentenoyloxy derivative at C-4a of milbemycin A3, respectively. Milbemycin α_{14}, 3-methyl-2-butenoyloxy derivative, especially possesses a potent acaricidal activity.

Milbemycins and avermectins, members of the class of sixteen membered macrolides which possess a potent antiparasitic activity were reviewed by DAVIES and GREEN in 1986[1]. After the first review on milbemycins and avermectins by DAVIES and GREEN, many new families of antiparasitic macrolide antibiotics have been isolated from *Streptomyces cyaneogriseus* subsp. *noncyanogenus*[2], *S. thermoarchaensis*[3], *S. hygroscopicus*[4], *Streptomyces* sp.[5,6], *S. hygroscopicus* subsp. *aureolacrimosus*[7,8], and also a hybrid microorganism obtained by protoplast fusion of *S. avermitilis* and *S. hygroscopicus*[9].

Scheme 1. Structures of milbemycins α_{11} to α_{15}.



	R_1	R_2
Milbemycin α_{11} (**1**)	CH_3	$COCH=C(CH_3)_2$
Milbemycin α_{12} (**2**)	CH_3	$COCH_2CH(CH_3)_2$
Milbemycin α_{13} (**3**)	CH_3	$COCH=C(CH_3)-CH_2CH_3$
Milbemycin α_{14} (**4**)	CH_2CH_3	$COCH=C(CH_3)_2$
Milbemycin α_{15} (**5**)	CH_2CH_3	$COCH_2CH(CH_3)_2$

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In search of new antiparasitic macrolides, many streptomycetes with gray color series and spiral spore chains which are morphologically related to the above producing strains were intensively isolated from soils collected all over Japan. We found that one such strain, *Streptomyces hygroscopicus* subsp. *aureolacrimosus* SANK 60286 produced a new family of milbemycins together with the other milbemycins. These compounds, α-series of milbemycins[10], named milbemycins α_{11} (**1**), α_{12} (**2**), α_{13} (**3**), α_{14} (**4**) and α_{15} (**5**), possess a potent antiparasitic activity, including acaricidal, insecticidal and nematocidal activities and were determined as acyloxy derivatives at C-4a in milbemycins (Scheme 1).

In this paper, we report the taxonomy and fermentation of the producing organism, and the isolation at C-4a acyloxy derivatives of milbemycins and antiacaricidal and antinematocidal activities.

Materials and Methods

Taxonomy

The producing organism, SANK 60286, was isolated from a soil sample collected in Miura, Kanagawa Prefecture, Japan.

Taxonomic studies were carried out according to the procedure of the International Streptomyces Project[11]. The color recorded for the mature culture was described according to "Guide to Color Standard"[12]. Diaminopimelic acid in the whole-cell hydrolysates was analyzed by the method of BECKER *et al.*[13].

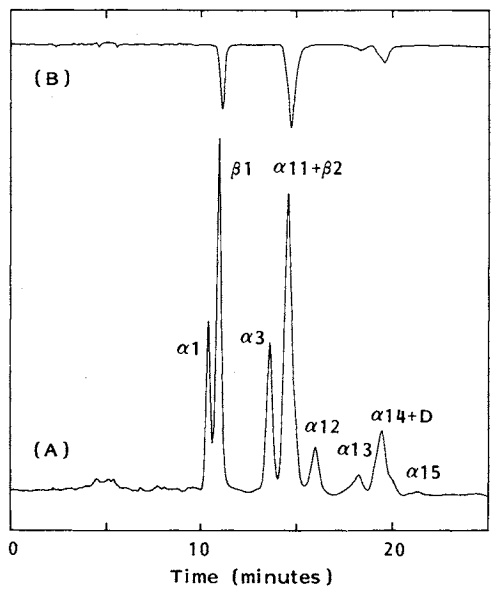
Fermentation

A loopful amount of the culture of strain SANK 60286 was inoculated to a 2-liter Erlenmeyer flask which contained 500 ml of the seed medium composed of sucrose 1%, Polypepton 0.35%, and K_2HPO_4 0.05%. The inoculated flasks were incubated on a rotary shaker at 28°C for 48 to 72 hours. Then a 1-liter aliquot of the culture was transferred into 30-liter jar fermentor containing 15 liters of the producing medium composed of sucrose 8%, soybean powder 1%, skimmed milk 1%, yeast extract 0.1%, meat extract 0.1%, $CaCO_3$ 0.3%, K_2HPO_4 0.03%, $MgSO_4 \cdot 7H_2O$ 0.1% and $FeSO_4 \cdot 7H_2O$ 0.005%, and adjusted to pH 7.2, before sterilization. Fermentation was carried out at 28°C for 12 days with an air-flow rate of 0.5 v/v/m and an agitation ratio of 40 to 180 rpm.

Isolation and Purification

The fermentation broth (32 liters) was filtered with the aid of Celite 545 (1.8 kg). The resulting cake was washed with water and both filtrate and wash were discarded. Methanol (20 liters) was used to extract the washed cake. The MeOH extract was concentrated to approximately 2 liter under reduced pressure and the resulting concentrate was extracted three times, with an equal volume of *n*-hexane. The combined hexane phase was washed with 20% NaOH solution and concentrated under reduced pressure to yield 38 g of oily substances. The residual oily substance was chromatographed on silica gel (300 g, Type 60, Mallinckrodt) and eluted with hexane followed by 3:1 mixture of hexane and

Fig. 1. HPLC profile of milbemycins.



Column: Senshu-pak, ODS, H-2151 (6 × 150 mm). Developing solvents: CH_3CN-H_2O (80:20). Flow rate: 1.5 ml/minute. Detection: A; 238 nm, B; differential spectra between 240 nm and 260 nm using photodiode array.

EtOAc. On the basis of HPLC analysis, fractions containing new milbemycins were combined to give 1.9 g of crude samples upon evaporation of the solvents. MeOH-H_2O (1:1) solution of crude samples was applied to a column of silanised-silica gel (160 g, Art 7719, Merck) packed with 50% MeOH. The column was eluted with elevating 60%, 70% and 80% MeOH. On HPLC analysis, fractions were combined and concentrated under reduced pressure to give 840 mg of a mixture of new milbemycins. HPLC chromatogram of the final crude sample is shown in Fig. 1. Chromatogram A was monitored at 238 nm, and B was monitored at the differential spectra between 240 nm and 260 nm. In the chromatogram B, ordinary milbemycins of the α-series were not detected, but new milbemycins, containing an α,β-unsaturated carboxyl ester in addition to the diene function of the macrolide core, were clearly detected.

Finally, each milbemycin was purified by preparative HPLC using Senshu-pak, (ODS, H-5251, 20×250 mm), with the developing solvent: 80% CH_3CN, and flow-rate: 9.9 ml/minute. Detection was by UV absorbance at 240 nm. New milbemycins, designated milbemycins α_{11} (**1**), α_{12} (**2**), α_{13} (**3**), α_{14} (**4**) and α_{15} (**5**) were obtained as 128 mg, 11.7 mg, 14.8 mg, 43 mg and 3 mg of white amorphous powder, respectively.

Antiparasitic Activity

a) Antiacaricidal Activity against Adult Mites

Sample solutions containing 0.01 ppm, 0.1 ppm, 0.3 ppm, 1 ppm or 3 ppm of individual compounds were prepared. Two-spotted spider mites (*Tetranychus urticae*), sensitive to organophosphorus insecticides, were inoculated on the primary leaves of cowpea plants (*Vigna sinensis* Savi). One day after inoculation, 7 ml of the sample solution containing 0.01% of detergent was sprayed by a rotary sprayer to give a sprayed amount of 3.5 mg/cm^2 of leaf. After being sprayed, the leaves were allowed to stand in a room kept at 25°C. After 3 days, whether the adult insects died or not was examined by a binocular microscope and the mortality (%) was calculated.

b) Antiacaricidal Acitivity against Mite Eggs

Sample solutions containing 0.1 ppm, 0.3 ppm, 1 ppm or 3 ppm of individual compounds were prepared. Female adult two-spotted spider mites were allowed to lay on the primary leaves of cowpea plants. The adult mites were removed to obtain test leaves each bearing about 50 eggs. In a similar manner to the preceding example, the sample solutions were applied to the test leaves. After standing for 2 weeks in a room kept at 25°C, the number of unhatched eggs was counted, and the unhatched egg rates (%) were calculated.

c) Antinematocidal Activity

A 0.1% methanol solution of individual compounds was diluted by 10 times with water to prepare a solution of 100 μg/ml. Then, an appropriate varying amount of the solution of the compounds was added to 1 ml of an aqueous suspension containing living nematodes, *Caenorhabditis elegans*, which was left at 25°C for 18 hours, after shaking. The number of the nematodes which were immobilized, and the total number of the nematodes tested were counted under a stereoscopic microscope. Immobilized rates (%) against the total number of tested nematodes were calculated.

Results and Discussion

Taxonomic Characteristics

The strain SANK 60286 grew relatively well on various agar media. The strain formed spiral sporophores branching monopodially on aerial hyphae. Spores were covered with a capsule-like membrane with a fairly irregular or rugose, possibly warty surface (Plate 1). Special structures such as sporangia, zoospores, ball-like bodies, or sclerotia were not observed on the media employed. The cultural characteristics on various agar media at 28°C for 14 days are shown in Table 1. The color of the vegetative mycelium was pale yellow to yellowish brown. The aerial mycelium was abundant and varied from white to yellowish gray mass color. Within 3 to 7 days, it formed a golden yellow globose accumulation liquid

Table 1. Cultural characteristics of strain SANK 60286.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Abundant, yellowish brown	Abundant, grayish white with yellowish patches and with black moistened patches	Pale yellow
Oatmeal agar (ISP-3)	Abundant, pale yellow	Abundant, grayish white with yellowish patches and with black moistened patches	Light olive gray
Inorganic salts-starch agar (ISP-4)	Abundant, light olive	Abundant, white to light olive gray with yellowish patches and with black moistened patches	None
Glycerol-asparagine agar (ISP-5)	Good, yellowish brown	Good, white to yellowish gray with yellowish patches	None
Tyrosine agar (ISP-7)	Good, light olive gray	Good, white to pale yellow with yellowish patches	None
Nutrient agar (Difco)	Good, yellowish gray	Scant, gray	None
Sucrose-nitrate agar	Good, grayish white	Good, white	None
Glucose-asparagine agar	Abundant, yellowish gray	Good, grayish white	None

Plate 1. Scanning electron micrograph of strain SANK 60286 (on potato extract-carrot extract agar, 28°C, 14 days).

Bar represents 10 μm.

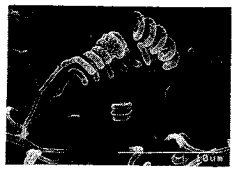


Table 2. Carbohydrate utilization pattern of strain SANK 60286.

Carbohydrate	Response
D-Glucose	++
L-Arabinose	++
Sucrose	++
D-Xylose	+
Inositol	++
D-Mannitol	++
D-Fructose	++
D-Rhamnose	++
Raffinose	++

++: Strongly positive utilization.
+: Positive utilization.

exudate in various media, and subsequent yellowish patches on the aerial mycelium. Sometimes, moist black, liquefied (hygroscopic) areas were also found in the aerial mycelium of older cultures. The soluble pigment was pale yellow to light olive gray. No melanoid pigment was produced. The strain grows within the temperature range of 18 to 37°C. The hydrolysis of starch and reduction of nitrate were positive. The liquefaction of gelatin and coagulation or peptonization of milk were weakly positive. Since strain SANK 60286 can grow weakly on basal medium without any added carbon source, it is difficult to describe exactly its ability for carbon utilization. The relative utilization is shown in Table 2. The whole-cell analysis of the strain showed the presence of LL-diaminopimelic acid, and it was classified as cell wall type I. Based on the taxonomic properties described above, the strain SANK 60286 was nearly identical with those of *S. hygroscopicus* subsp. *aureolacrimosus* B-41-146. Progeny of the type strain SANK 60286 of *S. hygroscopicus* subsp. *aureolacrimosus* has been deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan with an accession number of FERM BP-1190[14].

Physico-chemical Properties

The physico-chemical properties of milbemycins α_{11} (**1**), α_{12} (**2**), α_{13} (**3**), α_{14} (**4**) and α_{15} (**5**) are

Table 3. Physico-chemical properties of milbemycins α_{11} (**1**), α_{12} (**2**), α_{13} (**3**), α_{14} (**4**) and α_{15} (**5**).

	1	**2**	**3**	**4**	**5**
Molecular formula:	$C_{36}H_{50}O_9$	$C_{36}H_{52}O_9$	$C_{37}H_{52}O_9$	$C_{37}H_{52}O_9$	$C_{37}H_{54}O_9$
EI-MS (*m/z*):	626	628	640	640	642
Elemental analysis:					
Found (%):	C; 68.83	C; 67.04	C; 67.41	C; 67.62	
	H; 8.32	H; 8.01	H; 8.12	H; 7.84	
Calcd (%):	$C_{36}H_{52}O_9$	$C_{36}H_{52}O_9 \cdot H_2O$	$C_{37}H_{52}O_9 \cdot H_2O$	$C_{37}H_{52}O_9 \cdot H_2O$	
	C; 68.98	C; 66.85	C; 67.45	C; 67.45	
	H; 8.04	H; 8.42	H; 8.26	H; 8.26	
$[\alpha]_D^{23°}$ ($CHCl_3$):	+104.3°	+118.3°	+91.6°	+96.1°	
	(*c*, 1.05)	(*c*, 1.00)	(*c*, 0.89)	(*c*, 1.14)	
UV; λ (EtOH), nm (ε):	230 (sh)	238 (750)	230 (sh)	230 (sh)	
	238 (990)	244 (810)	237 (805)	237 (800)	
	244 (990)	253 (sh)	245 (795)	244 (810)	
	252 (sh)	253 (sh)	253 (sh)		

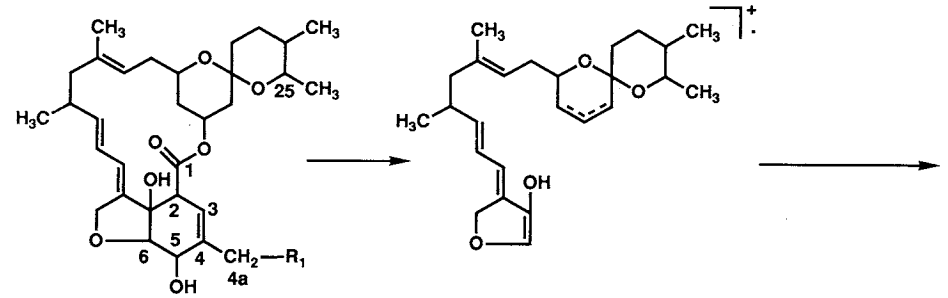
summarized in Table 3.

Their molecular weights and molecular formulae were determined by EI-MS spectrometry and elemental analyses.

Structure Elucidation

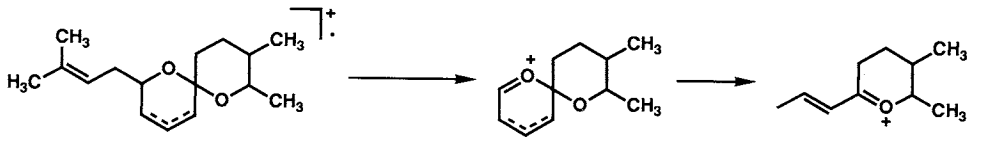
^{1}H NMR spectra of milbemycins α_{11} (**1**), α_{12} (**2**) and α_{13} (**3**) were very similar to that of milbemycin α_1 (A3). The doublet methyl signal at 1.15 ppm in **1** was coupled with the methine proton (3.28 ppm) at C-25. This coupling pattern was also observed in the spectra of **2** and **3**. These facts suggested that the C-25 substituent of **1**, **2** and **3** was a methyl group as in milbemycin A3. In addition, the relationship of **1**, **2** and **3** to milbemycin A3 was easily recognized by the appearance of characteristic fragment ions in their MS spectra. Ions at *m/z* 153 (**g**), 181 (**f**), 250 (**d**), and 400 (**b**) were observed in the spectra of **1**, **2**, **3** and A3. Structurally significant fragment ions *via* retro Diels-Alder cleavage and allylic cleavage are illustrated in Fig. 2. These ions **b**, **d**, **f** and **g** have previously been described for milbemycin A3[15]. The observation of the ion of type **b** provided evidence for the macrocyclic structure and suggested the presence of the substructural unit C-6 through C-25 plus side chain at C-25. In the similar way, the MS spectra of milbemycin α_{14} (**4**) and α_{15} (**5**) showed peaks at *m/z* 167, 195, 264 and 414. These fragment ions were larger by 14 mass units than **g**, **f**, **d** and **b**. This fact lead to the conclusion that **4** and **5** were derivatives of milbemycin A4[15]. Accordingly, the new substituent of **1** to **5** should exist in the counterpart with C-1 through C-5, derived from the retro Diels-Alder reaction of A-ring (Fig. 2). Further evidence for the substituent in **1** to **5** were provided by the comparison of their NMR data. The ^{1}H NMR spectra of **1** to **5** showed the absence of the allylic methyl signal at C-4a, which appeared at around 1.8 ppm in A3 and A4 and a newly appeared AB quartet signal around at 4.6 to 4.8 ppm due to a hydroxymethyl group. In the comparison of ^{13}C NMR spectra of **4** and A4, the C-4a methyl signal in A4 was replaced by a triplet signal of hydroxymethyl at 63.5 ppm in **4**. In addition, the structure was conclusively confirmed by the results of a ^{1}H, ^{13}C COLOC experiment which showed connectivities between C-4a and 3-H and between C-1′ in ester moiety and 4a-H[16]. These observations suggested that milbemycins α_{11}, α_{12} and α_{13} were acyloxy derivatives at the C-4a position of milbemycin A3, and milbemycins α_{14} and α_{15} were also acyloxy derivatives at the C-4a of milbemycin A4. The substituent at C-4a of **1** and **4** possessed an element C_5H_7O from comparison of molecular formulae between **1** and A3, and **4** and A4. The ^{1}H and ^{13}C NMR spectra

Fig. 2. Mass fragmentation pathways of milbemycins.



	R_1
α_{11} (1)	$OCOCH=C(CH_3)_2$
α_{12} (2)	$OCOCH_2CH(CH_3)_2$
α_{13} (3)	$OCOCH=C(CH_3)-CH_2CH_3$
α_1(A3)	H

b: $m/z=400$



d: $m/z=250$ **f**: $m/z=181$ **g**: $m/z=153$

Table 4. Acaricidal activities of milbemycins against adult mites.

Concentration (ppm)	Mortality (%)					
	α_{11}	α_{12}	α_{13}	α_{14}	A3/A4[a]	Avermectin[b]
3	100	100	100	100	25.5	
1	100	96.5	95.6	100	21.7	100
0.3	93.0	73.9	77.3	100		100
0.1	73.9	37.4	62.4	44.8		21.7
0.01	11.1	3.6	14.9	24.8		

[a] Milbemycins A3 and A4 mixtures (30 : 70).
[b] Avermectin was obtained from the commercially available insecticide AVID from Merck and Co., Inc.

Table 5. Acaricidal activities of milbemycins against mite eggs.

Concentration (ppm)	Unhatched (%)					
	α_{11}	α_{12}	α_{13}	α_{14}	A3/A4[a]	Avermectin[b]
3	93.6	97.8	94.3	94.5	1.8	
1	50.9	76.6	71.4	56.5	2.4	4.2
0.3	0	0	4.1	53.8		2.4
0.1	0	0	3.8	0		0

[a,b] See footnote to Table 4.

Table 6. Nematocidal activities of milbemycins against *Caenorhabditis elegans*.

Concentration (ppm)	Immobility (%)				
	α_{11}	α_{12}	α_{13}	α_{14}	D[c]
5	100	100	100	100	100
1	100	100	100	100	100
0.5	92	97	95	97	93
0.1	78	73	84	60	81

[c] Milbemycin D produced from Sankyo Co., Ltd., Japan.

of **1** and **4** showed the presence of two allylic methyl groups and two olefinic carbons, with a proton attached to one of them, suggesting a 3-methyl-2-butenoyl substructure. Thus, structures of **1** and **4** were deduced as 3′-methyl-2′-butenoyloxy derivative of milbemycin A3 and A4, respectively.

In the same manner, the substituent at C-4a of **2** and **5** possessed an element C_5H_9O. The ^1H NMR spectra of **2** and **5**, after decoupling experiments, revealed a new set of resonances corresponding to an isopropyl group, which could be attributed to the hydrogenated 3-methyl-2-butenoate side chain. The structures of **2** and **5** were deduced as 3′-methylbutyroyloxy derivatives of milbemycin A3 and A4, respectively.

The substituent at C-4a of **3** possessed an element C_6H_9O. The ^1H NMR spectrum of **3** showed an allylic methyl signal at 2.18 ppm and olefinic proton at 5.75 ppm. These signals were assigned to 3′-methylpent-2′-enoate moiety. The geometry of double bond of the 3′-methylpent-2′-enoate group was assigned as *E* configuration from the chemical shift of the allylic methyl signal, which appeared at unusually low field due to anisotropic effect of the carbonyl group. The structure of **3** was therefore deduced as 3′-methylpent-2′-enoyloxy derivative of milbemycin A3.

After we had applied for the patent for these compounds in 1986, patent applications for antibiotics MI 198 Z1, Z5, Z7, Z3 and Z6[8] and KSB-1939 H3, H2, H4 and S5[5], which seem to be identical with milbemycins α_{11}, α_{12}, α_{13}, α_{14} or α_{15}, were independently submitted.

Biological Activity

Milbemycins α_{11} to α_{14} possessed potent acaricidal and nematocidal activities (Tables 4, 5 and 6). Among them, especially, milbemycin α_{14} was more active than known milbemycins in acaricidal activity.

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