MILBEMYCINS, A NEW FAMILY OF MACROLIDE ANTIBIOTICS

STRUCTURE DETERMINATION OF MILBEMYCINS D, E, F, G, H, J AND K

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The milbemycins, a group of potent, broad-spectrum antiparasitic and pesticidal agents, are architecturally novel antibiotics of 16-membered macrocyclic lactone. Seven new milbemycin analogues designated as milbemycins D, E, F, G, H, J and K were isolated from the fermentation broth of the mutant strain of *Streptomyces hygroscopicus* subsp. *aureolacrimosus*. The structural determination of these new components was made mainly by comparing with mass spectra, and ^{1}H and ^{13}C NMR spectra of milbemycin α- and β-series previously published from our laboratory. Milbemycins D, E, F, G and H have characteristically an isopropyl side chain at C-25 which differs from the known milbemycin family bearing methyl or ethyl group at C-25. Milbemycins J and K possess a ketone group at C-5 instead of a hydroxyl or methoxy group.

Apart from X-ray crystallography, the *R*-configuration of the hydroxyl group at C-5 could be best explained both by application of CD allylic benzoate method to the *p-N,N*-dimethylaminobenzoate of milbemycin D and by comparison of the specific rotation of milbemycin D itself and its acetate with the epimeric isomers at C-5.

Milbemycin[1~3], which was found and structurally elucidated in 1975 in our laboratory as shown in Fig. 1, is a group of the fermentation products of *Streptomyces hygroscopicus* subsp. *aureolacrimosus* with potent, broad-spectrum anthelmintic, insecticidal and acaricidal activity, but is devoid of the antibacterial activity associated with macrolide derivatives. Common characteristic feature of the milbemycin is the presence of both a 6,6-membered spiroketal and a cyclohexene ring fused to the 16-membered macrocyclic lactone ring. Milbemycins can be divided structurally into two groups of α- and β-series: α-series has the tetrahydrofuran ring fused to the cyclohexene ring, β-series has no tetrahydrofuran ring. After several years of our finding milbemycins, the Merck group reported on the avermectins, isolated from the fermentation broth of *Streptomyces avermitilis*, which were glycosides with an α-L-oleandosyl-α-L-oleandosyl moiety at C-13 of milbemycin, and showed a similar biological activity to that of the milbemycin[4,5].

In the course of our fermentation development, novel milbemycin analogues designated as milbemycins D, E, F, G, H, J and K were obtained from the fermentation broth of the mutant strain of *S. hygroscopicus* subsp. *aureolacrimosus*. The fermentation, isolation and physico-chemical properties of these new metabolites have been reported in the previous papers[6,7].

In this paper we wish to report the structure determination of these new metabolites and the absolute configuration of milbemycin D.

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Results and Discussion

Structure of Milbemycins D, E, F, G, H, J and K

A number of over-all skeleton and structural elements of new components could be deduced mainly from analysis of their mass spectral fragmentation and ^{1}H and ^{13}C NMR spectral data. In the mass spectra of milbemycins, the appearance of a relatively intensive molecular ion and fragment ions resulted from allyl fission, ester elimination, α-cleavage adjacent to an oxygen atom and a retro Diels-Alder reaction was commonly observed. The formula of the molecular and diagnostically prominent ions were confirmed by high resolution mass spectral analysis. The interpretations of the mass spectral data shown in Schemes 1, 2, 3 and 4 were proved from the comparison with the mass spectra of the metabolites using ^{13}C-labelled precursor in the biosynthetic studies as described in the accompanying paper[8].

Milbemycin D

Milbemycin D, mp 186~188°C, $[\alpha]_D^{27}$ +107° (*c* 0.25, acetone), has the molecular formula of $C_{33}H_{48}O_7$ (Table 1). The IR (ν 3450 and 1710 cm^{-1}) and UV [λ_{max}^{EtOH} 238 nm (shoulder) and 244 nm (ε 31,000)] spectra indicated a marked resemblance to those of milbemycins α_1 and α_3.

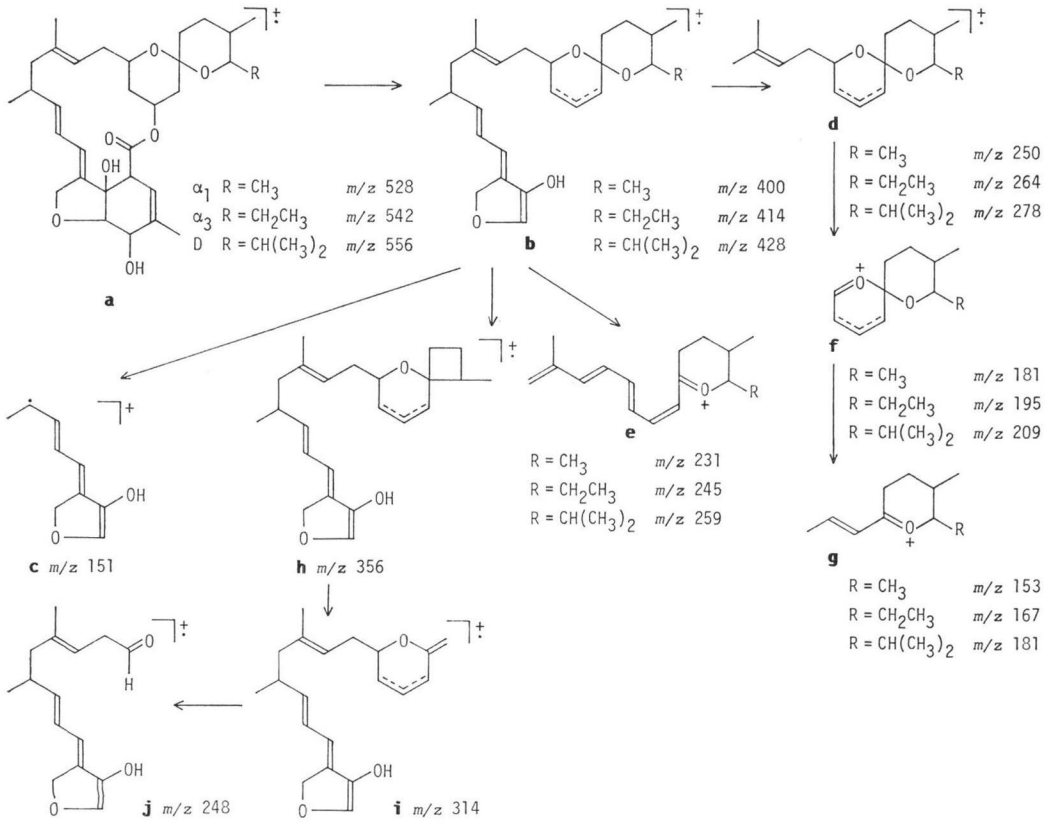
Upon electron impact on milbemycin D, a mass reaction of the type encountered already among milbemycins yielded species **b** at *m/z* 428. This fragment **b** undergoes homolysis at the allylically activated bond between C-12 and -13 to lead to the fragment ions **c**, **d** and **e** at *m/z* 151, 278 and 259, respectively, followed by the further fragmentation of the species **d** to the ion **g** (*m/z* 181) through the ion **f** (*m/z* 209). On one hand, it is obviously probable that bond rupture of the ion **b** occurs in the region of the spiroketal system to lead to the fragment ions **h**, **i** and **j** at *m/z* 356, 314 and 248 (Scheme 1). Comparison of the mass spectrum of milbemycin D with those of milbemycins α_1 ($C_{31}H_{44}O_7$, M^+ 528, **a**, R=CH_3) and α_3 ($C_{32}H_{46}O_7$, M^+ 542, **a**, R=C_2H_5) showed that the *m/z* values of ions **b**, **d**, **e**, **f** and **g**, which comprise the side chain at C-25, of milbemycin D were 28 and 14 mass units more than those of milbemycins α_1 and α_3, respectively, while the ions **h**, **i** and **j** were unaffected by changes in the spiroketal portion of the molecule. Thus it can be concluded that a C_3H_7 group is attached at C-25 instead of the ethyl group of milbemycin α_3 and the methyl group of milbemycin α_1.

The ^{1}H NMR spectrum of milbemycin D showed two methyl signals as doublets at δ 0.86 and 1.05 (each 3H) assignable to an isopropyl group. In contrast, the methyl signals of the side chain at C-25 in milbemycins α_1 (25-R=CH_3) and α_3 (25-R=CH_2CH_3) appeared as a doublet peak at δ 1.15 and a triplet peak at δ 0.85, respectively. This was confirmed by the comparison of ^{13}C NMR spectral data of these metabolites (α_1 and α_3). The D component showed two methyl signals at δ 14.2 and 21.0 *versus* at δ 19.4 of milbemycin α_1 and δ 10.1 of milbemycin α_3. The low field shift of the methine signal at C-25

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

Milbemycin	M^+	Fragment ions											Molecular formula
D	556		428	410	314	278	259	248		209	181	151	$C_{33}H_{48}O_7$
E	572	554	430		294	278	259			209	181		$C_{34}H_{52}O_7$
F	665		428	410	314	278	259	248		209	181	151	94 $C_{38}H_{51}NO_9$
G	570		428	410	314	278	259	248		209	181	151	$C_{34}H_{50}O_7$
H	540	522	504					245	227	209	181		$C_{33}H_{48}O_6$
J	526	508	401			259		241		181	153	151	$C_{31}H_{42}O_7$
K	540	522	415			259		241		195	167	151	$C_{32}H_{44}O_7$

Scheme 1. Diagnostic ion structures of mass spectra of milbemycins α_1, α_3 and D.



bearing ethereal oxygen atom was observed at δ 78.4 for D component, while milbemycins α_1 and α_3 showed it at δ 71.3 and 76.0, respectively.

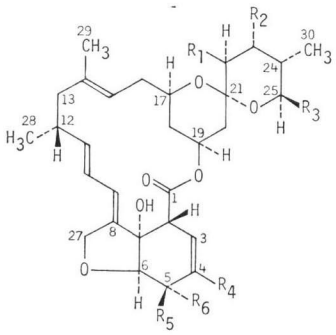
These data are consistent with the presence of an isopropyl group in milbemycin D as shown in Fig. 1 in place of the methyl group at C-25 of milbemycin α_1.

Milbemycin F

Milbemycin F, amorphous, has the molecular formula of $C_{38}H_{51}NO_9$ (Table 1). The IR (ν 3320, 1730 and 1710 cm^{-1}) and UV [λ_{max}^{EtOH} 245 nm (ε 24,100) and 253 nm (ε 21,400)] spectra of milbemycin F were substantially identical with those of milbemycins α_9 and α_{10}.

In the mass spectrum of milbemycin F, the bulk of the high mass fragments (ions **b** through **j**) agreed in detail with that of milbemycin D and the differentiation was the appearance of pyrrole carboxylic acid ($C_5H_5NO_2$, m/z 111) and pyrrole carbonyl (C_5H_4NO, m/z 94) radicals (Table 1). Accordingly the pyrrole ring should exist in the counterpart, suffered a retro Diels-Alder reaction, of species **b** (Scheme 1). Further evidence for the pyrrole ester chain in F component was provided by the comparison of the NMR data: the C-4 methyl broad singlet at δ 1.86 characteristic in D component was replaced by a broad singlet of the methylene group at δ 4.87 bearing oxygen atom. In both ^1H and ^{13}C NMR spectra, the existence of the pyrrole ring is confirmed by three multiplet signals at δ 6.18, 6.86 and 7.01 and by three peaks at δ 110.9, 116.6 and 121.3 assignable to β, β and α-protons and to the three carbons of pyrrole ring, respectively.

Fig. 1. Structures of milbemycins (1). α Series of milbemycin.



	R_1	R_2	R_3	R_4	R_5	R_6
α_1	H	H	CH_3	CH_3	OH	H
α_2	H	H	CH_3	CH_3	OCH_3	H
α_3	H	H	C_2H_5	CH_3	OH	H
α_4	H	H	C_2H_5	CH_3	OCH_3	H
α_5	OH	$OCOCHC_4H_9$ (CH$_3$)	CH_3	CH_3	OH	H
α_6	OH	$OCOCHC_4H_9$ (CH$_3$)	CH_3	CH_3	OCH_3	H
α_7	OH	$OCOCHC_4H_9$ (CH$_3$)	C_2H_5	CH_3	OH	H
α_8	OH	$OCOCHC_4H_9$ (CH$_3$)	C_2H_5	CH_3	OCH_3	H
α_9	H	H	CH_3	CH_2OCO–pyrrole	OH	H
α_{10}	H	H	C_2H_5	CH_2OCO–pyrrole	OH	H
D	H	H	$CH(CH_3)_2$	CH_3	OH	H
F	H	H	$CH(CH_3)_2$	CH_2OCO–pyrrole	OH	H
G	H	H	$CH(CH_3)_2$	CH_3	OCH_3	H
J	H	H	CH_3	CH_3	=O	
K	H	H	C_2H_5	CH_3	=O	

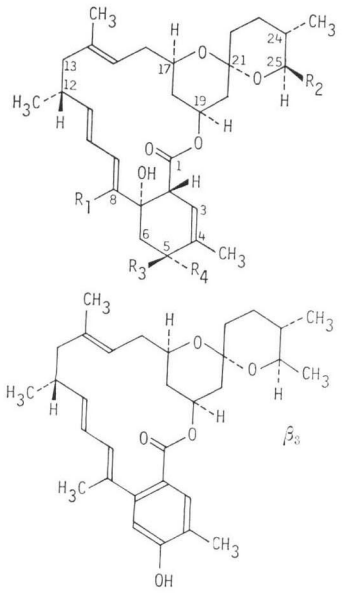
The above results allow the structure of milbemycin F to be formulated as shown in Fig. 1.

Milbemycin G

Milbemycin G, amorphous, $[\alpha]_D^{27}$ +108° (c 0.25, acetone), has the molecular formula $C_{34}H_{50}O_7$ (Table 1). The IR (ν 3450 and 1710 cm^{-1}) and UV [λ_{max}^{EtOH} 244 nm (ε 30,500)] spectra indicated a marked resemblance to those of milbemycins α_2 and α_4.

The mass spectra of milbemycins G and D coincided completely with each other except for their molecular ions, indicating that the CH_3 group of milbemycin G exists in the part of a fragment eliminated from the molecular ion by a retro Diels-Alder reaction. The ^1H and ^{13}C NMR were very similar to those of milbemycin D with the exception of the presence of one methyl signal of methoxy group at δ 3.48 (3H, s) and δ 58.77.

Fig. 1. Structures of milbemycins (2). β Series of milbemycin.



	R_1	R_2	R_3	R_4
β_1	CH_2OH	CH_3	OCH_3	H
β_2	CH_2OH	C_2H_5	OCH_3	H
E	CH_2OH	$CH(CH_3)_2$	OCH_3	H
H	CH_3	$CH(CH_3)_2$	=O	

These data are consistent with the presence of a methoxy group in milbemycin G in place of the hydroxyl group at C-5 of milbemycin D as shown in Fig. 1.

Milbemycin J

Milbemycin J, mp 213~215°C, $[\alpha]_D^{27}$ +40° (c 0.25, acetone), has the molecular formula $C_{31}H_{42}O_7$ (Table 1). The IR spectrum indicated an α,β-unsaturated ketone moiety at 1680 cm^{-1} and a tertiary hydroxyl group at 3510 cm^{-1} without a secondary hydroxyl group. The UV spectrum exhibited an absorption maximum at 240 nm (ε 28,000).

The ^1H NMR was very similar to that of milbemycin α_1, the differences being a methine at C-6 and vinylic proton at C-3 as singlets shifted to downfields at δ 4.02 and 6.52, respectively. Hence, in the structure of milbemycin J, the presence of a ketone functionality in place of a hydroxyl group at C-5 in milbemycin α_1 was inferred.

In the mass spectrum, an outstanding fragment ion was a stabilized aromatic ring species **b** formed by dehydration from the molecular ion. An interpretation of the mass spectrum was shown in Scheme 2. The ion **b** was followed by a bond cleavage of the allylically activated position and of the spiroketal region in a well-defined manner as described above.

Further evidence for the structure was provided by chemical reaction, *i.e.* reduction of milbemycin J with sodium borohydride gave stereoselectively and alcoholic compound having indistinguishable spectral data from milbemycin α_1.

Milbemycin K

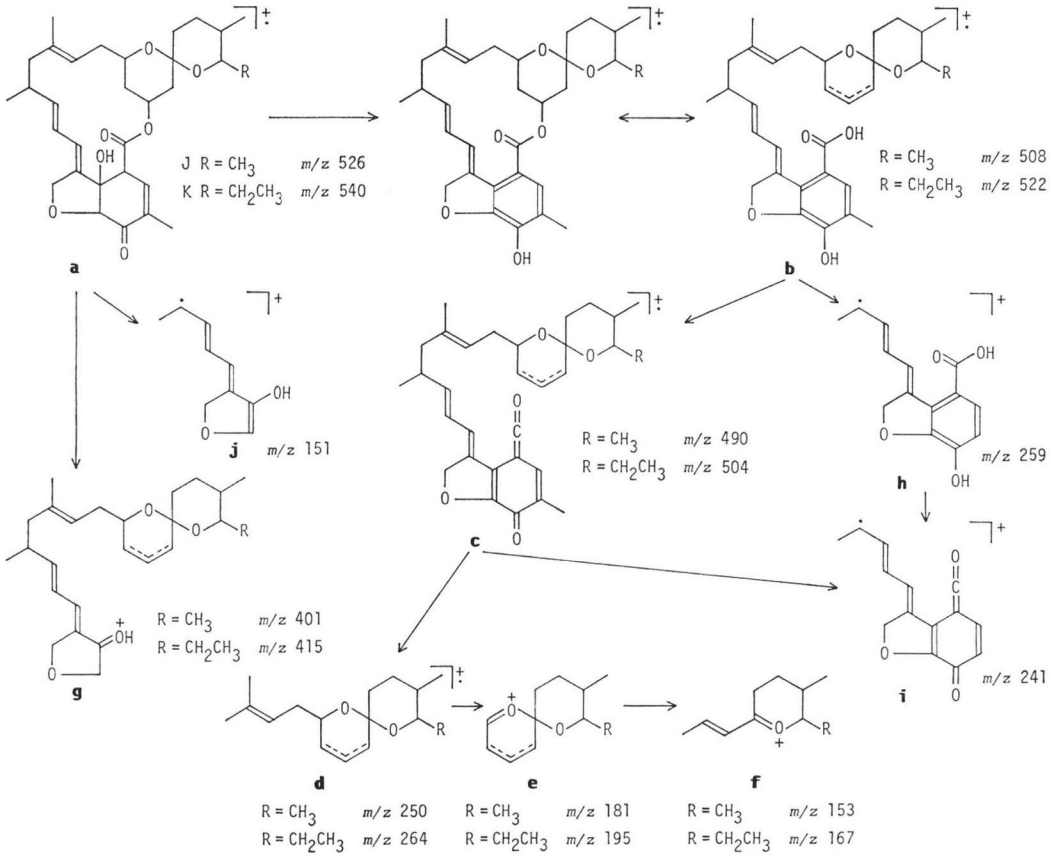
Milbemycin K, amorphous, $[\alpha]_D^{27}$ +42° (c 0.25, acetone), has the molecular formula $C_{32}H_{44}O_7$ (Table 1). The IR (ν 3510, 1730 and 1680 cm^{-1}) and UV [λ_{max}^{EtOH} 240 nm (ε 29,200)] spectra were substantially identical to those of milbemycin J.

The ^1H NMR was very similar to that of milbemycin α_3, the difference being as quite equal downfield shift of proton signals at C-3 and C-6 as observed in the ^1H NMR of milbemycin J.

In the mass spectrum as shown in Scheme 2, diagnostic ions **a**, **b**, **c**, **d**, **e**, **f**, and **g** at m/z 540, 522, 504, 264, 195, 167 and 415 were 14 mass unit more than those of milbemycin J, and another prominent ion peaks at m/z 259, 241 and 151 formed from ions **b**, **c** and **a** by cleavage of C_{12}-C_{13} bond were observed in both spectra. These data allows the conclusion that the structure of milbemycin K is a homo analogue at C-25 of milbemycin J as shown in Fig. 1.

Chemical proof for the structure of milbemycin K was obtained by sodium borohydride reduction

Scheme 2. Diagnostic ion structures of mass spectra of milbemycins J and K.



to give milbemycin α_3.

Milbemycin E

Milbemycin E, amorphous, $[\alpha]_D^{27}$ +157° (*c* 0.25, acetone), has the molecular formula $C_{34}H_{52}O_7$ (Table 1). The IR (ν 3490 and 1715 cm^{-1}) and UV [λ_{max}^{EtOH} 241 nm (ε 26,000)] spectra were essentially identical with those of milbemycins β_1 and β_2.

In the mass spectrum of milbemycin E, the fragment ions **b**, **c**, **d** and **e** derived from the rupture of mass spectrometrically fragile bond were observed at *m/z* 430, 278, 209 and 181 with the increased shift by 28 and 14 mass units compared with the corresponding ions in the spectrum of milbemycins β_1 and β_2, respectively, while one of the important peaks was observed as a prominent peak having a following ion structure at *m/z* 294, which was unaffected by changes in the spiroketal portions of the molecule (Scheme 3).

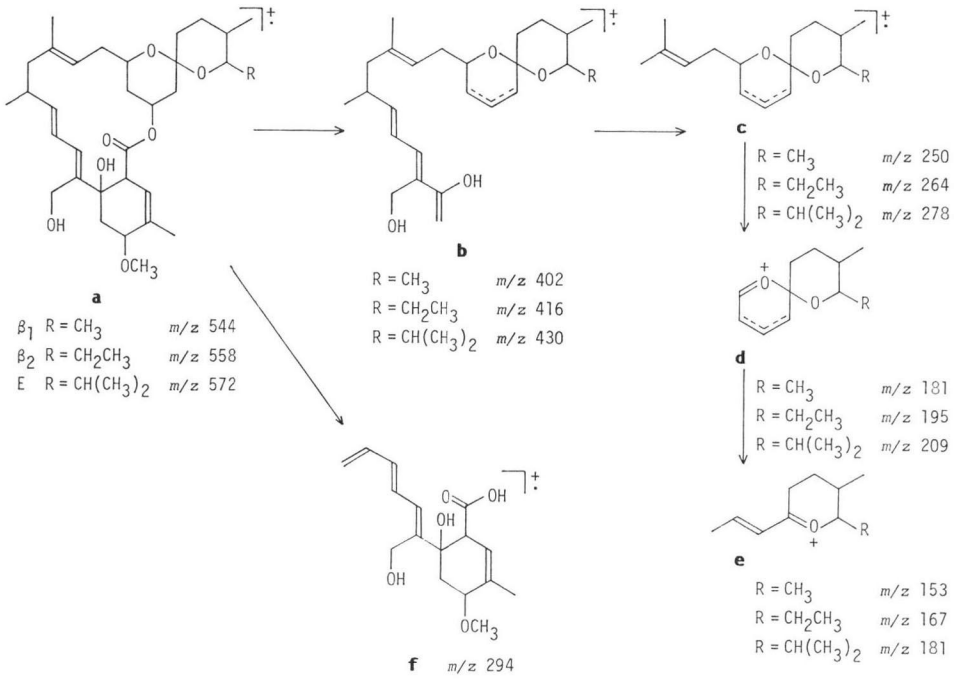
In accordance with the mass data, the ^1H NMR showed two doublets of methyl signals in the isopropyl group at δ 0.83 and 1.00 (each 3H, d).

These results clearly indicate that milbemycin E includes isopropyl radical as the side chain at C-25, and the structure was determined as depicted in Fig. 1.

Milbemycin H

Milbemycin H, amorphous, $[\alpha]_D^{27}$ +60° (*c* 0.25, acetone), has the molecular formula $C_{33}H_{48}O_6$

Scheme 3. Diagnostic ion structures of mass spectra of milbemycins β_1, β_2 and E.



(Table 1). The IR (ν 3490, 1715 and 1685 cm^{-1}) and UV [λ_{max}^{EtOH} 237 nm (ε 25,200)] spectra were essentially identical with those of milbemycins J and K, which have an α,β-unsaturated ketone in common. The molecular formula of this compound differs from other milbemycins by a shortage of one oxygen.

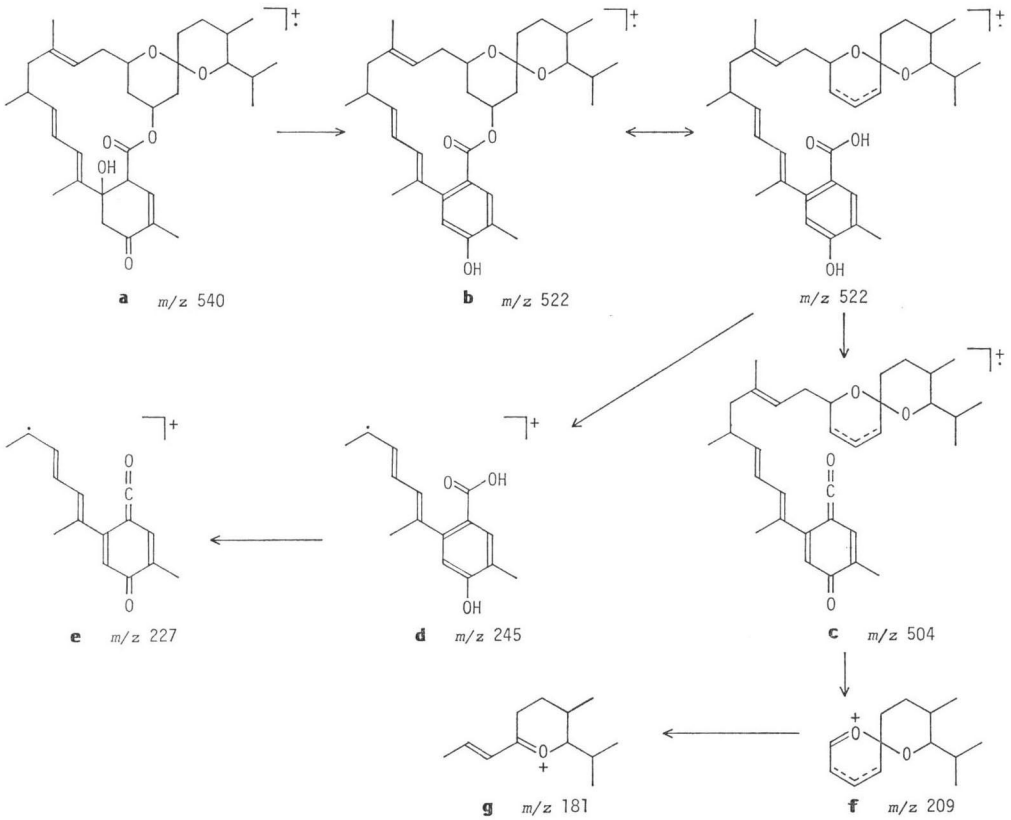
In the mass spectrum, the intense peaks of **a**, **b**, **c**, **d** and **e** could be explained by the formation of stabilized aromatic species resulted from the dehydration reaction of molecular ion, as shown in Scheme 4. The assemblage of the fragments allows to construct the overall structure of milbemycin H. Additional evidence for this structure is provided by the ^1H NMR spectrum, showing the methyl signal at C-8 at δ 1.74 (3H, s) and the methylene signal at C-6 adjacent to a carbonyl group at δ 2.45 and 2.74 (each 1H, d).

Absolute Configuration of Milbemycins

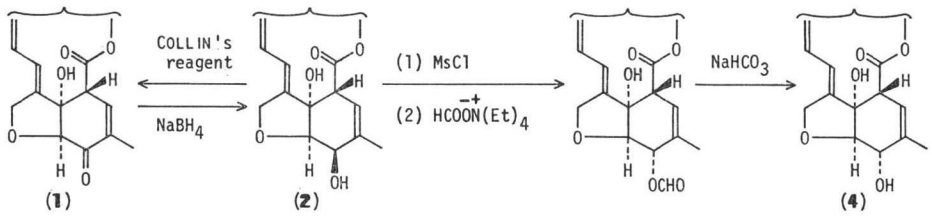
In the previous paper[1,2], the relative configuration of the milbemycins was determined by X-ray crystallography and a detailed analysis of the ^1H NMR data. The absolute stereochemistry, however, have not been confirmed. The Merck group recently reported that avermectins were chemically correlated to the milbemycins by dehydroxylation of C-13 hydroxyl group of avermectin aglycons[9] and the absolute configuration of avermectins was determined on the basis of the L-series of methyl oleandroside produced by methanolysis of avermectin A_{2a}[10].

In order to directly establish the absolute configuration of the hydroxyl group at C-5 incorporated in the cyclic milbemycin D skeleton, the MILLS' rule[11] was employed together with CD allylic benzoate method[12]. For the preparation of the C-5 epimeric alcohol, reduction of the α,β-unsaturated ketone derivative (**1**), obtained by oxidation of milbemycin D (**2**) with COLLIN's reagent, with sodium borohydride gave no epimeric alcohol, but stereoselectively the starting material, milbemycin D, probably

Scheme 4. Diagnostic ion structures of mass spectrum of milbemycin H.



Scheme 5.

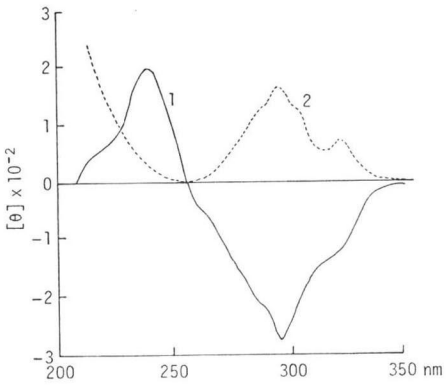


due to the steric hindrance of the tetrahydrofuran ring and/or the participation of the tertiary hydroxyl group at C-7. Alternatively, the 5-mesylate of milbemycin D was subjected to S_N2 displacement with tetraethylammonium formate followed by hydrolysis with $NaHCO_3$, giving the epimeric alcohol (**4**) as shown in Scheme 5.

The specific rotations of milbemycin D ($[\alpha]_D$ +105°) and its acetate (**5**) (+92°) were more levorotatory than the epimer ($[\alpha]_D$ +132°) and its acetate (**6**) (+119°), respectively, showing that the stereochemistry of C-5 in milbemycin D is the *R* configuration. The same conclusion was also supported by CD measurement of the *p-N,N*-dimethylaminobenzoates (**7** and **8**). The CD spectrum of the benzoate of natural milbemycin D gave rise to a negative Cotton effect at 295 nm and the epimer at C-5 positive (Fig. 2).

It comes to the conclusion that application of the empirical rules usually employed for deducing the absolute configurations of allylic alcohols to milbemycin D led to the formulations as shown in Fig. 1.

Fig. 2. CD spectra of the benzoate of milbemycin D (1) and its epimeric isomer at C-5 (2) in ethanol solution.



Experimental

The new milbemycin analogues were amorphous or foams and were therefore purified by high performance column chromatography [Waters Corasil A column with eluting solvents of *n*-hexane - MeOH (100: 3.5) or Waters Micro-Bondapak C_{18} reverse phase column with MeOH - water (100: 7.5) as solvent]. Silica gel (Kieselgel 60, 70~230 mesh, ASTM) was used for short column chromatography. Usual workup means two or three time extractions with the solvent specified and washing the extract with water, drying with Na_2SO_4, and concentration to a solid residue *in vacuo*. All compounds were characterized by 100 MHz or 90 MHz ^{1}H NMR spectra of a Varian EM-390 and HA-100 in $CDCl_3$ solution with tetramethylsilane as internal standard, by mass spectra on a Jeol MLS-01SG, by IR spectra on a Jasco IRA-2 spectrometer and by UV spectra on a Hitachi 200–20 spectrophotometer, and were in full agreement with the assigned structures.

5-Oxomilbemycin D (1)

To a stirred solution of the COLLIN's reagent, prepared from chromium (VI) oxide (5.0 g, 50 mmol) and pyridine (8.0 g, 101 mmol) in 115 ml of CH_2Cl_2, was added dropwise a solution of milbemycin D (2.78 g, 5 mmol) in 50 ml of CH_2Cl_2 in an ice bath over 30 minutes. After stirring for an hour at an ambient temperature, the reaction mixture was poured into 700 ml of *n*-hexane, and filtered by Celite. The filtrate was concentrated *in vacuo*, and the residue was purified by column chromatography on silica gel (50 g) by elution with *n*-hexane - ethyl acetate (EtOAc) (5: 1 to 4: 1) to give 1.498 g of **1** as an amorphous. ^{1}H NMR (100 MHz, $CDCl_3$) δ 6.50 (1H, br s, H-3), 3.80 (1H, s, H-6), 4.71 (2H, m, H_2-27); IR ν_{max}^{Nujol} cm^{-1} 3470 (*tert.* OH), 1740, 1715, 1685; MS *m/z* 554 (M^+), 536 (M^+-18), 209, 181.

Reduction of 5-Oxomilbemycin D by Sodium Borohydride

To a stirred solution of 5-oxomilbemycin D (20 mg, 0.036 mmol) in 1 ml of MeOH in an ice bath was added $NaBH_4$ (6 mg). The reaction mixture was additionally stirred for 20 minutes at an ambient temperature. To the cold reaction mixture was added 1 ml of dilute acetic acid. After the usual workup with EtOAc, 17 mg of a crude milbemycin D as a foam was obtained. It was purified by column chromatography on silica gel by elution with *n*-hexane - EtOAc (85: 15) to give 14 mg of milbemycin D (**2**), which was identified with the authentic sample.

5β-*O*-Methanesulfonylmilbemycin D (3)

To a stirred solution of milbemycin D (1.112 g, 2 mmol) and triethylamine (600 mg, 6 mmol) in 10 ml of anhydrous tetrahydrofuran in an ice bath was added dropwise a solution of methanesulfonyl chloride (573 mg, 5 mmol) in 5 ml of anhydrous tetrahydrofuran. After one hour at an ambient temperature, the usual workup with benzene gave 1.21 g of a crude **3** as a white amorphous product which was used to the further reaction. ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.52 (3H, s, CH_3-14), 1.85 (3H, br s, CH_3-4), 3.07 (3H, s, O–SO_2CH_3), 3.32 (1H, m, H-2), 4.07 (1H, d, J=6.0 Hz, H-6), 4.65 (2H, s, H_2-27); IR ν_{max}^{Nujol} cm^{-1}3500 (*tert.* OH), 1750, 1725, 1180.

5α-Hydroxymilbemycin D (4)

A solution of 5β-*O*-methanesulfonylmilbemycin D (1.011 g, 1.59 mmol) and tetraethylammonium

formate (534 mg, 3.62 mmol) in 30 ml of CH_2Cl_2 was stirred at room temperature for 3 days. The usual workup with benzene gave 980 mg of a crude formate. A solution of the formate (980 mg) and $NaHCO_3$ (300 mg) in 30 ml of MeOH and 1 ml of water was stirred at room temperature for 10 hours. After the usual workup with benzene, 786 mg of a viscous oily product was purified by column chromatography on silica gel by elution with *n*-hexane - EtOAc to give 446 mg of **4**. ^1H NMR (90 MHz, $CDCl_3$) δ 1.89 (3H, br s, CH_3-4), 3.03 (1H, m, H-2), 3.81 (1H, d, J=1.5 Hz, H-6), 4.00 (1H, m, H-5), 4.56 (2H, s, H_2-27); IR ν_{max}^{Nujol} cm^{-1} 3560 (*tert.* OH), 3380 (*sec.* OH), 1710; MS *m/z* 556 (M^+), 428, 410, 209, 181.

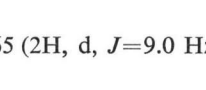
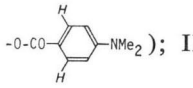
5β-*O*-Acetylmilbemycin D (**5**)

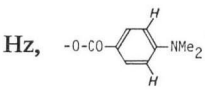
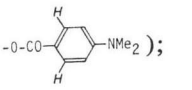
A solution of milbemycin D (400 mg, 0.719 mmol) and 1 ml of acetic anhydride in 3 ml of pyridine was allowed to stand at room temperature overnight. The reaction mixture was poured into water. The usual workup with EtOAc gave a crude product, which was purified by column chromatography on silica gel by elution with *n*-hexane - EtOAc (85: 15) to give 274 mg of **5**. ^1H NMR (90 MHz, $CDCl_3$) δ 1.53 (3H, s, CH_3-14), 1.76 (3H, br s, CH_3-4), 2.13 (3H, s, CH_3CO–O-5), 3.33 (1H, m, H-2), 4.05 (1H, d, J=6.0 Hz, H-6); IR ν_{max}^{Nujol} cm^{-1} 3470, 1740, 1710, 1230, 1160; MS *m/z* 598 (M^+), 428, 410, 209, 181.

5α-*O*-Acetylmilbemycin D (**6**)

A solution of 5β-*O*-methanesulfonylmilbemycin D (229 mg, 0.361 mmol) and tetraethylammonium acetate (188.5 mg, 0.72 mmol) in 10 ml of CH_2Cl_2 was stirred at room temperature for 2 days. The usual workup with benzene gave a crude product (211 mg), which was purified by the column chromatography on silica gel by elution with *n*-hexane - EtOAc (95: 5 to 75: 25) to give 173 mg of **6**. ^1H NMR (100 MHz, $CDCl_3$) δ 1.51 (3H, s, CH_3-14), 1.74 (3H, br s, CH_3-4), 2.10 (3H, s, CH_3CO–O-5), 3.04 (1H, m, H-2), 3.70 (1H, d, J=1.0 Hz, H-6); IR ν_{max}^{Nujol} cm^{-1} 3550, 1740, 1230, 1165; MS *m/z* 598 (M^+), 538, 428, 410, 209, 181.

5β- and 5α-*O*-*p*-(*N*,*N*-Dimethyl)aminobenzoylmilbemycin D (**7**, **8**)

A solution of milbemycin D (556 mg, 1 mmol) and *p*-*N*,*N*-dimethylaminobenzoyl chloride (472 mg, 2.57 mmol) in 5.5 ml of pyridine was allowed to stand at room temperature for 4 hours. The precipitated material was filtered and washed with benzene. The filtrate was washed successively with water and saturated NaCl solution, and dried over Na_2SO_4. After evaporation of the solvent *in vacuo*, the residue was chromatographed on silica gel by elution with *n*-hexane - EtOAc (90: 10 to 75: 25) to give 352 mg of **7** as an amorphous. ^1H NMR (90 MHz, $CDCl_3$) δ 1.53 (3H, s, CH_3-14), 1.80 (3H, br s, CH_3-4), 3.00 (6H, s, $-NMe_2$), 3.42 (1H, m, H-2), 6.65 (2H, d, J=9.0 Hz, ), 7.94 (2H, d, J=9.0 Hz, ); IR ν_{max}^{Nujol} cm^{-1} 3480, 1715, 1610, 1180, 1120; UV λ_{max}^{EtOH} nm 237 (ε 31,870), 244 (ε 31,570), 312 (ε 31,740); MS *m/z* 703 (M^+), 538, 209, 181.

In a similar manner as described above, 52 mg of **8** was obtained from 5α-hydroxymilbemycin D (66 mg) and *p*-*N*,*N*-dimethylaminobenzoyl chloride (40 mg) in 1 ml of pyridine. ^1H NMR (90 MHz, $CDCl_3$) δ 1.52 (3H, s, CH_3-14), 1.79 (3H, br s, CH_3-4), 3.01 (6H, s, $-NMe_2$), 3.10 (1H, m, H-2), 3.86 (1H, d, J=1.5 Hz, H-6), 6.62 (2H, d, J=9.0 Hz, ), 7.92 (2H, d, J=9.0 Hz, ); IR ν_{max}^{Nujol} cm^{-1} 3480, 1715, 1610, 1180, 1100; UV λ_{max}^{EtOH} nm 238 (ε 31,610), 244 (ε 31,000), 313 (ε 32,110); MS *m/z* 703 (M^+), 538, 520, 209, 181.

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