MILBEMYCIN DERIVATIVES: MODIFICATION AT THE C-5 POSITION

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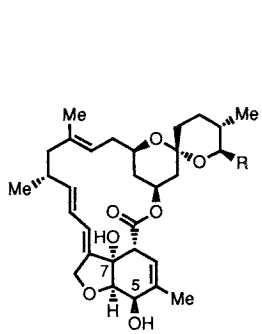
Protection of the hydroxyl group at the C-5 position of milbemycin A_4 and D was carried out to investigate the influence of the C-5 hydroxyl group on the anthelmintic potency of these derivatives. Moreover, the hydroxyl group was converted into amide groups as bioisosters. Biological activities of these derivatives were measured against *Nippostrongylus brasiliensis in vitro*, and minimal concentrations which induce 100% immotility in worms were determined for each derivative. Biological testing revealed that the hydroxyl group at C-5 is a structural requirement for retaining anthelmintic activity.

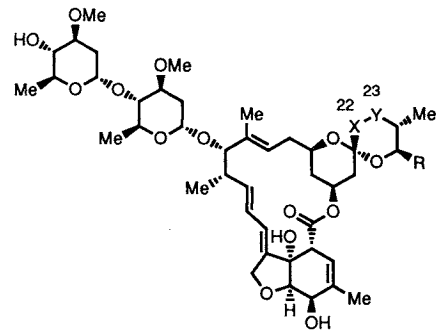
The milbemycins (**1**) are a family of 16-membered ring macrolides isolated from *Streptomyces hygroscopicus* subspecies *aureolacrimosus*[1~3]. Shortly after the discovery of the milbemycins, a Merck group reported on the isolation of the avermectins (**2**) from *Streptomyces avermitilis*[4,5]. These two families of compounds possess similar structures as illustrated in Fig. 1, and exhibit exceptionally potent acaricidal and insecticidal activities. Although a macrocyclic lactone structure is one of the features in the antibacterial macrolides and the antifungal macrocyclic polyenes, the avermectins and the milbemycins have surprisingly neither antibacterial nor antifungal activities. Since their discovery, the avermectins and the milbemycins have attracted considerable interest from several laboratories due to their potent activities and unique structures[6~9]. Furthermore numerous efforts have been made to develop naturally occurring compounds, or semisynthetic derivatives, as insecticides for agricultural use and as anthelmintics for

Fig. 1. Structures of milbemycins and avermectins.



1

Milbemycin	R	
A_4	Et	**1a**
D	*i*-Pr	**1b**
A_3	Me	**1c**



2

Avermectin	R	X–Y
B_{1a}	*s*-Bu	C=C
B_{1b}	*i*-Pr	C=C
B_{2a}	*s*-Bu	CH_2–CHOH
B_{2b}	*i*-Pr	CH_2–CHOH

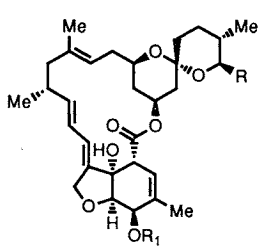
not only animal but also human use. Presently some avermectins and milbemycins are in common use for three particular applications. They are as follows: a combination of the 22,23-dihydro derivatives of avermectin B_{1a} and B_{1b} is effective as a veterinary endectocide, a mixture of milbemycin A_4 and A_3 as an agricultural miticide, and a combination of the oxime derivatives of milbemycin A_4 and A_3 as a canine parasiticide.

In the course of our program to develop an anthelmintic, we have investigated chemical transformations of milbemycins. The biochemical explanation as to why the avermectins and the milbemycins possess such activities is based on the argument that they interfere with the neurotransmission of invertebrates by their allosterical binding to GABA-binding-protein[10]. The hydroxyl groups at the C-5 and C-7 positions, the ester moiety, and the oxygen atoms in the spiroacetal moiety of the milbemycin skeleton must have important roles in the formation of this binding complex. Thus one would expect that protection of the hydroxyl groups of the milbemycin skeleton would be disadvantageous for the biological activities. However, many derivatives of avermectins and milbemycins have been synthesized by protecting the C-5 hydroxyl group with a proper functional group. These manipulations are due to the notion that the activities *in vivo* are influenced by variations in uptake and metabolism of the individual compounds. In our laboratories, a vitro assay which excludes the effects of uptake and metabolism has been used to examine the biological effect which those potent derivatives have. In this paper, we will describe the chemical transformations performed at the C-5 position of milbemycin A_4 and D, and their biological activities in this assay system.

Results and Discussion

Initially, we synthesized derivatives varying in functionality, namely esters, ethers, sulfonates and carbamates of the C-5 hydroxyl group, by standard chemistry as described in Scheme 1[11]. The synthesized derivatives and the parent compounds, milbemycin A_4 and D, were examined their potencies on *N. brasiliensis* motility *in vitro*, *i.e.* minimal concentrations required to cause 100% of immotile worms after 24 hours of incubation at 26°C were determined for each milbemycin analog, and the concentrations are described as MEC (μg/ml) values. As shown in Table 1, the milbemycin analogs synthesized showed lower activities than the parent compounds. The levels of activities of the derivatives were almost the same in each series of A_4 and D.

Scheme 1. Modifications of the C-5 hydroxyl group of milbemycins.



	R	R_1
1a	Et	H
1b	*i*-Pr	H

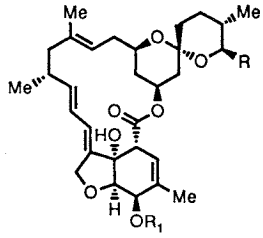
MeI/Ag_2O (or ethyl vinyl ether/H^+) → ethers
3 (R_1 = Me)
4 (R_1 = ethoxyethyl)

acid anhydride → esters
5 (R_1 = acetyl)
6 (R_1 = propionyl)
7 (R_1 = butyryl)

$MeSO_2Cl/Et_3N$ → sulfonates
8 (R_1 = $MeSO_2$)

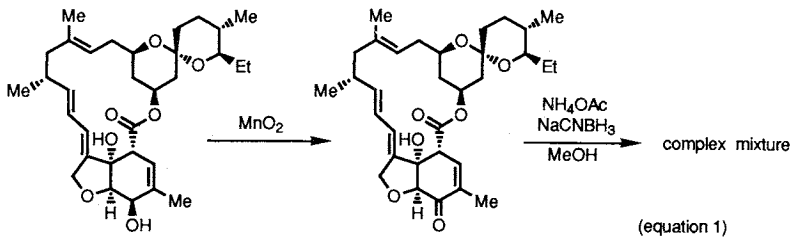
$MeNCO/cat.Ph_2SnCl_2$ → carbamates
9 (R_1 = MeNHCO)

Table 1. Efficiency of milbemycin analogs on *N. brasiliensis* motility *in vitro*.



Milbemycin	R_1	MEC[a] (μg/ml)	
		A_4 series (R=Et)	D series (R=*i*-Pr)
1a (A_4)	H	0.005	—
1b (D)	H	—	0.0025
3	Me	0.32	0.16
4	Ethoxyethyl	0.32	0.16
5	Acetyl	0.16	0.08
6	Propionyl	0.16	0.16
7	Butyryl	0.08	0.08
8	$MeSO_2$	0.32	0.16
9	MeNHCO	1.25	0.08

[a] MEC: Minimal concentrations required to cause 100% of immotile worms.



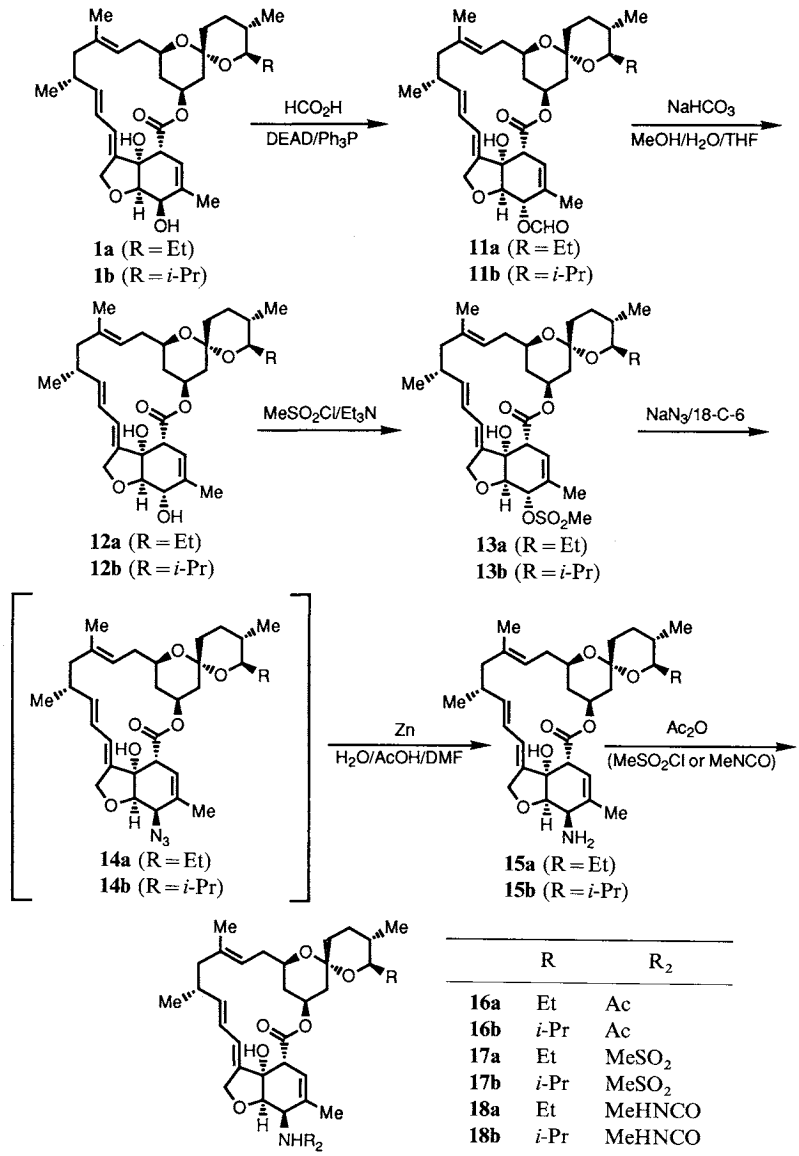
(equation 1)

1a **10a**

As the importance of the C-5 hydroxyl group in the milbemycin skeleton for biological activity became clear, we next focussed on a study of the corresponding bioisosters. Functional groups such as amide, sulfonamide and ureido are well known as the bioisosters for hydroxyl groups[12]; therefore, the C-5 hydroxyl group was replaced by these functionalities.

Firstly, we examined reductive amination to obtain the 5-amino derivative directly. Thus, the 5-oxomilbemycin A_4 (**10a**), which was easily obtained from milbemycin A_4 (**1a**) by the oxidation of the C-5 hydroxyl group with MnO_2[13], was treated with NH_4OAc and $NaCNBH_3$ in MeOH. The reaction, however, afforded a complex mixture (Equation 1). Consequently, we attempted a somewhat more elaborate sequence applying double inversion at the C-5 position of milbemycins (**1a, b**). Thus, the C-5 hydroxyl group was inverted by the Mitsunobu reaction (HCO_2H, Ph_3P, diethyl azodicarboxylate) and subsequent hydrolysis ($NaHCO_3$, H_2O-MeOH-THF) of the formate (**11a, b**) gave hydroxy derivative (**12a, b**). Methanesulfonylation ($MeSO_2Cl$, Et_3N) of the α-hydroxy derivative yielded α-methanesulfonate (**13a, b**), which was reacted with NaN_3 in CH_3CN at ambient temperature to give a crude mixture containing 5β-azido derivative (**14a, b**). Subsequently, the crude mixture was subjected to the reduction with Zn in DMF including a small amount of H_2O and AcOH. Purification of the reaction mixture by column chromatography gave the desired 5β-amino derivative (**15a, b**) in moderate yield (50 and 43%, respectively). Despite only attaining moderate levels of conversion to the amino derivatives, other reaction conditions to obtain these derivatives were not attested further. By reaction with Ac_2O, $MeSO_2Cl$ or MeNCO, the amino derivatives were converted into their corresponding acetamides (**16a, b**), methanesulfonamides (**17a, b**) or ureido derivatives (**18a, b**) in good yields (Scheme 2).

Scheme 2. Synthesis of 5-amino milbemycin derivatives.



1a (R = Et)
1b (R = *i*-Pr)

11a (R = Et)
11b (R = *i*-Pr)

12a (R = Et)
12b (R = *i*-Pr)

13a (R = Et)
13b (R = *i*-Pr)

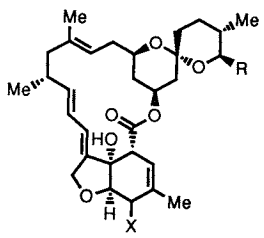
14a (R = Et)
14b (R = *i*-Pr)

15a (R = Et)
15b (R = *i*-Pr)

	R	R_2
16a	Et	Ac
16b	*i*-Pr	Ac
17a	Et	$MeSO_2$
17b	*i*-Pr	$MeSO_2$
18a	Et	MeHNCO
18b	*i*-Pr	MeHNCO

The results of the aforementioned biological test of the bioisosters and intermediates synthesized are described in Table 2. Contrary to our expectations, these bioisosters (**16**, **17** and **18**) required even higher concentrations than their corresponding precursors to induce full immotility in worms. Furthermore, their activities were lower than those of the *O*-protected derivatives as shown in Table 1. As the correlation between binding affinities of avermectin analogs and their biological activities on *Caenorhabditis elegans* motility was reported[14], the biological activities of mibemycin analogs against *N. brasiliensis* in this study would indicate the extent of their binding affinities to the receptor. Therefore one could postulate from the results of the biological test that the bioisosters have much lower binding affinities than the parent compounds or the *O*-protected derivatives in Scheme 1. Furthermore the introduced amide functionalities

Table 2. Efficiency of milbemycin analogs on *N. brasiliensis* motility *in vitro*.



Milbemycin	X-	MEC[a] (μg/ml)	
		A_4 series (R=Et)	D series (R=*i*-Pr)
16	AcNH	5	1.25
17	$MeSO_2NH$	2.5	0.63
18	MeHNCONH	>10	>10
11	HCO_2	2.5	1.25
12	HO	2.5	0.63
13	$MeSO_2O$	0.16	0.08
15	H_2N	2.5	2.5

[a] MEC: Minimal concentrations required to cause 100% of immotile worms.

in the bioisosters seemed to interfere with the binding to the receptor rather than act as bioisosters for the hydroxyl group. On the other hand, α-substituted derivatives were anticipated to have lower activities than β-substituted derivatives bacause the conformation and the shape of the milbemycin skeleton would be modified by the introduction of the α-substituent. Actually, the α-formyloxy derivatives (**11a, b**) and the α-hydroxy derivatives (**12a, b**) showed much lower activities than the β-substituted derivatives in Table 1. However, the α-methanesulfonyloxy derivatives (**13a, b**) showed nearly the same activities as those of the β-substituted derivatives as shown in Table 1, and even higher than the corresponding β-substituted sulfonates. Although the interpretation of these results were not in accord with the objective of this study, it is nonetheless an interesting one.

In conclusion, ethers, esters, sulfonates and carbamates of the C-5 hydroxyl group of milbemycin A_4 and D were synthesized as a means of masking the hydroxyl functionality. Furthermore amide functionalities as bioisosters were introduced into the C-5 position of the skeleton in place of the hydroxyl group. All derivatives synthesized were tested for their potencies on *N. brasiliensis* motility *in vitro*. Unfortunately, there were no derivatives which exhibited a higher biological activity than the parent milbemycin A_4 or D in our investigations. It was confirmed that the existence of the β-hydroxyl group at the C-5 position is essential for the preservation of the potent biological activities of the milbemycins.

Experimental

N. brasiliensis Motility Assay

The worms used were the *N. brasiliensis* L3 stage. The assay were conducted as follows: About 30 worms were suspended in the phosphate buffer solution (0.90 ml, pH 7.6) containing 1% of sterilized Bacto ager (DIFCO) in each test tube. The milbemycin analogs tested were dissolved in polyethylene glycol 400 (0.10 ml) and added to the test tubes containing the worms. After 24 hours of incubation at 26°C, the percentage of immotile worms was determined at several concentrations of each milbemycin analog. The minimal concentration caused 100% of immotile worms was described as a MEC (μg/ml) value.

General Methods

The natural products (milbemycin A_4 and D) were used as starting materials. Each compound was purified by column chromatography before the reactions and showed >96% purity by HPLC analysis. All compounds were characterized by NMR spectra on a JEOL GSX 400 or JEOL GX 270 spectrometer in $CDCl_3$ solution with tetramethylsilane as internal reference, by mass spectra on a JEOL JMS-AX505H model and by IR spectra on a JASCO FT/IR-830 and were in full agreement with the assigned structures. Unless otherwise indicated all common reagents and solvents were used as obtained from commercial

suppliers without further purification.

5-*O*-Methylmilbemycin A_4 (**3a**)

To a stirred solution of milbemycin A_4 (54 mg, 0.1 mmol) and Ag_2O (46 mg, 0.2 mmol) in CH_3CN (0.5 ml) at ambient temperature was added MeI (0.06 ml, 1.0 mmol) by syringe. After 64 hours, the mixture was filtered with Celite and the filtrate was evaporated *in vacuo*. The residue was chromatographed by flash chromatography (SiO_2, hexane-EtOAc, 5:1) to give 49 mg of impure compound **3a** as a white glass. Final purification by preparative HPLC (YMC 120A ODS, CH_3CN) gave 33 mg (59%) of 5-*O*-methylmilbemycin A_4: IR (KBr) cm^{-1} 3467, 1712, 1181, 1165; MS *m/z* 556 (M^+) $C_{33}H_{48}O_7$; ^1H NMR (400 MHz, $CDCl_3$) δ 4.03 (1H, d, J=5.8 Hz, 6-H), 3.98 (1H, br s, 5-H), 3.51 (3H, s, $-OCH_3$).

5-*O*-Methylmilbemycin D (**3b**)

Reaction of milbemycin D (56 mg, 0.1 mmol) with MeI (0.06 ml 1.0 mmol) and Ag_2O (46 mg, 0.2 mmol) according to the procedure for **3a** at ambient temperature for 17 hours and purification by flash chromatography (SiO_2, hexane-EtOAc, 4:1) followed by further purification by preparative HPLC (YMC 120A ODS, 90% CH_3CN) gave 33 mg (58%) of 5-*O*-methylmilbemycin D: IR (KBr) cm^{-1} 3470, 1712, 1181, 1165, 1010; MS *m/z* 570 (M^+) $C_{34}H_{50}O_7$; ^1H NMR (400 MHz, $CDCl_3$) δ 4.03 (1H, d, J=5.7 Hz, 6-H), 3.98 (1H, m, 5-H), 3.51 (3H, s, $-OCH_3$).

5-*O*-(1-Ethoxyethyl)milbemycin A_4 (**4a**)

A mixture of milbemycin A_4 (54 mg, 0.1 mmol), ethyl vinyl ether (0.02 ml, 0.25 mmol) and a catalytic amount of pyridinium *p*-toluenesulfonate (PPTS) in CH_2Cl_2 (0.5 ml) was stirred for 1 hour. The reaction mixture was poured into saturated $NaHCO_3$ and extracted with CH_2Cl_2, and the extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane-EtOAc, 5:1) to give 49 mg of impure mixture of the two diastereomers. Final purification by preparative HPLC (YMC 120A ODS, 90% CH_3CN) gave 30 mg (49%) of 5-*O*-(1-ethoxyethyl)milbemycin A_4 as a mixture of two diastereomers: IR (KBr) cm^{-1} 3473, 1737, 1712, 1180, 1166, 990; MS *m/z* 614 (M^+) $C_{36}H_{54}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 4.96 and 4.86 (1H, q, J=5.3 and 5.3 Hz, $-OCHCH_3OCH_2CH_3$), 4.47 and 4.29 (1H, m, 5-H), 3.99 and 3.96 (1H, d, J=5.8 and 5.6 Hz, 6-H), 3.62~3.69 (2H, m, $-OCHCH_3OCH_2CH_3$), 1.38 and 1.40 (3H, d, J=5.3 and 5.3 Hz, $-OCHCH_3OCH_2CH_3$), 1.19 and 1.20 (3H, t, J=7.2 and 7.2 Hz, $-OCHCH_3OCH_2CH_3$).

5-*O*-(1-Ethoxyethyl)milbemycin D (**4b**)

Reaction of milbemycin D (167 mg, 0.3 mmol) and ethyl vinyl ether (0.07 ml, 0.75 mmol) in the presence of PPTS according to the procedure for **4a**, and purification by flash chromatography (SiO_2, hexane-EtOAc, 6:1) followed by preparative HPLC (YMC 120A ODS, 90% CH_3CN) gave 75 mg (40%) of 5-*O*-(1-ethoxyethyl)milbemycin D as a mixture of two diastereomers: IR (KBr) cm^{-1} 3474, 1741, 1712, 1166, 1119, 1011, 998; MS *m/z* 628 (M^+) $C_{37}H_{56}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 4.96 and 4.86 (1H, q, J=5.4 and 5.4 Hz, $-OCHCH_3OCH_2CH_3$), 4.47 and 4.29 (1H, m, 5-H), 3.99 and 3.96 (1H, d, J=5.7 and 5.6 Hz, 6-H), 3.61~3.71 (2H, m, $-OCHCH_3OCH_2CH_3$), 1.38 and 1.40 (3H, d, J=5.4 and 5.4 Hz, $-OCHCH_3OCH_2CH_3$), 1.19 and 1.20 (3H, t, J=7.2 and 7.1 Hz, $-OCHCH_3OCH_2CH_3$).

5-*O*-Acetylmilbemycin A_4 (**5a**)

A mixture of milbemycin A_4 (108 mg, 0.2 mmol), Ac_2O (50 mg, 0.5 mmol) and a catalytic amount of dimethylaminopyridine (DMAP) in CH_2Cl_2 (1 ml) was stirred at ambient temperature for 2 hours. The reaction mixture was poured into saturated $NaHCO_3$ and extracted with CH_2Cl_2, and the extract was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane-EtOAc, 4:1) to give 110 mg (94%) of 5-*O*-acetylmilbemycin A_4 as glass: IR (KBr) cm^{-1} 3470, 1743, 1714, 1236, 1180; MS *m/z* 584 (M^+) $C_{34}H_{48}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.55 (1H, m, 5-H), 4.06 (1H, d, J=6.1 Hz, 6-H), 2.16 (3H, s, $-O_2CCH_3$).

5-*O*-Acetylmilbemycin D (**5b**)

According to the procedure for 5-*O*-acetylmilbemycin A_4 (**5a**), milbemycin D (56 mg, 0.1 mmol) was

treated with Ac_2O (0.02 ml, 0.25 mmol) in the presence of DMAP to give 52 mg (87%) of 5-*O*-acetylmilbemycin D: IR (KBr) cm^{-1} 3470, 1743, 1714, 1236, 1166; MS *m/z* 598 (M^+) $C_{35}H_{50}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.53~5.56 (2H, m, 5-H and 3-H), 4.06 (1H, d, J=6.0 Hz, 6-H), 2.16 (3H, s, $-O_2CCH_3$).

5-*O*-Propionylmilbemycin A_4 (**6a**)

As according to the procedure for 5-*O*-acetylmilbemycin A_4 (**5a**), milbemycin A_4 (54 mg, 0.1 mmol) was treated with propionic anhydride (33 mg, 0.25 mmol) in the presence of DMAP to give 56 mg (94%) of 5-*O*-propionylmilbemycin A_4: IR (KBr) cm^{-1} 3469, 1740, 1714, 1180, 1166; MS *m/z* 598 (M^+) $C_{35}H_{50}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.56 (1H, m, 5-H), 4.06 (1H, d, J=5.9 Hz, 6-H), 2.44 (2H, q, J=7.5, $-O_2CCH_2CH_3$), 1.19 (3H, t, J=7.5, $-O_2CCH_2CH_3$).

5-*O*-Propionylmilbemycin D (**6b**)

According to the procedure for 5-*O*-acetylmilbemycin A_4 (**5a**), milbemycin D (56 mg, 0.1 mmol) was treated with propionic anhydride (33 mg, 0.25 mmol) in the presence of DMAP to give 51 mg (83%) of 5-*O*-propionylmilbemycin D: IR (KBr) cm^{-1} 3473, 1741, 1714, 1180, 1011; MS *m/z* 612 (M^+) $C_{36}H_{52}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.56 (1H, m, 5-H), 4.06 (1H, d, J=6.0 Hz, 6-H), 2.44 (2H, q, J=7.5 Hz, $-O_2CCH_2CH_3$), 1.19 (3H, t, J=7.5 Hz, $-O_2CCH_2CH_3$).

5-*O*-Butyrylmilbemycin A_4 (**7a**)

In accordance with the procedure for 5-*O*-acetylmilbemycin A_4 (**5a**), milbemycin A_4 (54 mg, 0.1 mmol) was treated with butyric anhydride (40 mg, 0.25 mmol) in the presence of DMAP to give 54 mg (88%) of 5-*O*-butyrylmilbemycin A_4: IR (KBr) cm^{-1} 3474, 1739, 1714, 1179, 1166; MS *m/z* 612 (M^+) $C_{36}H_{52}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.55 (1H, m, 5-H), 4.06 (1H, d, J=6.1 Hz, 6-H), 2.39 (2H, t, J=7.4 Hz, $-O_2CCH_2CH_2CH_3$).

5-*O*-Butyrylmilbemycin D (**7b**)

According to the procedure for 5-*O*-acetylmilbemycin A_4 (**5a**), milbemycin D (56 mg, 0.1 mmol) was treated with butyric anhydride (40 mg, 0.25 mmol) in the presence of DMAP to give 55 mg (87%) of 5-*O*-butyrylmilbemycin D: IR (KBr) cm^{-1} 3474, 1739, 1714, 1178, 1011; MS *m/z* 622 (M^+) $C_{37}H_{54}O_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.56 (1H, m, 5-H), 4.06 (1H, d, J=6.0 Hz, 6-H), 2.40 (2H, t, J=7.4 Hz, $-O_2CCH_2CH_2CH_3$).

5-*O*-Mesylmilbemycin A_4 (**8a**)

To a stirred solution of milbemycin A_4 (32 mg, 0.06 mmol) and Et_3N (12 mg, 0.12 mmol) in CH_2Cl_2 (0.5 ml) at 0°C was added methanesulfonic anhydride (21 mg, 0.12 mmol) in CH_2Cl_2 (0.5 ml). After 30 minutes at 0°C, the reaction mixture was poured into water and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane - EtOAc, 3 : 1) to give 35 mg (96%) of 5-*O*-mesylmilbemycin A_4: IR (KBr) cm^{-1} 3470, 1713, 1455, 1363, 1176; MS *m/z* 620 (M^+) $C_{33}H_{48}SO_9$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.32 (1H, m, 5-H), 4.11 (1H, d, J=6.0 Hz, 6-H), 3.12 (3H, s, $-OSO_2CH_3$).

5-*O*-Mesylmilbemycin D (**8b**)

Reaction of milbemycin D (56 mg, 0.1 mol) with methanesulfonic anhydride (35 mg, 0.2 mmol) and Et_3N (20 mg, 0.2 mmol) according to procedure for **8a** gave 51 mg (80%) of 5-*O*-mesylmilbemycin D: IR (KBr) cm^{-1} 3475, 1713, 1457, 1365, 1176; MS *m/z* 634 (M^+) $C_{34}H_{50}SO_9$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.33 (1H, m, 5-H), 4.11 (1H, d, J=6.2 Hz, 6-H), 3.12 (3H, s, $-OSO_2CH_3$).

5-*O*-Methylcarbamoylmilbemycin A_4 (**9a**)

A mixture of milbemycin A_4 (54 mg, 0.1 mmol), methyl isocyanate (0.015 ml, 0.25 mmol) and a catalytic amount of Ph_2SnCl_2 in CH_2Cl_2 (1 ml) was stirred for 30 minutes. The reaction mixture was poured into water and extracted with CH_2Cl_2, and the extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane - EtOAc, 1 : 1) to give 51 mg (85%) of 5-*O*-methylcarbamoylmilbemycin A_4: mp 156~160°C; IR (KBr) cm^{-1} 3405, 1731, 1523, 1455,

1181, 1167; MS *m/z* 599 (M^+) $C_{34}H_{49}NO_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.53 (1H, m, 5-H), 4.06 (1H, d, J=5.9 Hz, 6-H), 2.83 (3H, d, J=5.0 Hz, $-NHCH_3$).

5-*O*-Methylcarbamoylmilbemycin D (**9b**)

According to the procedure for **9a**, reaction of milbemycin D (56 mg, 0.1 mmol) and methyl isocyanate (0.015 ml, 0.1 mmol) in the presence of catalytic amount of Ph_2SnCl_2 gave 43 mg (70%) of 5-*O*-methylcarbamoylmilbemycin D: mp 160~165°C; IR (KBr) cm^{-1} 3404, 1732, 1522, 1245, 1167, 1016; MS *m/z* 613 (M^+) $C_{35}H_{51}NO_8$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.53 (1H, m, 5-H), 4.06 (1H, d, J=5.9 Hz, 6-H), 2.84 (3H, d, J=4.6 Hz, $-NHCH_3$).

5-*Epi*-5-*O*-formylmilbemycin A_4 (**11a**)

To a stirred solution of milbemycin A_4 (0.53 g, 0.97 mmol), Ph_3P (1.57 g, 6.0 mmol) and HCO_2H (0.28 g, 6.0 mmol) in THF (7 ml) at ambient temperature was added diethyl azodicarboxylate (DEAD, 1.04 g, 6.0 mmol) in THF (3 ml). After 20 hours, the reaction mixture was concentrated *in vacuo* to dryness. The residue was purified by flash chromatography (SiO_2, hexane-EtOAc, 4:1) to give 0.53 g (92%) of 5-*epi*-5-*O*-formylmilbemycin A_4 as white crystals: mp 257~260°C; IR (KBr) cm^{-1} 3480, 1732, 1166, 990; MS *m/z* 570 (M^+) $C_{33}H_{46}O_8$; ^1H NMR (270 MHz, $CDCl_3$) δ 8.18 (1H, d, J=1.0 Hz, $-OCHO$), 5.41 (1H, m, 5-H), 3.75 (1H, d, J=1.0 Hz, 6-H).

5-*Epi*-5-*O*-formylmilbemycin D (**11b**)

In accordance with the procedure for **11a**, reaction of milbemycin D (0.56 g, 1.0 mmol), Ph_3P (2.10 g, 8.0 mmol), HCO_2H (0.47 g, 8.0 mmol) and DEAD (1.39 g, 8.0 mmol) gave 0.57 g (97%) of 5-*epi*-5-*O*-formylmilbemycin D: mp 241~249°C; IR (KBr) cm^{-1} 3480, 1734, 1164, 1009; MS *m/z* 584 (M^+) $C_{34}H_{48}O_8$; ^1H NMR (270 MHz, $CDCl_3$) δ 8.18 (1H, d, J=1.0 Hz, $-OCHO$), 5.41 (1H, m, 5-H), 3.75 (1H, d, J=1.0 Hz, 6-H).

5-*Epi*-milbemycin A_4 (**12a**)

A mixture of 5-*epi*-5-*O*-formylmilbemycin A_4 (171 mg, 0.3 mmol) and $NaHCO_3$ (50 mg, 0.6 mmol) in MeOH (1 ml), THF (2 ml) and H_2O (0.1 ml) was stirred at ambient temperature for 8 hours. The reaction mixture was poured into water and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane-EtOAc, 2:1) to give 162 mg (99%) of 5-*epi*-5-*O*-formylmilbemycin A_4: IR (KBr) cm^{-1} 3343, 1738, 1717, 1169, 989; MS *m/z* 542 (M^+) $C_{32}H_{46}O_7$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.03 (1H, br s, 5-H), 3.84 (1H, d, J=1.5 Hz, 6-H).

5-*Epi*-milbemycin D (**12b**)

According to the procedure for **12a**, reaction of 5-*epi*-5-*O*-formylmilbemycin D (292 mg, 1.0 mmol) and $NaHCO_3$ (84 mg, 1.0 mmol) gave 275 mg (99%) of 5-*epi*-milbemycin D: IR (KBr) cm^{-1} 3440, 1720, 1456, 1177, 1010; MS *m/z* 556 (M^+) $C_{33}H_{48}O_7$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.03 (1H, br s, 5-H), 3,84 (1H, d, J=1.5 Hz, 6-H).

5-*Epi*-5-*O*-mesylmilbemycin A_4 (**13a**)

Reaction of **12a** (151 mg, 0.28 mmol) and methanesulfonic anhydride (97 mg, 0.56 mmol) in the presence of Et_3N (57 mg, 0.56 mmol) according to the method for **8a**, and purification by flash chromatography (SiO_2, hexane-EtOAc, 3:1) gave 171 mg (98%) of 5-*epi*-5-*O*-mesylmilbemycin A_4: IR (KBr) cm^{-1} 3500, 1732, 1713, 1366, 1177, 989; MS *m/z* 620 (M^+) $C_{33}H_{48}SO_9$; ^1H NMR (270 MHz, $CDCl_3$) δ 5.03 (1H, m, 5-H), 3.97 (1H, d, J=1.5 Hz, 6-H), 3.12 (3H, s, $-OSO_2CH_3$).

5-*Epi*-5-*O*-mesylmilbemycin D (**13b**)

Reaction of **12b** (56 mg, 0.1 mmol), methanesulfonic anhydride (35 mg, 0.2 mmol) and Et_3N (20 mg, 0.2 mmol) according to the method for **8a** gave 53 mg (83%) of 5-*epi*-5-*O*-mesylmilbemycin D: IR (KBr) cm^{-1} 3487, 1716, 1456, 1367, 1178, 1010; MS *m/z* 634 (M^+) $C_{34}H_{50}SO_9$; ^1H NMR (400 MHz, $CDCl_3$) δ 5.04 (1H, br s, 5-H), 3.98 (1H, d, J=2.0 Hz, 6-H), 3.12 (3H, s, $-OSO_2CH_3$).

5-Deoxy-5β-aminomilbemycin A_4 (**15a**)

A mixture of **13a** (105 mg, 0.17 mmol) and NaN_3 (55 mg, 0.85 mmol) in CH_3CN (1.7 ml) containing a catalytic amount of 18-crown-6 ether was stirred at 40°C for 5 hours. The reaction mixture was poured into water and extracted with EtOAc. The extract was washed with water, dried over Na_2SO_4 and evaporated *in vacuo*. The residue was dissolved in DMF (2 ml), and 2 drops of water and 4 drops of AcOH were added to the solution. Zinc (56 mg, 0.85 mmol) was added in some portions to the solution cooled in an ice bath. After the addition, the ice bath was removed and the reaction mixture was stirred at ambient temperature for 1 hour. The insoluble material was removed by filtration, and the filtrate was dissolved in EtOAc, washed with saturated $NaHCO_3$, and water, dried, and concentrated *in vacuo*. The residue was purified by column chromatography (SiO_2, EtOAc - EtOH - Et_3N, 20 : 1 : 1) to give 46 mg (50%) of 5-deoxy-5β-aminomilbemycin A_4: IR (KBr) cm^{-1} 3480, 3390, 1732, 1713, 1178, 990; MS *m/z* 541 (M^+) $C_{32}H_{47}NO_6$; ^1H NMR (270 MHz, $CDCl_3$) δ 3.88 (1H, d, J=6.4 Hz, 6-H), 3.64 (1H, br, 5-H).

5-Deoxy-5β-aminomilbemycin D (**15b**)

As according to the procedure for **15a**, 5-deoxy-5β-aminomilbemycin D was obtained as glass (43%): IR (KBr) cm^{-1} 3470, 3387, 1714, 1174, 1011; MS *m/z* 555 (M^+) $C_{33}H_{49}NO_6$; ^1H NMR (270 MHz, $CDCl_3$) δ 3.87 (1H, d, J=5.9 Hz, 6-H), 3.52~3.68 (2H, m, 5-H and 17-H).

5-Deoxy-5β-acetaminomilbemycin A_4 (**16a**)

To a stirred solution of **15a** (35 mg, 0.06 mmol) and Et_3N (13 mg, 0.13 mmol) in CH_2Cl_2 (1 ml) at 0°C was added Ac_2O (13 mg, 0.13 mmol) in CH_2Cl_2 (0.4 ml). After 20 minutes at 0°C, the reaction mixture was poured into water and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane - EtOAc, 2 : 3) to give 36 mg (95%) of 5-deoxy-5β-acetaminomilbemycin A_4 as white crystals: mp 168~170°C; IR (KBr) cm^{-1} 3440, 3390, 1740, 1713, 1663, 1175, 989; MS *m/z* 583 (M^+) $C_{34}H_{49}NO_7$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.94~4.99 (2H, m, 5-H and 15-H), 3.81 (1H, d, J=5.9 Hz, 6-H), 2.05 (3H, s, $-NHCOCH_3$).

5-Deoxy-5β-acetaminomilbemycin D (**16b**)

According to the procedure for **16a**, reaction of **15b** (38 mg, 0.07 mmol), Ac_2O (14 mg, 0.14 mmol) and Et_3N (14 mg, 0.14 mmol) gave 36 mg (88%) of 5-deoxy-5β-acetaminomilbemycin D as white crystals: mp 164~168 °C; IR (KBr) cm^{-1} 3448, 3363, 1742, 1714, 1684, 1180, 1101; MS *m/z* 597 (M^+) $C_{35}H_{51}NO_7$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.90~5.02 (2H, m, 5-H and 15-H), 3.81 (1H, d, J=6.3 Hz, 6-H), 2.05 (3H, s, $-NHCOCH_3$).

5-Deoxy-5β-methanesulfonylaminomilbemycin A_4 (**17a**)

To a stirred solution of **15a** (40 mg, 0.07 mmol) and Et_3N (15 mg, 0.15 mmol) in CH_2Cl_2 (0.3 ml) at 0°C was added $MeSO_2Cl$ (17 mg, 0.15 mmol) in CH_2Cl_2 (0.3 ml). After 30 minutes at 0°C, the reaction mixture was poured into water and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane - EtOAc, 3 : 1) to give 32 mg (70%) of 5-deoxy-5β-methanesulfonylaminomilbemycin A_4 as white crystals: mp 162~166°C; IR (KBr) cm^{-1} 3468, 3301, 1715, 1335, 1181, 1159, 988; MS *m/z* 619 (M^+) $C_{33}H_{49}NSO_8$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.21 (1H, m, 5-H), 3.93 (1H, d, J=5.9 Hz, 6-H), 3.01 (3H, s, $-NHSO_2CH_3$).

5-Deoxy-5β-methanesulfonylaminomilbemycin D (**17b**)

According to the procedure for **17a**, reaction of **15b** (56 mg, 0.1 mmol), $MeSO_2Cl$ (23 mg, 0.2 mmol) and Et_3N (30 mg, 0.3 mmol) gave 54 mg (86%) of 5-deoxy-5β-methanesulfonylaminomilbemycin D as white crystals: mp 163~166°C; IR (KBr) cm^{-1} 3498, 3294, 1731, 1713, 1180, 1158, 1010, 997, 984; MS *m/z* 633 (M^+) $C_{34}H_{51}NSO_8$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.22 (1H, m, 5-H), 3.94 (1H, d, J=6.3 Hz, 6-H), 3.01 (3H, s, $-NHSO_2CH_3$).

5-Deoxy-5β-(3-methylureido)milbemycin A_4 (**18a**)

To a stirred solution of **15a** (40 mg, 0.07 mmol) in CH_2Cl_2 (0.2 ml) at 0°C was added methyl isocyanate (8.4 mg, 0.15 mmol) in CH_2Cl_2 (0.2 ml). The reaction mixture was stirred at ambient temperature for

2 hours; and poured into water and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by flash chromatography (SiO_2, hexane-EtOAc, 1:2) to give 32 mg (72%) of 5-deoxy-5β-(3-methylureido)milbemycin A_4 as white crystals: mp 189~191°C; IR (KBr) cm^{-1} 3400, 1737, 1714, 1673, 1560, 1165, 991; MS *m/z* 598 (M^+) $C_{34}H_{50}N_2O_7$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.79 (1H, br, 5-H), 3.85 (1H, d, J=5.9 Hz, 6-H), 2.80 (3H, d, J=5.3 Hz, $-NHCONHCH_3$).

5-Deoxy-5β-(3-methylureido)milbemycin D (**18b**)

As according to the procedure for **18a**, reaction of **15b** (40 mg, 0.07 mmol) and methyl isocyanate (12 mg, 0.21 mmol) gave 43 mg (97%) of 5-deoxy-5β-(3-methylureido)milbemycin D: mp 185~190°C; IR (KBr) cm^{-1} 3380, 1694, 1648, 1562, 1523, 1181, 1164, 1010, 998; MS *m/z* 612 (M^+) $C_{35}H_{52}N_2O_7$; ^1H NMR (270 MHz, $CDCl_3$) δ 4.77 (1H, br, 5-H), 3.85 (1H, d, J=5.9 Hz, 6-H), 2.80 (3H, br s, $-NHCONHCH_3$).

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