STRUCTURAL STUDIES OF NEW MACROLIDE ANTIBIOTICS, SHURIMYCINS† A AND B

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The structures of new antibiotics, shurimycins A and B produced by *Streptomyces hygroscopicus* A1491, were elucidated from the physico-chemical properties, 2D NMR techniques and chemical degradation experiments to be 36-membered macrolides related to azalomycins, scopafungin and guanidylfungins. Shurimycins were active against fungi and Gram-positive bacteria.

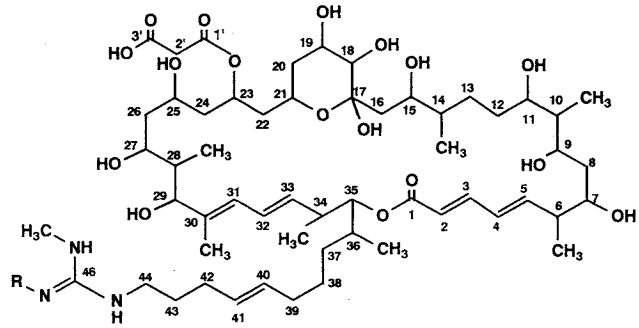
New antifungal antibiotics, shurimycins A and B (**1** and **2**), were found to be produced by *Streptomyces hygroscopicus* A1491. Their structures were determined by 2D NMR, HRFAB-MS and chemical degradations. These antibiotics consist of a 36-membered polyhydroxyl lactone ring, a guanidyl group and a monoester of malonic acid (Fig. 1).

In this paper, we report the fermentation of the producing strain, isolation, physico-chemical properties, structural elucidation and biological activities of **1** and **2**.

Fermentation

The producing strain, A1491, was isolated from a soil sample collected in Okinawa Prefecture, Japan and identified as *Streptomyces hygroscopicus*[1,2]. A spore suspension of this strain was inoculated into an Erlenmeyer flask (200 ml) containing 40 ml of the medium consisting of maltose syrup 4%, soybean oil 0.3%, soybean meal 2%, Pharmamedia (Traders Oil Mill Co., Texas) 1%, soluble vegetable protein 0.5%, $CaCO_3$ 0.3%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CoCl_2 \cdot 6H_2O$ 0.001% and $NiCl_2$ 0.001% (pH 7.0 before sterilization). The flask was incubated on a rotary shaker at 210 rpm for 4 days at 27°C. This seed culture was transferred

Fig. 1. The structures of shurimycins A (**1**) and B (**2**).



1 R=H
2 R=CH_3

† Shurimycins A and B were originally called MBA 028-24 A and B[1,2].

to a 30-liter jar fermentor containing 15 liters of the same medium. Fermentation was carried out at 27°C for 4 days with stirring at 200 rpm and at an air flow of 20 liters per minute.

Isolation

The mycelial cake separated from a cultured broth (30 liters) by centrifugation was extracted with methanol. The aqueous methanol extract was chromatographed on an HP-20 column with methanol. Fractions active against pathogenic fungi, such as *Alternaria mali*, *Fusarium oxysporum*, *Botrytis cinerea*, *Pyricularia oryzae* and *Rhizoctonia solani*, were subjected to reversed phase chromatography on an ODS-A60 column (1 liter, YMC) eluted with a linear gradient of methanol concentration from 60% to 100% in water. Fractions having antifungal activity were collected and purified by HPLC using an ODS column (3 × 25 cm) eluted with 90% methanol to give a mixture of **1** and **2** (800 mg). The mixture (400 mg) was further purified by HPLC using an ODS column (2 × 25 cm) eluted with 85% methanol to give **1** (120 mg) and **2** (160 mg).

Physico-chemical Properties

The physico-chemical properties of **1** and **2** are summarized in Table 1. The UV, IR, MP and optical rotation of **1** and **2** showed almost the same spectra or values for each compound. The strong broad IR band at 3340 cm^{-1} indicated the presence of several hydroxyl groups, and the absorptions at 1700 cm^{-1} and 1640 cm^{-1} showed the presence of carboxyl groups. Their UV absorption maxima at 240 nm (ε 37,800 and 37,300) and 264 nm (ε 19,900) indicated the presence of a diene conjugated to an acid or ester group. They were soluble in methanol and dimethyl sulfoxide, insoluble in water, acetone and acetonitrile. The molecular formula of **1** was determined to be $C_{56}H_{95}N_3O_{17}$ by HRFAB-MS (obsd *m/z* 1,082.6708, calcd 1,082.6740 $(M+H)^+$), and that of **2** to be $C_{57}H_{97}N_3O_{17}$ (obsd *m/z* 1,096.6915, calcd 1,096.6896 $(M+H)^+$).

Partial Structures of Shurimycin A (1)

The structure of **1** was mainly elucidated by 2D NMR measurements and chemical degradation experiments. The ^{13}C NMR spectrum contained 54 carbon signals with two sets of overlapping peaks, including three carboxylate carbons; one guanidino carbon and ten olefinic carbons indicating that **1** possessed two rings in the molecule because 11 degrees of unsaturation were required from its molecular formula. DEPT and ^{13}C-^1H COSY spectra revealed **1** containing one hemiketal carbon, twelve oxymethines, six non-oxygenated methine carbons, fifteen methylene carbons and eight methyl carbons involving one *N*-methyl group (Table 2). Fig. 2 shows the partial structures **a**~**e** elucidated from 2D NMR measurements

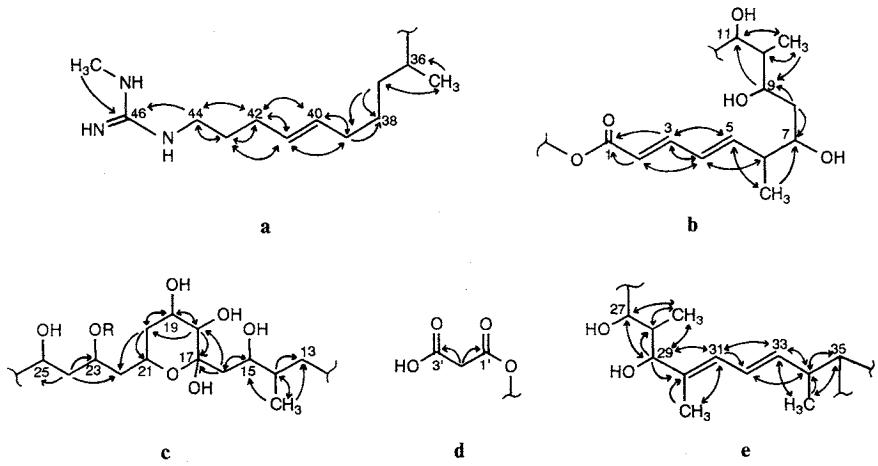
Table 1. Physico-chemical properties of shurimycins A (**1**) and B (**2**).

	1	**2**
Appearance	Colorless powder	Colorless powder
Molecular formula	$C_{56}H_{95}N_3O_{17}$	$C_{57}H_{97}N_3O_{17}$
HRFAB-MS (*m/z*)		
Calcd:	1,082.6740	1,096.6896
Found:	1,082.6708 $(M+H)^+$	1,096.6915 $(M+H)^+$
$[\alpha]_D^{25}$ (*c* 1.0, MeOH)	+60°	+53°
MP	130° (dec)	130° (dec)
UV λ_{max}^{MeOH} nm (ε)	240 (37,800), 264 (19,900)	240, (37,300), 264 (19,900)
IR (KBr) cm^{-1}	3340, 2960, 2940, 1700, 1640, 1590, 1460, 1380, 1240, 1140, 1090, 1070, 1000, 960	3340, 2960, 2940, 1700, 1640, 1590, 1460, 1380, 1240, 1140, 1090, 1070, 1000, 960

Table 2. NMR data of shurimycins A (**1**) and B (**2**) in CD_3OD.

Position	1		2	
	^{13}C	^{1}H	^{13}C	^{1}H
1	168.9 s	—	168.9 s	—
2	120.5 d	5.81 (1H, d, J=15 Hz)	120.5 d	5.81 (1H, d, J=15 Hz)
3	146.8 d	7.18 (1H, dd, J=15, 10 Hz)	146.8 d	7.18 (1H, dd, J=15, 10 Hz)
4	128.9 d	6.21 (1H, m)	128.9 d	6.21 (1H, m)
5	148.8 d	6.21 (1H, m)	148.7 d	6.21 (1H, m)
6	43.6 d	2.38 (1H, m)	43.6 d	2.38 (1H, m)
6-Me	12.3 q	1.08 (3H, d, J=7 Hz)	12.3 q	1.08 (3H, d, J=7 Hz)
7	76.0 d	3.85 (1H, m)	76.0 d	3.85 (1H, m)
8	38.3 t	1.50, 1.77 (2H, m)	38.3 t	1.50, 1.77 (2H, m)
9	76.0 d	3.85 (1H, m)	76.0 d	3.85 (1H, m)
10	45.2 d	1.48 (1H, m)	45.2 d	1.48 (1H, m)
10-Me	9.9 q	0.87 (3H, d, J=7 Hz)	9.9 q	0.87 (3H, d, J=7 Hz)
11	71.7 d	3.91 (1H, m)	71.7 d	3.91 (1H, m)
12	30.8 t	1.30 (2H, m)	30.8 t	1.30 (2H, m)
13	31.5 t	1.39 (2H, m)	31.5 t	1.39 (2H, m)
14	41.1 d	1.59 (1H, m)	41.0 d	1.59 (1H, m)
14-Me	13.5 q	0.91 (3H, d, J=6.8 Hz)	13.6 q	0.91 (3H, d, J=6.6 Hz)
15	72.7 d	3.84 (1H, m)	72.7 d	3.84 (1H, m)
16	42.0 t	1.83 (2H, m)	42.0 t	1.83 (2H, m)
17	99.5 s	—	99.5 s	—
18	76.4 d	3.35 (1H, d, J=8.5 Hz)	76.5 d	3.35 (1H, d, J=8.5 Hz)
19	69.6 d	3.85 (1H, m)	69.6 d	3.85 (1H, m)
20	41.3 t	1.27, 1.90 (2H, m)	41.4 t	1.27, 1.90 (2H, m)
21	64.8 d	4.07 (1H, m)	64.8 d	4.07 (1H, m)
22	45.1 t	1.62, 1.77 (2H, m)	45.1 t	1.62, 1.77 (2H, m)
23	70.0 d	5.33 (1H, m)	71.7 d	5.33 (1H, m)
24	43.9 t	1.28, 1.62 (2H, m)	43.8 t	1.28, 1.62 (2H, m)
25	65.3 d	3.86 (1H, m)	65.2 d	3.86 (1H, m)
26	41.8 t	1.63 (2H, m)	41.8 t	1.63 (2H, m)
27	67.2 d	4.25 (1H, m)	67.2 d	4.26 (1H, m)
28	42.1 d	1.50 (1H, m)	42.2 d	1.50 (1H, m)
28-Me	10.3 q	0.69 (3H, d, J=7 Hz)	10.3 q	0.67 (3H, d, J=7 Hz)
29	80.7 d	3.80 (1H, m)	80.7 d	3.80 (1H, m)
30	138.4 s	—	138.4 s	—
30-Me	11.6 q	1.57 (3H, s)	11.5 q	1.57 (3H, s)
31	128.1 d	5.86 (1H, d, J=11 Hz)	128.1 d	5.85 (1H, d, J=11 Hz)
32	128.8 d	6.17 (1H, dd, J=15, 11 Hz)	128.8 d	6.18 (1H, dd, J=15, 11 Hz)
33	136.5 d	5.32 (1H, dd, J=15, 9 Hz)	136.4 d	5.32 (1H, dd, J=15, 9 Hz)
34	41.0 d	2.47 (1H, m)	41.0 d	2.48 (1H, m)
34-Me	16.8 q	1.01 (3H, d, J=6.7 Hz)	16.8 q	1.01 (3H, d, J=6.7 Hz)
35	79.9 d	4.73 (1H, dd, J=9, 2.8 Hz)	79.9 d	4.73 (1H, dd, J=9, 2.6 Hz)
36	34.4 d	1.80 (1H, m)	34.4 d	1.80 (1H, m)
36-Me	13.7 q	0.95 (3H, d, J=6.8 Hz)	13.7 q	0.95 (3H, d, J=6.7 Hz)
37	34.7 t	1.17, 1.33 (2H, m)	34.6 t	1.17, 1.33 (2H, m)
38	27.9 t	1.38, 1.42 (2H, m)	27.9 t	1.38, 1.42 (2H, m)
39	33.6 t	1.98 (2H, m)	33.5 t	1.98 (2H, m)
40	132.3 d	5.43 (1H, m)	132.3 d	5.43 (1H, m)
41	130.1 d	5.43 (1H, m)	130.1 d	5.43 (1H, m)
42	30.5 t	2.06 (2H, m)	30.5 t	2.06 (2H, m)
43	29.7 t	1.65 (2H, m)	29.7 t	1.65 (2H, m)
44	42.0 t	3.15 (2H, t, J=7 Hz)	42.0 t	3.16 (2H, t, J=7 Hz)
1′	171.6 s	—	171.6 s	—
2′	46.0 t	3.22 (2H, m)	46.0 t	3.22 (2H, m)
3′	174.0 s	—	173.8 s	—
N–Me	28.3 q	2.84 (3H, s)	28.3 q	2.85 (3H, s)
N–Me′	—	—	28.3 q	2.85 (3H, s)
N=CN_2	158.1 s	—	157.1 s	—

Fig. 2. The partial structures of **1** and the ^{13}C-^{1}H long range couplings observed in the HMBC spectrum.



in CD_3OD as follows.

In the ^{1}H NMR spectrum of **1** in DMSO-d_6, three NH protons were observed at 7.40, 7.52 and 7.55 ppm (data not shown). ^{1}H-^{1}H COSY spectrum showed that the NH proton signal at 7.40 ppm correlated with the *N*-methylene protons and the signal at 7.52 ppm correlated with the *N*-methyl protons. This finding and the ^{13}C-^{1}H long range couplings measured in CD_3OD shown in Fig. 2 (**a**) indicated the presence of partial structure **a**. The presence of a diene system (C-2 to C-5) was indicated from the ^{1}H-^{1}H COSY spectrum. The spectrum and the ^{13}C-^{1}H long range couplings shown in Fig. 2 (**b**) revealed the linkage from C-1 to C-11, thus revealing the partial structure **b**. The connections from C-13 to C-25 were elucidated from the ^{1}H-^{1}H and the ^{13}C-^{1}H long range couplings shown in Fig. 2 (**c**). The presence of a six-membered hemiketal ring was supported by comparison with the published NMR data for azalomycins[3~6], copiamycin[7,8], RP 63834[9] and malolactomycins[10]. The low chemical shift (5.33 ppm) of 23-H suggested that the methine was acylated. Thus, the partial structure **c** was determined as shown in Fig. 2. The methylene protons (2′-H), which gave the isolated multiplet at 3.22 ppm in the ^{1}H NMR spectrum of **1**, were correlated to two carbonyl carbons at 171.6 ppm (C-1′) and 174.0 ppm (C-3′). These methylene protons were gradually exchanged by deuterium in CD_3OD solution[11]. Thus, this portion (C-1′ to C-3′) is most likely to be a malonate moiety, the partial structure **d** (Fig. 2). The presence of the partial structure **e** was indicated from ^{1}H-^{1}H COSY and HMBC spectra as shown in Fig. 2 (**e**).

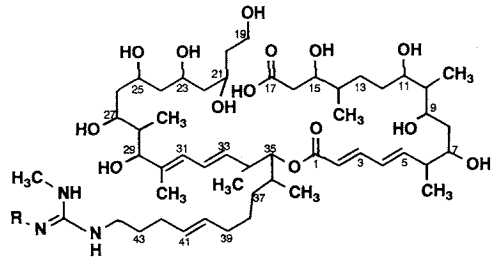
In the HMBC spectrum of **1**, ^{13}C-^{1}H long range couplings of 35-H to C-1 and 36-CH_3 to C-35 were observed. Thus, the partial structures **a** and **b** were connected to **e**. The partial structure **d** was determined to be connected to C-23 in the partial structure **c** as judged from the chemical shift of 23-H. However, the connections between the partial structures **c** and **e** (C-26), and between **b** and **c** (C-12) were still ambiguous. To elucidate the complete structure, the chemical degradation of **1** was performed and the structures of the degradation products were studied as described below.

Chemical Degradation of Shurimycin A (1)

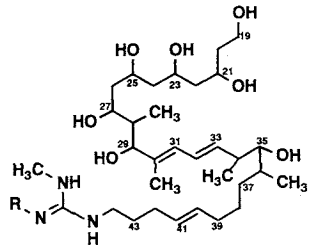
Periodate Oxidation of 1

Compound **1** was oxidized with $NaIO_4$ and reduced with $NaBH_4$ to give **3** (Fig. 3). The molecular formula of **3** was determined to be $C_{52}H_{93}N_3O_{14}$ by HRFAB-MS. In the ^{13}C NMR spectrum of **3**, several

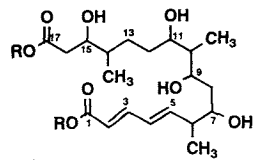
Fig. 3. The structures of the degradation products.



3 R=H
4 R=CH_3



5 R=H
6 R=CH_3



7 R=H
8 R=CH_3

carbon signals of **1**, 171.6 ppm (C-1'), 46.0 ppm (C-2'), 174.0 ppm (C-3') and 76.4 ppm (C-18), were not observed. The characteristic signal of a carboxyl group at 181.3 ppm was assigned to C-17 by the HMBC spectrum. The signal of C-19 shifted largely to the upfield (60.3 ppm in **3** while 69.6 ppm in **1**) and was found to be a methylene from the DEPT spectrum. These findings suggested that the $NaIO_4$ and $NaBH_4$ treatments resulted in the degradation of the six-membered hemiketal ring and the elimination of malonic acid of **1**.

Alkaline Hydrolysis of **3**

Compound **3** was hydrolyzed with 3 N KOH to give **5** and **7**. These two products were considered to result from the hydrolysis of the ester bond between C-1 and C-35. The molecular formula of **5** was determined to be $C_{32}H_{61}N_3O_7$ by HRFAB-MS. In the ^1H NMR spectrum of **5**, all the proton signals were well separated and clearly distinguished. In the ^1H-^1H COSY spectrum of **5**, the signals of 26-H (1.29 and 1.88 ppm) were correlated with 25-H (3.67 ppm) and 27-H (3.30 ppm). Thus, the linkages between C-25 and C-26, and between C-26 and C-27, which had been ambiguous during the structural analysis of **1** as described above, were confirmed. The structure of **5** was thus established to be that shown in Fig. 3.

Compound **7** was considered to be derived from the C-1 to C-17 part of **3**, thus considered to have two carboxyl groups. The molecular formula of **7** was determined to be $C_{20}H_{34}O_8$ from the HRFAB-MS of its dimethyl ester derivative **8**. In the ^1H NMR spectrum of **7**, the proton signals 11-H, 12-H, 13-H and 14-H were distinguished and completely assigned. The linkages C-11 to C-14, which had been ambiguous as described above, were thus confirmed, and the structure of **7** was determined as that shown in Fig. 3.

Structure of Shurimycin A (**1**)

The connectivities between partial structures **c** and **e** were clarified from the structure of **5**. Similarly,

we could connect the partial structures **b** and **c** from the structure of **7**. Thus, the structure of **1** was established to be a 36-membered macrolide as shown in Fig. 1.

Stereochemistries of the double bonds at C-2, C-4, C-30, C-32 and C-40 were determined in the following manner. In the ^{1}H NMR of **1**, the large coupling constants between 2-H and 3-H (J=15 Hz), and 32-H and 33-H (J=15 Hz) as well as the NOE of 32-H upon irradiation of 30-CH_3 indicated the olefinic linkages of C-2=C-3, C-32=C-33 and C-30=C-31 to be oriented in *E*-configurations. Stereochemistries of C-40=C-41 and C-4=C-5 were determined from the ^{1}H NMR spectra of **5** and **7**. The coupling constant between 40-H and 41-H was 15 Hz, and that of 4-H and 5-H was 16 Hz. Thus, the olefinic linkages of C-40=C-41 and C-4=C-5 were also determined to be *E*-configurations.

From the above mentioned results, the structure of **1** including its stereochemistry was established as that shown in Fig. 1. Compound **1** is a 36-membered macrolide antibiotic.

Structure of Shurimycin B (2)

The structure of **2** was similarly determined by NMR and chemical degradation. Compound **2** gave NMR spectra similar to those of **1** except for the presence of an extra *N*-methyl signal. Compound **2** was treated with $NaIO_4$ and $NaBH_4$ to give the degradation product, **4** (Fig. 3). The molecular formula of **4** was determined to be $C_{53}H_{95}N_3O_{14}$ by HRFAB-MS. Compound **4** was hydrolyzed with 3 N KOH to give **6** and **7**. Compound **7** was the common product of hydrolysis of **3** as already described. The structure of **6** was also determined as shown in Fig. 3 by FAB-MS and NMR. Thus, the difference between **1** and **2** was clarified to come from the *N*-methyl group at their terminal guanidine, and the structure of **2** was determined as shown in Fig. 1.

Biological Activities

The minimal inhibitory concentrations (MIC) of **1** and **2** against bacteria and fungi were determined by the agar dilution method. The results are given in Table 3. Compounds **1** and **2** were active against Gram-positive bacteria and fungi. However, compounds **3** and **4** lost antimicrobial activity (data not shown).

Discussion

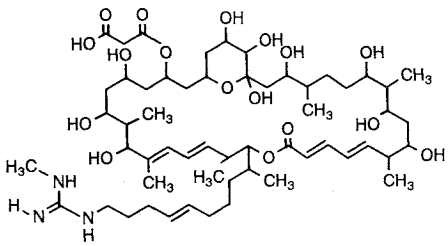
The new 36-membered macrolide antibiotics, shurimycins A and B, were isolated from the cultured broth of the actinomycete strain, A1491. They were inhibitory to Gram-positive bacteria and fungi. Their structures were related to those of azalomycins[3~6], scopafungin (niphimycin)[12~15], copiamycin[7,8], guanidylfungins[16,17], amycins[18], RP 63834[9] and malolactomycins[10] (Fig. 4). All of these antibiotics including shurimycins commonly consist of a macrocyclic polyhydroxyl lactone ring with a malonyl monoester, a hemiketal, and a side chain with a mono, di, or trisubstituted guanidine as their terminal moiety. The structures of these shurimycins closely resemble those of azalomycins. However, shurimycins differ from azalomycins by the absence of 2-CH_3 and the presence of 28-CH_3.

Compounds **3** and **4** were found to lose the antimicrobial activity of the original shurimycins.

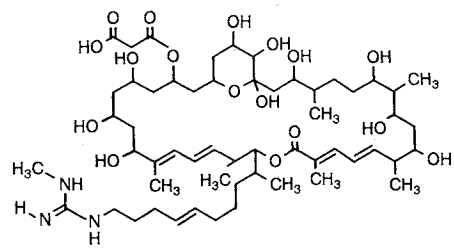
Table 3. Minimal inhibitory concentrations (MIC) of shurimycins A (**1**) and B (**2**).

Test organism	MIC (μg/ml)	
	1	**2**
Bacillus subtilis	3.1	3.1
Sarcina lutea	3.1	3.1
Staphylococcus aureus 209P	1.56	1.56
Bacteroides fragilis	6.2	12.5
Escherichia coli	>100	>100
Pseudomonas aeruginosa	>100	>100
Cryptococcus neoformans	1.56	1.56
Trichophyton mentagrophytes	3.1	3.1
Candida albicans	3.1	6.2
Aspergillus fumigatus	3.1	6.2
Alternaria mali	3.1	3.1
Fusarium oxysporum	12.5	12.5
Botrytis cinerea	0.78	0.78
Pyricularia oryzae	0.78	0.78
Rhizoctonia solani	0.78	1.56

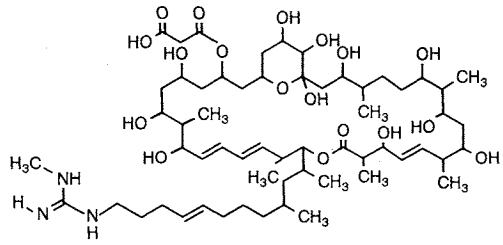
Fig. 4. The structures of shurimycin A and related antibiotics.



Shurimycin A



Azalomycin F_{4a}



Scopafungin

This finding strongly supports a previous report that the six-membered hemiketal ring in the macrolide of this kind is essential for antimicrobial activity[19].

Experimental

General

NMR spectra were recorded on a Bruker AM 500 spectrometer with TMS as an internal standard. FAB mass spectra were obtained on a JEOL HX-100 spectrometer with glycerol as matrix. IR spectra were recorded on a Jasco FT/IR-8000 spectrometer. UV spectra were obtained on a Shimadzu UV-3100S spectrometer. Optical rotations were measured with a Jasco DIP-370 polarimeter.

Periodate Oxidation of Shurimycins A (1) and B (2)

A solution of **1** (100 mg) and sodium periodate ($NaIO_4$) (100 mg) in 10 ml of methanol-water (3:2) was stirred for 1 hour at room temperature. Excess $NaIO_4$ was decomposed with ethylene glycol. After evaporation, the residue was extracted with methanol. The extract was treated with $NaBH_4$ (100 mg) for 12 hours at room temperature. The reaction mixture was then evaporated off *in vacuo* to give an oily produet. The product was purified by HPLC using an ODS column (2 × 25 cm) with a mobile phase of methanol-20 mM ammonium acetate (9:1) to give **3** (50 mg): FAB-MS *m/z* 984 $(M+H)^+$; HRFAB-MS $C_{52}H_{93}N_3O_{14}$ (obsd *m/z* 984.6738, calcd 984.6739 $(M+H)^+$); ^1H NMR (500 MHz, CD_3OD): δ 0.69 (3H, d, J=8 Hz, 28-Me), 0.91 (3H, d, J=8 Hz, 10-Me), 0.91 (3H, d, J=8 Hz, 14-Me), 0.91 (3H, d, J=8 Hz, 36-Me), 1.01 (3H, d, J=8 Hz, 34-Me), 1.08 (3H, d, J=8 Hz, 6-Me), 1.10 (1H, m, 37a-H), 1.20 (2H, m, 13-H), 1.30 (1H, m, 12a-H), 1.30 (1H, m, 37b-H), 1.35 (2H, m, 38-H), 1.42 (1H, m, 26a-H), 1.50 (1H, m, 8a-H), 1.50 (1H, m, 14-H), 1.51 (2H, m, 24-H), 1.52 (1H, m, 10-H), 1.52 (2H, m, 22-H), 1.55 (1H, m, 12b-H), 1.62 (1H, m, 26b-H), 1.63 (1H, m, 28-H), 1.64 (2H, m, 20-H), 1.65 (2H, m, 43-H), 1.68 (3H, s, 30-Me), 1.75 (1H, m, 8b-H), 1.75 (1H, m, 36-H), 1.98 (2H, dd, J=16 and 8 Hz, 39-H), 2.06 (2H, dd, J=12 and 8 Hz, 42-H), 2.20 (1H, dd, J=16 and 8 Hz, 16a-H), 2.33 (1H, dd, J=16 and 2 Hz, 16b-H), 2.40 (1H, m, 6-H), 2.59 (1H, m, 34-H), 2.84 (3H, s, N-Me), 3.16 (2H, t, J=8 Hz, 44-H), 3.70 (2H, t, J=8 Hz, 19-H), 3.72 (1H, m, 7-H), 3.75 (1H, m, 9-H), 3.75 (1H, m, 15-H), 3.85 (1H, m, 11-H), 3.91 (1H, d, J=10 Hz, 29-H), 4.00 (1H, m, 21-H), 4.06 (1H, m, 25-H), 4.11 (1H, m, 23-H), 4.22 (1H, t, J=4 Hz, 27-H), 4.80 (1H, m, 35-H), 5.44 (1H, m, 40-H), 5.44 (1H, m, 41-H), 5.51 (1H, dd, J=16 and 8 Hz, 33-H), 5.83 (1H, d,

J=16 Hz, 2-H), 5.91 (1H, d, J=12 Hz, 31-H), 6.18 (1H, m, 5-H), 6.27 (1H, m, 4-H), 6.27 (1H, dd, J=16 and 10 Hz, 32-H) and 7.23 (1H, dd, J=16 and 12 Hz, 3-H); ^{13}C NMR (125 MHz, CD_3OD): δ 10.6 (q, 10-Me), 11.1 (q, 28-Me), 11.7 (q, 30-Me), 14.5 (q, 36-Me), 15.0 (q, 6-Me), 15.7 (q, 14-Me), 18.1 (q, 34-Me), 27.9 (t, C-38), 28.4 (q, N–Me), 29.8 (t, C-43), 30.6 (t, C-42), 30.7 (t, C-12), 33.4 (t, C-13), 34.1 (t, C-39), 34.5 (t, C-37), 35.4 (d, C-36), 39.1 (t, C-8), 39.6 (d, C-14), 40.9 (d, C-34), 41.5 (t, C-16), 41.5 (t, C-20), 42.0 (d, C-28), 42.1 (t, C-44), 43.6 (t, C-26), 44.2 (d, C-6), 44.7 (d, C-10), 46.4 (t, C-24), 46.8 (t, C-22), 60.3 (t, C-19), 66.3 (d, C-21), 66.6 (d, C-25), 67.0 (d, C-23), 69.1 (d, C-27), 72.6 (d, C-11), 74.2 (d, C-15), 75.7 (d, C-7), 75.7 (d, C-9), 80.8 (d, C-35), 81.2 (d, C-29), 120.6 (d, C-2), 128.1 (d, C-32), 128.2 (d, C-31), 129.6 (d, C-4), 130.3 (d, C-41), 132.5 (d, C-40), 136.8 (d, C-33), 138.6 (s, C-30), 146.8 (d, C-3), 148.3 (d, C-5), 157.3 (s, CN_2=N), 168.6 (s, C-1) and 181.3 (s, C-17). Compound **2** was similarly treated with $NaIO_4$ and $NaBH_4$ to give the product **4**: FAB-MS m/z 998 $(M+H)^+$; HRFAB-MS $C_{53}H_{95}N_3O_{14}$ (obsd m/z 998.6894, calcd 998.6892 $(M+H)^+$). ^{1}H and ^{13}C NMR spectra are similar to those of **3** except for the presence of an extra *N*-methyl signal.

Alkaline Hydrolysis of **3** and **4**

To a suspension of **3** (25 mg) in methanol (16 ml) was added 3 N KOH (8 ml) and the solution was stirred at room temperature overnight, then neutralized with dilute aq HCl and evaporated to dryness. The hydrolysates **5** (2 mg) and **7** (3 mg) were purified by HPLC using an ODS column (2×25 cm) with a mobile phase of methanol-water (75:25) containing 0.1% trifluoroacetic acid. **5**: FAB-MS m/z 582 $(M+H-H_2O)^+$; HRFAB-MS $C_{32}H_{61}N_3O_7$ (obsd m/z 582.4485, calcd 582.4482 $(M+H-H_2O)^+$); ^{1}H NMR (500 MHz, CD_3OD): δ 0.80 (3H, d, J=8 Hz, 28-Me), 0.88 (3H, d, J=8 Hz, 36-Me), 1.00 (3H, d, J=8 Hz, 34-Me), 1.17 (2H, m, 37-H), 1.29 (1H, m, 26a-H), 1.30 (1H, m, 20a-H), 1.35 (1H, m, 38a-H), 1.40 (1H, m, 28-H), 1.42 (1H, m, 38b-H), 1.49 (2H, m, 22-H), 1.51 (1H, m, 24a-H), 1.55 (1H, m, 36-H), 1.61 (1H, m, 24b-H), 1.63 (1H, m, 20b-H), 1.65 (2H, m, 43-H), 1.70 (3H, s, 30-Me), 1.88 (1H, m, 26b-H), 1.99 (2H, m, 39-H), 2.07 (2H, m, 42-H), 2.40 (1H, m, 34-H), 2.85 (3H, s, N–Me), 3.13 (1H, m, 35-H), 3.15 (2H, t, J=8 Hz, 44-H), 3.30 (1H, m, 27-H), 3.37 (1H, m, 29-H), 3.67 (2H, m, 19-H), 3.67 (1H, m, 25-H), 3.96 (1H, m, 21-H), 4.03 (1H, m, 23-H), 5.40 (1H, ddd, J=15, 8 and 4 Hz, 40-H), 5.40 (1H, ddd, J=15, 8 and 4 Hz, 41-H), 5.70 (1H, dd, J=16 and 8 Hz, 33-H), 5.95 (1H, d, J=10 Hz, 31-H) and 6.30 (1H, dd, J=16 and 10 Hz, 32-H). **7**: FAB-MS molecular ion peak was not observed; ^{1}H NMR (500 MHz, CD_3OD): δ 0.91 (3H, d, J=8 Hz, 10-Me), 0.91 (3H, d, J=8 Hz, 14-Me), 1.08 (3H, d, J=8 Hz, 6-Me), 1.29 (2H, m, 13-H), 1.35 (1H, m, 12a-H), 1.50 (1H, m, 10-H), 1.51 (1H, m, 8a-H), 1.54 (1H, m, 14-H), 1.56 (1H, m, 12b-H), 1.76 (1H, m, 8b-H), 2.32 (1H, dd, J=16 and 6 Hz, 16a-H), 2.39 (1H, m, 6-H), 2.48 (1H, dd, J=16 and 3 Hz, 16b-H), 3.72 (1H, ddd, J=10, 6 and 3 Hz, 7-H), 3.79 (1H, ddd, J=10, 6 and 3 Hz, 9-H), 3.84 (1H, ddd, J=10, 3 and 2 Hz, 11-H), 3.89 (1H, ddd, J=10, 6 and 3 Hz, 15-H), 5.82 (1H, d, J=16 Hz, 2-H), 6.18 (1H, dd, J=16 and 8 Hz, 5-H), 6.30 (1H, dd, J=16 and 12 Hz, 4-H) and 7.26 (1H, dd, J=16 and 12 Hz, 3-H). Compound **4** was similarly hydrolyzed with 3 N KOH to give **6** and **7**. **6**: FAB-MS m/z 596 $(M+H-H_2O)^+$; HRFAB-MS $C_{33}H_{63}N_3O_7$ (obsd m/z 596.4642, calcd 596.4638 $(M+H-H_2O)^+$); ^{1}H NMR spectrum is similar to **5** except for the presence of an extra *N*-methyl signal at 2.85 ppm.

Methyl Esterification of **7**

Compound **7** (2 mg) in methanol was treated with trimethylsilyldiazomethane, and the dimethyl ester derivative, **8** (1.5 mg) was purified by HPLC using an ODS column (0.46×25 cm) with a mobile phase of methanol-water (75:25) containing 0.1% trifluoroacetic acid. **8**: FAB-MS m/z 431 $(M+H)^+$; HRFAB-MS $C_{22}H_{38}O_8$ (obsd m/z 431.2654, calcd 431.2645 $(M+H)^+$).

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