Structures of New Pseudo-Peptide Antibiotics, Sperabillins

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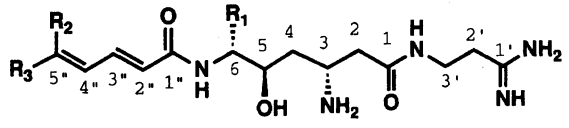
Sperabillins, novel antibiotics, were isolated from the culture filtrate of *Pseudomonas fluorescens* YK-437. The structure of sperabillin A was elucidated to be 3-[[(3*R*,5*R*)-3-amino-6-[(2*E*,4*Z*)-2,4-hexadienoylamino]-5-hydroxyhexanoyl]amino]propanamidine dihydrochloride. Sperabillin B has a methyl substituent at the C-6 position of sperabillin A. Sperabillin C and D are (2*E*,4*E*)-isomers of the 2,4-hexadienoyl moiety of sperabillin A and B, respectively.

In a screening program for new inhibitors of cell wall synthesis from bacterial strains, we isolated a novel antibiotic lactivicin[1—3] which shows similar biological activities to β-lactam antibiotics in spite of the absence of a β-lactam ring in the molecule. During the course of the modified program using lactivicin-sensitive strains, we found four new antibiotics in the culture filtrate of *Pseudomonas fluorescens* YK-437. They were basic, water-soluble substances and named sperabillin A (**1**), B (**2**), C (**3**), and D (**4**).[4] These antibiotics are active in vitro against Gram-positive and Gram-negative bacteria, including antibiotic-resistant strains, and show much stronger activities in vivo than expected from the in vitro potencies.[4] Their chemical structures were determined from spectral analyses and degradation studies as shown in Fig. 1. Their unique structures are composed of 2,4-hexadienoic acid, 3,6-diamino-5-hydroxyhexanoic (or -heptanoic) acid and 2-aminoethanamidine in binding with amide bonds.

This paper deals with the chemical characterization and structure determination of sperabillins.

Results and Discussion

The antibiotics were isolated by column chromatographies using cation-exchange resins, cation-exchange Sephadex and activated carbon, which are methods used to isolate basic, water-soluble antibiotics. Further purification was carried out by preparative HPLC using an ODS column. The main components were **1** and **2**, and the minor ones were **3** and **4**. Details of the isolation procedure were described in a previous paper.[4]



Sperabillin	R_1	R_2	R_3
A (1)	H	CH_3	H
B (2)	CH_3	CH_3	H
C (3)	H	H	CH_3
D (4)	CH_3	H	CH_3

Fig. 1. Structures of sperabillin A, B, C, and D.

The physicochemical properties of **1**—**4** are summarized in Table 1 along with the retention times on HPLC. The signs of the specific rotations of **1** and **3** were opposite to those of **2** and **4**. The molecular formulas were determined to be $C_{15}H_{27}N_5O_3 \cdot 2HCl$ for **1** and **3** and $C_{16}H_{29}N_5O_3 \cdot 2HCl$ for **2** and **4** from elemental analyses, molecular ion peaks in SI-MS and the number of carbons in ^{13}C NMR spectra.

The UV spectra of sperabillins showed the maximum at 262—266 nm (ε about 28000). This characteristic absorption suggested that each sperabillin component has a dienone chromophore in common. The IR spectra of sperabillins had strong absorption bands at 1660—1670 and 1550 cm^{-1} which appeared to be similar to the pattern of peptide compounds. The ^{13}C and ^1H NMR spectral data of sperabillins (**1**—**4**) are shown in Tables 2 and 3, respectively. The ^{13}C NMR signals suggested the presence of two olefins, five methylenes, two methines and one methyl in **1**. In addition, three quaternary carbons were observed in the carbonyl region. When the ^1H NMR spectrum was measured in DMSO-d_6, two exchangeable triplets were observed at δ=8.28 (J=5.9 Hz) and 8.54 (J=5.6 Hz), which were attributable to amide protons. The proton spin-decoupling study revealed the existence of three moieties in **1**. The dienone moiety consisted of the (2″*E*, 4″*Z*) form, CH_3–CH^{Z}=CH–CH^{E}=CH–C(=O)–, on the basis of coupling constants ($J_{2''-3''}$=15.1 and $J_{4''-5''}$=11.0 Hz), and the other two moieties were presumed to be –(C=O)NH–CH_2–CH_2– and –(C=O)NH–CH_2–$\underset{|}{CH}$–CH_2–$\underset{|}{CH}$–CH_2–.

To confirm the functional groups of **1**, several reactions were carried out (Scheme 1). Protection of the primary amino group gave the *t*-butoxycarbonylamino (*N*-Boc) derivative **5**. In the ^1H NMR spectrum of **5**, the H-3 methine signal of **1** at δ=3.86 shifted to δ=4.28. The existence of an amidino group was revealed by transformation of **5** into the dimethylpyrimidine derivative **6**, and hence one of the signals in the carbonyl region of **1** was attributed to the amidine carbon. Furthermore, the hydroxyl group in **5** was converted to ketone **7** by Swern oxidation followed by deprotection. These reactions re-

Table 1. Physicochemical Properties and Retention Time on HPLC of Sperabillins (**1**—**4**)

	1	**2**	**3**	**4**
Appearance	White powder	White powder	White powder	White powder
$[\alpha]_D$ (c)[a]	−11° (1.1)	+56° (1.0)	−11° (0.7)	+30° (0.5)
SI-MS $(M+H)^+$	326	340	326	340
Molecular formula	$C_{15}H_{27}N_5O_3\cdot 2HCl(H_2O)$	$C_{16}H_{29}N_5O_3\cdot 2HCl(1.5H_2O)$	$C_{15}H_{27}N_5O_3\cdot 2HCl(0.5H_2O)$	$C_{16}H_{29}N_5O_3\cdot 2HCl(0.5H_2O)$
UV λ_{max} /nm (ε)[b]	266 (27400)	264 (28400)	262 (27600)	262 (27500)
IR (KBr) /cm^{-1}	1700, 1670, 1550	1700, 1660 1610, 1550	1660, 1630 1540	1660, 1635 1530
HPLC t_R /min[c]	5.3	5.8	5.7	6.2

a) Measured in water at 23—25°C. b) Measured in water. c) Column, YMC-Pack A-312 (ODS); mobile phase, 30% CH_3CN/10 mM 1-octanesulfonic acid-20 mM phosphoric acid (pH 3.0); flow rate, 2.0 ml min^{-1}; detection, UV absorbance at 254 nm.

Table 2. ^{13}C NMR Data of Sperabillins (**1**—**4**) (100 MHz, δ/ppm, in D_2O)[a]

	1	**2**	**3**	**4**
C=O[b]	174.7 s	174.7 s	174.7 s	174.7 s
C=O[b]	172.3 s	171.6 s	172.3 s	171.7 s
C=O[b]	171.7 s	171.6 s	171.6 s	171.6 s
2′	35.2 t	35.2 t	35.2 t	35.3 t
3′	39.1 t	39.1 t	39.1 t	39.2 t
2	39.8 t	39.9 t	39.7 t	39.9 t
3	49.2 d	49.3 d	49.2 d	49.3 d
4	38.1 t	37.5 t	38.0 t	37.5 t
5	69.2 d	72.6 d	69.2 d	72.5 d
6	47.8 t	52.3 d	47.7 t	52.2 d
6-CH_3	—	18.8 q	—	18.8 q
2″	125.3 d	125.5 d	123.1 d	123.2 d
3″	139.3 d	139.3 d	145.1 d	145.3 d
4″	129.6 d	129.6 d	132.0 d	132.0 d
5″	139.3 d	139.2 d	143.0 d	143.0 d
6″	16.0 q	16.0 q	20.6 q	20.7 q

a) Assignments were confirmed by ^1H-^{13}C COSY.
b) One of them is attributable to the amidine carbon.

vealed the C-3 amino, C-5 hydroxyl and C-1′ amidine substituents of **1**. Taking the molecular formula of **1** into consideration, the three moieties could be linked through amide bonds, therefore, the gross structure of **1** was deduced as shown in Fig. 1.

Acidic hydrolyses of **1** gave important information for confirming the structure and stereochemistry (Scheme 2). When **1** was hydrolyzed in 2 M (1 M=1 mol dm^{-3}) hydrochloric acid at 110°C for 6 h, (2*E*,4*E*)-2,4-hexadienoic acid (**8**), 2-aminoethanamidine (**9**)[5] and (3*R*,5*R*)-3,6-diamino-5-hydroxyhexanoic acid (**10**)[6,7] were obtained. The (2*E*,4*Z*)-2,4-hexadienoyl moiety of **1** was considered to be isomerized to the more stable (2*E*,4*E*)-form during the reaction. Amino acid **10** was identical with that obtained from the antibiotic negamycin[8] in all respects, including the specific rotation value. By another type of acidic hydrolysis in 2 M hydrochloric acid at 110°C for 15 min, **1** was selectively transformed into an amino acid **11** with liberation of **9**. Thus, the structure of **1** was established unambiguously to be 3-[[(3*R*,5*R*)-3-amino-6-[(2*E*,4*Z*)-2,4-hexadienoylamino]-5-hydroxyhexanoyl]amino]propanamidine dihydrochloride (**1**) (Fig. 1).

Sperabillin B (**2**) lacked the C-6 methylene and had additional methine and methyl groups when compared with **1**. This showed that **2** was the C-6 methyl congener of **1**. When **2** was hydrolyzed stepwise with acid, **12** and 3,6-diamino-5-hydroxyheptanoic acid (**13**) were obtained (Scheme 2). Amino groups of **13** were acetylated with acetic anhydride in aq sodium hydrogencarbonate, and then treated with dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBT) to afford the lactone **14**. The ^1H NMR analysis of **14** revealed that the lactone ring had *cis*-diequatorial substituents. A positive Cotton effect at 220 nm in the ORD curve and at 225 nm in the CD spectrum of **14** suggested the 5*R* (and hence 3*R*) configuration, which was same as that of **1**, from the application of Klyne's lactone sector rule.[6,9,10]

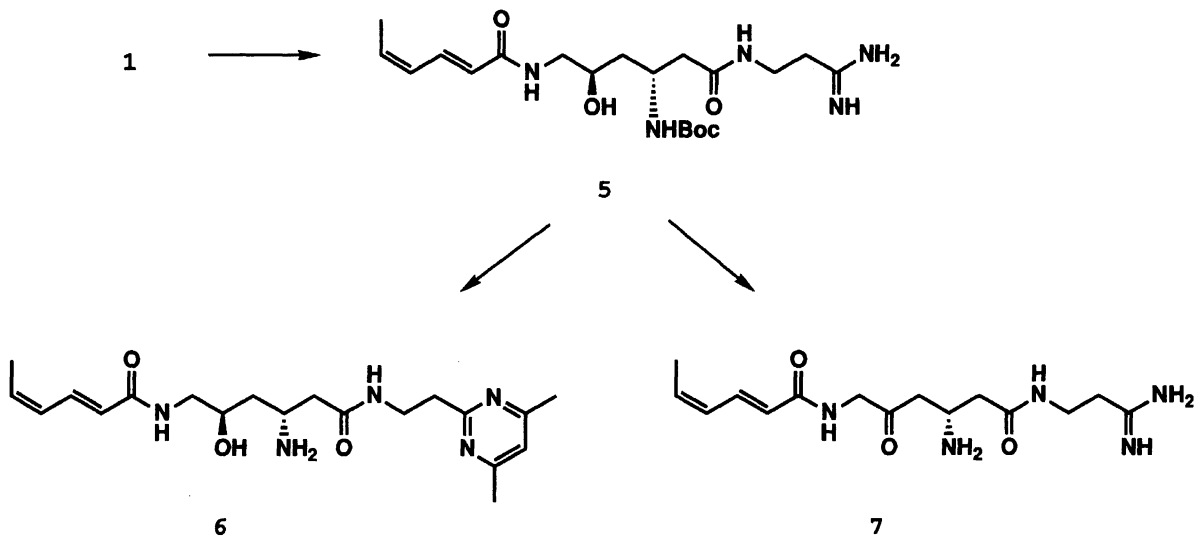
In order to confirm the structure of **2** and to investigate the possibility of transformation from **1** into **2**, we tried to introduce a methyl group into C-6 of **15**, which was easily obtained from **11** (Scheme 3). The keto ester **16** was obtained from **15** by protection of the carboxyl group and subsequent Swern oxidation. Methylation was carried out with **16** in the presence of methyl iodide and lithium diisopropylamide (LDA) to give a mixture of two diastereomers. One diastereomer was isolated in pure form by crystallization after transformation into a methyl ester (**17**), which was identical with that derived from **12**, including the optical rotation. This finding confirmed the 3*R* configuration of **2**. Furthermore, reduction of **17** with diisobutylaluminum hydride gave two diastereomers (**18** and **19**), with the minor one (**18**) being identical with that derived from **12**. This compound could be easily converted to **2**. The absolute configuration at the C-6 position remained unknown, however, until Hashiguchi et al. established it as being of the *R* configuration during study of the total synthesis of sperabillins.[11]

Sperabillin C (**3**) has the same molecular formula

Table 3. ^{1}H NMR Data of Sperabillins (**1**—**4**) (in D_2O)[a]

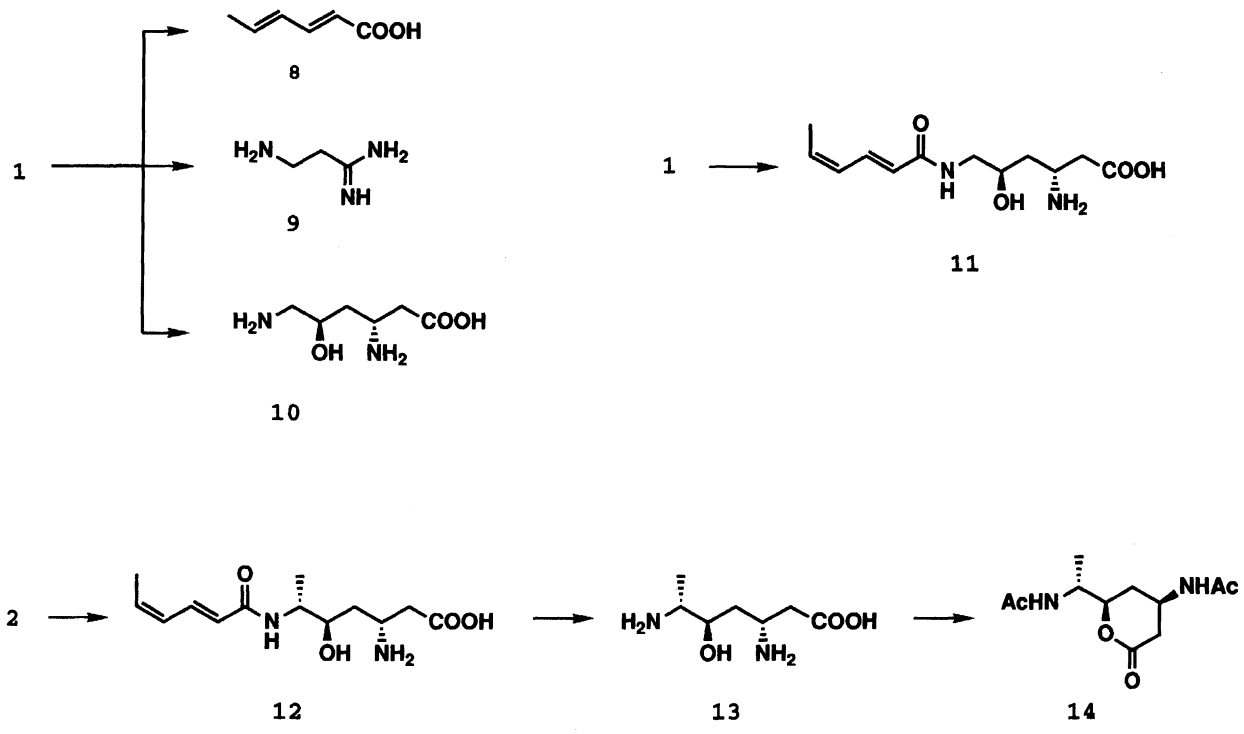
	1[b]	**2**[b]	**3**[c]	**4**[c]
2′	2.68 2H t (6.7)	2.65 2H t (6.8)	2.68 2H t (6.7)	2.65 2H t (6.7)
3′a	3.54 dt (14.0, 6.7)	3.52 dt (14.2, 6.8)	3.56 2H m	3.54 2H m
3′b	3.59 dt (14.0, 6.7)	3.57 dt (14.2, 6.8)		
2a	2.72 dd (7.0, 16.0)	2.71 2H d (6.8)	2.73 2H d (7.0)	2.71 2H d (6.8)
2b	2.76 dd (6.0, 16.0)			
3	3.86 m	3.85 m	3.86 m	3.84 m
4a	1.77 ddd (4.6, 10.0, 15.0)	1.73 ddd (4.6, 10.2, 14.9)	1.76 ddd (4.5, 10.0, 15.0)	1.72 ddd (4.5, 10.0, 15.0)
4b	1.87 m	1.83 ddd (3.2, 7.6, 14.9)	1.87 ddd (3.3, 7.5, 15.0)	1.80 ddd (3.5, 8.0, 15.0)
5	4.00 m	3.85 m	3.98 m	3.84 m
6a	3.33 dd (6.5, 14.0)	4.01 dq (3.7, 6.8)	3.31 dd (6.5, 14.0)	3.99 dq (3.5, 7.0)
6b	3.39 dd (4.4, 14.0)		3.36 dd (4.0, 14.0)	
2″	6.07 d (15.1)	6.07 d (14.9)	5.97 d (15.5)	5.97 d (15.5)
3″	7.56 dd (11.6, 15.1)	7.55 dd (11.5, 14.9)	7.12 dd (9.5, 15.5)	7.12 dd (9.5, 15.5)
4″	6.23 ddq (11.0, 11.6, 1.5)	6.22 ddq (10.7, 11.5, 1.7)	6.26 m	6.26 m
5″	6.02 dq (11.0, 7.2)	6.02 dq (10.7, 7.3)	6.26 m	6.26 m
6″	1.87 3H dd (1.5, 7.2)	1.87 3H dd (1.7, 7.3)	1.83 3H d (5.0)	1.82 3H d (5.5)
6-CH_3		1.19 3H d (6.8)		1.18 3H d (7.0)

a) Chemical shifts are in ppm downfield from sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4; coupling constants in Hz are in parenthesis. b) Recorded on a JEOL GX-400 instrument (400 MHz). c) Recorded on a Bruker AC-300 instrument (300 MHz).



Scheme 1.

with **1**. Their ^{1}H and ^{13}C NMR spectra, however, showed differences in the 2,4-hexadienoyl moiety; the other moieties showed good agreement. The coupling constant (J=15.5 Hz) of the doublet at δ=5.97 indi-



Scheme 2.

cated the 2″*E*-form, and hence the configuration at the C-4″ position was considered to be *E* in spite of overlapping of the H-4″ and H-5″ signals. Sperabillin D (**4**) apparently had the methyl group at the C-6 position like **2** and the hexadienoyl moiety of **4** was the same as that of **3** from NMR analysis. This compound was also derived from **2**.[12] The structures of **3** and **4** were further confirmed by the synthesis of **3** and **4** from **1** and **2**, respectively.[12]

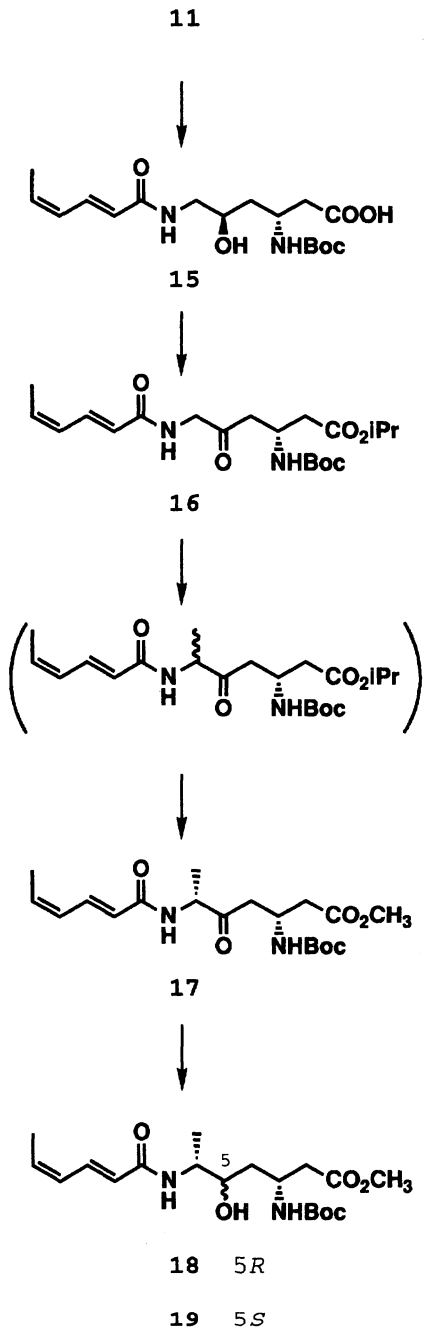
In this report, the unique structures of the novel antibiotics, sperabillin A—D (**1**—**4**), were revealed as shown in Fig. 1. We have been interested in their biological activities and tried to modify them to obtain more potent ones. The results will be described in a forthcoming paper.[12]

Experimental

Melting points were determined using a Yamato MP-21 melting point apparatus, and are uncorrected. UV spectra were taken on a Hitachi 320 spectrophotometer in water. Optical rotations in water at 20—26°C were obtained with a JASCO DPI-181 digital polarimeter, unless otherwise stated. ORD curves and CD spectra in MeOH at 20—26°C were recorded on a JASCO J-20A equipped with a DP-501N. IR spectra were measured with a Hitachi 285 grating IR spectrophotometer using KBr pellets. ^{1}H NMR spectra in D_2O were recorded on a Varian EM-390 spectrometer (90 MHz); chemical shifts (δ) are reported in ppm downfield from tetramethylsilane; coupling constants are reported in Hz, unless otherwise stated. ^{13}C NMR spectra in D_2O were recorded on a JEOL GX-400 (100 MHz) spectrometer; chemical shifts (δ) are reported in ppm downfield from sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4. SI-MS spectra were measured with a Hitachi M-80 A mass spectrometer using a xenon ion beam source. EI-MS spectra were measured with a JEOL JMS-DX303 instrument.

***N*-Boc Sperabillin A (5):** To a solution of **1** (1.25 g, purity 83%, 2.6 mmol) in 50% aq dioxane (50 ml) were added Et_3N (0.5 ml, 3.6 mmol) and 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON, 818 mg, 3.3 mmol) and the mixture was stirred for 5.5 h at room temperature with keeping at pH 8—9. The reaction mixture was adjusted to pH 7.2 and concentrated. The concentrate was diluted with H_2O and washed with hexane–ether (1:1). The aqueous layer was concentrated and chromatographed on Diaion HP-20 (50—100 mesh, 70 ml), eluting with 50% MeOH and 50% MeOH-5 mM HCl. The pure fraction was freeze-dried to give **5** (834 mg) as a white powder. $[\alpha]_D$ −23.7° (*c* 0.52); UV 264 nm (ε 29900); SI-MS *m/z* 426 $(M+H)^+$; ^{1}H NMR δ=1.65 (9H, s), 1.80 (2H, m), 2.08 (3H, d, *J*=6 Hz), 2.65 (2H, d, *J*=6 Hz), 2.92 (2H, t, *J*=6 Hz), 3.52 (2H, d, *J*=6 Hz), 3.75 (2H, t, *J*=6 Hz), 4.05 (m), 4.28 (m), 6.00—6.60 (3H, m), and 7.73 (dd, *J*=11, 15 Hz). Found: C, 49.08; H, 8.07; N, 14.44; Cl 7.26%. Calcd for $C_{20}H_{35}N_5O_5 \cdot HCl \cdot 1.5H_2O$: C, 49.12; H, 8.04; N, 14.32; Cl, 7.25%.

2,4-Dimethylpyrimidine Derivative (6): A solution of **5** (96 mg, 0.20 mmol), acetylacetone (25 μl, 0.24 mmol) and K_2CO_3 (52 mg, 0.38 mmol) in water (0.3 ml) was allowed to stand at room temperature for 8 d. The reaction mixture was diluted with 2% aqueous K_2CO_3 and extracted with EtOAc. The organic layer was concentrated and the residue obtained was washed with ether–hexane, and the insoluble substance was filtered off. The filtrate was concentrated and the residue was precipitated from ether–hexane to give a white powder. This powder was dissolved in TFA (0.1 ml) and the mixture was allowed to stand for 20 min at room temperature. The reaction mixture was concen-



Scheme 3.

trated and the residue was diluted with water. The aqueous solution was concentrated and freeze-dried to give a white powder of **6** (TFA salt 17 mg, 17%): UV 265 nm (ε 23000); SI-MS m/z 390 $(M+H)^+$; ^{1}H NMR δ=2.00 (2H, m), 2.05 (3H, d, J=6 Hz), 2.87 (2H, d, J=5 Hz), 2.90 (6H, s), 3.43 (2H, t, J=6 Hz), 3.55 (2H, d, J=6 Hz), 3.90 (2H, t, J=6 Hz), 3.96 (m), 4.16 (m), 6.0—6.6 (3H, m), 7.73 (dd, J=10, 15 Hz), and 7.83 (1H, s).

Ketone 7: To a solution of DMSO (1.0 ml) in CH_2Cl_2 (4.0 ml) was added oxalyl dichloride (0.11 ml, 1.28 mmol) dropwise at −70°C with stirring under argon. Stirring was continued for 5 min at −70°C followed by addition of a solution of **5** (197 mg, 0.42 mmol) in DMSO–CH_2Cl_2 (1:1, 1.2 ml). The reaction mixture was stirred for 20 min at −70°C and Et_3N (0.71 ml, 5.1 mmol) was added, and then warmed up to 0°C during 20 min. The mixture was poured into 80 mM HCl (32 ml) and washed with ether. The aqueous layer was concentrated and chromatographed on Diaion HP-20 (50—100 mesh, 13 ml), eluting with water and 20—50% aq MeOH. The pure fraction was concentrated and freeze-dried to give a white powder (74 mg).

A solution of the powder (70 mg) in TFA (0.4 ml) was allowed to stand for 40 min at room temperature. The mixture was evaporated and poured into ether. The precipitates dissolved in water (7 ml) was passed through IRA-402 (Cl^-, 7 ml), concentrated and freeze-dried to give a powder of **7** (56 mg, 43% from **5**). $[\alpha]_D$ −3.0° (c 0.53); UV 265 nm (ε 22700); SI-MS m/z 324 $(M+H)^+$; IR 1660 cm^{-1}; ^{1}H NMR δ=2.10 (3H, d, J=6 Hz), 2.90 (2H, t, J=6 Hz), 2.94 (2H, d, J=6.5 Hz), 3.29 (2H, d, J=6 Hz), 3.80 (2H, t, J=7 Hz), 4.29 (m), 4.45 (2H, s), 6.40 (3H, m), and 7.80 (dd, J=15, 10 Hz). Found: C, 43.63; H, 7.01; N, 16.88; Cl, 16.45%. Calcd for $C_{15}H_{25}N_5O_3 \cdot 2HCl \cdot H_2O$: C, 43.48; H, 7.05; N, 16.90; Cl, 17.11%.

Acidic Hydrolysis of 1: 1) A solution of **1** (1.94 g, 4.9 mmol) in 2 M HCl (193 ml) was refluxed for 6 h. The reaction mixture was extracted with $CHCl_3$ (2×200 ml). The combined organic layers were evaporated to dryness and crystallized from ether–hexane to give (2*E*,4*E*)-2,4-hexadienoic acid (**8**) (84 mg, 15%). The aqueous layer was evaporated to dryness and dissolved in water (55 ml). The solution was chromatographed on Dowex 50W×2 (H^+, 50—100 mesh, 100 ml), eluting with 0.5—1.5 M HCl. The fraction, mainly containing **9** was concentrated and crystallized from ether–MeOH to give **9** (2HCl salt, 426 mg, 55%) as colorless crystals. The fraction, mainly containing **10**, was evaporated to dryness. The residue was chromatographed on Dowex 50 W×2 (H^+, 50—100 mesh, 30 ml), with elution of 0.8—1.0 M HCl. The pure fraction was concentrated and freeze-dried to give **10** (2HCl salt, 301 mg, 31%) as a white powder.

8: Mp 125°C; UV 257 nm (ε 24100); EI-MS m/z 112 (M^+). The IR and ^{1}H NMR spectra and elemental analysis were in accord with those of the standard sample.

9: SI-MS m/z 88 $(M+H)^+$; ^{1}H NMR δ=3.20 (2H, m), and 3.66 (2H, m).

10: $[\alpha]_D$ −18.3° (c 0.85) and −5.7° (c 0.60, 2 M HCl); SI-MS m/z 163 $(M+H)^+$. The IR, ^{1}H NMR spectra and elemental analysis were identical with those of the sample prepared from negamycin.

2) A solution of **1** (5.02 g, purity 83 %, 10.5 mmol) in 2 M HCl (500 ml) was refluxed for 15 min. The reaction mixture was evaporated to dryness and the residue was diluted with water. The solution adjusted to pH 6.8 was chromatographed on Diaion HP-20 (50—100 mesh, 1.0 dm^3), eluting with water and 10% MeOH. The pure fraction was freeze-dried to give a white powder of **11** (1.79 g, 67%). $[\alpha]_D$ −22.3° (c 0.52); UV 265 nm (ε 27800); SI-MS m/z 257 $(M+H)^+$; IR 1660, 1630, 1610, and 1540 cm^{-1}; ^{1}H NMR δ=2.06 (2H, m), 2.07 (3H, d, J=5 Hz), 2.80 (2H, dd, J=4, 6 Hz), 3.60 (2H, dd, J=4, 6 Hz), 3.94 (m), 4.23 (m), 6.45 (3H, m), and 7.76 (dd, J=10, 15 Hz). Found: C, 53.81; H, 8.11; N, 10.46%. Calcd for $C_{12}H_{20}N_2O_4 \cdot 0.5H_2O$: C, 54.33; H, 7.98; N, 10.56%.

Acidic Hydrolysis of 2: 1) A solution of **2** (1.25 g,

purity 86%, 2.6 mmol) in 2 M HCl (125 ml) was refluxed for 18 min. The reaction mixture was treated by the same method with that used for the preparation of **11** to give a white powder of **12** (449 mg, 64%). $[\alpha]_D$ +84.7° (*c* 0.42); UV 265 nm (ε 32500); SI-MS *m/z* 271 $(M+H)^+$; IR 1610 and 1540 cm^{-1}; ^1H NMR δ=1.40 (3H, d, *J*=7 Hz), 2.04 (5H, m), 2.77 (2H, dd, *J*=6, 7 Hz), 4.10 (3H, m), 6.30 (3H, m), and 7.75 (dd, *J*=11, 16 Hz). Found: C, 54.55; H, 8.15; N, 9.72%. Calcd for $C_{13}H_{22}N_2O_4 \cdot H_2O$: C, 54.15; H, 8.39; N, 9.72%.

2) A solution of **12** (310 mg, 1.1 mmol) in 2 M HCl (31 ml) was refluxed for 6 h. The reaction mixture was treated by the same method with that used for the preparation of **10** to give a white powder of **13** (2HCl salt, 48 mg, 27%). $[\alpha]_D$ −2.7° (*c* 0.58); SI-MS *m/z* 177 $(M+H)^+$; ^1H NMR δ= 1.47 (3H, d, *J*=7 Hz), 2.05 (2H, m), 2.74 (2H, d, *J*=7 Hz), 3.43 (m, *J*=7 Hz), and 3.94 (2H, m). Found: C, 33.81; H, 7.81; N, 11.20; Cl, 29.52%. Calcd for $C_7H_{16}N_2O_3 \cdot 2HCl$: C, 34.02; H, 6.53; N, 11.34; Cl, 28.69%.

Lactone 14: To a solution of **13** (108 mg, 0.61 mmol) in 3% $NaHCO_3$ (15 ml) was added Ac_2O (0.17 ml) and the mixture was stirred for 3 h at room temperature. The mixture was stirred for 30 min after addition of Dowex 50W×2 (H^+, 10 ml). The resin was filtered off and washed with water (40 ml). The combined aqueous solutions were concentrated and freeze-dried to give a crude powder, which was purified by silica-gel chromatography, eluting with EtOAc–hexane. Crystallization from acetone afforded colorless crystals of the *N,N'*-diacetyl derivative (44 mg, 28%): Mp 160°C; $[\alpha]_D$ +66.7° (*c* 0.50, MeOH); SI-MS *m/z* 261 $(M+H)^+$; IR 1725, 1620, and 1540 cm^{-1}; ^1H NMR δ=1.34 (3H, d, *J*=7 Hz), 1.84 (2H, dd, *J*=6.5, 8 Hz), 2.17 (3H, s), 2.20 (3H, s), 2.80 (2H, dd, *J*=6.5, 8 Hz), 3.85 (m), 4.04 (m), and 4.60 (m). Found: C, 50.68; H, 7.80; N, 10.50%. Calcd for $C_{11}H_{20}N_2O_5$: C, 50.76; H, 7.74; N, 10.76%.

To a solution of the crystals (19 mg, 0.073 mmol) in DMF (0.7 ml) were added HOBT (15 mg, 0.11 mmol) and DCC (23 mg, 0.11 mmol) at 0°C and the mixture was stirred for 18 h at room temperature. The reaction mixture was evaporated to dryness, then dissolved in water (10 ml) and filtered. The filtrate was washed with EtOAc (10 ml), concentrated and freeze-dried to give an oily compound. Crystallization from ether gave **14** (15 mg, 85%) as colorless crystals. $[\alpha]_D$ +67.0° (*c* 0.44); ORD $[\Phi]_{220}$ +2200°; CD $[\theta]_{225}$ +5290; SI-MS *m/z* 243 $(M+H)^+$; IR 1730, 1650, and 1540 cm^{-1}; ^1H NMR δ=1.43 (3H, d, *J*=7 Hz), 1.80 (m), 2.19 (3H, s), 2.23 (3H, s), 2.40 (m), 2.65 (dd, *J*=9, 15 Hz), 3.20 (dd, *J*=6.5, 15 Hz), 4.33 (dd, *J*=7, 10 Hz), 4.37 (m), and 4.69 (dt, *J*=12, 3 Hz).

***N-t*-Butoxycarbonylation of 11:** To a solution of **11** (3.4 g, 13 mmol) in 50% aq acetone (114 ml) were added Et_3N (7.35 ml, 53 mmol) and Boc-ON (3.92 g, 15.9 mmol) and the mixture was stirred for 5 h at room temperature. Normal workup gave an oily residue, and colorless crystals of **15** (4.12 g, 87%) were obtained from EtOAc–ether–hexane: Mp 129°C; $[\alpha]_D$ −26.5° (*c* 0.50, MeOH); UV 260 nm (ε 29300); SI-MS *m/z* 357 $(M+H)^+$; IR 1700, 1660, and 1510 cm^{-1}; ^1H NMR (CD_3OD) δ=1.30 (9H, s), 1.60 (2H, m), 1.86 (3H, d, *J*=7 Hz), 2.50 (2H, d, *J*=7 Hz), 3.30 (2H, m), 3.70 (m), 4.10 (m), 6.00 (3H, m), and 7.55 (dd, *J*=10, 15 Hz). Found: C, 57.34; H, 7.72; N, 7.98%. Calcd for $C_{17}H_{28}N_2O_6$: C, 57.29; H, 7.92; N, 7.86%.

Ketone 16: To a solution of **15** (1.21 g, 3.4 mmol) in isopropyl alcohol (24 ml) was added (+)-10-camphorsulfonic acid (39 mg, 0.17 mmol) and the mixture was stirred for 6 h at room temperature. The reaction mixture was concentrated to a small volume and poured into a mixture of ether (30 ml) and 5% $NaHCO_3$ (30 ml). The organic layer was concentrated to give an oily product (1.28 g). The product was oxidized by a method similar to that used in the preparation of **7**. The reaction mixture obtained was poured into a mixture of 1 M HCl (7.8 ml) and sat. NH_4Cl (25 ml), and then extracted with ether. The organic layer was concentrated to give an oily residue. Crystallization from CH_2Cl_2–ether–hexane afforded **16** as colorless crystals (933 mg, 73%): $[\alpha]_D$ +1.6° (*c* 0.50, EtOH); EI-MS *m/z* 396 (M^+); UV (MeOH) 260 nm (ε 26800); IR 1730, 1700, 1660, 1630, and 1610 cm^{-1}; ^1H NMR ($CDCl_3$) δ=1.23 (6H, d, *J*=6 Hz), 1.40 (9H, s), 1.87 (3H, d, *J*=6 Hz), 2.58 (2H, d, *J*=6 Hz), 2.80 (2H, d, *J*=6 Hz), 4.33 (m), 4.62 (2H, d, *J*=5 Hz), 5.00 (m), 5.30 (br d, *J*=8 Hz), 5.9 (3H, m), 6.37 (m), and 7.58 (dd, *J*=11, 15 Hz). Found: C, 60.58; H, 8.12; N, 7.00%. Calcd for $C_{20}H_{32}N_2O_6$: C, 60.59; H, 8.14; N, 7.07%.

Methylation of 16: To a 0.32 M solution of LDA in THF (18 ml, 5.7 mmol) was dropwise added a solution of **16** (702 mg, 1.8 mmol) in THF (3 ml) and hexamethylphosphoramide (2.0 ml) in 15 min at −78°C with stirring under argon. After 20 min, CH_3I (0.144 ml, 2.3 mmol) was added to the mixture, which was gradually warmed up to 0°C during 2.5 h, and further stirred for 40 min at 0°C. The reaction mixture was poured into a mixture of ether (30 ml), 1 M HCl (9.2 ml) and sat. NH_4Cl (30 ml). The aqueous layer was extracted with ether (2×40 ml). The combined organic layers were washed with aq NH_4Cl (pH 2, 2×50 ml) and concentrated to give a crude oil. The oil was purified by silica-gel chromatography followed by crystallization from CH_2Cl_2–ether–hexane to afford a mixture of epimers (200 mg) as colorless crystals.

A solution of the mixture in MeOH (6.8 ml) and NaOMe (28% in MeOH, 0.144 ml) was stirred for 3.5 h at room temperature. The reaction mixture was poured into aq NH_4Cl (pH 4, 20 ml) and extracted with ether. The extract was concentrated to give an oily residue. Crystallization from ether–MeOH–hexane gave colorless crystals of **17** (82 mg, 43%): $[\alpha]_D$ +87.9° (*c* 0.55, EtOH); UV (MeOH) 261 nm (ε 26800); ^1H NMR ($CDCl_3$) δ=1.35 (3H, d, *J*=6.5 Hz), 1.43 (9H, s), 1.84 (4H, br t, *J*=6 Hz), 2.63 (2H, br d, *J*=6 Hz), 2.90 (2H, d, *J*=6 Hz), 3.67 (3H, s), 4.30 (2H, m), 4.66 (m, *J*=6.5 Hz), 5.30 (m), 5.90 (3H, m), 6.30 (br d, *J*=7 Hz), and 7.58 (dd, *J*=11, 15 Hz). Found: C, 59.80; H, 7.89; N, 7.37%. Calcd for $C_{19}H_{30}N_2O_6$: C, 59.67; H, 7.91; N, 7.32%. HPLC; column, ODS, YMC A-312; mobile phase, 35% CH_3CN/H_2O; flow rate, 2.0 ml min^{-1}; retention time, 14.6 min.

Reduction of 17: To a solution of **17** (111 mg, 0.29 mmol) in THF (3.7 ml) was added a 1.5 M solution of diisobutylaluminum hydride in toluene (0.60 ml, 0.90 mmol) dropwise at −70°C under nitrogen with stirring. The mixture was gradually warmed up to −40°C during 2.5 h. The solution was diluted with ether (10 ml) and poured into 2% aq citric acid (10 ml). The aqueous layer was extracted with ether and the extract was washed with water and brine, and evaporated to afford a crude oil (105 mg). The oil was purified by preparative HPLC (column, YMC Pack SIL SH-043;

mobile phase, 6—9% *i*-PrOH–hexane) to give **18** (11 mg, 10%) and its epimer (**19**) (35 mg, 31%) as colorless oils.

18: ^{1}H NMR ($CDCl_3$) δ=1.23 (3H, d, J=7 Hz), 1.44 (9H, s), 1.52 (2H, m), 2.53 (2H, m), 2.86 (3H, d, J=6.5 Hz), 3.63 (2H, m), 3.70 (3H, s), 4.07 (2H, m), 5.50 (m), 5.94 (3H, m), and 7.57 (dd, J=11, 15 Hz). HPLC; column, ODS, YMC A-312; mobile phase, 30% CH_3CN/H_2O; flow rate, 2.0 ml min^{-1}; retention time, 13.1 min.

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