Formamicin, a Novel Antifungal Antibiotic Produced by a Strain of *Saccharothrix* sp.

II. Structure Elucidation of Formamicin

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A novel antifungal antibiotic, formamicin, was isolated from the culture broth of *Saccharothrix* sp. MK27-91F2.

The absolute structure of formamicin was determinated by spectroscopic and X-ray crystallographic analysis and degradation study.

Formamicin (**1**) is a novel 16-membered macrolide antibiotic, produced by *Saccharothrix* sp. MK27-91F2, which showed strong antifungal activity against phytopathogenic fungi. The taxonomic study of the producing strain and the fermentation, isolation and biological activity of **1** is reported in the preceding paper[1].

In this paper, we report the physico-chemical properties, degradation study and structural elucidation of **1**.

Results and Discussion

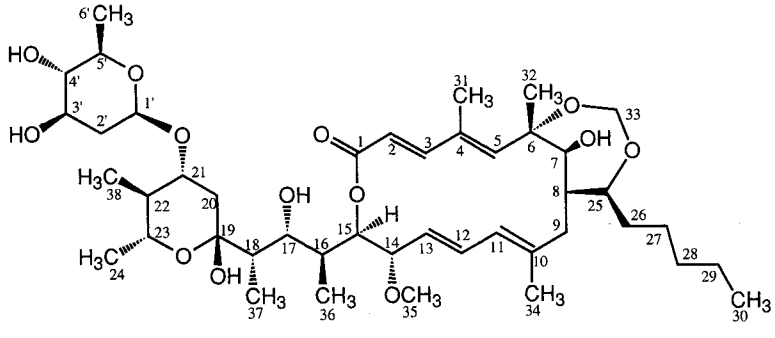
Structure Determination

The molecular formula of formamicin was established as $C_{44}H_{72}O_{13}$ on the basis of HRFAB-MS and NMR spectral analysis. In the UV spectrum, **1** showed strong absorption at 239 (37,200 sh), 245 (40,900) and 275 nm (ε 13,900) in EtOH. The UV and IR (1685 cm^{-1}) spectra suggested the presence of α,β,γ,δ-unsaturated lactone and diene residue in the molecule. These data resembled to those of bafilomycin-concanamycin group antibiotics which belonged to 16- and 18-membered macrolide group antibiotics. The physico-chemical properties of **1** are summarized in Table 1.

The multiplicities of carbon signals were determined by DEPT experiment. All bond correspondings between ^1H and ^{13}C signals were determined by DEPT and heteronuclear multiple quantum coherence (HMQC) experiments. The ^{13}C NMR spectrum of **1** showed 44 carbon signals. The DEPT and HMQC experiments revealed the presence of ten methyls, eight methylenes, fifteen methines, six olefinic methines, two sp^3 quaternary carbons, two olefinic quaternary carbons and one carbonyl carbon. The ^1H and ^{13}C NMR data of **1** were similar to those of leucanicidin[2] or bafilomycin A_1[3] except for NMR data of sugar, hemiacetal, methoxy, alkyl side-chain and methylenedioxy moieties. The ^1H and ^{13}C NMR spectral data of **1** in $CDCl_3$: benzene-d_6 = 4 : 1 are shown in Table 2.

The ^1H-^1H COSY and HMBC spectra of **1** were suggested **1** contained seven partial structures (**a**, **b**, **c**, **d**, **e**, **f** and **g**) as shown in Fig. 2. The connections among

Fig. 1. Structure of formamicin.



16-membered lactone ring residues **a**, **b**, **c** and C-6 were revealed by the HMBC spectrum. A methine proton at δ_H 4.96 (15-H) and an olefinic proton at δ_H 5.69 ppm (2-H) were coupled to a carbonyl carbon at δ_C 169.6 (C-1). A methyl proton at δ_H 1.33 (32-H) coupled to an olefinic carbon at δ_C 142.6 (C-5), a sp^3 quaternary carbon at δ_C 80.1 (C-6), and a methine carbon bearing a hydroxyl group at δ_C 74.8 (C-7). A methyl proton at δ_H 1.93 ppm (34-H) coupled to a methylen carbon at δ_C 34.8 (C-9) and two olefinic carbon at δ_C 143.4 (C-10) and δ_C 125.3 (C-11).

Table 1. Physico-chemical properties of formamicin.

Appearance	Colorless plate
MP (°C)	201~202
Molecular formula	$C_{44}H_{72}O_{13}$
FAB-MS (*m/z*)	831 $(M+Na)^+$ 807 $(M-H)^-$
HRFAB-MS (*m/z*)	
Calcd :	831.4871
Found :	831.4859 $(M+Na)^+$
$[\alpha]_D^{24}$	+15.5 (*c* 1, EtOH)
$UV\lambda_{max}^{EtOH}$ nm(ε)	239 (37,200 sh) 245 (40,900) 275 (13,900)
$IR\nu_{max}$(KBr)cm^{-1}	3423, 2969, 2933, 1685, 1452, 1385, 1268, 1066
Color reaction positive :	I_2, vanilline-sulfuric acid, molybdophosphoric acid-sulfuric acid
Silicagel TLC*	Rf 0.39 ($CHCl_3$:MeOH=9:1)

* Kieselgel 60 F254, art 5715, Merck

A methin proton at δ_H 3.78 (23-H) of the partial structre **e** was coupled to an anomeric carbon at δ_C 99.4 (C-19). A hydroxy proton at δ_H 5.71 (19-OH) was coupled to the anomeric carbon at δ_C 99.4 (C-19), a methylen carbon at δ_C 40.2 (C-20) and a methine carbon at δ_C 42.7 (C-18). A methine bearing an oxygen atom at δ_H 4.96 (15-H) of the lactone ring moiety was coupled to a methine carbon at δ_C 37.3 (C-16) as shown in Fig. 3. This linkage was not determined from the ^1H-^1H COSY spectrum because of small coupling constant between 15-H and 16-H.

The presence of sugar moiety (**f**) and the position of

Table 2. ^{13}C and ^1H NMR data of formamicin (**1**) in $CDCl_3$ - benzene-d_6 = 4 : 1.

Position	δC[a]	δH[b]	*J* (Hz)	Position	δC[a]	δH[b]		*J* (Hz)
1	169.6 s			22	43.7 d	1.2~1.3	m	
2	115.0 d	5.69 d	15.4	23	69.8 d	3.78	m	6.4, 9.5
3	153.8 d	7.27 d	15.4	24	19.5 q	1.15	d	6.4
4	134.6 s			25	78.0 d	3.61	m	1.0, 6.4, 7.0
5	142.6 d	5.71 s		26	30.2 t	1.25~1.27	m	
6	80.1 s			27	25.8 t	1.25~1.30	m	
7	74.8 d	3.56 dd	5.2, 9.0	28	32.0 t	1.25~1.35	m	
	OH	1.66 d	5.2	29	22.8 t	1.25~1.35	m	
8	47.2 d	2.65 m		30	14.2 q	0.91	t	6.8
9	34.8 t	1.83 m		31	11.7 q	1.83	d	0.8
		1.97 d	14.0	32	24.9 q	1.33	s	
10	143.4 s			33	86.5 t	4.63	d	8.4
11	125.3 d	5.76 d	10.6			4.73	d	8.4
12	132.9 d	6.53 dd	10.6, 15.0	34	20.2 q	1.93	s	
13	127.8 d	5.20 dd	10.0, 15.0	35	55.7 q	3.23	s	
14	82.3 d	3.91 t	9.0, 10.0	36	9.7 q	0.83	d	7.0
15	75.8 d	4.96 dd	1.0, 9.0	37	7.3 q	1.08	d	7.0
16	37.3 d	2.19 m		38	13.3 q	0.99	d	6.6
17	70.5 d	4.20 ddd	2.0, 3.2, 10.0	1'	96.8 d	4.53	dd	9.4, 2.0
	OH	4.87 d	3.2	2'	39.7 t	1.57	ddd	9.4, 12.4, 13.0
18	41.9 d	1.76 m				2.06	m	2.0, 5.0, 13.0
19	99.4 s			3'	72.0 d	3.44	m	3.0, 5.0, 8.8, 12.4
	OH[c]	5.71 s			OH	2.18	d	3.0
20	40.2 t	1.17 m		4'	77.9 d	2.97	ddd	3.0, 8.8, 10.0
		2.34 dd	4.6, 12.0		OH	2.18	d	3.0
21	76.2 d	3.81 m	4.6, 10.5, 11.0	5'	71.4 d	3.17	dq	6.2, 9.4
				6'	17.7 q	1.25	d	6.2

a: 125MHz, chemical shifts in ppm, multipulicity.

b: 500MHz, chemical shifts in ppm, multipulicity.

c: The hydroxy proton was observed isolate singlet signal at δ 6.05 in benzen-d_6.

Fig. 2. Partial structures (**a**, **b**, **c**, **d**, **e**, **f** and **g**) of formamicin.

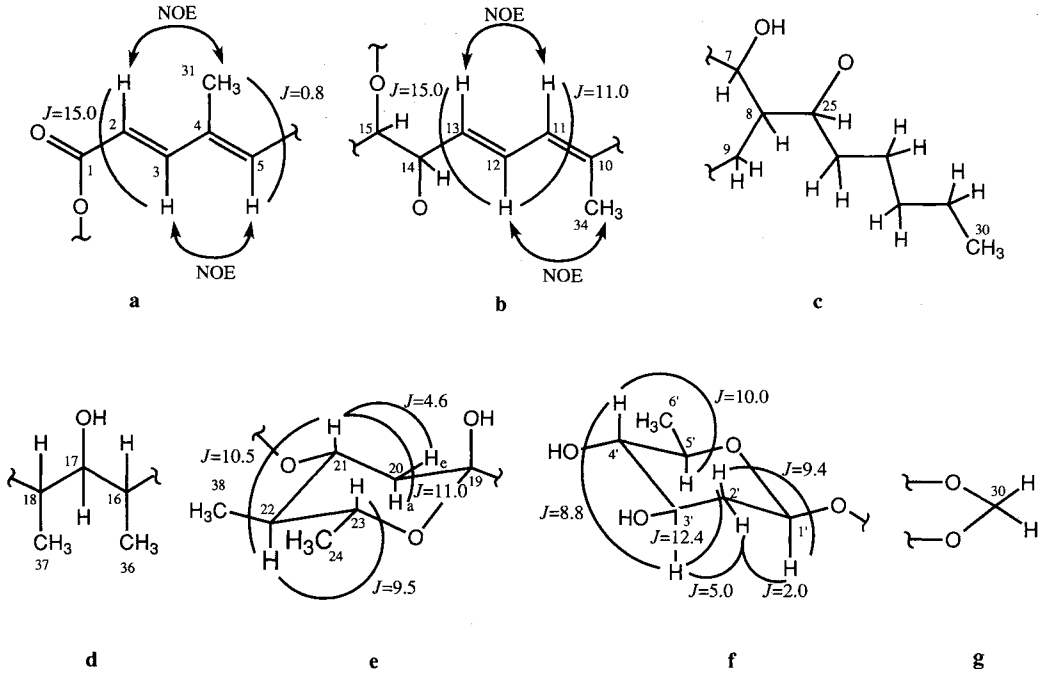
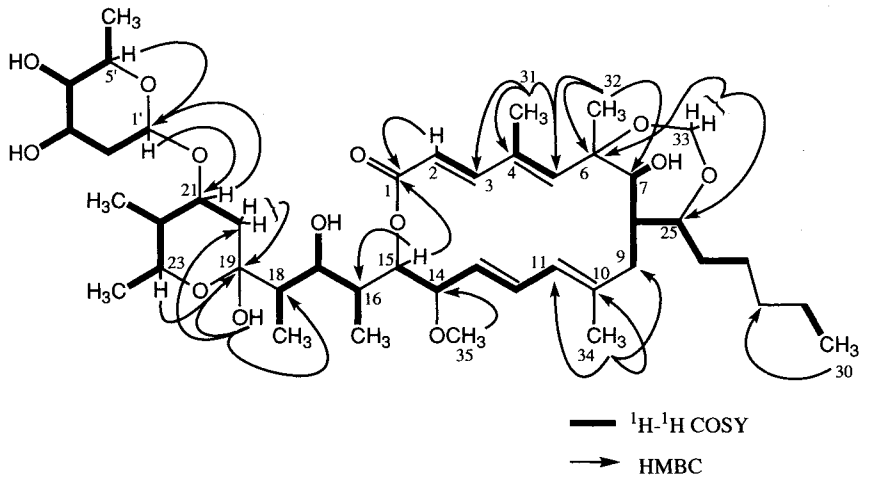


Fig. 3. ^{1}H-^{1}H COSY and HMBC experiments of formamicin ($CDCl_3$: benzene-d_6 = 4 : 1).



glycosidic linkage were also revealed by the HMBC spectrum as shown in Fig 3. A methin proton at δ_H 3.17 (5′-H) was coupled to an anomeric carbon at δ_C 96.8 (C-1′). A methine proton at δ_H 3.81 (21-H) was coupled to the anomeric carbon at δ_C 96.8 (C-1′).

The long-range ^{13}C-^{1}H couplings from the methylenedioxy protons (**g**) showed the presence of seven membered ring containing the methylenedioxy group. The methylenedioxy protons at δ_H 4.63 and 4.73 (H-33) were coupled to two methine carbons bearing oxygen atom at δ_C 80.1 (C-6) and δ_C 78.0 (C-25). The above described result of HMBC experiment is summarized in Fig. 3.

Configurations for four olefins at C-2, C-4, C-10 and C-12 in the 16-membered lactone ring were shown to be all *trans* by their large spin coupling constants and NOE experiments (Fig. 2). The relative stereochemistry of the 6-membered hemiacetal ring moiety (**e**) and sugar moiety (**f**) were suggested by the analysis of coupling constants as shown in Fig. 2.

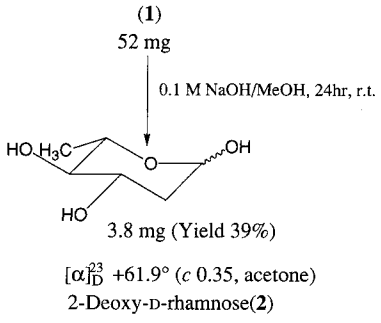
Stereochemistry of Formamicin

The absolute structure of **1** was determined by degradation study and X-ray structure analysis.

Alkaline hydrolysis of **1** with 0.1 M NaOH at room temperature for 24 hours gave a 2-deoxy-rhamnose (**2**, Fig. 4). The structure of **2** was elucidated to be α,β-anomeric mixture of **2** by the MS and NMR spectra[4]. The configuration of the anomeric center (C-1′) of **1** was determined to be β by its spin coupling constants (2.0 and 9.8 Hz) and the chemical shift (δ_H 4.58 and δ_C 97.1) data which were almost identical with those of the β anomer of **2**. The optical rotation of **2** was dextro-rotatory $[\alpha]_D^{23}$ +61.9° (*c* 0.35, acetone). Therefore, **2** was determined to be ***D*** series by the comparison with those of optical rotation values for α and β anomers of 2-deoxy-D-rhamnose in the literature, $[\alpha]_D^{23}$ +60.8° (*c* 0.977, acetone)[5].

The absolute structure of **1** was determined by X-ray structure analysis on the basis of stereochemistry of **2**. The ORTEP drawing of **1** is shown in Fig. 5.

Fig. 4. Alkaline degradation of formamicin.



Experimental

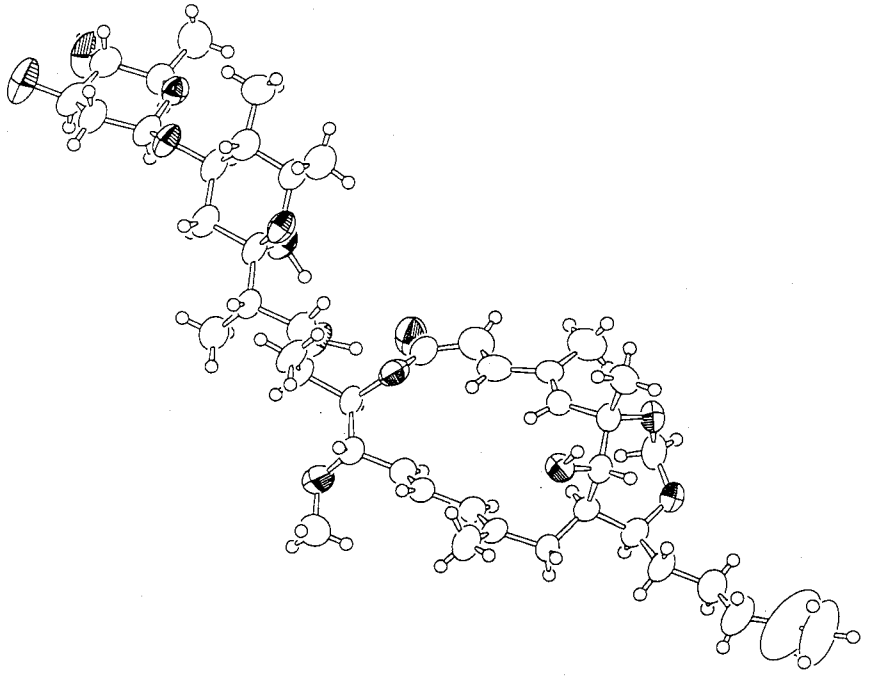
General

Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. UV spectra were recorded with a Hitachi 557 spectrophotometer. IR spectra were recorded with a Horiba FT-210 fourier transform infrared spectrometer. The ^1H and ^{13}C NMR spectra were measured with a JEOL JNM-A500 spectrometer at 24°C, using TMS ($\delta=0$ ppm) as internal reference. The mass spectrum was recorded with a JEOL JMS-SX102 mass spectrometer.

Alkaline Hydrolysis of Formamicin (1)

Alkaline hydrolysis of formamicin (54 mg, 0.067 mmol) was carried out with 0.1 M NaOH in MeOH (5.0 ml) at room temperature for 24 hours. The reaction mixture was detected by molybdophosphoric acid-sulfuric acid positive spot arising from **2** on a silica gel TLC (Kieselgel 60 F_{254}, art 5715, Merck) developing with $CHCl_3$-MeOH (4 : 1) as a solvent system. **2** showed the Rf value of 0.40. The reaction mixture of alkaline hydrolysate was neutralized with 1 M HCl (0.5 ml) and then concentrated. The concentrated material was suspended in H_2O and washed with EtOAc. The aqueous layer was concentrated and extracted with MeOH. The MeOH extract was separated by a Sephadex LH-20 column chromatography developing with 80% aq MeOH. The fraction containing **2** was concentrated and extracted with acetone (5 ml).

Fig. 5. ORTEP drawing of formamicin.



The acetone extract was concentrated to give pure 3.8 mg of **2** (0.26 mmol, yield 39%). 2: FABMS *m/z* 147 $(M-H)^-$; Molecular formula $C_6H_{12}O_4$; $[\alpha]_D^{23}$ +61.9° (*c* 0.35, acetone); ^1H NMR (500 MHz, D_2O): α anomer δ 1.26 (3H, d, 6.0), 1.71 (ddd, 3.6, 11.2, 13.0), 2.13 (ddd, 1.0, 5.0, 13.0), 3.11 (1H, dd, 9.0, 10.0), 3.88 (2H, m), 5.32 (br d, 3.0): β anomer δ 1.28 (3H, d, 6.0), 1.51 (1H, ddd, 10.0, 11.2, 12.6), 2.26 (1H, ddd, 2.0, 5.0, 12.6), 3.05 (1H, dd, 9.0, 10.0), 3.67 (1H, ddd, 5.0, 9.0, 11.2), 3.42 (1H, m), 4.91 (1H, dd, 2.0, 10.0); ^{13}C NMR (125 MHz, D_2O): α anomer δ 17.6 q, 38.3 t, 68.4 t, 68.7 t, 77.6 t, 94.0 d: β anomer δ 17.6 q, 40.5 t, 70.9 t, 72.6 t, 77.0 t, 91.9 d.

X-Ray Crystallographic Analysis

1 was recrystallized from a diethylether solution. A colorless plate crystal having approximate dimensions of $0.02 \times 0.30 \times 0.35$ mm was chosen for X-ray crystallography. All measurements were made on Rigaku AFC7R diffractometer with graphite monochromated Cu-Kα radiation and a rotation anode generator. The crystal data are as follows: Empirical formula; $C_{44}H_{72}O_{13}$. F.W.; 809.04. Crystal system; Orthorhombic. Space group, $P2_12_12_1$. Lattice parameters; a = 11.149(3) Å, b = 44.610(3) Å, c = 9.200(3) Å, V = 4575(1) Å^3. Z value; 4. Dcalc; 1.174 g/cm^3. μ(CuKα); 6.97 cm^{-1}. Of the 4662 reflections which were collected, 4449 were unique. No decay correction was applied. The structure was solved by direct method (SHELX86)[6] and expanded using Fourier techniques (DIRDIF92)[7]. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 2152 observed reflections ($I > 1.5\sigma(I)$) and 514 variable parameters and converged with unweighted agreement factors of R = 0.056 and Rw = 0.072. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.21 and $-0.25\,e^-/Å^3$, respectively. All calculations were performed using the teXsan crystallographic software package of Molecular Structure Corporation.

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