NEW CIRRAMYCIN-FAMILY ANTIBIOTICS F-1 AND F-2

SELECTION OF PRODUCER MUTANTS, FERMENTATION, ISOLATION, STRUCTURAL ELUCIDATION AND ANTIBACTERIAL ACTIVITY

YOSUKE SAWADA, TAKASHI TSUNO, TAKEO MIYAKI, TAKAYUKI NAITO and TOSHIKAZU OKI

Bristol-Myers Research Institute, Ltd., Tokyo Research Center, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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Two new 16-membered macrolides, cirramycins F-1 and F-2, were isolated from the culture filtrate of a mutant strain B-1425 of *Streptomyces cirratus* JTB-3. The antibiotics were also produced by bio-transformation of cirramycin A_1 using a blocked mutant strain A-0033. Structures of F-1 and F-2 have been elucidated by spectral interpretation and analysis of acid degradation products. Both involved isomeric modification of a neutral sugar; F-1 contained L-rhodinose, and F-2 L-amicetose. Based on spectral data, cirramycin F-1 and antibiotic A6888C were found to be identical. Cirramycins F-1 and F-2 are active against Gram-positive bacteria, but less active than cirramycin A_1.

In the course of studying the microbial transformation of macrolide antibiotics, we have isolated various mutants and variants of *Streptomyces cirratus* JTB-3, a producer of cirramycins A_1 and B,[1,2] after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment. Among them, a mutant strain B-1425 was found to produce new cirramycin-family antibiotics designated as F-1 and F-2, and a blocked mutant strain A-0033 was capable of transforming cirramycin A_1 to both F-1 and F-2. This paper deals with the selection of mutants, and the fermentation, isolation, structural determination and antibacterial activity of cirramycins F-1 and F-2.

Materials and Methods

MNNG Treatment

The spores of *S. cirratus* strain JTB-3[1,2] grown on modified BENNETT's agar (soluble starch 0.5%, glucose 0.5%, fish meat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6%, pH 7.0) at 28°C for 10 days were suspended in saline and fragmented by ultrasonic vibration in an ice-bath into single-cell units. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes. The cell pellet was resuspended in 10 mM Tris-HCl, pH 9.0, mixed with MNNG to a final concentration of 1,000 μg/ml, and incubated with gentle shaking at 28°C for 1 hour. Cells were washed once with saline, resuspended in saline, plated on fresh agar in a Petri-dish and incubated at 28°C for 10 days to grow mutant colonies. Cell survival was 1×10^{-4} compared with MNNG-untreated cells.

Selection of Mutants

Individual colonies (2,494) on the agar plate were cut into agar pieces with a cork borer (6 mm i.d.) and the agar pieces were put on an agar plate inoculated with spores of *Bacillus subtilis* PCI 219 at pH 8.0. Colonies on agar pieces showing bigger inhibition zones than that of the parent strain or no inhibition zone were selected and cultivated on fresh modified BENNETT's agar plates.

Fermentation Medium

The production (or seed) medium contained oatmeal 6%, Pharmamedia (Traders Protein) 0.4%,

$CaCO_3$ 0.2%, $CaSO_4 \cdot 2H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05%, $ZnSO_4 \cdot 7H_2O$ 0.03%, $MgSO_4 \cdot 7H_2O$ 0.01%, $FeSO_4 \cdot 7H_2O$ 0.1%, pH 7.0. Mature spores on a slant culture were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the medium and cultivated at 28°C on a rotary shaker at 200 rpm. In the case of submerged fermentation in a 200-liter tank fermentor, 2 liters of the seed culture were transferred to a tank fermentor containing 120 liters of the medium.

Antibacterial Activity

Antibacterial activity in the fermentation broth was determined by the paper-disc agar diffusion method using *B. subtilis* PCI 219 as the test organism at pH 8.0, and the activity measured against a cirramycin A_1 free base standard.

MIC was determined using Mueller-Hinton agar medium in which about 1×10^6 cells/ml were inoculated.

Bio-conversion of Protyronolide to Active Compounds

Mutants which were blocked in the macrolide ring closure were tested for the ability to convert protyronolide to active compounds. Each mutant was grown on the modified BENNETT's agar medium containing protyronolide at 1,000 μg/ml at 28°C for 7 days and an agar piece from each mutant was provided for testing antibacterial activity against *B. subtilis* PCI 219, pH 8.0.

Bio-conversion of Cirramycin A_1 by Blocked Mutants

A blocked mutant was grown in the fermentation medium (10 ml) for 2 days. Cirramycin A_1 dissolved in MeOH (150 μl) was added to the culture at the final concentration of 200 μg/ml and the shaking culture was continued for 1 day at 28°C. Antibiotics were extracted with 10 ml of EtOAc at pH 9.0 and compared with authentic samples by bioautography after TLC (EtOAc - MeOH, 1 : 1) using *B. subtilis* PCI 219 as the test organism. A selected strain A-0033 was grown in the fermentation medium for 2 days. Cirramycin A_1 (200 μg/ml) was fed to the culture and fermentation was continued for 1 day at 28°C. Isolation and purification of F-1 and F-2 were achieved by preparative silica gel TLC (E. Merck $60F_{254}$ plates) using EtOAc - MeOH (1 : 1). Antibiotics were visualized by exposing the plate to short wavelength UV. Each antibiotic was extracted from the silica gel with EtOAc - MeOH (1 : 1) and lyophilized. Yield: F-1 (4.0 mg) and F-2 (2.5 mg).

Partial Hydrolysis of F-1 and Antibiotic A447C

A solution of 15 mg of F-1 (or A447C) in 1 ml of 0.1 N HCl was heated on a water bath at 85°C for 30 minutes. The reaction mixture was neutralized by 0.1 N NaOH and concentrated *in vacuo*. The residue was extracted once with a mixture of $CHCl_3$ - MeOH (1 : 2) and the extract was subjected to preparative TLC using the same solvent system. A part of the plate was sprayed with 5% *p*-anisaldehyde and heated at 95°C. The band corresponding to rhodinose was extracted with $CHCl_3$ - MeOH (1 : 1) and evaporated *in vacuo*. Comparison of the sugar from F-1 with rhodinose obtained from A447C showed them to be identical (Table 5).

Preparation of L-Amicetose

Cirramycin B (free base, 150 mg) was dissolved in MeOH (50 ml) and sodium borohydride (300 mg) was added. The reaction mixture was gently stirred for 1 hour at room temperature and evaporated *in vacuo*. The product was extracted twice with EtOAc and the extract was evaporated to yield a white powder (135 mg) of crude hexahydrocirramycin B.[3] A solution of 15 mg of F-2 (or hexahydrocirramycin B) in 1 ml of 0.1 N HCl was heated at 85°C for 30 minutes. L-Amicetose was recovered from both hexahydrocirramycin B and F-2 as described in Table 5.

Preparation of Methyl α-L-Amicetoside

Hexahydrocirramycin B (100 mg) in 10% HCl in absolute MeOH (1 ml) was heated at 80°C for 30 minutes. The reaction mixture was evaporated to give an oil from which methyl α-L-amicetoside (3 mg) was obtained by preparative silica gel TLC with *n*-hexane - acetone (4 : 1): Colorless liquid; ^{1}H NMR ($CDCl_3$) δ 4.63 (1H, br s), 1.67~1.80 (2H, m, 2-H_{ax} and 3-H_{ax}), 1.80~1.88 (2H, m, 2-H_{eq} and 3-H_{eq}), 3.27 (1H, dt, J=9.0 and 5.3 Hz, 4-H), 1.36 (1H, d, J=5.3 Hz, 4-OH), 3.57 (1H, dq, J= 9.0 and 6.2 Hz, 5-H), 1.27 (3H, d, J=6.2 Hz, 6-H), 3.35 (3H, s, OCH_3) (ref 4); $[\alpha]_D^{24}$ −89° (*c* 0.11, $CHCl_3$) (ref 4, $[\alpha]_D^{24}$ −113° (*c* 0.13, $CHCl_3$)).

F-2 (50 mg) dissolved in dry methanol (1 ml) containing 10% HCl was heated at 80°C for 30 minutes. The reaction mixture was evaporated *in vacuo* and α-L-amicetoside (1.3 mg, $[\alpha]_D^{25}$ −90° (*c* 0.07, $CHCl_3$)) was obtained by preparative silica gel TLC as described above. Methyl α-L-amicetoside from F-2 was also identical with an authentic sample by chromatography on a silica gel plate (Table 5). The methanolysis product of F-1 gave no methyl amicetosides.

General

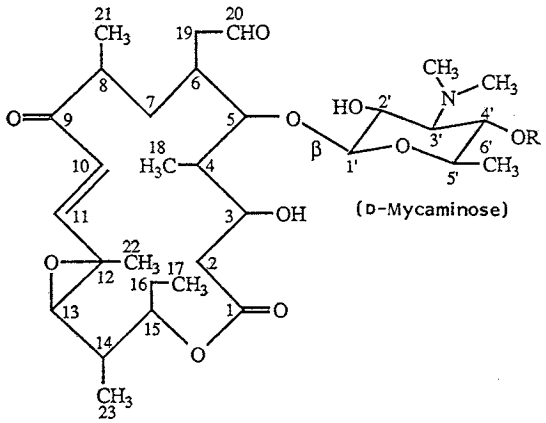
MP's were determined by a Yanagimoto micro melting point apparatus type MP-S3. IR absorption spectra were measured with an Analect fx-6160 FT-spectrometer (KBr pellet) and mass spectra with a Shimadzu LKB 9000A mass spectrometer which has been modified for desorption chemical ionization (D/CI) operation; the source temperature was maintained at 170~190°C with the ionizing gas pressure at 5×10^{-5} Torr.[5] NMR spectra were recorded on a Jeol GX-400 spectrometer. The sample was dissolved in $CDCl_3$ with TMS as the internal standard. Optical rotations were determined using a Jasco DIP-140 polarimeter.

Results

Selection of the Producing Strains and Fermentation

We selected a high producer B-1425 and a mutant (A-0077) blocked in unspecified steps prior to formation of the intact macrolide ring. A blocked mutant A-0033 was used as a positive converter organism of cirramycin A_1 to antibiotics F-1 and F-2. B-1425 and A-0033 showed no significant changes in cultural and physiological properties in comparison with the parent strain JTB-3 (data not shown).

Fig. 1. Structures of cirramycins.



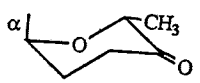
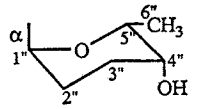
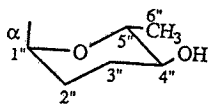
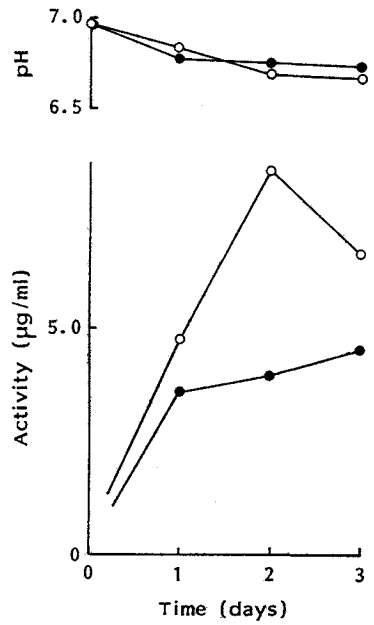
Cirramycin	R
A_1	H
B	 (L-Cinerulose A)
F-1	 (L-Rhodinose)
F-2	 (L-Amicetose)

Fig. 2. Time course of the flask fermentation of strains B-1425 (○) and JTB-3 (●).

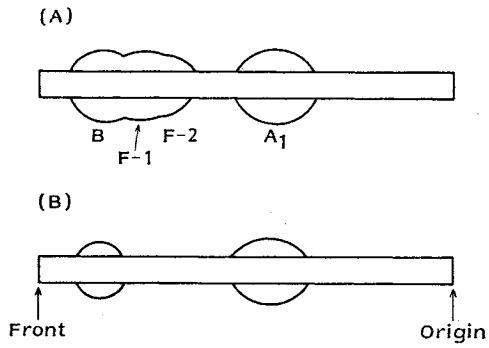


A high producer strain B-1425 and the parent strain JTB-3 were shake-cultured in 500-ml Erlenmeyer flasks containing 100 ml of the medium. A typical time course of fermentation is illustrated in Fig. 2. The production of antibiotics in mutant strain B-1425 reached a maximum 2 days after inoculation. Bioautograms of antibiotics produced by both strains are shown in Fig. 3.

Isolation and Purification of F-1 and F-2

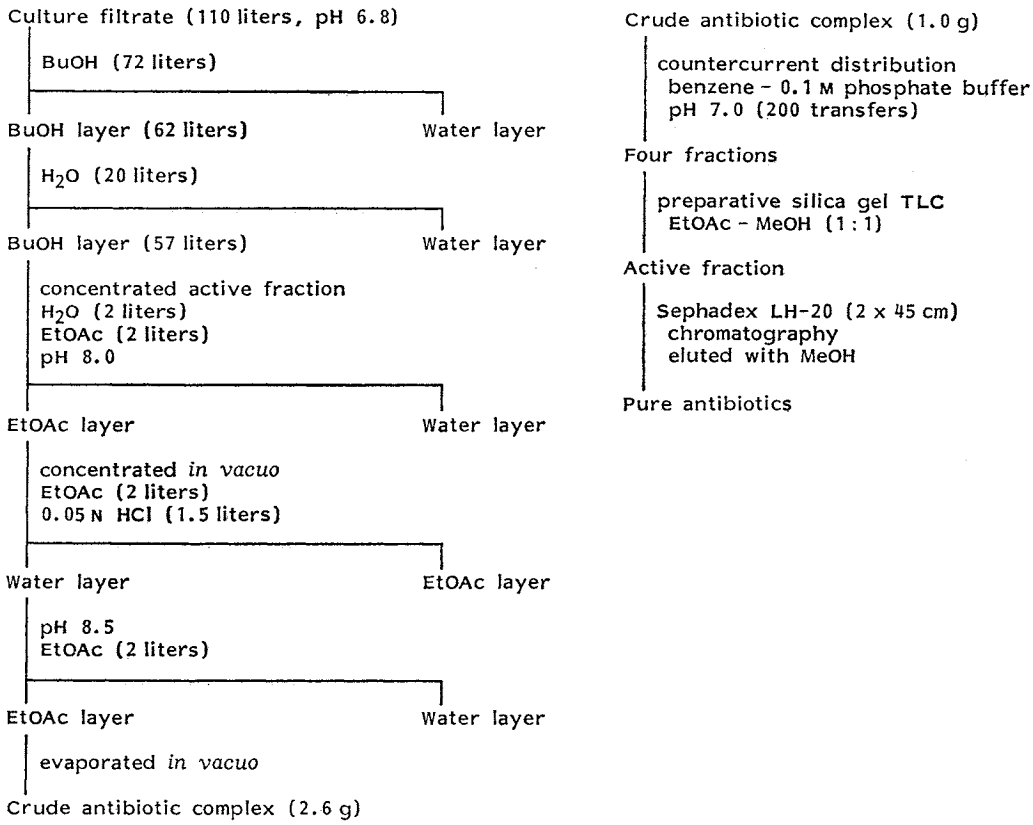
The flow diagram for the isolation of the antibiotics is shown in Fig. 4. Antibiotics in the 2-day culture (110 liters) were extracted with butanol. Antibiotics were transferred to an aqueous phase at pH 2.0 and transferred back to ethyl acetate at pH 8.5. Ethyl acetate extracts were evaporated *in vacuo* to yield a crude powder (2.6 g) which was subjected to countercurrent distribution using benzene - 0.1 M phosphate buffer (pH 7.0). From 1 g of crude antibiotic complex, cirramycins A_1 (94 mg, tubes 30~67), F-2 (42 mg), F-1 (65 mg) and B (560 mg) were obtained as semi-pure antibiotics. Antibiotics

Fig. 3. Bioautograms of antibiotics produced by strains B-1425 (A) and JTB-3 (B).



Solvent system: EtOAc - MeOH (1 : 1).
Test organism: *Bacillus subtilis* PCI 219.

Fig. 4. Isolation and purification.



F-1 and F-2 were purified by preparative silica gel TLC using a solvent system of ethyl acetate-methanol (1:1). The antibiotic was visualized by exposing the plate to UV light and extracted with the same solvent system. Each antibiotic was further purified by Sephadex LH-20 column chromatography eluted with 99% methanol. Yield: F-1 (32 mg) and F-2 (26 mg).

Acid-stability Test

The antibiotic (1.0 mg) was dissolved in 100 μl of MeOH, mixed with 900 μl of rat gastric juice (pH 2.0), and incubated at 37°C. Residual antibiotic activity was determined as described above. Acid-stability of antibiotics F-1 and F-2 in rat gastric juice (pH 2.0) was examined. F-1 and F-2 as well as cirramycin A_1 were stable under these conditions, while erythromycin A (EM-A) was rapidly inactivated (Fig. 5).

Structural Characteristics of F-1 and F-2

The physico-chemical properties of F-1 and F-2 are quite similar to each other (Table 1). Both F-1 and F-2 are white amorphous powders having a molecular formula of $C_{37}H_{61}NO_{12}$ on the basis of NMR and MS spectra. The UV and IR spectra of F-1 and F-2 are almost identical and resemble that of cirramycin A_1.[1] From the UV spectra, both antibiotics were assumed to have an epoxyenone structure. Differentiation of F-1 and F-2 was observed only on silica gel TLC (Table 2).

The D/CI-MS of F-1 and F-2 using isobutane (*iso*-C_4H_{10}) as the reagent gas gave exactly the same fragmentation pattern. These spectra showed the protonated molecular ion peak $(MH)^+$ at *m/z* 712 and the base ion peak at *m/z*

Fig. 5. Stability of antibiotics in rat gastric juice.
● F-1, F-2 and A_1, ○ erythromycin A.

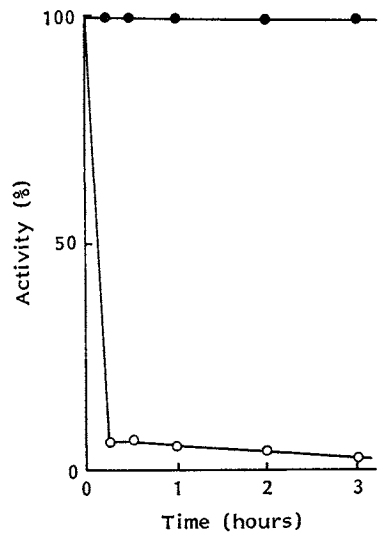


Table 1. Physico-chemical properties of F-1 and F-2.

	F-1	F-2
$[\alpha]_D^{25}$ (*c* 0.23, MeOH)	−41°	−46°
MP (°C, dec)	148~152	148~152
Appearance	White amorphous powder	White amorphous powder
UV λ_{max}^{MeOH} nm (ε)	240 (13,000)	240 (13,000)
IR (KBr) cm^{-1}	3443, 2786, 2739, 1720, 1694, 1622, 1186, 1061	3444, 2785, 2739, 1720, 1694, 1621, 1185, 1061, 1053
MW (D/CI-MS) (*m/z*)	712 $(MH)^+$	712 $(MH)^+$
Molecular formula[a]	$C_{37}H_{61}NO_{12}$	$C_{37}H_{61}NO_{12}$
Solubility		
Soluble:	MeOH, EtOAc, benzene, $CHCl_3$, 0.01 N HCl	MeOH, EtOAc, benzene, $CHCl_3$, 0.01 N HCl
Insoluble:	Hexane, ether, H_2O	Hexane, ether, H_2O
Color reaction		
Positive:	TOLLEN's reagent	TOLLEN's reagent
Negative:	Fehling reagent	Fehling reagent

[a] Based on D/CI-MS and ^{13}C NMR spectra.

768 corresponding to the $(M+iso\text{-}C_4H_9)^+$. Aglycone-derived ions were observed at *m/z* 407 $(A-OH)^+$ and *m/z* 425 $(A+H)^+$, respectively. The cleavage of a glycosidic carbon-oxygen bond of the terminal sugar led to the ions of aglycone-mycaminose $(A-MA+2H)^+$ at *m/z* 598 as reported[6,7] and the terminal sugar at *m/z* 115. The ions at *m/z* 306, 304 and 288 were also from the fragmentations of the glycosidic linkages (Fig. 6). Consequently, F-1 and F-2 were found to resemble a cirramycin-family antibiotic A6888C produced by *Streptomyces flocculus* NRRL 11459.[8] The ^{13}C NMR with polarization transfer experiments (DEPT) of F-1 and F-2 showed 37 carbons including 2 anomeric, 7 *C*-methyl carbons and 3 carbonyl carbons. The assignments of the carbon signals of the aglycone-aminosugar moiety in F-1 or F-2 (Table 3) were based on the comparison with ^{13}C NMR chemical shifts of cirramycin A_1[9] in which the D-mycaminose glycosidic linkage was shown to be in the β-orientation. The chemical shifts of the anomeric carbons of mycaminose in F-1 and F-2 were each at δ 103.8, indicating that these antibiotics possess a β-D-mycaminosyl linkage. The assignments of the terminal sugar were based on the comparison with ^{13}C NMR chemical shifts of the L-rhodinose-containing antibiotics, aclacinomycin N1,[4] sulfurmycins F and G[10] and A447 C[11] and the L-amicetose-containing antibiotics, aclacinomycin M1,[4] and sulfurmycin E.[10] As a result of these assignments, F-1 is believed to have an L-rhodinose as the distal sugar moiety, whereas F-2 has an L-amicetose. Chemical shifts for the anomeric carbons (C-1″) at δ 97.9 (F-1) and δ 97.1 (F-2) indicate that the linkage of these sugars is in the α-configuration. Assignments of

Table 2. Rf values on silica gel TLC.

Cirramycin	Rf value	
	Solvent 1	Solvent 2
B	0.88	0.20
F-1	0.71	0.36
F-2	0.62	0.22
A_1	0.32	0.14

Solvent system: 1; EtOAc - MeOH (1 : 1), 2; $CHCl_3$ - MeOH (9 : 1).

Fig. 6. D/CI-MS spectral fragmentations of F-1 and F-2.

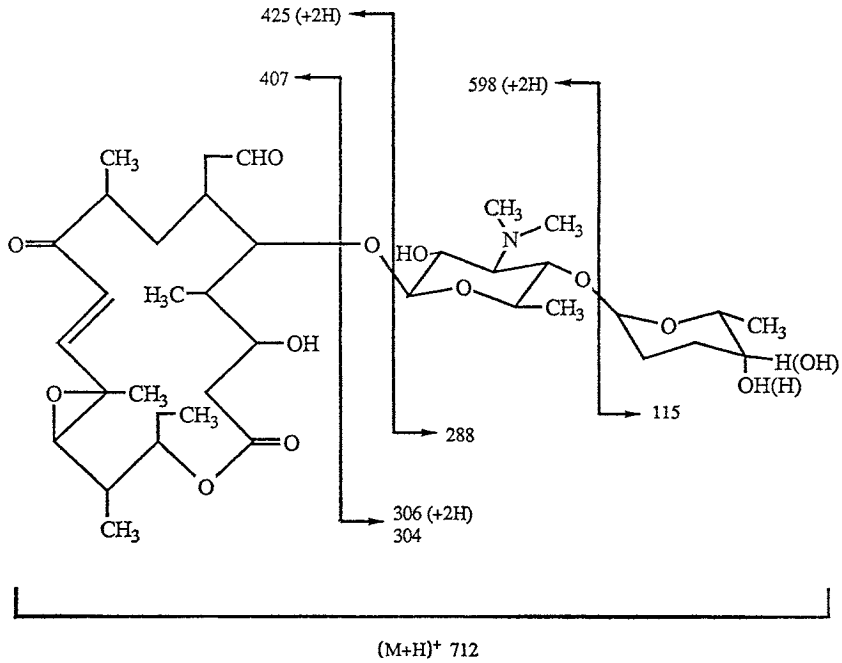


Table 3. ^{13}C NMR chemical shifts (ppm) in $CDCl_3$ at 100 MHz.

Position	F-1	F-2	A_1[9]	A6888C[8]
C-1	173.5 s	173.5 s	173.5	173.4
C-2	39.6 t	39.6 t	39.7	
C-3	70.6 d	70.6 d	71.1	
C-4	45.1 d	45.1 d	45.2	
C-5	81.3 d	81.4 d	81.3	
C-6	31.2 d	31.2 d	31.8	
C-7	31.7 t	31.8 t	31.8	
C-8	37.8 d	37.8 d	37.8	
C-9	200.2 s	200.2 s	200.2	200.3
C-10	122.7 d	122.7 d	122.9	122.9
C-11	151.0 d	151.0 d	151.0	151.0
C-12	59.7 s	59.7 s	59.7	
C-13	66.8 d	66.8 d	67.1	
C-14	41.0 d	41.6 d	40.9	
C-15	78.1 d	78.1 d	77.1	
C-16	24.7 t	24.7 t	24.8	
C-17	9.0[a]q	9.0[a]q	9.0[a]	
C-18	9.1[a]q	9.1[a]q	9.3[a]	
C-19	43.7 t	43.8 t	43.9	
C-20	202.7 d	202.8 d	202.5	202.7
C-21	17.4 q	17.4 q	17.5	
C-22	15.0[b]q	15.0[b]q	15.1[b]	
C-23	14.5[b]q	14.5[b]q	14.5[b]	
C-1′	103.8 d	103.8 d	104.2	
C-2′	69.1 d	69.1 d	70.3	
C-3′	67.9 d	67.9 d	71.1	
C-4′	76.9 d	76.9 d	68.0	
C-5′	73.6 d	73.6 d	73.5	
C-6′	18.6 q	18.6 q	17.8	
$N(CH_3)_2$	41.3 q	41.2 q	41.7	
C-1″	97.9 d	97.1 d		
C-2″	23.9 t	27.3 t		
C-3″	25.7 t	29.7 t		
C-4″	67.2 d	70.5 d		67.3
C-5″	67.4 d	71.9 d		
C-6″	16.9 q	17.9 q		16.8

[a,b] Assignments may be reversed.

The prime symbols are applied for the mycaminose and the double prime symbols for either rhodinose or amicetose.

the ^{13}C NMR signals of antibiotic A6888C were, in part, reported.[8] Signals for C-4″ and C-6″ were described as 67.3 and 16.8 ppm. Comparing to the values of 67.2 and 16.9 ppm for cirramycin F-1 and 70.5 and 17.9 ppm for cirramycin F-2, the distal sugar of antibiotic A6888C seemed to be identical with that of cirrmaycin F-1. ^1H NMR spectra of F-1 and F-2 were also measured (Fig. 7). Chemical shift assignments have been compared with those of antibiotics A447 C[11] and M-4365 A2.[12] We attempted to assign spectra of the terminal sugar moiety in F-1 or F-2 using the ^1H-^1H COSY and the relayed COSY methods. The 4″-H and 5″-H of L-rhodinose or L-amicetose in either F-1 or F-2 were assigned unambiguously (Table 4). The coupling constant (J=8.8 Hz) between 4″-H and 5″-H of F-2 indicates the diaxial orientation, while that of F-1 is very small (J<1 Hz) which supports the equatorial (4″-H)–axial (5″-H) configuration. The anomeric protons (1″-H) of both F-1 and F-2

Table 4. ^{1}H NMR chemical shift-assignments (400 MHz).

Position	F-1			F-2			M-4365 A2[12]			A6888C[8] (δ)	A447 C[11] (δ)
	δ	m	J=Hz	δ	m	J=Hz	δ	m	J=Hz		
2-H	2.08	d-like	16.9	2.08	d	16.9					
	2.64	dd	16.9, 10.6	2.64	dd	16.9, 10.6					
3-H	3.89	d	10.6	3.89	d	10.6	3.92	br d	10		
5-H	3.70	d	9.5	3.70	d	10.3	3.71	br d	10		
10-H	6.44	d	15.8	6.46	d	15.8	6.37	d	16	6.40	
11-H	6.55	d	15.8	6.55	d	15.8	6.55	d	16	6.59	
13-H	2.82	d	9.9	2.82	d	9.9	2.83	d	9		
15-H	4.88	dt	9.5, 2.5	4.85	dt	9.5, 2.5	4.88	br t			
17-H	0.89	t	7.3	0.89	t	7.0	0.89	t	8		
18-H	1.10	d	7.0	1.11	d	6.6					
19-H	2.99	m		2.99	m						
20-H	9.70	s		9.70	s		9.64	s		9.72	
21-H	1.17	d	7.0	1.17	d	7.0					
22-H	1.43	s		1.43	s		1.43	s		1.42	
23-H	1.14	d	7.0	1.16	d	7.0					
1′-H	4.24	d	7.3	4.24	d	7.7	4.22	d	7	4.24	
6′-H	1.26	d	7.0	1.21	d	7.0					
$N(CH_3)_2$	2.53	s		2.54	s		2.27	s		2.52	
1″-H	4.94	br s		4.88	br s						4.80
2″-H_{ax}	1.4~1.6	m		1.6~1.8	m						
2″-H_{eq}	1.8~2.0	m		1.9~2.0	m						
3″-H_{ax}	1.6~1.8	m		1.6~1.8	m						
3″-H_{eq}	1.8~2.0	m		1.9~2.0	m						
4″-H	3.61	br s		3.2~3.3	m						3.58
5″-H	4.30	q	6.6	3.95	dq	6.8, 8.8					4.06~4.07
6″-H	1.14	d	6.6	1.21	d	6.2					1.15~1.17

m: Multiplicity.

Fig. 7. ^1H NMR spectra of F-1 (A) and F-2 (B) in $CDCl_3$ at 400 MHz.

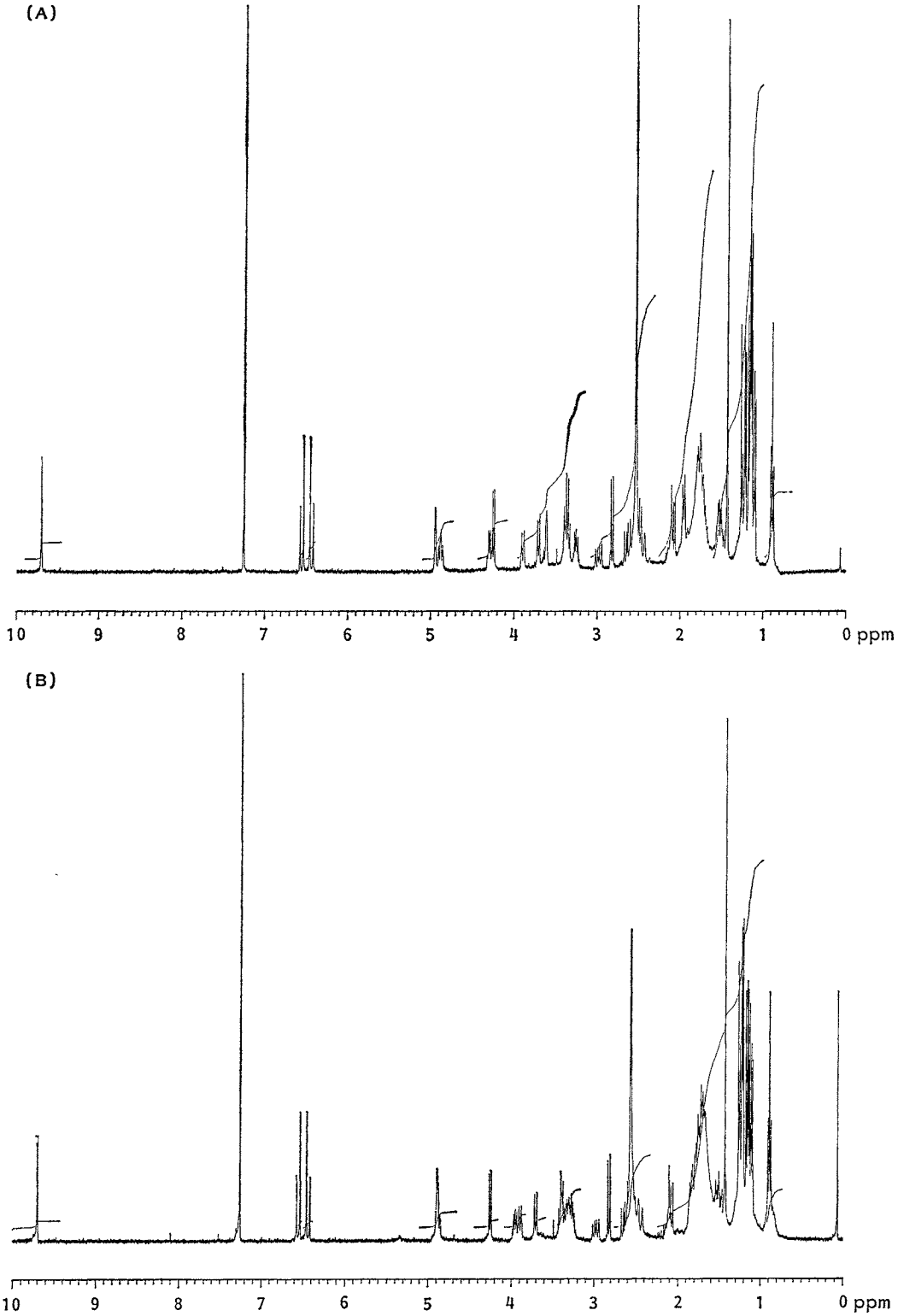


Table 5. Silica gel TLC of sugar components.

Solvent	Sample	Rf value	Color
I	L-Rhodinose[a]	0.67	Green
	Acid hydrolysate of F-1	0.67	Green
	L-Amicetose[b]	0.70	Yellowish brown
	Acid hydrolysate of F-2	0.70	Yellowish brown
II	Methyl α-L-amicetoside[c]	0.32	
	Methanolysis product of F-2	0.32	

Solvent system: I; Butanol - acetic acid - H_2O (4 : 1 : 1), 5% *p*-anisaldehyde, II; *n*-hexane - acetone (4 : 1), 5% sulfuric acid.

[a] Acid hydrolysate of A447 C.[11]

[b] Acid hydrolysate of tetrahydrocirramycin B.[3]

[c] Methanolysis product of tetrahydrocirramycin B.[3]

Table 6. *In vitro* antibacterial activity of cirramycins A_1, F-1, F-2, kitasamycin (LM) and erythromycin A (EM-A).

Organism	MIC (μg/ml)				
	A_1	F-1	F-2	LM	EM-A
Staphylococcus aureus Smith	0.2	1.6	1.6	0.4	0.2
S. aureus A9497	0.2	1.6	1.6	0.4	0.1
S. aureus Terajima	0.2	1.6	1.6	0.2	0.1
S. aureus A9534	0.2	1.6	1.6	0.4	0.1
S. aureus A9601	0.2	1.6	1.6	0.4	0.1
S. aureus 209P JC-1	<0.05	0.4	0.8	<0.05	<0.05
S. aureus MS353	0.4	1.6	1.6	0.4	0.2
S. aureus Russell	>25	>25	>25	>100	100
S. aureus A20978	>25	>25	>25	>100	>100
S. aureus MS15026	>25	>25	>25	>100	>100
S. aureus MS15009 pMS99	>25	>25	>25	>100	>100
S. aureus MS15009 pMS98	0.2	0.8	>25	0.4	>100
S. aureus MS15027	0.2	0.8	1.6	0.4	1.6
S. epidermidis A20017	0.2	1.6	1.6	0.2	0.1
S. epidermidis A22316	0.2	1.6	1.6	0.2	0.1
S. epidermidis A22348	0.2	1.6	1.6	0.2	0.1
S. epidermidis A22349	0.2	1.6	1.6	0.4	0.1
S. epidermidis A22314	0.2	0.8	1.6	0.2	>100
S. epidermidis A22317	>25	>25	>25	>100	>100
S. epidermidis A22318	>25	>25	>25	>100	>100
Enterococcus faecalis A9808	0.4	1.6	3.1	0.4	0.8
E. faecalis A9809	0.4	0.8	1.6	0.4	0.4
E. faecium A24817	0.1	0.8	0.4	0.4	0.8
E. faecium A24885	0.2	0.8	1.6	0.4	1.6
Escherichia coli NIHJ JC-2	>25	>25	>25	>100	100
E. coli K-12 C600	>25	>25	>25	>100	50
Klebsiella pneumoniae PCI 602	1.6	12.5	12.5	6.3	6.3
Salmonella enteritidis G 14	>25	>25	>25	>100	100
S. paratyphi-A 1015	>25	>25	>25	100	25
S. typhi 901	>25	>25	>25	100	50

appear as broad singlet signals. On the basis of NMR data, the terminal sugars of F-1 and F-2 were determined to be α-L-rhodinose and α-L-amicetose, respectively. Reported ^{1}H NMR chemical shifts of 4″-H (3.58 ppm) and 6″-H (1.15~1.17 ppm) of antibiotic A447 C were very closed to 4″-H (3.61 ppm) and 6″-H (1.14 ppm) of cirramycin F-1.

Identification of Acid Hydrolyzed Products

F-1 and F-2 yielded L-rhodinose and L-amicetose upon mild hydrolysis with dilute hydrochloric acid, respectively (Table 5). Upon methanolysis, F-2 gave methyl α-L-amicetoside as a major product, whereas F-1 gave no significant neutral methyl glycosides because methyl rhodinosides are known to be volatile.[4] Thus, the structures of F-1 and F-2 were elucidated and are depicted in Fig. 1.

Bio-conversion of Cirramycin A_1 by A-0033

Cirramycin A_1 was fed to a culture of blocked mutant A-0033 and microbially converted to cirramycins B, F-1 and F-2. Conversion ratio of F-1 and F-2 was approximately 6:4, determined by silica gel TLC scanned at 254 nm. Isolated F-1 and F-2 were identical to authentic samples produced by strain B-1425 in terms of TLC behavior and the UV spectra.

Antibacterial Activity of F-1 and F-2

The MICs of F-1 and F-2 in comparison with cirramycin A_1, kitasamycin and EM-A are given in Table 6. F-1 and F-2 were equally active but less active than cirramycin A_1, kitasamycin and erythromycin.

Discussion

Cirramycins A_1 and B, and a few closely related antibiotics have been isolated from the fermentation broths of *S. cirratus* JTB-3.[1,2] *Streptomyces fradiae* var. *acinicolor* B-58941,[3] *Streptomyces griseoflavus*,[13] *Nocardiopsis* sp. M 119[14] and *S. flocculus* NRRL 11459.[8] Cirramycin B possesses an acid-unstable 4-keto-sugar, L-cinerulose A, as the distal sugar in the molecule.[3] We found that a high producer strain, B-1425, co-produced new acid-stable antibiotics F-1 and F-2 which turned out to be the hydrogenated products of cirramycin B. The sugar moiety of F-1 was shown to be α-L-rhodinosyl-D-mycaminose, whereas that of F-2 was α-L-amicetosyl-D-mycaminose, both of which are unique among macrolide antibiotics. The structure of an antibiotic B-58941,[3] identical to cirramycin B, has been determined to include an L-cinerulose A as a neutral sugar. CELMER[15] described the 4-keto group in L-cinerulose A as an important biosynthetic intermediate and suggested how the L-form can arise from the D-form. Antibiotics B-58941,[3] acumycin[13] and cirramycin B[2] were found to have the 4-keto sugar, L-cinerulose A. In this study, the absolute configuration of rhodinose and amicetose was determined to be α-L being the same as that of L-cinerulose A in antibiotic B-58941[3] and other anthracycline antibiotics.[4,10,11] The L-cinerulose A in cirramycin B might be stereo-nonspecifically reduced to produce both F-1 and F-2, so that the absolute configuration of rhodinose or amicetose in F-1 or F-2 could be retained. NASH *et al.*[8] reported only an antibiotic A6888C isolated from the fermentation broth of *S. flocculus* NRRL 11459. Although no stereochemistry of the antibiotic was not determined, it seemed to be identical with cirramycin F-1 based on two ^{13}C signals and two ^1H chemical shifts.

Bio-conversion experiments with cirramycin A_1 using a blocked mutant A-0033 showed that cirramycin A_1 is the precursor of cirramycins B, F-1 and F-2. Component ratio of F-1 to F-2 in the biotransformation mixture was approximately 6:4. The ratio of F-1 to F-2 in the crude antibiotic complex of strain B-1425 was also about the same. These results suggest that both F-1 and F-2 are derived from cirramycin B by reduction of the L-cinerulose A moiety in a stereo-nonspecific manner.

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