Notes

MYCINAMICINS, NEW MACROLIDE ANTIBIOTICS

XI. ISOLATION AND STRUCTURE ELUCIDATION OF A KEY INTERMEDIATE IN THE BIOSYNTHESIS OF THE MYCINAMICINS, MYCINAMICIN VIII

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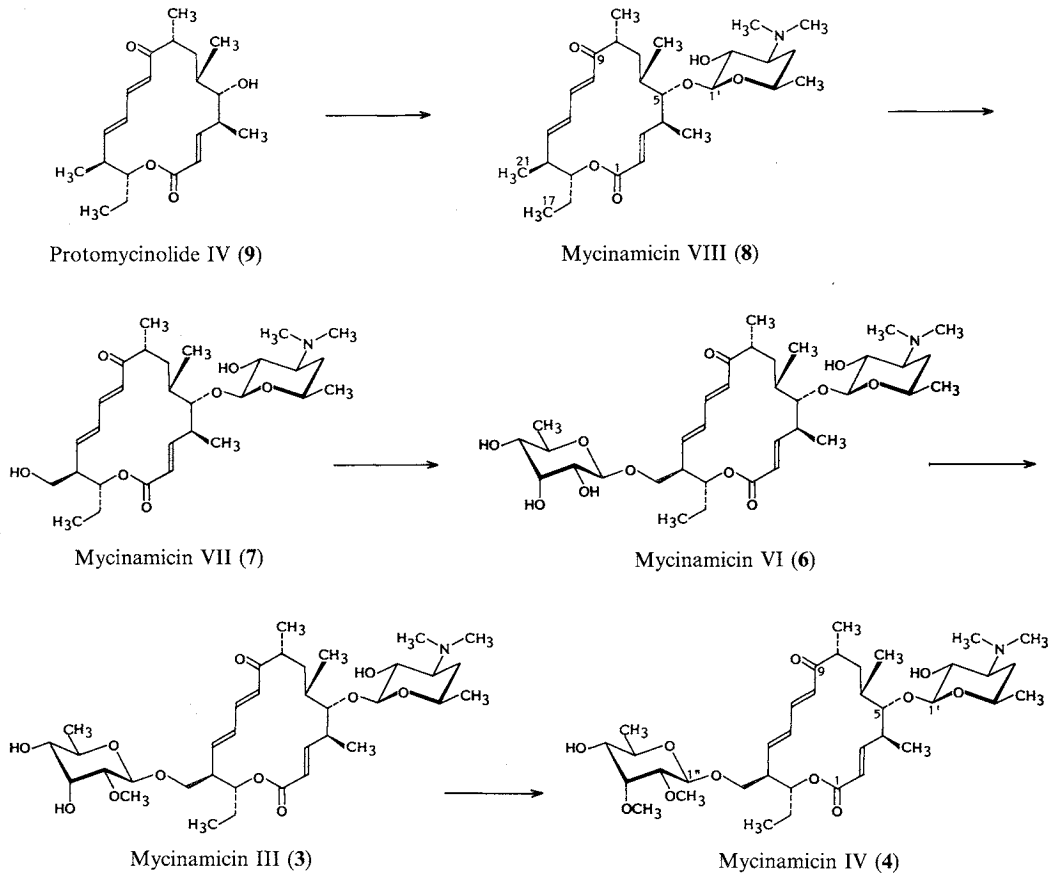
The mycinamicins are 16-membered macrolide antibiotics produced by *Micromonospora griseorubida* sp. nov., which have strong antibacterial activity against Gram-positive bacteria[1]. The complex consists of seven components; mycinamicins I (**1**), II (**2**), III (**3**), IV (**4**), V (**5**), VI (**6**) and VII (**7**)[2~4]. During the search for new antibiotics from the fermentation broth of mycinamicin-producing strains, we have discovered a new minor component designated mycinamicin VIII (**8**). Recently, the same product has been obtained in much higher yield from the fermentation of a mutant strain C-7-1, isolated during on going mutagenic studies of *M. griseorubida* sp. nov. In addition, this mutant was found to accumulate protomycinolide IV (**9**)[5]. These compounds (**8** and **9**) are considered to be key intermediates in the biosynthesis of the mycinamicins. In this communication we describe the isolation and identification of the new metabolite, compound **8**, which provided definitive information about the biosynthetic pathway from protomycinolide IV (**9**) to mycinamicin IV (**4**).

Table 1. ^1H and ^{13}C NMR chemical shifts (ppm) in $CDCl_3$ for mycinamicin VIII (**8**).

Proton	^1H (multiplicity, J=Hz)	Carbon	^{13}C (multiplicity)
		C-1	166.3 (s)
2-H	5.75 (d, $J_{2,3}$=15.6)	C-2	121.1 (d)
3-H	6.61 (dd, $J_{3,2}$=15.6, $J_{3,4}$=5.9)	C-3	151.5 (d)
4-H	2.75 (m)	C-4	41.3 (d)
5-H	3.29 (d, $J_{5,4}$=10.7)	C-5	88.0 (d)
6-H	~1.24 (m)	C-6	34.0 (d)
7-H	~1.68 (m)/~1.59 (m)	C-7	32.6 (t)
8-H	2.56 (m)	C-8	44.9 (d)
		C-9	203.8 (s)
10-H	6.21 (d, $J_{10,11}$=15.1)	C-10	123.0 (d)
11-H	7.09 (dd, $J_{11,10}$=15.1, $J_{11,12}$=11.2)	C-11	141.9 (d)
12-H	6.09 (dd, $J_{12,11}$=11.2, $J_{12,13}$=15.1)	C-12	131.7 (d)
13-H	5.70 (dd, $J_{13,12}$=15.1, $J_{13,14}$=9.8)	C-13	145.1 (d)
14-H	~2.28 (m)	C-14	43.3 (d)
15-H	4.64 (ddd, $J_{15,14}$=12.2, $J_{15,16}$=9.8, 2.4)	C-15	77.2 (d)
16-H	1.81 (m)/~1.51 (m)	C-16	24.7 (t)
17-H	0.94 (t, $J_{17,16}$=7.3)	C-17	9.6 (q)
18-H	1.24 (d, $J_{18,4}$=6.8)	C-18	19.5 (q)
19-H	1.00 (d, $J_{19,6}$=6.8)	C-19	17.4 (q)
20-H	1.14 (d, $J_{20,8}$=6.8)	C-20	17.7 (q)
21-H	1.08 (d, $J_{21,14}$=6.3)	C-21	15.7 (q)
1'-H	4.24 (d, $J_{1',2'}$=7)	C-1'	105.0 (d)
2'-H	3.24 (dd, $J_{2',1'}$=7, $J_{2',3'}$=10.2)	C-2'	70.4 (d)
3'-H	2.47 (m)	C-3'	65.9 (d)
4'-H	~1.64 (m)/~1.27 (m)	C-4'	28.2 (t)
5'-H	2.49 (m)	C-5'	69.5 (d)
6'-H	1.24 (d, $J_{6',5'}$=5.9)	C-6'	21.2 (q)
$N(CH_3)_2$	2.27 (s)	$N(CH_3)_2$	40.3 (q)

Fig. 1. The proposed biosynthetic pathway of mycinamicins.



Protomycinolide IV (**9**)

Mycinamicin VIII (**8**)

Mycinamicin VII (**7**)

Mycinamicin VI (**6**)

Mycinamicin III (**3**)

Mycinamicin IV (**4**)

Results and Discussion

The molecular formula of mycinamicin VIII (**8**) was determined to be $C_{29}H_{47}NO_6$ based on high-resolution chemical ionization (HRCI)-MS ($(M+H)^+$, m/z 506.3465; Calcd for $C_{29}H_{48}NO_6$: 506.3481) and elemental analysis (C 68.88, H 9.37, N 2.77, Found: C 68.73, H 9.54, N 2.60). The UV spectrum suggested the presence of α,β-unsaturated lactone (215 nm) and $\alpha,\beta,\gamma,\delta$-unsaturated ketone (281 nm) chromophores. The IR spectrum also showed the presence of hydroxyl (3420 cm^{-1}), α,β-unsaturated lactone (1715, 1660 cm^{-1}) and α,β, γ,δ-unsaturated ketone (1685, 1635, 1595 cm^{-1}) moieties. These data are very similar to those for compound **7**. The presence of a desosamine moiety was shown by the fragment ion at m/z 158, 174 and 176 in the CI-MS. The protonated molecular ion (m/z 506) appeared at 16 units lower than the corresponding ion of **7** (m/z 522). The ^1H and ^{13}C NMR spectra of **8** in $CDCl_3$ were shown in Table 1. The assignments were made on the basis of the ^1H-^1H correlation spectroscopy (COSY) and ^1H-^{13}C chemical shifts correlated with the 2D NMR experiments. The ^1H NMR spectrum ($CDCl_3$) of **8** was very similar to that of **7**. However, the 21-CH_2OH signal observed at δ_H 3.76 in **7** disappeared, while a new methyl signal appeared at δ_H 1.08 in **8**. The structure was confirmed by ^{13}C NMR spectral data. The spectroscopic data mentioned above support the conclusion.

Mycinamicin VIII (**8**) may be an early stage intermediate in the biosynthetic pathway of the mycinamicins. The components of the culture filtrate were determined by analytical HPLC (compound **8**, 73%; compound **9**, 24%; other minor components <3%), and from these result,it seems likely that the C-7-1 strain is blocked at the step of the oxidation at the C-21 position. Compound **9** is a 16-membered lactone with the fundamental carbon skeleton of the mycinamicin aglycones, and may be the first biosynthetic intermediate. A summary of the proposed pathway from protomycinolide IV (**9**) to mycinamicin IV (**4**) is shown as Fig. 1. According

Table 2. Antibacterial spectra of mycinamicins VIII (**8**) and IV (**4**).

Test organism	MIC (μg/ml)	
	8	**4**
Staphylococcus aureus ATCC 6538P	6.3	0.1
S. aureus MS353	6.3	0.1
S. epidermidis sp-al-1	3.1	<0.05
Streptococcus pyogenes N. Y. 5	1.6	<0.05
Micrococcus luteus ATCC 9341	1.6	<0.05
M. luteus ATCC 10240	3.1	<0.05
Corynebacterium diphtheriae P. W. 8	6.3	1.6
Bacillus subtilis ATCC 6633	6.3	0.4
Escherichia coli NIHJ JC-2	>100	>100
Pseudomonas aeruginosa IAM 1095	>100	>100

to isolation experiments of each component from the fermentation broths of mycinamicin-producing strains, we suggest the biosynthetic pathway **9**→**8**→**7**→**6**→**3**→**4** for mycinamicins. When this biosynthetic pathway for the mycinamicins is compared to that of tylosin[6], it seems that there are some similarities. However, the presence of neutral intermediates (mycinolide IV and dedesosaminyl-derivatives)[5] in culture filtrates of *M. griseorubida* sp. nov. does not fit into the proposed biosynthetic scheme. Possibly, a different scheme must be considered, one that proceeds through neutral as well as basic intermediates. If so, the relationship to tylosin biosynthesis may require revision. In order to clarify this situation, bioconversion experiments are being carried out.

Mycinamicin VIII (**8**) exhibited antibacterial activity against Gram-positive bacteria. As shown in Table 2, the antibacterial activities of **8** were much less than that of **4**.

Experimental

General Procedure

The IR spectra were taken with a Hitachi 260-50 IR spectrophotometer. The UV spectra were recorded on a Shimadzu UV-365 spectrometer. The NMR spectra were obtain with a Jeol JNM-GSX400 spectrometer at 400 (^{1}H) and 100 (^{13}C) MHz with TMS as an internal reference. The MS were taken with a Jeol JMS-SX102 spectrometer. Analytical HPLC was carried out with a Shimadzu LC-6A system, a YMC-GEL ODS 5 μm, stainless steel column (Yamamura Chemical Institute, Ltd., Kyoto), 150 mm × 4 mm i.d. Flow rate of the mobile phase (0.1 M NaH_2PO_4-methanol-acetonitrile, 55: 31: 14) was 0.8 ml/minute and operated at 40°C.

Fermentation

The mutant strain C-7-1 was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment of mycinamicin-producing strains of *M. griseorubida* A11725. Culture conditions and production media were as described previously[1].

Isolation and Purification

The culture filtrate (1 liter) of a mutant was extracted at pH 9.0 with equal volumes of ethyl acetate. The mycinamicins in the organic extract were transferred to a dilute hydrochloric acid solution (pH 3.0). The acidic aqueous layer was extracted with ethyl acetate at pH 9.0. The ethyl acetate phase was dried (Na_2SO_2) and concentrated *in vacuo* to a solid residue and gave a crude powder (*ca.* 100 mg) of the neutral component, protomycinolide IV (**9**). The acidic aqueous layer was extracted with ethyl acetate at pH 9.0 and this organic extract was concentrated to afford the mycinamicin VIII (**8**) as a crude powder (350 mg). This compound **8** was purified by preparative HPLC (YMC-GEL ODS I-25/44, 600 mm × 20 mm i.d.) using 0.1 M NaH_2PO_4 (pH 2.5, adjusted with 20% H_3PO_4)-methanol (4: 6) as solvent system with detection at 220 nm. Fractions (20 ml) were collected at a flow rate of 10 ml/minute. Individual fractions were assayed by analytical HPLC. The retention time of **8** was 46.3 minutes. When the area of **8** peak exceeded 95% of all other peaks in the HPLC trace, the fractions were collected and combined, and the methanol was removed *in vacuo*. The aqueous solutions were extracted with ethyl acetate at pH 9.0. The ethyl acetate extract was dried (Na_2SO_4) and concentrated *in vacuo*. Mycinamicin VIII (**8**) was crystallized from *n*-hexane and acetone to afford colorless prisms. The crystals were collected by filtration, washed with *n*-hexane, and dried *in vacuo* to give **8** (260 mg).

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