NOVEL ANTIBIOTICS, AMYTHIAMICINS

II. STRUCTURE ELUCIDATION OF AMYTHIAMICIN D

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The structure of a unique polythiazole-containing cyclic peptide antibiotic, amythiamicin D, was elucidated by chemical degradations and NMR spectral analyses. Acid hydrolysis of amythiamicin D gave one mole of glycine and three new amino acids. Structures of *N*-acetyl-*O*-methyl derivatives of these new amino acids were determined by NMR and UV spectral analyses. Connectivities of these amino acids were determined by HMBC experiments.

Amythiamicin D, a new antimicrobial antibiotic, has been isolated from the fermentation broth of *Amycolatopsis* sp. MI481-42F4. In the preceding paper[1] we have described the taxonomy and fermentation of the producing strain, as well as the purification and biological properties of amythiamicins. In this paper, we report on the structure elucidation of amythiamicin D (Fig. 1).

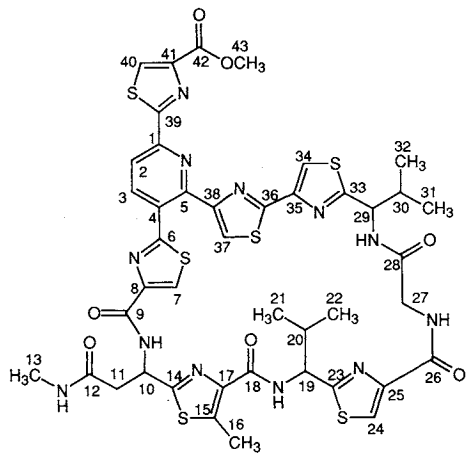
Acid Hydrolysis of Amythiamicin D

Amythiamicin D was hydrolyzed with 6 N HCl at 110°C for 16 hours in a sealed tube. One mole of glycine and three unknown amino acids were detected by amino acid autoanalysis. These were isolated as *N*-acetyl-*O*-methyl derivatives **1**, **2** and **3** by HPLC (Fig. 2).

Structure of Compound **1**

The molecular formula of **1** was deduced to be $C_{12}H_{16}N_2O_5S$ by HRFAB-MS. In the ^1H NMR spectrum, two methyl ester protons and *N*-acetyl protons were observed. Connectivities from the 10-NH to the 11-CH_2 were confirmed by the ^1H-^1H COSY spectrum.

Fig. 1. Structure of amythiamicin D.



^1H-^{13}C long-range couplings in the heteronuclear multiple bond correlation (HMBC)[2] spectrum of **1** are shown in Fig. 3. Cross peaks from the 10-CH (δ_H 6.15) and the 11-CH_2 (δ_H 3.25, 3.47) to C-14 (δ_C 167.7) showed the connectivity between C-14 and C-10/11. Another cross peaks from the 16-CH_3 (δ_H 2.57) to C-15 (δ_C 141.3) and C-17 (δ_C 145.4) indicated the connectivity between C-16 and C-15/17. The existence of a trisubstituted thiazole ring C bearing a carbomethoxy group (–C(18)$OOCH_3$), a methyl group (16-CH_3) and a C_3 unit (C-10, C-11, C-12) was suggested by the remaining elements

Table 1. Physico-chemical properties of amythiamicin D.

Appearance	Colorless needles
MP	>300°C (dec)
FAB-MS (*m/z*)	Positive: 1031 $(M+H)^+$
	Negative: 1030 (M^-)
HRFAB-MS (*m/z*)	Calcd for $C_{43}H_{43}N_{12}O_7S_6$: 1,031.1702
	Found: 1,031.1688 $(M+H)^+$
Molecular formula	$C_{43}H_{42}N_{12}O_7S_6$
Elemental analysis calcd. for	$C_{43}H_{42}N_{12}O_7S_6$
Calcd:	C 50.08, H 4.11, N 16.30, O 10.86, S 18.66
Found:	C 49.72, H 4.20, N 16.34, O 10.98, S 18.32
Optical rotation	$[\alpha]_D^{22}$ +179°C (*c* 0.5, MeOH)
UV λ_{max}^{MeOH} nm (log ε)	204 (4.72), 224 (4.79), 250 (sh 4.56), 307 (4.49), 345 (sh 4.04)
$\lambda_{max}^{MeOH-HCl}$	205 (4.72), 224 (4.80), 250 (sh 4.57), 307 (4.50), 345 (sh 4.04)
$\lambda_{max}^{MeOH-NaOH}$	204 (4.78), 224 (4.79), 250 (sh 4.57), 308 (4.49), 345 (sh 4.04)
IR ν_{max}^{KBr} cm^{-1}	3385, 3115, 2960, 1725, 1665, 1535, 1495, 1220, 1025, 755
Solubility Soluble:	MeOH, DMSO
Insoluble:	*n*-Hexane, H_2O
Color reaction Positive:	Phosphomolybdate-H_2SO_4, Rydon-Smith
Negative:	Ninhydrin, Dragendorff

consisting of three carbon atoms (C-14, C-15, C-17), one nitrogen atom and one sulfur atom.

The position of methyl ester group and methyl group on thiazole ring C was confirmed by comparison of its UV spectrum of **1** with those of synthesized methyl 2,5-dimethylthiazole-4-carboxylate[3] (**5**) and ethyl 2,4-dimethylthiazole-5-carboxylate[4]. The UV spectrum of **1** showing maxima at 205 nm (log ε 4.42), 242 nm (log ε 3.98) in MeOH was very similar to that (λ_{max} (MeOH) 204 nm (log ε 4.25), 243 nm (log ε 3.87)) of synthesized **5**. On the other hand the UV maximum of synthesized ethyl 2,4-dimethylthiazole-5-carboxylate was shifted 18 nm in MeOH (λ_{max} 260 nm (log ε 4.06)). Furthermore, the ^{13}C and ^1H chemical shifts of **1** were similar to those of **5** (Fig. 3). These data revealed the connectivity between C-17 and C-18.

The connectivity between C-14 and C-10 was solved by comparison of the ^{13}C chemical shifts of **1** with those of **4** which was obtained by acid hydrolysis of amythiamicin D followed by methanolysis. The chemical shift, δ_C 173.2 (C-14) in **4**, was shifted to δ_C 167.7 in **1** (Fig. 3). This β-shift due to acetylation of amino group suggested that C-10 of the side chain was linked to C-14 of thiazole ring. From the above described data, the structure of **1** was deduced as shown in Fig. 3.

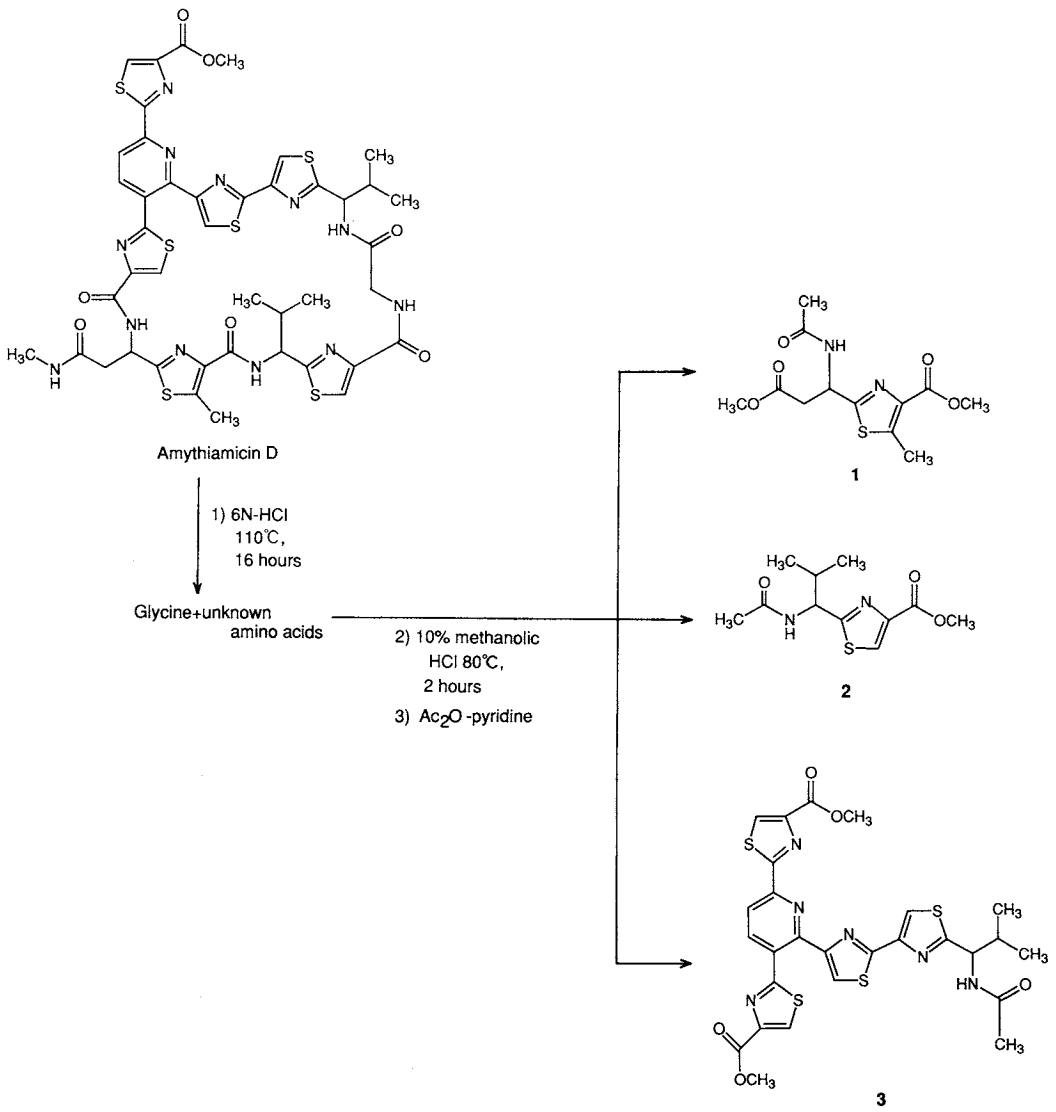
Structure of Compound 2

The molecular formula of **2** was deduced to be $C_{11}H_{16}N_2O_3S$ by HRFAB-MS. In the ^1H NMR spectrum, estermethyl protons and *N*-acetyl protons were observed. Connectivities from the 19-NH to the 22-CH_3 were confirmed by the ^1H-^1H COSY spectrum.

The ^1H-^{13}C long-range couplings from the 19-CH (δ_H 5.62), the 19-NH (δ_H 9.41) and the 20-CH (δ_H 2.48) to C-23 (δ_C 173.7) were observed in the HMBC spectrum. This result supported the connectivity between C-19 and C-23 (Fig. 4). The presence of a disubstituted thiazole ring D was suggested from the consideration of the remaining elements consisting of three carbon atoms (C-23, C-24, C-25), one aromatic proton 24-CH (δ_H 8.36) which had long-range couplings with C-23 and C-25 (δ_C 147.2), one nitrogen atom and one sulfur atom.

The position of the methyl ester group on the thiazole ring D was confirmed by comparison of its UV spectrum of **2** with that of degradation products derived from micrococcin P[5]. The UV absorption maximum (λ_{max} (MeOH) 236 nm (log ε 3.86)) of **2** was consistent with that (λ_{max} (95% EtOH) 235 nm (log

Fig. 2. Acid hydrolysis of amythiamicin D and its *N*-acetyl-*O*-methyl derivatives **1**, **2** and **3**.



ε 3.81)) of 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid hydrochloride reported in micrococcin P. Furthermore, the ^{13}C and ^{1}H chemical shift data of **2** were in good agreement with those of a thiazole residue, Thz-2 in thiostrepton[6] (Fig. 4). From these results, the structure of **2** was determined as shown in Fig. 4.

Structure of Compound 3

The molecular formula of **3** was deduced to be $C_{27}H_{24}N_6O_5S_4$ by HRFAB-MS. In the ^{1}H NMR spectrum of **3**, two methyl ester protons and *N*-acetyl protons were observed. The ^{1}H-^{1}H COSY spectrum revealed the two partial structures (29-NH–29-H–30-H–31-H(32-H)) and (2-H–3-H) (Fig. 5).

Four characteristic singlet protons at δ_H 8.30 (7-CH), δ_H 7.43 (34-CH), δ_H 8.04 (37-CH) and δ_H 8.34 (40-CH) showed ^{1}H-^{13}C long-range couplings with two quaternary sp^2 carbons, C-6 (δ_C 166.0) and C-8 (δ_C 146.6), C-33 (δ_C 171.2) and C-35 (δ_C 148.9), C-36 (δ_C 162.0) and C-38 (δ_C 153.6), and C-39 (δ_C 169.0)

Table 2. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectral data for **1** and **4** in C_5D_5N.

Position	**1**		**4**	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
10	48.5 d	6.15 dt (7.6, 8.2)*	51.6 d	4.78 dd (4.3, 8.2)
11	38.7 t	3.25 dd (7.6, 16.2), 3.47 dd (7.6, 16.2)	42.6 t	3.01 dd (8.2, 16.2), 3.30 dd (4.3, 16.2)
12	171.1 s		172.0 s	
14	167.7 s		173.2 s	
15	141.3 s		141.5 s	
16	12.9 q	2.57 s	13.0 q	2.62 s
17	145.4 s		145.2 s	
18	163.0 s		163.3 s	
12-OCH_3	51.7 q	3.55 s	51.5 q	3.57 s
18-OCH_3	51.6 q	3.79 s	51.5 q	3.81 s
$COCH_3$	170.0 s			
$COCH_3$	22.9 q	2.12 s		
10-NH		9.71 d (8.2)		

* The coupling constants (Hz) are in parentheses.

Fig. 3. ^{1}H and ^{13}C NMR data for **1**, **4** and **5**.

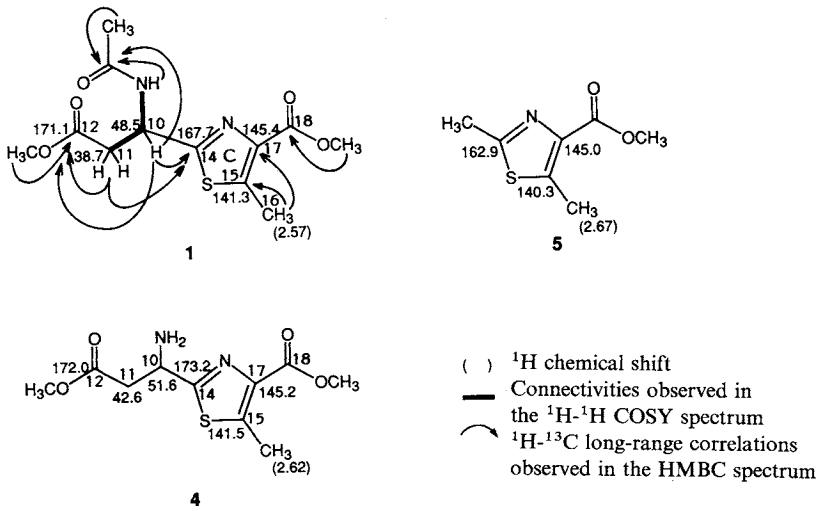


Table 3. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectral data for **2** in C_5D_5N.

Position	δ_C (ppm)	δ_H (ppm)	Position	δ_C (ppm)	δ_H (ppm)
19	57.1 d	5.62 dd (6.7, 8.9)*	25	147.2 s	
20	33.3 d	2.48 m	26	162.0 s	
21	19.8 q	0.95 d (6.7)	26-OCH_3	51.9 q	3.82 s
22	18.5 q	1.00 d (6.7)	$COCH_3$	170.1 s	
23	173.7 s		$COCH_3$	22.8 q	2.13 s
24	128.2 d	8.36 s	19-NH		9.41 d (8.9)

* The coupling constants (Hz) are in parentheses.

and C-41 (δ_C 148.2), respectively. Since these data were similar to those in **2** and **3** contained four sulfur atoms, the presence of four disubstituted thiazole rings was suggested. The other long range correlations are as follows: i) from the 40-CH to C-42 (δ_C 161.8) showed the connectivity between C-41 (thiazole ring

Fig. 4. ^{1}H and ^{13}C NMR data for **2** and Thz-**2**.

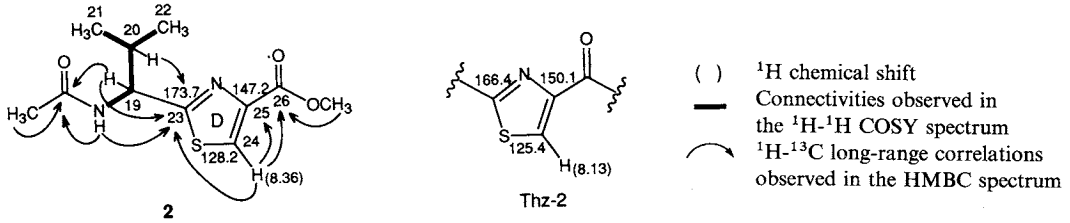


Fig. 5. ^{1}H and ^{13}C NMR data for **3** and **6**.

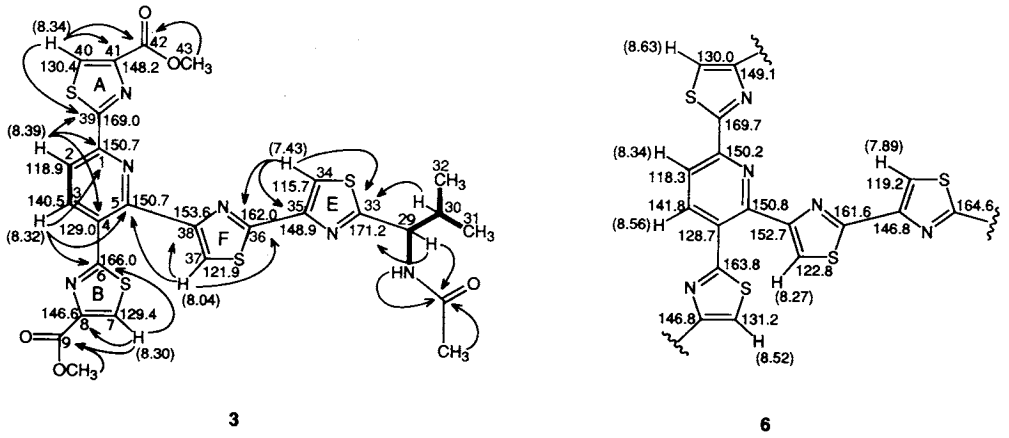


Table 4. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectral data for **3** in $CDCl_3$.

Position	δ_C (ppm)	δ_H (ppm)	Position	δ_C (ppm)	δ_H (ppm)
1	150.7 s		34	115.7 d	7.43 s
2	118.9 d	8.39 d (8.2)*	35	148.9 s	
3	140.5 d	8.32 d (8.2)	36	162.0 s	
4	129.0 s		37	121.9 d	8.04 s
5	150.7 s		38	153.6 s	
6	166.0 s		39	169.0 s	
7	129.4 d	8.30 s	40	130.4 d	8.34 s
8	146.6 s		41	148.2 s	
9	161.9 s		42	161.8 s	
29	56.1 d	5.22 dd (6.4, 8.9)	9-OCH_3	52.6 q	3.97 s
30	33.4 d	2.37 m	43-OCH_3	52.6 q	4.02 s
31	19.2 q	0.98 d (6.4)	$COCH_3$	169.7 s	
32	18.0 q	0.99 d (6.4)	$COCH_3$	23.4 q	2.11 s
33	171.2 s		29-NH		6.27 d (8.9)

* The coupling constants (Hz) are in parentheses.

A) and C-42 , ii) from the 7-CH to C-9 (δ_C 161.9) showed the connectivity between C-8 (thiazole ring B) and C-9, iii) from the 29-CH (δ_H 5.22) and the 30-CH (δ_H 2.37) to C-33 showed the linkage between C-29 (iso-butylamino residue) and C-33 (thiazole ring E), iv) from the 34-CH to C-36 showed the linkage

between thiazole ring E and thiazole ring F.

The existence of a pyridine ring was suggested by the remaining elemental composition which contained five carbon atoms, C-1 (δ_C 150.7), C-2 (δ_C 118.9), C-3 (δ_C 140.5), C-4 (δ_C 129.0), C-5 (δ_C 150.7) and one nitrogen atom. Among these carbons, C-1 and C-5 should be adjacent carbons to the nitrogen because of lower chemical shifts. The long-range correlation observed from the 37-CH (thiazole ring F) to C-5 showed the connectivity between the thiazole ring F and the pyridine ring. The substituted positions, C-1 and C-4 for two 4-carbomethoxythiazolyl residues (thiazole rings A and B) on the pyridine ring were established by the long-range connectivities from two protons, the 2-CH and the 3-CH which coupled each other to C-39 and C-6, respectively. The above spectral data indicated that the thiazole rings A, B and the bithiazole ring (E-F) are attached to C-1, C-4 and C-5 of the pyridine ring, respectively. Furthermore, the characteristic UV spectrum (λ_{max} (MeOH), 220 nm (log ε 4.72), 303 nm (log ε 4.53), 335 nm (sh) (log ε 4.20)), ^{1}H and ^{13}C NMR spectral data (Fig. 5) of **3** were in good agreement with those (λ_{max} (EtOH) 304 nm (log ε 4.73), 340 nm (sh) (log ε 4.41), Fig. 5) of the chromophore (**6**) contained in GE 2270A[7]. On the basis of these results described above, the structure of **3** was proposed as shown in Fig. 5.

Structure of Amythiamicin D

The physico-chemical properties of amythiamicin D are summarized in Table 1. The molecular formula of amythiamicin D was established to be $C_{43}H_{42}N_{12}O_7S_6$ by HRFAB-MS, ^{13}C NMR spectrum and elemental analysis. Chemical shifts in the ^{1}H and ^{13}C NMR spectra are shown in Table 5.

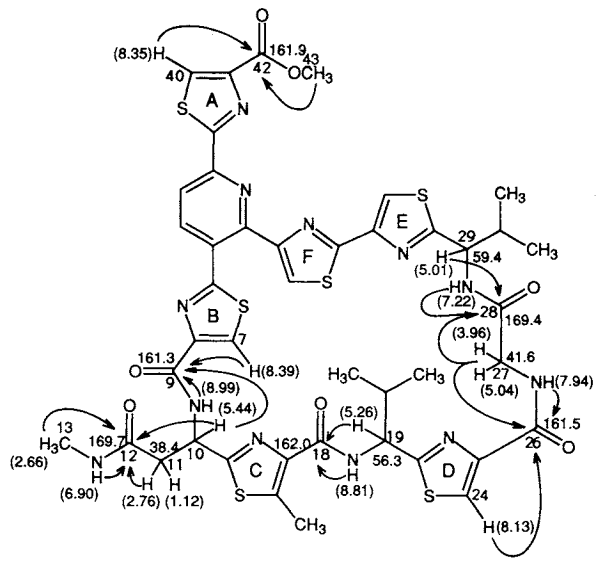
The structures of three unusual amino acids **1**, **2** and **3** were elucidated as shown in Figs. 3, 4 and 5, respectively. The remaining problems to be solved were to prove the connections among **1**, **2**, **3** and glycine.

Table 5. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectral data for amythiamicin D in $CDCl_3$.

Position	δ_C (ppm)	δ_H (ppm)	Position	δ_C (ppm)	δ_H (ppm)
1	150.6 s		25	148.4 s	
2	118.7 d	8.36 d (8.2)*	26	161.5 s	
3	140.4 d	8.13 d (8.2)	27	41.6 t	3.96 dd (3.4, 17.4),
4	127.7 s				5.04 dd (9.4, 17.4)
5	150.2 s		28	169.4 s	
6	165.0 s		29	59.4 d	5.01 dd (7.0, 7.8)
7	125.3 d	8.39 s	30	33.3 d	2.09 m (7.0, 7.8)
8	150.3 s		31	19.2 q	0.93 d (7.0)
9	161.3 s		32	19.2 q	1.11 d (7.0)
10	48.4 d	5.44 dt (3.7, 9.1)	33	173.1 s	
11	38.4 t	1.12 m,	34	114.3 d	7.25 s
		2.76 dd (3.7, 16.5)	35	148.8 s	
12	169.7 s		36	160.0 s	
13	26.3 q	2.66 d (4.9)	37	123.2 d	8.24 s
14	167.7 s		38	154.4 s	
15	140.6 s		39	169.1 s	
16	12.4 q	2.67 s	40	130.5 d	8.35 s
17	142.2 s		41	148.2 s	
18	162.0 s		42	161.9 s	
19	56.3 d	5.26 dd (4.6, 7.9)	43-OCH_3	52.6 q	4.02 s
20	34.7 d	2.30 m	10-NH		8.99 d (9.1)
21	18.3 q	1.00 d (6.9)	12-NH		6.90 q (4.9)
22	18.1 q	0.90 d (6.9)	19-NH		8.81 d (7.9)
23	168.7 s		27-NH		7.94 dd (3.4, 9.4)
24	123.9 d	8.13 s	29-NH		7.22 d (7.0)

* The coupling constants (Hz) are in parentheses.

Fig. 6. ^{1}H-^{13}C long-range correlations of amythiamicin D obtained from the HMBC experiment.



There were a total of four amino groups and six carboxyl groups derived from these amino acids. The negative ninhydrin reaction and characteristic absorption bands (1665, 1495 cm^{-1}) in the IR spectrum showed the presence of four amide bonds, at least, in the molecule. These four peptide bonds were elucidated by HMBC spectral data as shown below. The cross peaks, i) from the 10-NH (δ_H 8.99) and the 10-CH (δ_H 5.44) to C-9 (δ_C 161.3) showed the amide bond between C-9 and the 10-NH, ii) from the 19-NH (δ_H 8.81) and the 19-CH (δ_H 5.26) to C-18 (δ_C 162.0) showed the amide bond between C-18 and the 19-NH, iii) from the 27-NH (δ_H 7.94) and the 27-CH_2 (δ_H 3.96, 5.04) to C-26 (δ_C 161.5) showed the amide bond between C-26 and the 27-NH, iv) from the 29-NH (δ_H 7.22) and the 29-CH (δ_H 5.01) to C-28 (δ_C 169.4) showed the amide bond between C-28 and the 29-NH as shown in Fig. 6. These data indicated the cyclic structure; **1**→**2**→Glycine→**3**→**1**.

For the other two carboxyl groups (C-12 of compound **1** and C-42 of compound **3**), HMBC spectral data were also applicable. The cross peaks, i) from the 13-NH (δ_H 6.90) and the 13-CH_3 (δ_H 2.66) to C-12 (δ_C 169.7) showed the amide bond between C-12 and the 13-$NHCH_3$, ii) from the 43-OCH_3 (δ_H 4.02) to C-42 (δ_C 161.9) showed the connectivity between C-42 and the 43-OCH_3 as shown in Fig. 6. These data showed that an *N*-methylcarboxamide group was attached to C-11 and a methoxycarbonyl group was attached to C-41, respectively. From these results, the structure of amythiamicin D was proposed as shown in Fig. 1.

Amythiamicin D belongs to the group of antibiotics classified by BERDY[8] as thiazolyl peptides. The clarification of stereochemistry is now in progress.

Experimental

General

NMR spectra were obtained on a JEOL JNM-A500 spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Chemical shifts are given in ppm using TMS as an internal standard. All NMR experiments for amythiamicin D were performed using 15 mg of amythiamicin D dissolved in 0.6 ml of

$CDCl_3$ at 24°C. Mass spectra were obtained on a JEOL JMS-SX102 spectrometer. UV spectra were recorded on a Hitachi U-3210 spectrometer. IR spectra were measured on a Hitachi I-5020 FT-IR spectrometer and optical rotation was determined by a Perkin-Elmer 241 polarimeter. Amino acid autoanalysis was performed on a Hitachi L-8500 amino acid autoanalyzer.

Acid Hydrolysis and *O*-Methylation and *N*-Acetylation

Amythiamicin D (110 mg) was hydrolyzed with 6 N HCl (10 ml) at 110°C for 16 hours in a sealed tube. The reaction mixture was concentrated under reduced pressure. A suspension of the residue in 10% anhydrous HCl-MeOH (10 ml) was heated at 80°C for 2 hours in a sealed tube. The reaction mixture was evaporated to dryness. Acetic anhydride (0.15 ml) was added to a solution of the residue in pyridine (3 ml), and the mixture was stirred for 3 hours at room temperature. After the reaction mixture was concentrated under reduced pressure, the residue was chromatographed on a Capcell Pak C_{18} column (Shiseido Co., Ltd.) with a linear gradient from 25% CH_3CN to CH_3CN, giving **1** (22 mg, colorless needles), **2** (20 mg, colorless oil) and **3** (53 mg, colorless needles).

1: FAB-MS *m/z* 301 $(M+H)^+$. HRFAB-MS *m/z* 301.0849 $(M+H)^+$ (Calcd for $C_{12}H_{17}N_2O_5S$ 301.0858). IR (KBr) cm^{-1} 3295, 1740, 1715, 1650, 1530, 1440, 1375, 1325, 1280, 1230, 1175, 1065.

2: FAB-MS *m/z* 257 $(M+H)^+$. HRFAB-MS *m/z* 257.0957 $(M+H)^+$ (Calcd for $C_{11}H_{17}N_2O_3S$ 257.0960). IR (KBr) cm^{-1} 3435, 2965, 1725, 1655, 1535, 1375, 1225, 1100, 990, 760.

3: FAB-MS *m/z* 641 $(M+H)^+$. HRFAB-MS *m/z* 641.0789 $(M+H)^+$ (Calcd for $C_{27}H_{25}O_5N_6S_4$ 641.0770). IR (KBr) cm^{-1} 3275, 1735, 1715, 1640, 1545, 1435, 1375, 1325, 1250, 1210, 1095, 740.

Preparation of Compound **4**

Amythiamicin D (22.5 mg) was hydrolyzed with 6 N HCl (3 ml) at 110°C for 13 hours in a sealed tube. The reaction mixture was concentrated under reduced pressure. A suspension of the residue in 4% anhydrous HCl-MeOH (2.7 ml) was heated at 80°C for 2 hours in a sealed tube. After the reaction mixture was concentrated under reduced pressure, the residue was chromatographed on a Capcell Pak C_{18} column with 13% CH_3CN containing 0.1% TFA. Colorless oil thus obtained was dissolved in $CHCl_3$ and washed with saturated aqueous $NaHCO_3$, then, with water. The organic layer was concentrated under reduced pressure to give **4** (3.5 mg) as a colorless oil.

4: FAB-MS *m/z* 259 $(M+H)^+$. HRFAB-MS *m/z* 259.0742 $(M+H)^+$ (Calcd for $C_{10}H_{15}N_2O_4S$ 259.0752).

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