CEPHABACIN $M_{1\sim6}$, NEW 7-METHOXYCEPHEM ANTIBIOTICS OF BACTERIAL ORIGIN

II. ISOLATION, CHARACTERIZATION AND STRUCTURAL DETERMINATION

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(Received for publication May 31, 1985)

Six components of new cephem antibiotics, cephabacin $M_{1\sim6}$, were isolated from the culture filtrate of *Xanthomonas lactamgena* YK-431 by various types of column chromatographies and preparative reverse-phase HPLC. Their structures were determined by spectroscopic analyses and degradation studies. They consist of 7-methoxydeacetylcephalosporin C as a nucleus and a tri- to heptapeptide including a new amino acid, which is bound at the 3-position with an ester bond.

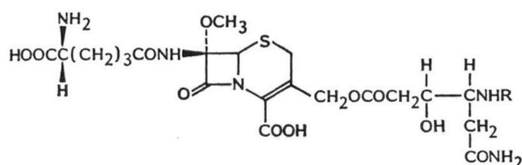
In previous papers, we reported the isolation of 15 new cephem antibiotics[1,2], cephabacin $F_{1\sim9}$ and $H_{1\sim6}$, which were produced by three new bacterial strains[3], *Lysobacter lactamgenus* YK-90, *Xanthomonas lactamgena* YK-280 and *X. lactamgena* YK-278. They have unique structures[4] with the oligopeptides including new amino acids binding to 7-formylaminodeacetylcephalosporin C or deacetylcephalosporin C at the 3-position. The antibiotics show moderate antimicrobial activities *in vitro* and protective effects *in vivo*[5]. Cephabacin F components are remarkably stable to various types of β-lactamases due to the presence of the formylamino group at the 7-position[5]. Recently, 7-formamidocephalosporins have been isolated as acetyl derivatives (SQ 28516 and 28517) from *Flavobacterium* sp. SC 12,154[6] and chitinovorins A, B and C from *Flavobacterium chitinovorum* sp. nov.[7].

We isolated the new cephem antibiotics cephabacin $M_{1\sim6}$ from the culture filtrate of a new bacterial strain, *X. lactamgena* YK-431[8]. These antibiotics have a 7-methoxydeacetylcephalosporin C skeleton and oligopeptides, including a new amino acid, as side chains. They display biological characteristics between those of cephabacin F and H components[5]. This paper describes the isolation, characterization and structural elucidation of cephabacin $M_{1\sim6}$ as shown in Table 1[9].

Isolation and Characterization

The isolation of cephabacin $M_{1\sim6}$ (**1**~**6**, respectively) was carried out by a procedure similar to that used for cephabacin F and H components[2]. These amphoteric (basic as a whole molecule), water-soluble antibiotics were isolated as hydrochlorides by column chromatographies using cation-exchange resins, activated carbon, high-porous resins and cation-exchange Sephadex. Gross separation into two groups was accomplished with Diaion HP-20 chromatography. The fractions were successively separated into three components each by CM-Sephadex chromatography. Each crude component was finally purified by preparative reverse-phase HPLC. The active fractions were detected by antimicrobial activity using strains hypersensitive to β-lactam antibiotics *Escherichia coli*

Table 1. Structures of cephabacin $M_{1\sim6}$.



Compound	R
M_1 (**1**)	←L-Val←L-Orn
M_2 (**2**)	←L-Val←L-Orn←L-Ser
M_3 (**3**)	←L-Val←L-Orn←L-Ser←L-Ala
M_4 (**4**)	←L-Val←L-Orn←L-Val←L-Orn
M_5 (**5**)	←L-Val←L-Orn←L-Val←L-Orn←L-Ser
M_6 (**6**)	←L-Val←L-Orn←L-Val←L-Orn←L-Ser←L-Ala

Table 2. Mobilities of cephabacin $M_{1\sim6}$ on TLC and HPLC.

TLC	Rf value					
Solvent system	**1**	**2**	**3**	**4**	**5**	**6**
CH_3CN - 3% $(NH_4)_2SO_4$ (1:1)	0.49	0.49	0.52	0.51	0.51	0.52

Adsorbent: Cellulose f (Tokyo Kasei).
Detection: Bioautography using *Pseudomonas aeruginosa* C 141 and UV absorbance at 254 nm.

HPLC	Retention time (minutes)					
Mobile phase	**1**	**2**	**3**	**4**	**5**	**6**
5% MeOH - 0.01 M P.B. (pH 3), 2 ml/minute	5.1	5.4	6.7	—	—	—
10% MeOH - 0.01 M P.B. (pH 3), 2 ml/minute	2.2	2.2	2.4	5.2	6.4	7.8

Column: ODS-Sil, YMC-Pack A-312 (Yamamura Chem. Lab.).
Detection: UV absorbance at 254 nm.
Equipment: Model 6000A/660/440 (Waters).

Table 3. Stabilities of **1**, **4**, and **7** in aqueous solutions.

Component	Half-life time (hours)			
	pH 3.0	pH 5.0	pH 7.0	pH 9.0
1	1.8	1.8	1.5	0.37
4	1.7	1.8	1.2	0.35
7	2.0	25.4	29.0	6.5

Concentration: 100 μg/ml in 0.01 M P.B.
Temperature: 60°C.
Detection: HPLC method.

PG 8 and *Pseudomonas aeruginosa* C141 and by TLC and HPLC. Their mobilities are shown in Table 2.

Cephabacin $M_{1\sim6}$ were obtained as freeze-dried white powders. They show positive color reactions for ninhydrin and potassium permanganate reagents and negative color reactions for Sakaguchi

Table 4. Physico-chemical properties of cephabacin $M_{1\sim6}$.

	Component (Hydrochloride)					
	1	2	3	4	5	6
$[\alpha]_D$ (c)	+80.9° (0.53)	+57.8° (0.50)	+44.2° (0.53)	+33.8° (0.50)	+16.5° (0.52)	+4.6° (0.52)
SI-MS (MH^+)	775	862	933	988	1,075	1,146
Molecular formula	$C_{31}H_{50}N_8O_{13}S \cdot 2HCl \cdot 3H_2O$	$C_{34}H_{55}N_9O_{15}S \cdot 2HCl \cdot 3H_2O$	$C_{37}H_{60}N_{10}O_{16}S \cdot 2HCl \cdot 3H_2O$	$C_{41}H_{69}N_{11}O_{15}S \cdot 2HCl \cdot 3H_2O$	$C_{44}H_{74}N_{12}O_{17}S \cdot 2HCl \cdot 4H_2O$	$C_{47}H_{79}N_{13}O_{18}S \cdot 2HCl \cdot 4H_2O$
Anal	Found Calcd	Found Calcd	Found Calcd	Found Calcd	Found Calcd	Found Calcd
C	41.74, 41.29	41.48, 41.30	41.93, 41.93	44.37, 44.16	42.19, 43.31	43.11, 43.72
H	6.28, 6.48	6.30, 6.42	6.75, 6.47	7.15, 6.96	6.48, 6.94	6.97, 6.95
N	12.59, 12.43	12.81, 12.75	13.09, 13.21	13.90, 13.82	13.56, 13.78	13.96, 14.10
O	28.39	29.12	28.68	25.83	27.54	27.26
S	3.15, 3.56	2.57, 3.24	2.81, 3.02	2.52, 2.88	2.09, 2.63	2.09, 2.48
Cl	7.97, 7.86	5.86, 7.17	5.12, 6.69	6.55, 6.36	7.17, 5.81	6.56, 5.79
UV λ_{max} nm (ε)	264 (9,000) 240 (sh, 7,200)	264 (9,200) 240 (sh, 7,400)	264 (9,300) 240 (sh, 7,500)	264 (8,800) 240 (sh, 7,200)	264 (9,600) 240 (sh, 7,900)	264 (8,800) 240 (sh, 7,200)
CD [θ] (nm)	−58,800 (234), +49,100 (260)	−61,200 (234), +50,600 (260)	−64,700 (234), +45,500 (260)	−59,800 (234), +43,200 (260)	−58,200 (234), +34,200 (260)	−59,600 (234), +49,000 (260)
IR ν_{max} (cm^{-1}) β-CO	1780	1775	1770	1770	1770	1770
Ester	1740	1740	1730	1735	1735	1730
Amide	1680	1680	1670	1660	1660	1660
Amino acid analysis (6 N HCl, 110°C, 11~14 hours)	D-α-AAA (1.1) L-Val (1.0) L-Orn (1)	D-α-AAA (0.9) L-Val (1.2) L-Orn (1) L-Ser (0.9)	D-α-AAA (1.0) L-Val (1.0) L-Orn (1) L-Ser (1.3) L-Ala (1.2)	D-α-AAA (1.1) L-Val (2.0) L-Orn (2)	D-α-AAA (1.1) L-Val (1.7) L-Orn (2) L-Ser (1.2)	D-α-AAA (1.1) L-Val (2.0) L-Orn (2) L-Ser (1.4) L-Ala (1.3)
	Gly (0.4)	Gly (0.4)	Gly (0.4)	Gly (0.4)	Gly (0.4)	Gly (0.4)

Table 5. ^{1}H NMR spectra of cephabacin $M_{1\sim6}$.

Position	1	2	3
2-CH$_2$	3.33 (d, 18.1) 3.66 (d, 18.1)	3.34 (d, 18.0) 3.67 (d, 18.0)	3.33 (d, 18.0) 3.67 (d, 18.0)
6-CH	5.17 (s)	5.18 (s)	5.18 (s)
9-CH$_2$	4.73 (d, 12.6) 4.94 (d, 12.6)	4.74 (d, 12.4) 4.94 (d, 12.4)	4.74 (d, 12.6) 4.96 (d, 12.6)
12-CH$_2$	2.48 (t, 7.3)	2.49 (t, 7.2)	2.49 (t, 7.2)
13-CH$_2$	1.64~1.84 (m)	1.66~1.84 (m)	1.66~1.84 (m)
14-CH$_2$	1.84~2.02 (m)	1.84~2.00 (m)	1.84~2.00 (m)
15-CH	3.80 (t, 5.1)	3.75 (t, 6.1)	3.75 (t, 6.1)
17-CH$_3$	3.53 (s)	3.54 (s)	3.55 (s)
19-CH$_2$	2.46~2.54 (m) 2.69 (dd, 3.2, 15.4)	2.46~2.54 (m) 2.70 (dd, 2.9, 15.9)	2.45~2.53 (m) 2.70 (dd, 3.1, 15.5)
20-CH	4.10 (m)	4.09 (ddd, 2.9, 7.0, 10.0)	4.09 (m)
21-CH	4.20 (ddd, 4.1, 6.5, 10.4)	4.19 (m)	4.20 (ddd, 4.0, 7.0, 10.0)
22-CH$_2$	2.46~2.54 (m) 2.70 (dd, 4.1, 14.9)	2.46~2.54 (m) 2.72 (dd, 3.9, 14.9)	2.45~2.53 (m) 2.73 (dd, 4.0, 14.8)
25-CH	4.09 (d, 7.8)	4.02 (d, 8.1)	4.02 (d, 8.1)
26-CH	2.04 (m)	2.03 (m)	2.03 (m)
27-CH$_3$	0.95 (d, 6.6)	0.94 (d, 6.6)	0.94 (d, 6.8)
28-CH$_3$	0.95 (d, 6.6)	0.95 (d, 6.8)	0.94 (d, 6.8)
30-CH	4.10 (m)	4.45 (t, 7.1)	4.41 (dd, 6.1, 7.8)
31-CH$_2$	1.72~1.96 (m)	1.74~1.95 (m)	1.76~1.92 (m)
32-CH$_2$	1.64~1.78 (m)	1.66~1.78 (m)	1.66~1.80 (m)
33-CH$_2$	3.04 (t, 7.5)	3.04 (t, 7.4)	3.05 (t, 7.3)
45-CH		4.19 (dd, 4.2, 5.7)	4.52 (t, 5.9)
46-CH$_2$		3.96 (dd, 5.7, 12.2) 4.01 (dd, 4.2, 12.2)	3.88 (d, 5.9)
48-CH			4.13 (q, 7.1)
49-CH$_3$			1.55 (d, 7.1)

Position	4	5	6
2-CH$_2$	3.33 (d, 18.2) 3.66 (d, 18.2)	3.32 (d, 17.8) 3.66 (d, 17.8)	3.33 (d, 18.0) 3.67 (d, 18.0)
6-CH	5.17 (s)	5.17 (s)	5.18 (s)
9-CH$_2$	4.73 (d, 12.5) 4.93 (d, 12.5)	4.72 (d, 12.3) 4.94 (d, 12.3)	4.74 (d, 12.5) 4.96 (d, 12.5)
12-CH$_2$	2.49 (t, 7.1)	2.48 (t, 7.3)	2.49 (t, 7.2)
13-CH$_2$	1.62~1.85 (m)	1.64~1.84 (m)	1.65~1.86 (m)
14-CH$_2$	1.85~2.00 (m)	1.84~2.00 (m)	2.86~2.00 (m)
15-CH	3.75 (dd, 5.6, 6.6)	3.74 (dd, 5.6, 6.3)	3.75 (t, 6.1)
17-CH$_3$	3.54 (s)	3.54 (s)	3.55 (s)
19-CH$_2$	2.45~2.54 (m) 2.67 (dd, 3.2, 15.9)	2.44~2.53 (m) 2.68 (dd, 3.7, 16.0)	2.45~2.54 (m) 2.69 (dd, 3.1, 15.4)
20-CH	4.07 (ddd, 3.2, 7.0, 10.0)	4.06 (m)	4.08 (m)
21-CH	4.19 (ddd, 3.9, 7.0, 10.5)	4.20 (m)	4.21 (ddd, 4.1, 6.8, 10.2)
22-CH$_2$	2.45~2.54 (m) 2.72 (dd, 3.9, 14.9)	2.44~2.53 (m) 2.71 (dd, 3.7, 14.5)	2.45~2.54 (m) 2.73 (dd, 4.1, 15.1)
25, 35-CH	4.02 (d, 7.8) 4.15 (d, 7.3)	4.02 (d, 7.8) 4.07 (d, 7.6)	4.04 (d, 7.8) 4.09 (d, 7.6)
26, 36-CH	2.06 (m)	2.04 (m)	2.04 (m)
27, 37-CH$_3$	0.93 (d, 7.1) 0.95 (d, 6.6)	0.92 (d, 6.6) 0.93 (d, 6.9)	0.93 (d, 6.6) 0.93 (d, 6.6)
28, 38-CH$_3$	0.96 (d, 6.6) 0.97 (d, 6.4)	0.94 (d, 6.7) 0.95 (d, 6.8)	0.94 (d, 6.8) 0.94 (d, 6.8)
30, 40-CH	4.12 (t, 6.6) 4.39 (t, 7.1)	4.38 (t, 7.0) 4.48 (dd, 6.0, 7.8)	4.39 (t, 6.8) 4.43 (dd, 5.6, 8.5)
31, 32-CH$_2$	1.62~2.00 (m)	1.64~2.00 (m)	1.65~2.00 (m)
41, 42-CH$_2$	1.62~2.00 (m)	1.64~2.00 (m)	1.65~2.00 (m)
33, 43-CH$_2$	3.04 (t, 7.3) 3.05 (t, 7.6)	3.03 (t, 7.3)	3.04 (t, 7.5) 3.05 (t, 7.5)
45-CH		4.18 (dd, 4.3, 5.6)	4.51 (t, 6.0)
46-CH$_2$		3.96 (dd, 5.6, 12.0) 4.01 (dd, 4.3, 12.0)	3.87 (d, 6.0)
48-CH			4.10 (q, 7.2)
49-CH$_3$			1.53 (d, 7.2)

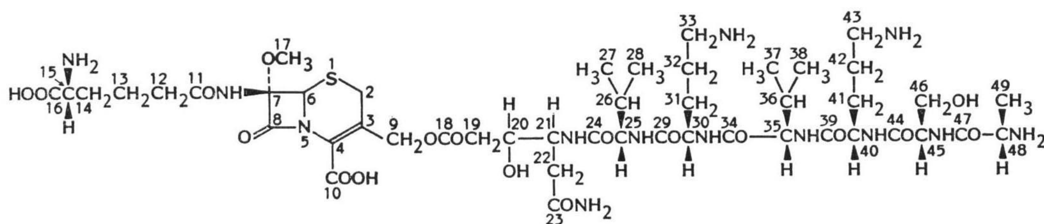
Table 6. ^{13}C NMR spectra of cephabacin $M_{1\sim6}$.

Carbon	1	2	3	4	5	6
11	180.17 s	180.09 s	180.13 s	180.13 s	180.18 s	180.07 s
23	178.63 s	178.58 s	178.63 s	178.62 s	178.62 s	178.57 s
16	176.92 s	177.06 s	177.07 s	177.10 s	177.13 s	177.02 s
18	175.81 s	175.80 s	175.82 s	175.81 s	175.89 s	175.88 s
24, 34	175.30 s	175.51 s	175.56 s	175.51 s	175.54 s	175.49 s
				*175.51 s	*175.54 s	175.46 s
29, 39	171.95 s	175.56 s	175.71 s	175.64 s	175.83 s	175.83 s
				172.39 s	175.81 s	175.77 s
44		171.07 s	174.18 s		171.07 s	174.23 s
47			173.86 s			173.90 s
10	170.66 s	170.68 s	170.71 s	170.73 s	170.77 s	170.67 s
8	163.40 s	163.25 s	163.27 s	163.31 s	163.28 s	163.25 s
4	134.47 s	134.55 s	134.58 s	134.65 s	134.57 s	134.60 s
3	119.38 s	118.82 s	118.85 s	118.86 s	118.83 s	118.79 s
7	97.89 s	97.76 s	97.79 s	97.82 s	97.79 s	97.78 s
20	72.37 d	72.25 d	72.32 d	72.30 d	72.32 d	72.31 d
9	67.07 t	66.99 t	67.02 t	67.07 t	67.04 t	67.01 t
6	65.59 d	65.45 d	65.48 d	65.48 d	65.46 d	65.45 d
46		63.18 t	63.93 t		63.18 t	63.89 t
25, 35	62.94 d	62.90 d	62.90 d	62.80 d	62.79 d	62.71 d
				62.60 d	62.45 d	62.34 d
45		57.35 d	58.31 d		57.36 d	58.25 d
15	57.29 d	*57.35 d	57.39 d	57.39 d	*57.36 d	57.36 d
17	56.16 q	56.14 q	56.07 q	56.10 q	56.07 q	56.05 q
30, 40	55.27 d	56.05 d	56.05 d	55.87 d	*56.07 d	55.94 d
				55.24 d	55.78 d	55.76 d
21	54.16 d	54.02 d	54.07 d	54.06 d	54.06 d	54.01 d
48			51.85 d			51.82 d
33, 43	41.71 t	41.72 t	41.77 t	41.82 t	41.79 t	41.77 t
				41.63 t	41.72 t	41.71 t
19	41.64 t	41.54 t	41.58 t	41.56 t	41.57 t	41.55 t
22	38.49 t	38.65 t	38.71 t	38.60 t	38.62 t	38.60 t
12	37.63 t	37.55 t	37.58 t	37.59 t	37.58 t	37.55 t
26, 36	32.87 d	32.73 d	32.77 d	32.97 d	32.96 d	32.96 d
				32.92 d	32.90 d	32.87 d
14	32.77 t	32.73 t	32.77 t	32.77 t	32.76 t	32.74 t
31, 41	30.85 t	30.80 t	30.76 t	30.83 t	30.80 t	30.72 t
				*30.83 t	*30.80 t	30.65 t
2	28.59 t	28.41 t	28.43 t	28.48 t	28.45 t	28.41 t
32, 42	25.14 t	25.97 t	26.04 t	26.10 t	26.08 t	26.04 t
				25.21 t	26.04 t	*26.04 t
13	23.60 t	23.50 t	23.53 t	23.55 t	23.53 t	23.51 t
27, 37	21.39 q	21.28 q	21.31 q	21.35 q	21.35 q	21.32 q
				21.20 q	21.22 q	21.21 q
28, 38	20.71 q	20.61 q	20.58 q	20.66 q	20.72 q	20.61 q
				20.62 q	20.59 q	20.52 q
49			19.38 q			19.38 q

* Duplicate.

and Barton reagents. The antibiotics are easily soluble in water, soluble in dimethyl sulfoxide, and sparingly soluble in methanol or acetone. The stabilities of **1**, **4** and 7-methoxydeacetylcephalosporin C (**7**) at 60°C in phosphate buffer (P.B.) solutions of various pH are shown in Table 3. Components

Fig. 1. Numbering system for the assignment of NMR spectra.



1 and **4** are relatively stable in the acidic and neutral pH regions and unstable in the basic pH region, while, **7** is very stable in the neutral and basic pH regions.

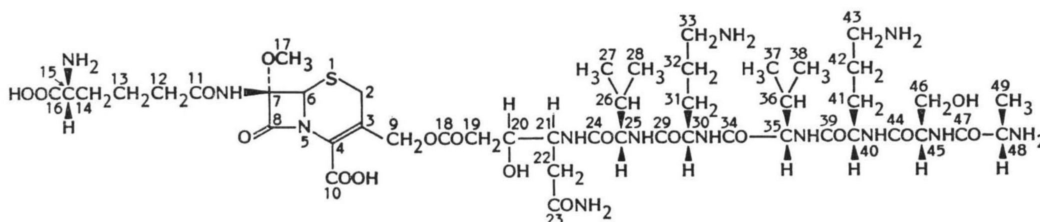
The physico-chemical and spectral data of **1~6** are summarized in Table 4. The specific rotations indicated regularly smaller values according to the increase in the molecular weight. The molecular formulae were determined from elemental analyses, molecular ion peaks in secondary ion mass spectrometry (SI-MS) and carbon numbers in ^{13}C NMR spectrometry. The UV spectra showed maxima at 264 nm (ε 8,800~9,600). The CD spectra had two characteristic contrary Cotton effects at 234 nm (negative) and at 260 nm (positive). This unusual shift from 228 nm in deacetylcephalosporin C to 234 nm in **7** is due to the presence of the 7-methoxy group[10], which was not observed in the cephabacin F components[2]. These spectral data clearly showed that cephabacin M components have the same chromophore as A 16886 B[11] (cephamycin C)[12]. The IR spectra of **1~6** indicated strong absorptions at 1780~1770 (β-lactam), 1740~1730 (ester) and 1680~1660 (amide) cm^{-1}.

Cephabacin $M_{1\sim6}$ were assumed to have 7-methoxy and peptide moieties from their ^{1}H and ^{13}C NMR spectra as shown in Tables 5 and 6. The numbering system for the assignment of the NMR signals is shown in Fig. 1. The existence of a methoxy group was clarified by the signals at δ 3.53~3.55 ppm (3H, s) in the ^{1}H NMR spectra and the signals at 56.05~56.16 ppm (q) in the ^{13}C NMR spectra. Amino acid analyses of the hydrolysates in 6 N HCl gave the following data (Table 4); one mol of valine and ornithine each were detected in **1~3** and two mol in **4~6**. One mol of serine was detected in **2**, **3**, **5** and **6**, and one mol of alanine in **3** and **6**. One mol of α-aminoadipic acid (α-AAA) was also detected in all samples. About 0.4 mol of glycine was detected in the components as certified by BRANNON *et al.*[13]. The absolute configurations of valine, ornithine, serine, alanine and α-aminoadipic acid were determined to be of the L-, L-, L-, L- and D-form, respectively, by the modified HPLC method using a chiral mobile phase[14]. The presence of L-Ser and L-Ala in the components was clarified by the difference from the molecular ion peaks in the SI-MS and by the increase of signals in the ^{13}C NMR spectra (Tables 4 and 6).

Structural Determination

Fig. 2 shows the reaction and degradation pathways of cephabacin M components. Compound **1** gave a tri-*N*-benzoyl derivative, **8**, $[\alpha]_D$ +51.4° (*c* 0.48), SI-MS; *m/z* 1,131 $(M+H)^+$, by benzoyl chloride in 3% $NaHCO_3$. On mild alkaline hydrolysis, **1** afforded **7**[15], $[\alpha]_D$ +177° (*c* 0.49), SI-MS; *m/z* 426 $(M+H)^+$, and a tripeptide, **9**, $[\alpha]_D$ −21.7° (*c* 0.53), SI-MS; *m/z* 390 $(M+H)^+$. Amino acid analysis showed that **9** contains L-Val and L-Orn as known amino acids. By the same reactions, **3** gave **7** and a pentapeptide, **10**, $[\alpha]_D$ −63.1° (*c* 0.42), SI-MS; *m/z* 548 $(M+H)^+$. Also, **4** gave **7** and another pentapeptide, **11**, $[\alpha]_D$ −50.3° (*c* 0.52), SI-MS; *m/z* 603 $(M+H)^+$. Pentapeptide **10** contained L-Val, L-Orn, L-Ser and L-Ala, and **11** had two mol of L-Val and L-Orn. When the molecular formulae of **7**

Fig. 1. Numbering system for the assignment of NMR spectra.



1 and 4 are relatively stable in the acidic and neutral pH regions and unstable in the basic pH region, while, 7 is very stable in the neutral and basic pH regions.

The physico-chemical and spectral data of **1~6** are summarized in Table 4. The specific rotations indicated regularly smaller values according to the increase in the molecular weight. The molecular formulae were determined from elemental analyses, molecular ion peaks in secondary ion mass spectrometry (SI-MS) and carbon numbers in ^{13}C NMR spectrometry. The UV spectra showed maxima at 264 nm (ε 8,800~9,600). The CD spectra had two characteristic contrary Cotton effects at 234 nm (negative) and at 260 nm (positive). This unusual shift from 228 nm in deacetylcephalosporin C to 234 nm in **7** is due to the presence of the 7-methoxy group[10], which was not observed in the cephabacin F components[2]. These spectral data clearly showed that cephabacin M components have the same chromophore as A 16886 B[11] (cephamycin C)[12]. The IR spectra of **1~6** indicated strong absorptions at 1780~1770 (β-lactam), 1740~1730 (ester) and 1680~1660 (amide) cm^{-1}.

Cephabacin $M_{1\sim6}$ were assumed to have 7-methoxy and peptide moieties from their ^{1}H and ^{13}C NMR spectra as shown in Tables 5 and 6. The numbering system for the assignment of the NMR signals is shown in Fig. 1. The existence of a methoxy group was clarified by the signals at δ 3.53~3.55 ppm (3H, s) in the ^{1}H NMR spectra and the signals at 56.05~56.16 ppm (q) in the ^{13}C NMR spectra. Amino acid analyses of the hydrolysates in 6 N HCl gave the following data (Table 4); one mol of valine and ornithine each were detected in **1~3** and two mol in **4~6**. One mol of serine was detected in **2**, **3**, **5** and **6**, and one mol of alanine in **3** and **6**. One mol of α-aminoadipic acid (α-AAA) was also detected in all samples. About 0.4 mol of glycine was detected in the components as certified by BRANNON *et al.*[13]. The absolute configurations of valine, ornithine, serine, alanine and α-aminoadipic acid were determined to be of the L-, L-, L-, L- and D-form, respectively, by the modified HPLC method using a chiral mobile phase[14]. The presence of L-Ser and L-Ala in the components was clarified by the difference from the molecular ion peaks in the SI-MS and by the increase of signals in the ^{13}C NMR spectra (Tables 4 and 6).

Structural Determination

Fig. 2 shows the reaction and degradation pathways of cephabacin M components. Compound **1** gave a tri-*N*-benzoyl derivative, **8**, $[\alpha]_D$ +51.4° (*c* 0.48), SI-MS; *m/z* 1,131 $(M+H)^+$, by benzoyl chloride in 3% $NaHCO_3$. On mild alkaline hydrolysis, **1** afforded **7**[15], $[\alpha]_D$ +177° (*c* 0.49), SI-MS; *m/z* 426 $(M+H)^+$, and a tripeptide, **9**, $[\alpha]_D$ −21.7° (*c* 0.53), SI-MS; *m/z* 390 $(M+H)^+$. Amino acid analysis showed that **9** contains L-Val and L-Orn as known amino acids. By the same reactions, **3** gave **7** and a pentapeptide, **10**, $[\alpha]_D$ −63.1° (*c* 0.42), SI-MS; *m/z* 548 $(M+H)^+$. Also, **4** gave **7** and another pentapeptide, **11**, $[\alpha]_D$ −50.3° (*c* 0.52), SI-MS; *m/z* 603 $(M+H)^+$. Pentapeptide **10** contained L-Val, L-Orn, L-Ser and L-Ala, and **11** had two mol of L-Val and L-Orn. When the molecular formulae of **7**

Table 7. ^{13}C NMR data of the decomposition products.

Carbon	7[a]	9	10	11	12[a]
2	27.9 t				
3	123.3 s				
4	131.8 s				
6	65.0 d				
7	97.1 s				
8	162.6 s				
9	63.2 t				
10	170.4 s				
11	179.5 s				
12	37.1 t				
13	23.1 t				
14	32.3 t				
15	56.8 d				
16	176.4 s				
17	55.5 q				
18		182.03 s	181.83 s	182.07 s	180.4 s
19		44.10 t	43.90 t	44.08 t	41.2 t
20		73.41 d	73.36 d	73.42 d	86.0 d
21		54.28 d	54.24 d	54.22 d	53.0 d
22		38.63 t	38.88 t	38.82 t	36.6 t
23		178.81 s	178.89 s	178.86 s	—*
24, 34		175.22 s	175.53 s	175.45 s 175.46 s	175.1 s
25, 35		63.00 d	62.90 d	62.57 d 62.87 d	62.5 d
26, 36		32.74 d	32.86 d	32.94 d 32.94 d	32.2 d
27, 37		21.29 q	21.34 q	21.17 q 21.35 q	20.8 q
28, 38		20.66 q	20.58 q	20.64 q 20.67 q	20.4 q
29, 39		172.23 s	175.64 s	172.71 s 175.62 s	171.8 s
30, 40		55.23 d	55.99 d	55.25 d 55.86 d	54.7 d
31, 41		30.94 t	30.81 t	30.80 t 30.99 t	30.5 t
32, 42		25.08 t	26.02 t	25.23 t 26.07 t	24.7 t
33, 43		41.62 t	41.76 t	41.62 t 41.79 t	41.2 t
44			174.14 s		
45			58.34 d		
46			63.89 t		
47			173.85 s		
48			51.83 d		
49			19.37 q		

* Not detected.
[a] Varian XL-100 (25 MHz).

and the known amino acids were subtracted from those of **1**, **3** or **4**, the residual moiety was found to consist of $-C_6H_{10}N_2O_3-$. The ^{13}C and ^{1}H NMR spectral data shown in Tables 7 and 8 support this finding.

Table 8. ^{1}H NMR data of the decomposition products.

Proton	7[a]	9	12	14
2-CH_2	3.38 (d, 18.0)			
	3.70 (d, 18.0)			
6-CH	5.20 (s)			
9-CH_2	4.28 (s)			
12-CH_2	2.51 (t, 7.0)			
13-CH_2	1.60~2.10 (m)			
14-CH_2	1.60~2.10 (m)			
15-CH	3.80 (t, 5.5)			
17-CH_3	3.56 (s)			
19-CH_2		2.29 (dd, 9.7, 15.4)	2.61 (dd, 8.5, 15.9)	2.63 (dd, 7.9, 15.4)
		2.46 (dd, 3.4, 15.4)	2.69 (dd, 5.0, 15.9)	2.69 (dd, 5.8, 15.4)
20-CH		4.03 (ddd, 3.4, 6.4, 9.7)	4.79 (dt, 5.0, 8.5)	4.83 (dt, 5.8, 7.9)
21-CH		4.19 (ddd, 3.8, 6.4, 10.4)	4.41 (dt, 5.0, 8.8)	4.47 (dt, 5.8, 8.8)
22-CH_2		2.49 (dd, 10.4, 14.9)	2.70 (dd, 5.0, 18.3)	2.69 (dd, 5.8, 18.3)
		2.71 (dd, 3.8, 14.9)	3.13 (dd, 8.8, 18.3)	3.15 (dd, 8.8, 18.3)
25-CH		4.11 (d, 7.8)	4.08 (d, 7.8)	3.77 (d, 5.9)
26-CH		2.05 (m)	2.07 (m)	2.23 (m)
27-CH_3		0.97 (d, 6.8)	0.98 (d, 8.3)	1.03 (d, 7.1)
28-CH_3		0.97 (d, 6.8)	1.00 (d, 8.3)	1.04 (d, 6.8)
30-CH		4.11 (t, 6.3)	4.10 (t, 7.8)	
31-CH_2		1.90~2.00 (m)	1.90~2.00 (m)	
32-CH_2		1.64~1.77 (m)	1.70~1.80 (m)	
33-CH_2		3.04 (t, 7.8)	3.03 (dt, 7.5, 15.0)	
			3.07 (dt, 7.5, 15.0)	

[a] Varian XL-100 (100 MHz).

Proton spin-decoupling studies of **3** were carried out to clarify the structure of the C_6 moiety. After assignment of the signals from the 7-methoxycephalosporin skeleton and the known amino acid moieties (Table 5), the remaining signals were irradiated as the following. When the methine signal at δ 4.20 ppm (1H, ddd) was irradiated, the proton signal of the methylene at 2.73 (1H, dd, J=4.0, 14.8 Hz) collapsed the doublet (J=14.8 Hz), and the proton signal of the methylene at 2.45~2.53 (2H, dd×2) and the methine signal at 4.09 (1H, m) showed different patterns from the original ones. On irradiation of the signal at 4.09, the proton signal of the methylene at 2.70 (1H, dd, J=3.1, 15.5 Hz) collapsed the doublet (J=15.5 Hz), and the proton signal of the methylene at 2.45~2.53 showed distinguishable patterns. By irradiation of the methylene signals at 2.714 or 2.476, the coupling constant between two methines was determined to be J=7.0 Hz. From these findings, the partial structure for the C_6 moiety was assumed to be –C(=O)CH_2CHCHCH_2C(=O)–. Furthermore chemical shifts and splitting patterns of two methine signals suggest that these methines combine to a hydroxyl or an amino group as analogy in cephabacin F and H components[4].

When compound **9** was hydrolyzed at 110°C in 2 N HCl for 2 hours, a lactone compound was obtained (Fig. 2), **12**, $[\alpha]_D$ +4.2° (c 0.50), SI-MS; m/z 373 (M+H)$^+$. The formation of a 5-membered lactone ring after deamination was easily elucidated from the difference (m/z 17) between **9** and **12** in SI-MS, a newly occurring absorption at 1770 cm^{-1} in **12** and a large downfield shift from 73.41 in **9** to 86.0 ppm in **12** (Table 7). By a similar procedure, **11** gave its lactone derivative, **13**, $[\alpha]_D$ −36.4° (c 0.50), SI-MS; m/z 586 (M+H)$^+$. These lactonic compounds contained the same known amino acids

Table 9. *N*-Terminal amino acids of cephabacin $M_{1\sim6}$ by DNP-method.

DNP-compound	M_1	M_2	M_3	M_4	M_5	M_6
DNP-α-AAA	+	+	+	+	+	+
Di-DNP-Orn	+	−	−	+	−	−
N^{ε}-DNP-Orn	−	+	+	+	+	+
DNP-Ser	−	+	−	−	+	−
DNP-Ala	−	−	+	−	−	+

as the starting materials. Another acidic hydrolysis of **9** by refluxing in 6 N HCl for 3 hours afforded a lactonic dipeptide, **14**, $[\alpha]_D$ +38.5° (*c* 0.47), SI-MS; *m/z* 259 $(M+H)^+$, and L-Orn, **15**.

The following experiments confirmed the position of the hydroxyl group at C_{20}. Compound **9** was converted into an arylamido compound, **16**, SI-MS; *m/z* 675 $(M+H)^+$, by amidation using 7-amino-1,3-naphthalenedisulfonic acid. After protection of the free carboxylic acid, lactonization was carried out under acidic conditions and gave compound **17**, SI-MS; *m/z* 658 $(M+H)^+$, IR; 1780 cm^{-1} (5-membered lactone ring).

The ^{13}C NMR spectrum of **1** indicated that it consists of compounds **7** and **9**. The DNP method for determinating the *N*-terminal amino acid groups shown in Table 9 yielded the dinitrophenyl derivatives of L-Orn and D-α-AAA from **1**. The sequence of the amino acids was finally determined by Edman degradation method[16] for **9** to be L-Orn, L-Val and the new amino acid, in this order. The carboxyl group in **9** apparently combines with the 3-hydroxymethyl group in **7** with an ester bond which is consistent with the absorption at 1740 cm^{-1} in the IR spectrum of **1**. Thus, the structure of **1** was determined to be 3-(5-carbamoyl-4-L-ornithyl-L-valylamino-3-hydroxy)valeryl-7-methoxy-deacetylcephalosporin C.

Compound **2** was hydrolyzed under mild basic conditions followed by acidic hydrolysis in 2 N HCl, giving a lactonic tetrapeptide, **18**, $[\alpha]_D$ −23.0° (*c* 0.53), SI-MS; *m/z* 460 $(M+H)^+$, and a dipeptide, L-ornithyl-L-valine, which was converted to the *N*-dibenzoyl derivative, **19**, SI-MS; *m/z* 427 $(M+H)^+$. These degradation and *N*-terminal analysis data from the DNP method (Table 9) confirmed the structures of **2** and **3** (Table 1).

The amino acid sequence of **11** was determined by Edman degradation method to be L-Orn, L-Val, L-Orn and L-Val, in this order. These findings, the *N*-terminal analysis and spectral data confirmed the structures of **4**~**6** (Table 1).

After the discovery of cephalosporin C from *Cephalosporium* in 1961[17], A 16886 B (cephamycin C) was isolated from *Streptomyces* in 1971. This discovery had a great impact on antibiotic researchers[11,18]. We found three types of new cephem antibiotics, cephabacin $H_{1\sim6}$ (7-H type), $M_{1\sim6}$ (7-methoxy type) and $F_{1\sim9}$ (7-formylamino type) from four bacterial strains and determined their unique peptidyl cephem structures. The findings showed that the organisms producing cepham antibiotics are widely distributed among fungi, *Streptomyces* and bacteria.

Cephabacin $M_{1\sim6}$ are active against Gram-negative and Gram-positive bacteria *in vitro*[8]. Cephabacin $M_{1\sim4}$ showed stronger protective effects at ED_{50} 14~32 mg/kg than **7** (>100 mg/kg) on experimentally infected mice using *E. coli* O-111 by subcutaneous injection. The preliminary acute toxicities (LD_{50}) of these antibiotics were more than 1,000 mg/kg in mice by subcutaneous administration.

From the viewpoint of the biosynthesis of cephabacins, it is of interest to know how the formyl-

amino or methoxy group is introduced into the active site at the 7-position and how the new amino acids are formed as having with a common structure from the 18- to the 22-position. These are subjects for future studies.

Experimental

The specific rotations, UV and CD spectra were measured at approx 25°C in H_2O unless otherwise stated. The IR spectra were measured in KBr pellet. The δ values in the ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra using Jeol GX-400 were recorded in ppm downfield from DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate). All spectra were measured in D_2O unless otherwise stated. The SI-mass spectra were measured on Hitachi M-80 A mass spectrometer with xenon ion beam source. The samples were supplied by glycerol matrix.

Isolation of Cephabacin $M_{1\sim6}$ (1~6)

The culture broth of *X. lactamgena* YK-431 (1,150 liters) was filtered at pH 6.5 with Hyflo-Super Cel. The filtrate (1,300 liters) cooled below 7°C was loaded onto Diaion SK-102 (Na^+ type, 100 liters) and active substances were eluted with 2 M NaCl (700 liters). The eluate was applied to activated carbon chromatography (25 liters) eluting with 8% *iso*-BuOH - 0.01 N HCl (175 liters). The concentrate (1.5 liters, pH 6.5), was chromatographed on Diaion HP-20 (4 liters). The components were separated into 2 groups at this stage by the elution using 50% MeOH - H_2O (8 liters) and 50% MeOH - 0.01 N HCl (12 liters). The concentrate (3 liters) of the former eluate, mainly containing **1~3**, was applied to CM-Sephadex C-25 (NH_4^+ type, 1 liter) and eluted with 0.02 M $AcONH_4$ (11 liters) and 0.05 M $AcONH_4$ (7 liters). Two eluates were separately desalted with activated carbon to give the crude powder of **3** (15.8 g) from the 0.02 M eluate and the mixed powder of **1** and **2** (12.3 g) from the 0.05 M eluate. The mixture of **1** and **2** was again loaded on CM-Sephadex and eluted with 0.02~0.05 M $AcONH_4$. Each fraction detected by HPLC was individually desalted with activated carbon to give freeze-dried **2** (4.39 g), the mixture of **1** and **2** (2.63 g) and **1** (2.13 g).

The latter eluate, mainly containing **4~6** was neutralized and concentrated. The concentrate (1.6 liters) was applied to CM-Sephadex C-25 (1 liter) and eluted with 0.1 M and 0.2 M NaCl. Three divided fractions were separately desalted with activated carbon to afford the crude powders of **6** (1.49 g), the mixture of **5** and **6** (0.64 g) and **4** (1.45 g). The mixture of **5** and **6** was again loaded on CM-Sephadex and eluted with 0.05 M $AcONH_4$. The fractions were analyzed by HPLC to divide 2 groups and each fraction was individually desalted with activated carbon to give the crude powder of **5** (220 mg) and **6** (285 mg). The part of the crude powders were purified with preparative HPLC using YMC-Pack S-30 (Yamamura Chem. Lab.) with the mobile phase of 2% MeOH - 0.01 M P.B. (pH 3). The pure fractions were desalted with activated carbon to give freeze-dried white powders. The yields are as follows:

Compound	Crude powder (mg)	Pure powder (mg)	Compound	Crude powder (mg)	Pure powder (mg)
1	600	301	**4**	1,660	667
2	600	246	**5**	220	98
3	310	122	**6**	175	95

Alkaline Hydrolysis of 1, 3, 4 (7, 9, 10, 11)

A soln of **1** (1.77 g) in H_2O (250 ml) was adjusted to pH 9.4 with 2 N NaOH. The soln was stirred for 20 hours at room temp with keeping at pH 9.2~9.7. The reaction mixture was diluted with H_2O (250 ml) and adjusted to pH 7.0. The soln was applied to QAE-Sephadex A-25 (Cl^- type, 300 ml) and eluted with 0.02 M NaCl. The eluate was desalted with activated carbon (50 ml) and followed by concentration and freeze-drying to give white powder of sodium salt of **7** (275 mg).

The effluent was desalted by activated carbon to afford crude **9**, which was purified on CM-Sephadex C-25 (Na^+ type, 150 ml) eluting with 0.02 M NaCl. The pure fractions were desalted with

activated carbon to afford white powder of **9** (378 mg).

7; UV λ_{max} 240 nm (ε 6,700) and 264 (8,100); IR ν_{max} 1770, 1610, 1530 cm^{-1}.

Anal Calcd for $C_{15}H_{20}N_3O_5SNa \cdot H_2O$: C 40.63, H 5.00, N 9.48, O 32.47, S 7.23, Na 5.18.
Found: C 40.74, H 5.24, N 9.58, S 6.44.

9; IR ν_{max} 1680, 1560 cm^{-1}.

Anal Calcd for $C_{16}H_{31}N_5O_6 \cdot 2HCl \cdot H_2O$: C 40.00, H 7.34, N 14.58, O 23.31, Cl 14.76.
Found: C 39.82, H 7.28, N 14.37, Cl 12.95.

Similar procedure gave hydrolysates as follows:

Starting material	Hydrolysate
3 (1.0 g)	**7** (130 mg), **10** (186 mg)
4 (500 mg)	**7** (80 mg), **11** (143 mg)

10; IR ν_{max} 1670, 1550 cm^{-1}.

Anal Calcd for $C_{22}H_{41}N_7O_9 \cdot 1.5HCl \cdot H_2O$: C 42.60, H 7.23, N 15.81, O 25.79, Cl 8.57.
Found: C 42.32, H 7.43, N 15.64, Cl 8.63.

11; IR ν_{max} 1675, 1650, 1560 cm^{-1}.

Anal Calcd for $C_{26}H_{50}N_8O_8 \cdot 3HCl \cdot 4H_2O$: C 39.82, H 7.84, N 14.29, O 24.48, Cl 13.56.
Found: C 39.25, H 7.43, N 14.03.

N-Tribenzoyl Cephabacin M_1 (**8**)

To a soln of **1** (100 mg) in 3% $NaHCO_3$ (10 ml) was added benzoyl chloride (80 μl) and the mixture was stirred for 3 hours at 4°C with keeping at pH 7.5~8.5. The reaction mixture was diluted with H_2O (50 ml) and washed with EtOAc (50 ml). The aqueous layer was adjusted to pH 6.6 and concentrated. The concentrate was chromatographed on Diaion HP-20 (50~100 mesh, 20 ml) and eluted with 30% MeOH. The pure fractions were freeze-dried to give white powder of **8** (89 mg). UV λ_{max} 228 nm (ε 40,100), 265 (12,300, sh); IR ν_{max} 1770, 1670, 1640, 1535 cm^{-1}.

Anal Calcd for $C_{52}H_{60}N_8O_{16}SNa_2 \cdot 3H_2O$: C 52.70, H 5.61, N 9.46, O 25.65, S 2.71, Na 3.88.
Found: C 52.23, H 5.85, N 9.70, S 2.74.

Lactonization (**12**, **13**, **18**)

a) A soln of **9** (400 mg) in 2 N HCl (30 ml) was stirred for 2 hours at 110°C. The concentrate of the reaction mixture at pH 4.5 was chromatographed on Dowex 50 WX2 (Na^+ type, 50~100 mesh, 50 ml) and eluted with 2 M NaCl. The eluate was applied to activated carbon (100 ml) and eluted with 8% *iso*-BuOH - 0.005 N HCl. The eluate was purified with preparative HPLC using YMC-Pack SH-343, S-10 by the solvent system of 0.01 M P.B. (pH 6.3). The pure fractions were desalted with activated carbon (30 ml) to give white powder of **12** (204 mg); IR ν_{max} 1770, 1665, 1560 cm^{-1}.

Anal Calcd for $C_{16}H_{28}N_4O_6 \cdot 2HCl \cdot 1.2H_2O$: C 41.15, H 6.99, N 12.00, O 24.67, Cl 15.19.
Found: C 41.01, H 7.09, N 12.05, Cl 15.69.

By similar procedure, a tetrapeptide obtained by alkaline hydrolysis of **2** (950 mg) afforded white powder of **18** (109 mg); IR ν_{max} 1775, 1660, 1550 cm^{-1}.

Anal Calcd for $C_{19}H_{33}N_5O_8 \cdot 2HCl \cdot 1.5H_2O$: C 40.79, H 6.85, N 12.52, O 27.17, Cl 12.68.
Found: C 40.72, H 6.72, N 12.62, Cl 13.48.

b) A soln of **11** (72 mg) in 2 N HCl (15 ml) was stirred for 8 hours at room temp. The reaction mixture was evaporated and purified with preparative HPLC using YMC-Pack SH-343, S-10 by the solvent system of 4% MeOH - 0.01 M P.B. (pH 3.0). The pure fractions were desalted with Diaion HP-20 (50~100 mesh, 20 ml) to give white powder of **13** (56 mg); IR ν_{max} 1785, 1650, 1555 cm^{-1}.

Anal Calcd for $C_{26}H_{47}N_7O_8 \cdot 3HCl \cdot 2.5H_2O$: C 42.19, H 7.49, N 13.25, O 22.70, Cl 14.37.
Found: C 42.33, H 7.19, N 12.91.

Acid Hydrolysis

A soln of **9** (240 mg) in 6 N HCl (10 ml) was refluxed for 3 hours. The reaction mixture was evaporated and chromatographed on Dowex 50 WX2 (H^+ type, 50~100 mesh, 15 ml). The eluate of 0.5 N HCl and 1.0 N HCl was separated and the former was desalted with Diaion SP-207 (20 ml) to

afford white powder of **14** (11 mg). The latter was chromatographed on Diaion SP-207 (10 ml) to afford white powder of **15** (25 mg). This sample was identical with L-Orn by the specific rotation, SI-MS, IR and NMR spectra.

14; IR ν_{max} 1780, 1670, 1610, 1530 cm^{-1}.

Anal Calcd for $C_{11}H_{18}N_2O_5 \cdot 0.9H_2O$: C 48.13, H 7.27, N 10.21, O 34.39.
Found: C 48.13, H 7.00, N 10.04.

Amidation

To a soln of **9** (240 mg) in H_2O (20 ml) at pH 4.0 were added *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide·HCl (192 mg) and 7-amino-1,3-naphthalenedisulfonic acid (purity 85%, 356 mg). The resulting soln was stirred for 4.5 hours at room temp. The reaction mixture was concentrated and purified with preparative HPLC using YMC-Pack SH-343, S-10 by the solvent system of 6% MeOH - 0.01 M P.B. (pH 3.0). The pure fractions were desalted with Diaion HP-20 (100~200 mesh, 80 ml) to give white powder of **16** (284 mg); IR ν_{max} 1675, 1580, 1550, 1200, 1040 cm^{-1}.

Anal Calcd for $C_{26}H_{35}N_6O_{11}S_2 \cdot 1.5H_2O$: C 44.50, H 5.89, N 11.98, O 28.50, S 9.14.
Found: C 44.70, H 5.82, N 11.85, S 8.79.

A soln of **16** (180 mg) in 2 N HCl (15 ml) was stirred for 7 hours at room temp. Upon treatment with lactonization described above, **17** (94 mg) was obtained as a freeze-dried white powder; IR ν_{max} 1780, 1680, 1625, 1585, 1535, 1200, 1040 cm^{-1}.

Anal Calcd for $C_{26}H_{35}N_5O_{11}S_2 \cdot 2.5H_2O$: C 44.44, H 5.74, N 9.97, O 30.74, S 9.12.
Found: C 44.34, H 5.29, N 9.86, S 8.91.

Acknowledgment

We are grateful to Drs. K. MORITA, Y. NAKAO and H. OKAZAKI in our Central Research Division for their encouragement throughout this work and to Dr. M. KONDO for his bioassay. Thanks are also due to the members of large-scale production and physical analysis sections for their support and to Mr. Y. NOHARA for his skillful assistance.

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