CEPACIDINE A, A NOVEL ANTIFUNGAL ANTIBIOTIC PRODUCED BY *Pseudomonas cepacia*

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE ELUCIDATION

YOONGHO LIM, JUNG-WOO SUH, SUNGHO KIM, BONGCHUL HYUN, CHOONGSUP KIM and CHUL-HOON LEE*

R & D Center, Cheil Foods & Chemicals Inc.,
522-1 Dokpyong-Ri, Majang-Myon, Ichun-Kun, Kyunggi-Do, South Korea

(Received for publication June 20, 1994)

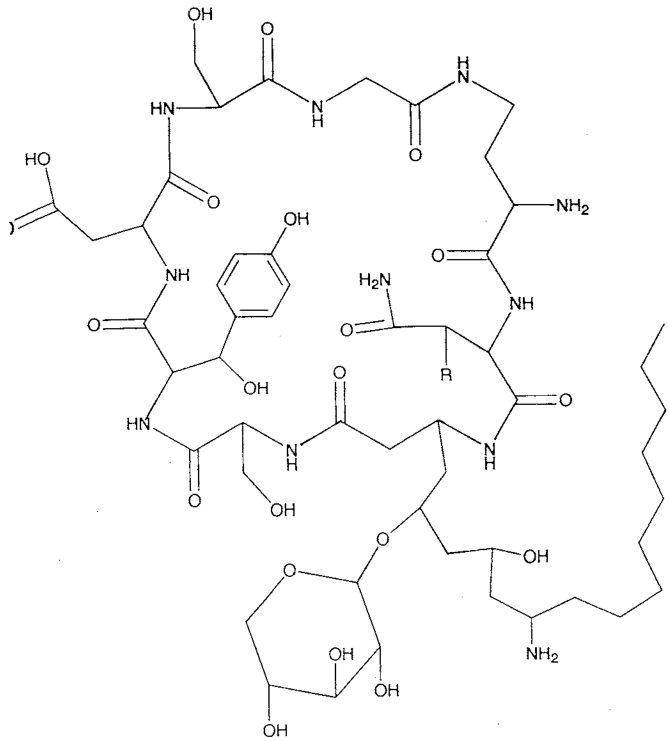
Cepacidine A is a novel glycopeptide with a potent antifungal activity, which is produced by *Pseudomonas cepacia* AF 2001. Its molecular weight was determined by FAB-MS (*m/z* 1215). The compound is comprised of glycine (1), serine (2), 2,4-diaminobutyric acid (1), aspartic acid (1), β-hydroxy tyrosine (1), β-hydroxy asparagine (1), xylose (1) and 5,7-dihydroxy-3,9-diamino-octadecanoic acid (1). Unfortunately, cepacidine A is a mixture of A_1 and A_2, either of which is barely distinguishable. Cepacidine A_2 includes asparagine (1) instead of β-hydroxy asparagine (1) of cepacidine A_1. The MS data and the NOESY, TOCSY and HMBC spectra show that cepacidine A is a cyclic peptide and xylose is connected to 5,7-dihydroxy-3,9-diaminooctadecanoic acid.

Cepacidine A is a new metabolite of *Pseudomonas cepacia* AF 2001 discovered in the author's screening program for antifungal substances. Its discovery, taxonomy, fermentation, isolation and biological properties were reported in the previous paper.[1] This paper describes the physico-chemical properties and structure elucidation of cepacidine A. (Fig. 1)

Physico-chemical Properties

The physico-chemical properties of cepacidine A is summarized in Table 1. Cepacidine A is isolated as white powders. The melting point is ranged between 210°C and 214°C. Cepacidine A is insoluble in ethyl acetate, hexane, ether and benzene, and is hardly soluble in water, methanol, ethanol, isopropanol, butanol and acetone, while soluble in DMSO, alkali aqueous solution and acidic aqueous solution. 50% aqueous solution of alcohol increases solubility. Cepacidine A shows positive color reactions to aniline and ninhydrin reagents. The Rf value of cepacidine A on silica gel, TLC, developed with *n*-butanol - acetic acid - water (3 : 1 : 1) was 0.18. However, the Rf values of cepacidine A on silica gel, TLC, developed with isopropanol - water - saturated aqueous ammonia (4 : 1 : 2), were separated as 0.53 and 0.58 so that the compound having the Rf value of 0.53 was named cepacidine A_1, and the compound having the Rf value of 0.58 was named cepacidine A_2. Since cepacidine A_1 and cepacidine A_2 each were barely obtainable by using prep-HPLC, it was unfortunate, that the mixture, cepacidine A, was used for all spectrometric analysis with the exception of TLC and amino acid which were analyzed by HPLC. The mixture has the 9 : 1 ratio of cepacidine A_1 and cepacidine A_2. The UV spectrum of cepacidine A dissolved in water showed two maximum absorption peaks at 232 nm and 274 nm, and the spectrum in DMSO showed only one peak at 278 nm. Cepacidine A is very stable in an aqueous solution ranging between pH 2 and pH 11, and it is unstable in aqueous solution above pH 11.5, and it loses its antifungal activities readily. The molecular formula of cepacidine A_1 was determined to be $C_{52}H_{85}O_{22}N_{11}$ by HRFAB-MS, ^{13}C NMR and elemental analysis (calcd: C 51.4, H 7.0, N 12.7, O 29.0; found: C 51.5, H 8.0, N 11.0, O 29.5),

Fig. 1. The structures of cepacidine A.



Cepacidine A_1 R=OH
Cepacidine A_2 R=H

Table 1. Physico-chemical properties of cepacidine A.

	Cepacidine A_1	Cepacidine A_2
Appearance	White powders	White powders
MP	210~214°C	210~214°C
UV λ_{max} nm (log ε) in H_2O	232 (2.8), 274 (1.7)	232 (2.8), 274 (1.7)
in DMSO	278 (1.2)	278 (1.2)
IR (KBr) ν_{max} cm^{-1}	3352, 2924, 2854, 1666, 1539, 1412, 1252, 1069, 557	3352, 2924, 2854, 1666, 1539, 1412, 1252, 1069, 557
$[\alpha]_D^{25}$ H_2O	+20.8	+20.8
TLC Rf value (*n*-BuOH - AcOH - H_2O, 3:1:1)	0.18	0.18
(*iso*-PrOH - H_2O - satd NH_4OH, 4:1:2)	0.53	0.58
Molecular formula	$C_{52}H_{85}O_{22}N_{11}$	$C_{52}H_{85}O_{21}N_{11}$
HRFAB-MS $(M+H)^+$ Calcd:	1216.5949	1200.5999
Found:	1216.5999	1200.5978

cepacidine A_2, $C_{52}H_{85}O_{21}N_{11}$. The molecular ions of cepacidine A_1 and cepacidine A_2 by HRFAB-MS were shown at *m*/*z* 1216.5999 (MH^+, calcd: 1216.5949) and 1200.5978 (MH^+, calcd: 1200.5999), respectively.

Structure Elucidation

Since cepacidine A showed positive color reaction to ninhydrin reagent, amino acid analysis was carried out by TLC and HPLC after acid hydrolysis. The analysis revealed that cepacidine A_1 consists of β-hydroxy Asp or/and Asn, Ser, Gly and 2,4-diaminobutyric acid (1 : 1 : 2 : 1 : 1), and cepacidine A_2, Asp or Asn, Ser, Gly and 2,4-diaminobutyric acid (2 : 2 : 1 : 1). The solution obtained from acid hydrolysis was eluted through octadecyl column. The column was washed with 50% isopropanol and the remnant collected for NMR experiments. The inspection of NMR experiments revealed the remnant to be octadecanoic acid derivative. From the ^{1}H NMR, ^{13}C NMR, COSY, HETCOR, NOESY, HOHAHA and HMBC experiments, the acid was determined to be a kind of amino acid with three functional groups

Fig. 2. The ^{1}H NMR spectrum of cepacidine A.

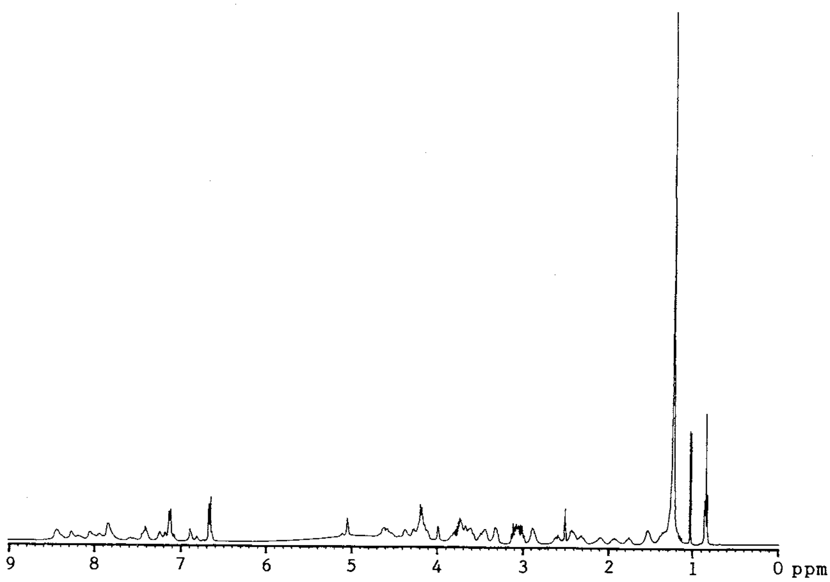


Fig. 3. The ^{13}C NMR spectrum of cepacidine A.

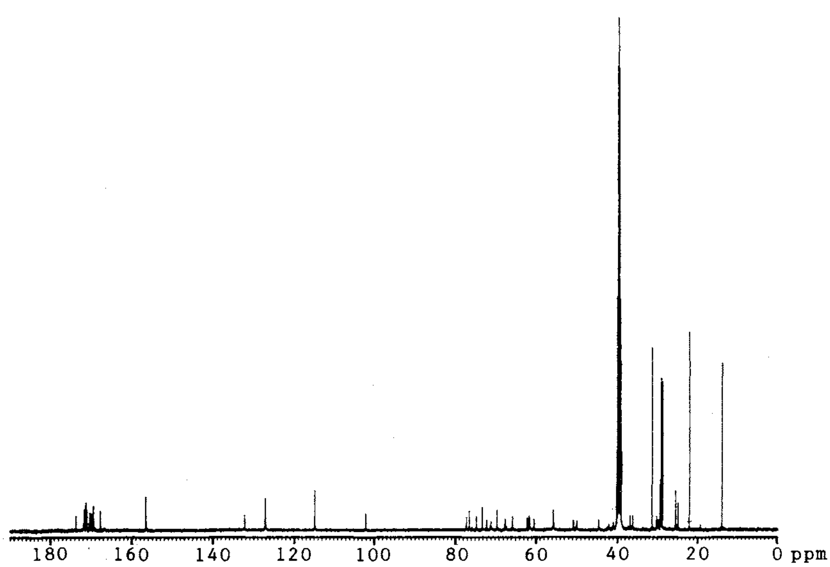
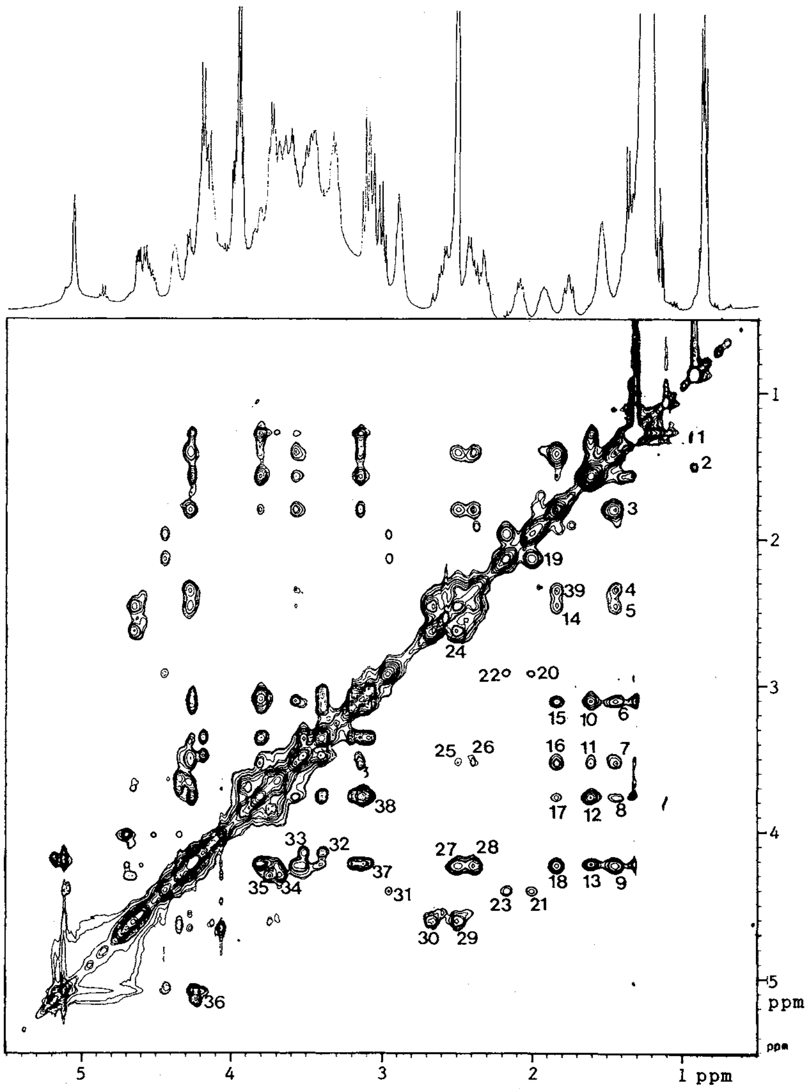


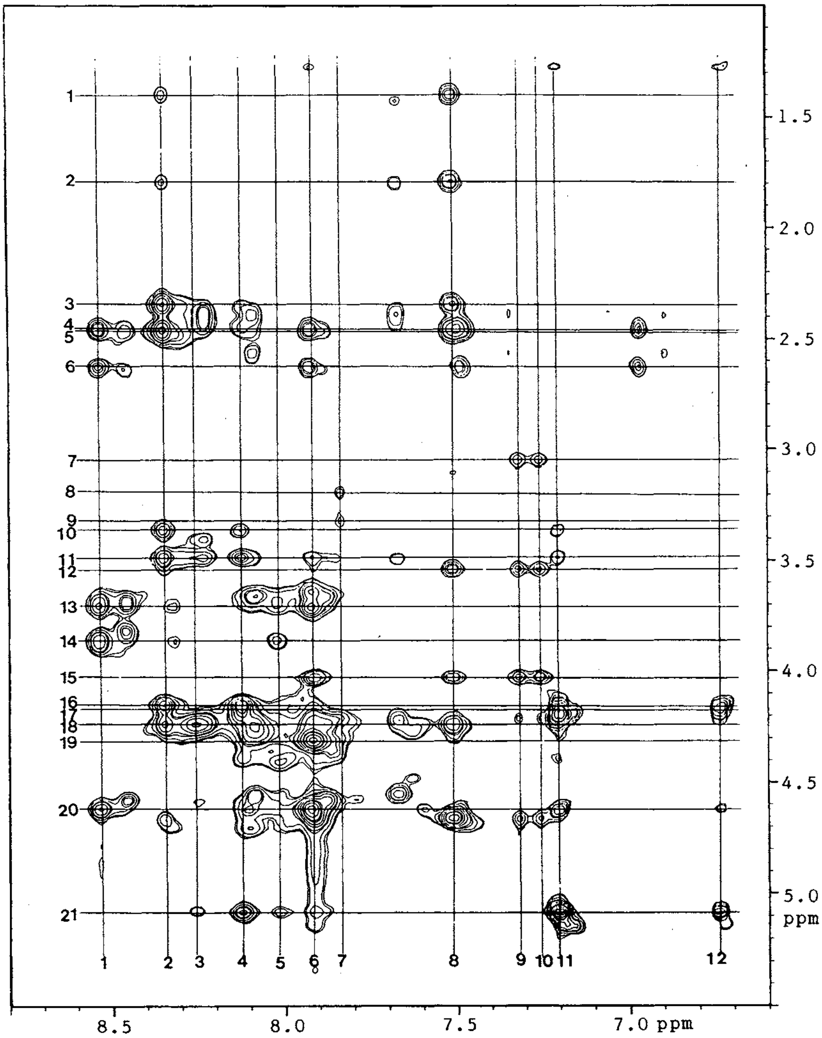
Fig. 4. The fingerprint region of the NOESY spectrum of cepacidine A.



consisting of one primary amine and two hydroxyl groups. As shown in Fig. 1, the acid is named 5,7-dihydroxy-3,9-diaminooctadecanoic acid. Because cepacidine A showed positive color reaction to aniline reagent, saccharide analysis was carried out by cellulose TLC and HPLC after acid hydrolysis. The analysis revealed both cepacidine A_1 and cepacidine A_2 to include xylose.

The spectrum of cepacidine A obtained from FAB-MS shows only MH^+ ions of cepacidine A_1 (*m/z* 1216) and Cepacidine A_2 (*m/z* 1200) except for a few small fragments and an xylose fragment. This phenomenon suggests that cepacidine A can be a cyclic peptide. The NMR experiments such as NOESY, HOHAHA and HMBC clarified this suggestion. The ^1H NMR and ^{13}C NMR spectra of cepacidine A are shown in Figs. 2 and 3. The fingerprint region of the NOESY spectrum of cepacidine A are shown in Fig. 4, and the cross peaks are assigned as listed in Table 2. In addition, the NOESY cross peaks among α, β and NH protons are shown in Fig. 5 and assigned as listed in Table 3.

Fig. 5. The NOESY cross peaks among α, β and NH protons of cepacidine A.



The sum of the calculated number of carbons of components obtained from amino acid analysis and saccharide analysis, and the number of carbons of 5,7-dihydroxy-3,9-diaminooctadecanoic acid, is only 43. However, the ^{13}C NMR spectrum gives 50 peaks. Therefore, the presence of the other components can be considered. The ^{1}H NMR spectrum of cepacidine A reveals the presence of an aromatic ring. Since the four carbon signals at 114.70, 127.00, 132,20 and 156.50 ppm are characteristic peaks caused by *para*-hydroxy phenyl group, the presence of Tyr can be expected, but amino acid analysis does not show the peak of Tyr, therefore, the presence of a derivative of Tyr cannot be considered. In the COSY spectrum, cross peaks among 4.19, 5.06, 6.67 and 7.14 ppm are observed. In addition, HETCOR shows four correlated peaks such as 4.19/60.40, 5.06/70.96, 6.67/114.70 and 7.14/127.00 (^{1}H δ/^{13}C δ). These phenomena suggest that one β-protons of Tyr is substituted with a hydroxyl group. In order to clarify this, a chemical experiment was carried out. The UV spectrum of cepacidine A in DMSO shows one peak at 278 nm. An addition of TFA into cepacidine A caused bathochromic shift of λ_{max} to 312 nm. An elimination of

Table 2. Assignments of the fingerprint region of the NOESY spectrum of cepacidine A.

No.	Assignments	No.	Assignments
1	C_{18}AA 18CH_3/C_{18}AA CH_{2s}	21	DAB β_1/DAB α
2	C_{18}AA CH_3/C_{18}AA CH_{2s}	22	DAB β_2/DAB γ
3	C_{18}AA 2CH_2/C_{18}AA 6CH_2	23	DAB β_2/DAB α
4	C_{18}AA 2CH_2/C_{18}AA 6$CH_{2'}$	24	Asp β_1/Asp β_2
5	C_{18}AA 2CH_2/C_{18}AA 6CH_2	25	C_{18}AA 6$CH_{2'}$/C_{18}AA 3CH_2
6	C_{18}AA 2CH_2/Xyl 5	26	C_{18}AA 6CH_2/C_{18}AA 3CH_2
7	C_{18}AA 2CH_2/C_{18}AA 3CH_2	27	C_{18}AA 6$CH_{2'}$/Xyl 1
8	C_{18}AA 2CH_2/Xyl 5′	28	C_{18}AA 6CH_2/Xyl 1
9	C_{18}AA 2CH_2/Xyl 1	29	Asp β_1/Asp α
10	C_{18}AA 4CH_2/Xyl 5	30	Asp β_2/Asp α
11	C_{18}AA 4CH_2/C_{18}AA 3CH_2	31	DAB α/DAB γ
12	C_{18}AA 4CH_2/Xyl 5′	32	Ser 1 β_1/Ser 1 α
13	C_{18}AA 4CH_2/Xyl 1	33	Ser 1 β_2/Ser 1 α
14	C_{18}AA 4CH_2/C_{18}AA 6$CH_{2'}$	34	Ser 2 β_1/Ser 2 α
15	C_{18}AA 4CH_2/Xyl 5	35	Ser 2 β_2/Ser 2 α
16	C_{18}AA 4CH_2/C_{18}AA 3CH_2	36	Tyr α/Tyr β
17	C_{18}AA 4CH_2/Xyl 5′	37	Xyl 1/Xyl 5
18	C_{18}AA 4CH_2/Xyl 1	38	Xyl 5/Xyl 5′
19	DAB β_1/DAB β_2	39	C_{18}AA 2$CH_{2'}$/C_{18}AA 6CH_2
20	DAB β_1/DAB γ		

Table 3. Assignments of the NOESY cross peaks of cepacidine A.

Row	Assignments	Row	Assignments	Column	Assignments
1	C_{18}AA 2CH_2	13	Ser 2 β_1	1	Asp NH
2	C_{18}AA 2$CH_{2'}$	14	Ser 2 β_2	2	Ser 1 NH
3	C_{18}AA 6CH_2	15	Xyl 4	3	Hydroxy Asn NH
4	Asp β_1	16	Ser 1 α	4	Tyr NH
5	C_{18}AA 6$CH_{2'}$	17	Tyr α	5	Gly NH
6	Asp β_2	18	Hydroxy Asn α	6	Ser 2 NH
7	Xyl 2	19	Ser2 α	7	DAB NH
8	DAB γ NH_2	20	Asp α	8	C_{18}AA NH
9	DAB γ $NH_{2'}$	21	Tyr β	9	C_{18}AA 9NH_2
10	Ser 1 β_1			10	C_{18}AA 9$NH_{2'}$
11	Ser 1 β_2			11	Tyr δ
12	C_{18}AA 3CH			12	Tyr ε

α-proton and β-proton of Tyr can cause a conjugation as mentioned by G. S. BISACCHI *et. al.*[2] Then, two carbon peaks at 114.70 and 127.00 in the ^{13}C NMR spectrum must be the ε and δ carbons of Tyr and they denote two carbon intensities each. As a result, the number of carbon in cepacidine A is not 50 as shown in the ^{13}C NMR spectrum, but it is 52. The ^{1}H NMR and ^{13}C NMR spectra are assigned as listed in Table 4. The carbonyl carbons of peptide bonds were assigned from the HMBC spectrum. (Fig. 6)

As mentioned previously, the molecular formula of cepacidine A_1 and cepacidine A_2 are determined to be $C_{52}H_{85}O_{22}N_{11}$ and $C_{52}H_{85}O_{21}N_{11}$ by HRFAB-MS and elemental analysis. In order to determine Asx, pyrolysed GC was carried out. Chromatograms of Asn and Asp as references are shown in Fig. 7. Two chromatograms can be distinguished by the characteristic peaks at a 47 minute retention time. Since the peaks of cepacidine A at the 47 minute retention time are the same as those of Asp, Asx contained in cepacidine A must be Asp. NOESY, and HOHAHA experiments revealed that xylose is connected to one of hydroxyl groups of 5,7-dihydroxy-3,9-diaminooctadecanoic acid. (Fig. 8) Until now Gly (1), Ser

Table 4. ^{1}H NMR and ^{13}C NMR assignments of cepacidine A.

No.	^{13}C Chemical shift	Multiplicity	^{1}H Chemical shift	Assignments
1	13.84	q	0.83 (t, 7.0)	C_{18}AA 18CH_3
2	22.01	t	1.25	C_{18}AACH_2
3	24.80	t	1.30 (d, 7.0, 20.0)	C_{18}AA 8CH_2
4	28.62	t	1.22	C_{18}AA CH_2
5	28.94	t	1.22	C_{18}AA CH_2
6	28.97	t	1.22	C_{18}AA CH_2
7	29.00	t	1.22	C_{18}AA CH_2
8	29.01	t	1.22	C_{18}AA CH_2
9	29.26	t	1.22	C_{18}AA CH_2
10	29.69	t	1.94 (ddt 6.0, 8.0, 8.0), 2.12 (ddt 6.0, 8.0, 7.0)	2,4-DAB β
11	30.07	t	1.56 (s)	C_{18}AA 4CH_2
12	31.22	t	1.22	C_{18}AA CH_2
13	36.02	t	2.90 (dd 7.0, 8.0)	2,4-DAB γ
14	36.69	t	2.42 (dd 6.0, 8.0) 2.66 (dd 6.0, 10.0)	Asp β
15	38.76	t	1.37 (dd 10.0, 12.0), 1.78 (dd 10.0, 12.0)	C_{18}AA 2CH_2
16	40.91	t	2.31 (m), 2.46 (m)	C_{18}AA 6CH_2
17	42.03	t	3.84 (d 11.0), 3.88 (d 11.0)	Gly α
18	44.52	d	4.22 (d 3.0)	Hydroxy Asn α
19	49.89	d	4.62 (dd 8.0, 10.0)	Asp α
20	50.74	d	4.38 (dd 6.0, 8.0)	2,4-DAB α
21	55.61	d	4.28 (dd 5.0, 6.0)	Ser 2 α
22	55.71	d	4.11 (dd 4.0, 6.0)	Ser 1 α
23	60.40	d	4.19 (d 3.0)	Hydroxy Tyr α
24	61.43	t	3.32 (dd 4.0, 11.0), 3.46 (dd 6.0, 11.0)	Ser 1 β
25	61.60	t	3.63 (dd 5.0, 10.5),	3.73 (m) Ser 2 β
26	62.06	d	3.79 (dd 11.0, 16.0)	C_{18}AA 9NH_2
27	65.67	t	3.03 (dd 10.0, 8.5) 3.73 (dd 7.0, 8.5)	Xyl 5
28	67.45	d	3.51 (t 10.0)	C_{18}AA 3CH
29	69.45	d	3.33 (m)	C_{18}AA 7OH
30	70.96	d	5.06 (d 3.0)	Hydroxy Tyr β
31	72.00	d	4.01 (ddd 10.0, 7.0, 4.0)	Xyl 4
32	73.10	d	3.00 (dd 8.5, 4.0)	Xyl 2
33	74.59	d	3.09 (d 3.0)	Hydroxy Asn β
34	76.43	d	3.14 (dd 8.5, 4.0)	Xyl 3
35	77.15	d		C_{18}AA 5OH
36	102.00	d	4.21 (d 4.0)	Xyl 1
37	114.70	d	6.67 (d 8.0)	Hydroxy Tyr ε
38	127.00	d	7.14 (d 8.0)	Hydroxy Tyr δ
39	132.20	s		Hydroxy Tyr γ
40	156.50	s		Hydroxy Tyr ζ
41	167.70	s		C_{18}AA C=O
42	169.30	s		Gly C=O
43	169.80	s		Hydroxy Asn C=O
44	170.30	s		Hydroxy Tyr C=O
45	171.00	s		Asp C=O
46	171.10	s		Ser C=O
47	171.20	s		2,4-DAB C=O
48	171.50	s		Ser C=O
49	171.60	s		Asp COOH
50	173.60	s		Hydroxy Asn $CONH_2$

Fig. 6. The HMBC spectrum of cepacidine A.

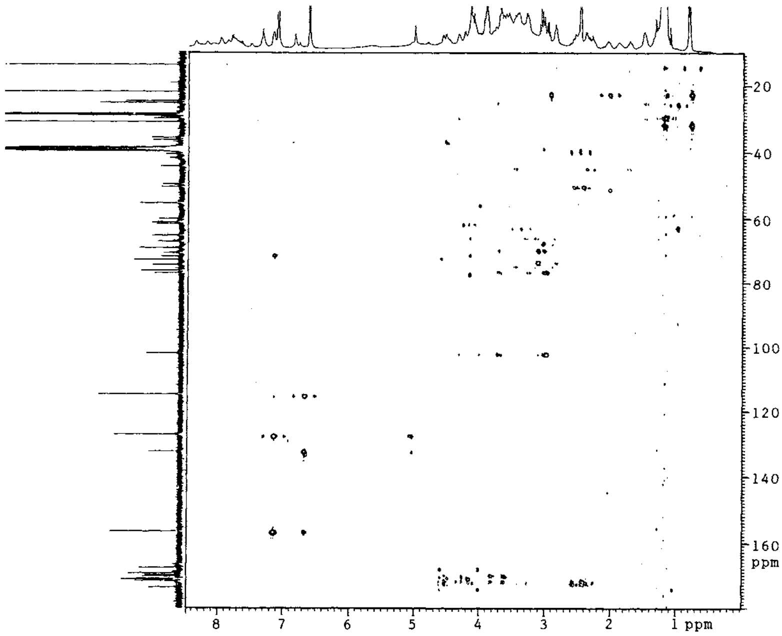


Fig. 7. The chromatograms of Asp (top), Asn (middle) and cepacidine A (bottom).

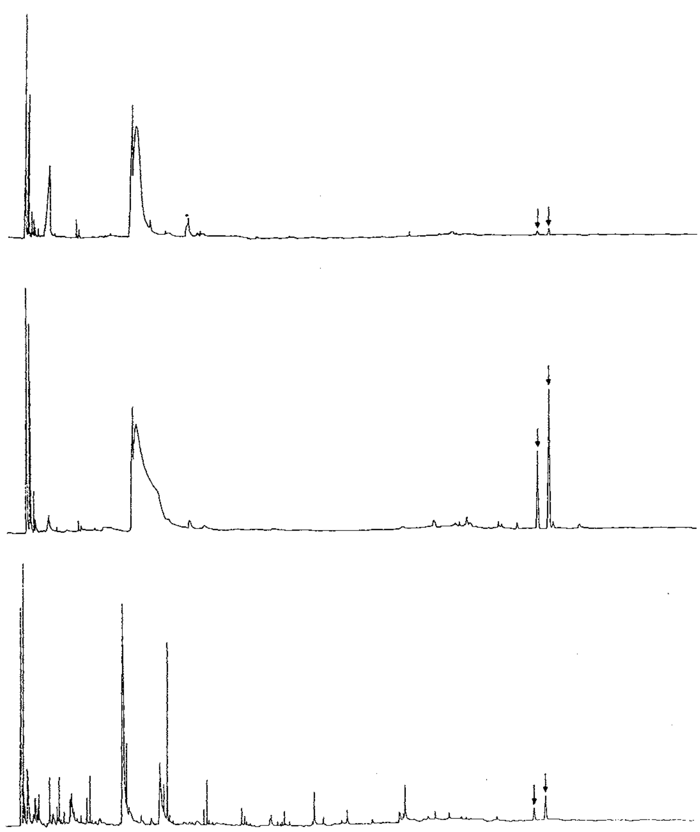
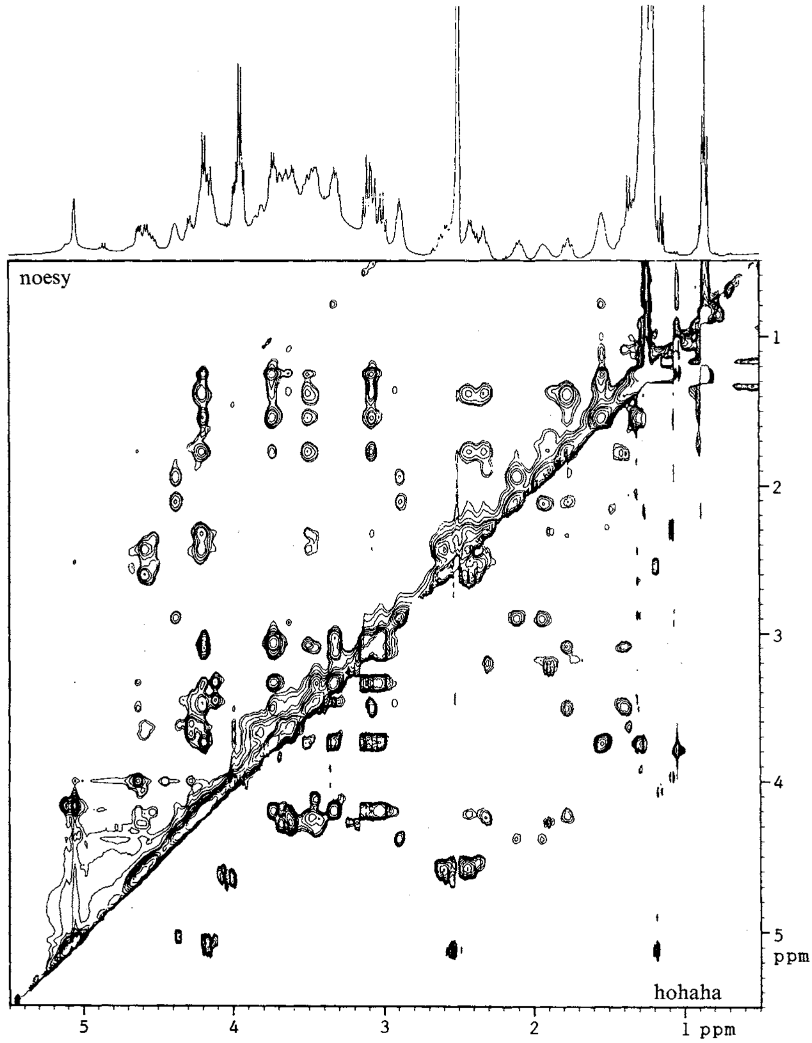


Fig. 8. The NOESY and HOHAHA spectra of cepacidine A.



(2), Asp (1), β-hydroxy Tyr (1), 2,4-diaminobutyric acid (1), xylose (1), and 5,7-dihydroxy-3,9-diaminooctadecanoic acid ($C_{18}H_{38}O_4N_2$) were determined and the sum of elements contained in those components are $C_{48}H_{95}O_{27}N_9$. However, because cepacidine A is a cyclic peptide and xylose is connected to 5,7-dihydroxy-3,9-diaminooctadecanoic acid, the formula must be $C_{48}H_{79}O_{19}N_9$. The difference between this formula and that obtained from HRFAB-MS gives $C_4H_6O_3N_2$ for cepacidine A_1. Therefore, the undetermined components, β-hydroxy Asx of cepacidine A_1, must be β-hydroxy Asn, and Asx of cepacidine A_2, Asn, respectively.

Table 5. Components of cepacidine A_1 and cepacidine A_2.

Components	Cepacidine A_1	Cepacidine A_2
Glycine	1	1
Serine	2	2
Aspartic acid	1	1
Asparagine	0	1
β-Hydroxy asparagine	1	0
2,4-Diaminobutyric acid	1	1
β-Hydroxy tyrosine	1	1
C_{18} Amino acid	1	1
Xylose	1	1

Fig. 9. The proton spin networks obtained by NOESY.

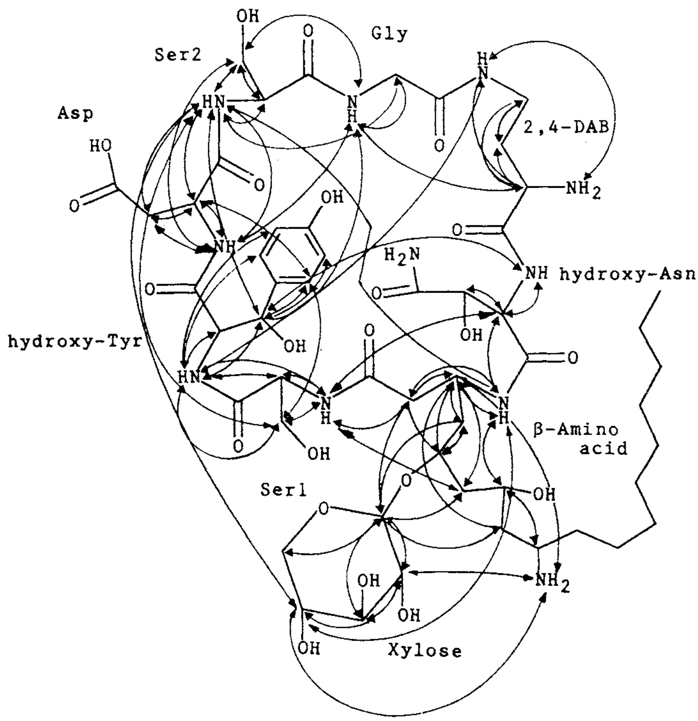


Table 6. Parameters of two-dimensional experiments.

Experiments	$t_2 \times t_1$	Number of scans	Dummy scans	Temperature (°C)	Acquisition time (seconds)
COSY	2,048 × 256	160	2	20	0.254
NOESY	2,048 × 512	72	2	20	0.252
HOHAHA	2,048 × 256	24	2	20	0.274
HETCOR	2,048 × 256	128	2	20	0.074
HMBC	2,048 × 512	64	4	20	0.236

Experiments	Spectral width (Hz)	Zero-filled	Window function	Mixing time (mseconds)	Methods
COSY	4,000	2K × 2K	sine 0		Magnitude[3]
NOESY	4,000	2K × 2K	sq. sine 2	300	TPPI[4]
HOHAHA	3,730	2K × 2K	sq. sine 3	110	TPPI[5]
HETCOR	26,400 × 4,350	2K × 256	sq.sine 2		Ref 6
HMBC	4,350 × 26,400	2K × 512	sq. sine 6		Ref 7

The components of cepacidine A_1 and cepacidine A_2 are listed in Table 5. Fig. 9 shows the proton spin networks obtained by NOESY.

Experimental

FAB-MS was measured on JEOL DX 303 spectrometer. UV and IR were recorded on Beckman DU-70, and on Bruker IFS 66, respectively. NMR spectra were recorded on a Bruker ARX 400 spectrometer in DMSO-d_6 and 95% DMSO-d_6/5% D_2O. Two dimensional experiments were performed and processed as listed in Table 6. EA data were obtained on Foss Heraeus CHN-O-Rapid. TLC was performed on pre-coated silica gel plates (Merck catalog No. 5642). Pyrolysed GC was measured on Schimadzu GC 15A and JHP-35 Pyrolyser with CBP-5 column. For amino acid analysis, cepacidine A was hydrolyzed with 6 N HCl at 105°C for eight hours, and Waters amino acid conversion kit and Waters amino acid analysis column were used with Waters Fluorescence 420, as a detector. For saccharide analysis, cepacidine A was hydrolyzed with 10% H_2SO_4 at 100°C for 1 hour, and the Waters carbohydrate analysis system and Waters carbohydrate column were used with Waters RI 410, as a detector.

Acknowledgments

We thank Mr. YOUNGKEUN MIN of Samsung Advanced Institute of Technology for the pyrolysed GC experiments.

References

1) LEE, C. H.; S. H. KIM, B. C. HYUN, J. W. SUH, C. YON, C. O. KIM, Y. H. LIM & C. S. KIM: Cepacidine A, a novel antifungal antibiotic produced by *Pseudomonas cepacia*. I. Taxonomy, production, isolation and biological activity. J. Antibiotics 47: 1402~1405, 1994
2) BISACCHI, G. S.; D. R. HOCKSTEIN, W. H. KOSTER, W. L. PARKER, M. L. RATHNUM & S. E. UNGER: Xylocandin: a new complex of antifungal peptides. II. Structural studies and chemical modifications. J. Antibiotics 40: 1520~1529, 1987
3) NAGAYAMA, K.; A. KUMAR, K. WÜTHRICH & R. R. ERNST: Experimental techniques of two-dimensional correlated spectroscopy. J. Magn. Reson. 40: 321~334, 1980
4) MARION, D. & K. WÜTHRICH: Applications of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of proton–proton spin–spin coupling constants in proteins. Biochem. Biophys. Res. Comm. 113: 967~974, 1983
5) BAX, A. & D. G. DAVIS: Mlev-17-based two-dimensional homonuclear magnetization transfer spectroscopy. J. Magn. Reson. 60: 355~360, 1985
6) BAX, A. & G. A. MORRIS: An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. J. Magn. Reson. 42: 501~505, 1981
7) BAX, A. & M. F. SUMMERS: ^1H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986