HETEROCYCLES, Vol. 53, No. 7, 2000, pp. 1533 - 1549, Received, 17th April, 2000

LI-F ANTIBIOTICS, A FAMILY OF ANTIFUNGAL CYCLIC DEPSIPEPTIDES PRODUCED BY *BACILLUS POLYMYXA* L-1129

Jun Kuroda,[1] Toshio Fukai,[1] Masataka Konishi,[1] Jun Uno,[2] Keiji Kurusu,[3] and Taro Nomura,[1]*

[1]*Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan*, [2]*Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan*, [3]*Biological Science Laboratories, Lion Co., 202 Tajima, Odawara, Kanagawa 256-0811, Japan*

Abstract: Twelve antifungal depsipeptide antibiotics named as LI-F03a, LI-F03b, LI-F04a, LI-F04b, LI-F05a, LI-F05b, LI-F06a, LI-F06b, LI-F07a, LI-F07b, LI-F08a and LI-F08b have been isolated from the fermentation broth of *Bacillus polymyxa* L-1129 by use of extensive preparative HPLC. Not only their intrinsic antifungal and anti-Gram positive bacterial activity, they also showed unique synergistic antifungal effect in combination with the azole group antifungal agents. The structural studies disclosed that they are cyclic depsipeptides composed of six amino acids (three of them, L-threonine, D-*allo*-threonine and D-alanine being in common among all components and other three amino acids variable) on which is subtituted by a 15-guanidino-3-hydroxypentadecanoic acid.

Recent increase of the opportunistic fungal infections especially in the immuno-compromised hosts such as cancer, AIDS, diabetics and elder patients has encouraged us further and urgent search for safe and effective antifungal agents in particular, fungicidal agents have been strongly requested.[1, 2] Many antifungal compounds have been identified, but safe and effective antifungal drugs are not found yet because of high similarity of fungi and mammalian cells.[3] Thus, amphotericin B developed many years ago is still widely used in spite of its serious side effects.[1b, 2, 4, 5] In our efforts to seeking new antifungal agents with novel modes of actions, we have found that a strain of *Bacillus polymyxa* named as L-1129 produced a complex of closely related antifungal antibiotics, LI-F antibiotics. The taxonomy of the producing strain and fermentation, isolation, characterization and biological properties of the five major components (LI-F03, LI-F04, LI-F05, LI-F07 and LI-F08) of the LI-F complex have been presented in the previous paper.[6] These antibiotics exhibited inhibitory activity against Gram-positive bacteria, mycobacteria, and a wide range of fungi and yeasts, but no activity against Gram-negative bacteria, and relatively low acute toxicity for ddY mice (LD_{50}, 100 — 200 mg/kg). In addition, it was found in recent biological studies that the antibiotics produced synergistic antifungal effect with the commercially

important azole group antifungal agents such as ketoconazole, clotrimazole, miconazole and econazole.[7] A similar strong synergistic antifungal activity of the azole group agents and copiamycin family of macrocyclic-lactone antibiotics possessing a long side chain with a terminal guanidino group has been reported.[8 — 10] Interesting enough, copiamycin not only potentiated the antifungal activity of the azoles but also made them fungicidal. In the previous studies of LI-F antibiotics, FAB-MS analysis of the five components isolated by HPLC revealed that they were still mixtures of two very closely related components, although their separation could not be achieved by various chromatographic procedures including preparative HPLC.[6] In our continuing studies on this group of antibiotics with interesting antifungal activity and the synergistic effect, we have succeeded in isolation of ten components, LI-F04a, LI-F04b, LI-F05a, LI-F05b, LI-F06a, LI-F06b, LI-F07a, LI-F07b, LI-F08a and LI-F08b, from the complex by careful selection of chromatographic conditions and repetition of reversed phase preparative HPLC. Structural studies of the new antibiotics showed that they are cyclic depsipeptides composed of six amino acids (four D-series amino acids and two L-series amino acid) and a unique 15-guanidino-3-hydroxypentadecanoic acid (GHPD, **1a**) as a side chain. The separation of LI-F03a and LI-F03b had not been successful and the structure determination of them was therefore performed using the mixture. In this paper, we report the isolation, structure elucidation and bioactivity of the LI-F components.

Results

Amino acid and fatty acid analysis: For the amino acid analysis, each antibiotic component (0.5 mg) was hydrolyzed with 6 mol/L HCl at 110°C. for 24 h in a sealed tube. The reaction solution was then evaporated *in vacuo* and the residue was partitioned between ethyl acetate (EtOAc) and water. The water layer was subjected to the amino acid analysis and also to determination of their stereochemistry by HPLC using a chiral column. These hydrolysis results demonstrated that LI-F antibiotics contained four D-series amino acids, two L-series amino acids and a GHPD (**1a**). Three amino acids, L-threonine (Thr), D-*allo*-Thr and D-alanine (Ala) and **1a** are observed in common in all LI-F antibiotics while three amino acids are variable among them. The difference of the LI-F sub-group (a-group and b-group) antibiotics resided in only one of D-amino acids [D-aspartic acid (Asp) in the a-group and D-glutamic acid (Glu) in the b-group]. The results suggested a close resemblance of their structures to those of fusaricidin group antibiotics (Figure 1)[11, 12] and KT-6291A and -6291B.[13]

Structure determination of LI-F04a and 04b: The amino acid analysis and HPLC analysis by the chiral column showed that LI-F04a had L-Thr, D-*allo*-Thr, D-Ala, L-valine (Val), D-Val and D-Asp. Electrospray ionization (ESI) MS analysis of the EtOAc extract of the acid hydrolysate indicated the presence of dehydrated GHPD (**1b**), *m/z* 298.4 $[M + H]^+$. The molecular formula of LI-F04a established by high-resolution matrix assisted laser desorption/ionization time-of-flight (HR-MALDI-TOF) MS in combination of amino acid analysis and NMR data was $C_{41}H_{74}N_{10}O_{11}$ which indicated the presence of D-asparagine (Asn) instead of D-Asp in the original antibiotic. The accuracy of the MALDI-TOF instrument is less than those of FAB- and FT-ICR MS instruments. Nevertheless, when combined with the data of NMR spectrometry and amino acid analysis, MALDI is useful enough for determination of the

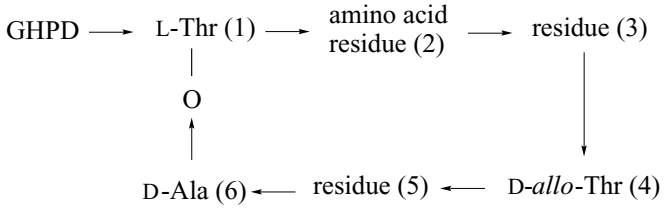
elemental composition of low molecular weight peptides. A procedure for the determination of molecular formula by MALDI-TOF MS was recently reported by us.[14] The presence of ester linkage was deduced by production of an open chain peptide with GHPD (**4c**) upon hydrolysis with MeOH-H_2O-28% NH_4OH (4:1:1, pH 9.0) at room temperature for 24 h. Compound (**4c**) gave a protonated molecule $[M + H]^+$ at *m/z* 901.5 (18 dalton (Da) larger than that of LI-F04a) measured by positive ESI-MS. ^1H and ^{13}C NMR study (in DMSO-d_6 at 50 °C) indicated the presence of the above six amino acids (Table 1) and a GHPD (EXPERIMENTAL) in the molecule. The sequence of the six amino acids and GHPD in LI-F04a was determined to be GHPD → Thr (1) → Val (2) → Val (3) → Thr (4) → D-Asn (5) → D-Ala (6) → O- by ESI-MS fragmentations of **4c** assisted with collision-induced dissociation (CID) MS of the protonated molecule with ion trap ESI MS spectrometer.[15] The MS showed types b and y fragment ions cleaved at each peptide linkage (rupture at CO-NH of peptide bond,[17] Table 2). The other some fragment ions of type a and a few fragments (types c and z)[17] were also informative for the elucidation of amino acid sequence (data not shown). The fragment ions also confirmed the presence of D-Asn and not D-Asp in the intact antibiotic. The peptide thus obtained was deduced to be hydrolyzed at the ester linkage between the carbonyl of Ala (6) and hydroxyl of Thr (1) but not the hydroxyl of GHPD and Thr (4) of the original antibiotic by the following evidence. Each network of spin-spin coupling of the amino acids was assigned with COSY spectrum and the connection of these residues was deduced with HMBC and NOESY spectra. The chemical shift of β-proton of Thr (1) appeared at δ 5.34 (1.38 ppm lower field than that of Thr (4), δ 3.96, Table 1) showing that the hydroxyl group of Thr (1) was esterified to cause a downfield shift. The primary structure of LI-F04a was same as those of fusaricidin A (Figure 1) isolated from *Bacillus polymyxa* KT-8[11] and KT-6291A from an unidentified strain *Bacillus* sp. KB-291.[13] The NMR data of LI-F04a were very similar to those of fusaricidin A[11] (Table 1). In order to establish the identify of LI-F04a with fusaricidin A and/or KT-6291A, stereochemistry of two threonines, residues (1) and (4), and two valines, residues (2) and (3), had to be determined. A partial hydrolysis of LI-F04a was and 6 mol/L HCl at 50 °C for 8 h in a sealed tube. The hydrolysate contained several peptide fragments as detected by TLC. They were purified by preparative TLC and analyzed with ESI-MS. Two peptide compounds, **2a** containing amino acid residues 3 – 5 and **2b** containing amino acid residues 3 – 6, were isolated. Compound (**2a**) gave a molecule ion $[M]^+$ at *m/z* 332.5 (base peak) measured in ESI-MS.[18] This MS result together with the amino acid analysis data by the chiral column HPLC indicated that **2a** was a tripeptide containing L-Val, D-*allo*-Thr, and D-Asn (detected as Asp in the amino acid analyses). Similarly, **2b** was identified to be a tetrapeptide composed of L-Val, D-*allo*-Thr, D-Asn and D-Ala by ESI-MS (m/z 403.5 $[M]^+$), by the amino acid analysis and HPLC using the chiral column. These results taking into consideration of the amino acid sequence determined for LI-F04a clearly showed that Thr (1) is L-Thr, Val (2) D-form, Val (3) L-form and Thr (4) D-*allo*-Thr. Thus, the amino acid sequence of LI-F04a was was unambiguously established as GHPD → L-Thr → D-Val → L-Val → D-*allo*-Thr → D-Asn → D-Ala → O-. The D-Ala links to the hydroxyl of L-Thr forming a cyclic lactone ring in the antibiotic LI-F04a, which revealed identity with fusaricidin A (Figure 1)[11] and may be identical with KT-6291A (stereochemistry of KT-6291A has not been reported).[13]

The constituent amino acids of LI-F04b, $C_{42}H_{76}N_{10}O_{11}$, were determined to be L-Thr, D-*allo*-Thr, D-Ala,

Table 1. ^{1}H and ^{13}C NMR Data of Amino Acid Residue of LI-F04a (Fusaricidin A) and LI-F04b (Fusaricidin B) in DMSO-d_6 measured at 50 °C

	LI-F04a		Fusaricidin A [a]		LI-F04b		Fusaricidin B [b]	
position	δ_C	δ_H (mult. *J* in Hz)	δ_C	δ_H	δ_C	δ_H (mult. *J* in Hz)	δ_C	δ_H
L-Thr (1)								
NH		8.12 (d, 8.5)		8.24		7.39 (d, 8)		7.50
α-CH	56.8	4.44 (dd, 8.5, 2)	56.8	4.40	56.6	4.42 (br)	56.7	4.43
β-CH	70.3	5.34 (dq, 2, 8)	70.2	5.32	70.2	5.34 (m)	70.1	5.34
γ-CH_3	16.1	1.19 (d, 8)	16.2	1.15	16.3	1.16 (d, 7)	16.4	1.16
CO	168.4		168.3		168.1		168.1	
D-Val (2)								
NH		7.18 (d, 9)		7.26		7.21 (d, 9)		7.30
α-CH	57.0	4.41 (dd, 9, 2)	56.9	4.42	56.8	4.44 (m)	56.7	4.45
β-CH	31.3	1.85 (m)	31.4	1.83	31.4	1.86 (m)	31.5	1.85
γ-CH_3	18.0	0.78 (d, 8)	18.2	0.76	18.0	0.75 (d, 7)	17.9	0.75
γ-CH_3	18.8	0.84 (d, 8)	19.0	0.82	19.0	0.81 (d, 7)	19.0	0.81
CO	170.5		170.8		170.9		171.0	
L-Val (3)								
NH		8.26 (d, 8)		8.37		8.30 (d, 8)		8.40
α-CH	58.0	4.21 (dd, 7, 8)	57.8	4.23	58.7	4.09 (m)	58.4	4.12
β-CH	29.7	1.99 (m)	30.0	1.99	29.3	1.99 (m)	29.5	2.00
γ-CH_3	17.9	0.87 (d, 7)	18.0	0.87	18.2	0.89 (d, 8)	18.2	0.86
γ-CH_3	19.1	0.89 (d, 7)	19.2	0.85	19.3	0.87 (d, 8)	19.2	0.88
CO	172.8		172.9		172.0		172.2	
D-*allo*-Thr (4)								
NH		8.29 (d, 7)		8.43		8.26 (d, 7)		8.36
α-CH	60.2	3.98 (br)	60.2	3.92	59.5	4.05 (br)	59.4	4.03
β-CH	65.8	3.96 (br)	65.6	3.93	65.8	3.98 (m)	65.6	3.96
β-OH		4.97 (br s)		4.97		5.01 (br s)		5.01
γ-CH_3	19.3	1.11 (d, 8)	19.5	1.10	19.5	1.12 (d, 7)	19.6	1.10
CO	170.3		170.2		170.2		170.3	
Residue (5)		(D-Asn)				(D-Gln)		
NH		7.95 (d, 8)		8.07		7.74 (d, 8)		7.87
α-CH	50.4	4.32 (dt, 8, 6)	50.3	4.28	52.8	3.92 (m)	52.7	3.92
β-CH	36.4	2.61 (dd, 16, 6)	36.5	2.56	26.3	1.99 (m)	26.1	1.99
β-CH		2.75 (dd, 16, 6)		2.77		2.14 (m)		2.13
CO	169.4		169.6	(γ-CH_2)	31.7	2.24 (m)	31.8	2.09 – 2.25
γ-CO	172.5		172.4	(CO)	170.0		170.4	
				(δ-CO)	174.3		174.2	
NH_2		6.91 (s)		7.03 (s)		6.73 (s)		6.84 (s)
		7.40 (s)		7.45 (s)		7.20 (s)		7.25 (s)
D-Ala (6)								
NH		7.28 (d, 8)		7.23		7.43 (d, 8)		7.39
α-CH	47.9	4.00 (m)	47.7	4.01	48.0	3.97 (dq, 7, 7)	47.8	4.07
β-CH_3	16.9	1.19 (d, 8)	17.2	1.15	16.9	1.22 (d, 7)	17.1	1.21
CO	170.3		170.5		170.4		170.5	

[a] Data from Y. Kajimura and M. Kaneda (ref. 11).
[b] Data from Y. Kajimura and M. Kaneda (ref. 12).



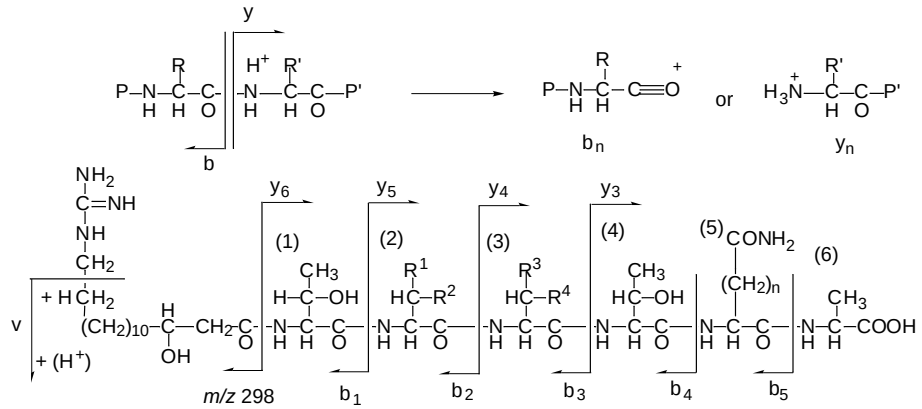
GHPD means 15-guanidino-3-hydroxypentadecanoic acid.

Residue	(2)	(3)	(5)	Residue	(2)	(3)	(5)
LI-F03a (Fusaricidin C)	D-Val	L-Tyr	D-Asn	LI-F03b (Fusaricidin D)	D-Val	L-Tyr	D-Gln
LI-F04a (Fusaricidin A)	D-Val	L-Val	D-Asn	LI-F04b (Fusaricidin B)	D-Val	L-Val	D-Gln
LI-F05a	D-Val	L-Ile	D-Asn	LI-F05b	D-Val	L-Ile	D-Gln
LI-F06a	D-*allo*-Ile	L-Val	D-Asn	LI-F06b	D-*allo*-Ile	L-Val	D-Gln
LI-F07a	D-Val	L-Phe	D-Asn	LI-F07b	D-Val	L-Phe	D-Gln
LI-F08a	D-Ile	L-*allo*-Ile	D-Asn	LI-F08b	D-Ile	L-*allo*-Ile	D-Gln

D-*allo*-Thr is (2*R*,3*R*)-*allo*-threonine, and L-*allo*-Ile is (2*S*,3*R*)-2-amino-3-methylpentanoic acid.

Figure 1. Structures of LI-F antibiotics.

Table 2. Fragment Ions (*m/z*) on CID Spectra of protonated Molecule of Ring-opened Peptides



R^1 = Me or Et, R^2 = Me, R^3 = Ph(OH), Me, Et or Ph, R^4 = H or Me, n = 1 or 2

Fragmentation	**3c**	**3d**	**4c**	**4d**	**5c**	**5d**	**6c**	**6d**	**7 c**	**7d**	**8c**	**8d**
$[M + H]^+$	965	979	901	915	915	929	915	929	949	963	929	943
$[MH - H_2O]^+$	947 s	961 s	883 s	897 s	897 s	911 s	897 s	911 s	931 s	945 s	911 s	925 s
$[MH–CO_2]^+$	921 s	935 s	857 s	871 s	871 s	885 s	871 s	885 s	905 s	919 s	885 s	899 s
v	894 s	908 s	830 s	844 s	844 s	858 s	844 s	858 s	878 s	892 s	858 s	872 s
(*m/z* 298)	–––	w	w	w	–––	w	–––	w	–––	–––	–––	–––
b_1	399	399	399	399	399	399	399	399 w	399	399	399 w	399
b_2	498	498	498 w	498	498 w	498 w	512	512 w	498	498	512	512
b_3	661	661	597	597	611	611	611	611	645	645	625 w	625
b_4	762	762	698	698	712	712	712	712	746	746	726	726
b_5	876	890	812	826	826	840	826	840	860	874	840	854
y_3	305	319	–––	319	305	319	305 w	319	305	319	305	319
y_4	468	482	404	418	418	432 w	404	418	452	466	418	432
y_5	567	581	503	517	517	531	517	531	551	565	531	545
y_6	666 w	680 w	604 w	618	618 w	632	–––	632 w	650	–––	632 w	646 w

s means strong peak and w is much weaker signal.

L-Val, D-Val and D-Glu by the amino acid analysis and the chiral column HPLC, and the fatty acid to be GHPD (**1a**) by ESI-MS of the complete acid hydrolysate of LI-F04b. The NMR data of LI-F04b were similar to those of fusaricidin B[12] (Table 1). These data indicated LI-F04b to be identical with fusaricidin B (Figure 1). On consideration of biosynthetic analogy of cyclic peptides,[19] optical sequence of the amino acid of LI-F04b may be same as that of LI-F04a. The shapes of CD curve of LI-F04a and -F04b were very similar each other allowing the assignment of the same optical sequence of the amino acids.

LI-F03a and LI-F03b: Structure determination of LI-F03a ($C_{45}H_{74}N_{10}O_{12}$) and LI-F03b ($C_{46}H_{76}N_{10}O_{12}$) was performed on their mixture (LI-F03) due to difficulty of their separation (ratio of LI-F03a and LI-F03b was 3 : 2 by HPLC). Amino acid analysis of LI-F03 and HPLC using the chiral column disclosed the presence of one molar each of D-Val, L-Thr, D-*allo*-Thr, D-Ala, and L-tyrosine (Tyr) and about three-fifth molar of D-Asp and two-fifth molar of D-Glu. Summing up these experimental results and CID MS of each protonated molecules of mixture of ring-opened peptides **3c** and **3d** (Table 2), LI-F03a was considered to be GHPD → Thr → D-Val → L-Tyr → Thr → D-Asn → D-Ala → O- while LI-F03b was a very similar peptide containing D-Gln in place of D-Asn of LI-F03a. The NMR spectra of the LI-F03 mixture (data not shown) were very similar to those of mixture of fusaricidins C and D.[12] Thus, LI-F03a and LI-F03b are considered to be fusaricidin C and fusaricidin D (Figure 1), respectively. KT-6291B (stereochemistry of the amino acids has not been reported)[13] may also be identical with LI-F03a.

LI-F05a and LI-F05b: Amino acid analysis and chiral HPLC analysis established that LI-F05b ($C_{43}H_{78}N_{10}O_{11}$) consisted of L-Thr, D-*allo*-Thr, D-Ala, D-Val, L-isoleucine (Ile) and D-Glu (D-Gln in the original peptide). The lipophilic acid (dehydrated GHPD, **1b**, *m/z* 298 $[M + H]^+$) was again obtained from the EtOAc extract of the acid hydrolysate. ^1H and ^{13}C NMR data of LI-F05b are illustrated in Table 3. The assignments were performed by ^1H-^1H COSY and *J*-resolved 2D spectra to establish the spin-spin network of all amino acid and GHPD residues. The chemical shifts of protons of Val of the antibiotic resembled to those of Val (2) of LI-F04b rather than those of Val (3) suggesting that the residue (2) of LI-F05b is Val. An alkaline hydrolysis of LI-F05b in MeOH-H_2O-28% NH_4OH (4:1:1) pH 9.0 at room temperature for 24 h afforded a water soluble product (**5d**) which yielded a protonated molecule $[M+H]^+$ at *m/z* 929.4 (18 Da larger than that of LI-F05b) in ESI-MS. The sequence of amino acids and **1a** in LI-F05b was established by analysis of the fragment ions in ESI-MS of **5d** assisted by CID-MS. The fragment ions were analyzed as shown in Table 2 establishing GHPD → Thr → Val → Ile → Thr → Gln → AlaOH sequence. This sequence was confirmed by HMBC and NOESY spectra (Figure 2). The β-proton of Thr (1) again resonated at lower field, δ 5.34 indicating an ester linkage from D-Ala (6) to the hydroxyl of Thr (1). These results established the structure of LI-F05b as shown in Figure 1. Thus, it differs presumably from LI-F04b (fusaricidin B) only at amino acid residue (3), where LI-F05b being L-Ile while LI-F04b L-Val. From the consideration of the biosynthetic pathway of these cyclic peptides,[19] the residue (1) was considered to be L-Thr, and the residue (4) D-*allo*-Thr. CD spectrum of LI-F05b exhibited a positive Cotton effect at 219 nm and a negative Cotton effect at 196 nm. Those Cotton effects are similar to those of LI-F04b, which showed a positive Cotton effect at 219 nm and a negative Cotton effect at 199 nm reflecting that they have same stereochemical sequences. From the above data, the structure of LI-F05b was determined as shown in Figure 1. The stereochemistry at C-3 of the side chain remained to

Table 3. NMR Data of Amino Acid Residues of LI-F05a, LI-F05b, LI-F06a and LI-F06b in DMSO-d_6 measured at 50 °C [or 30 °C]

	LI-F05a		LI-F05b			LI-F06a	LI-F06b (30 °C)
position	δ_H (mult. *J* in Hz)		δ_C	δ_H (mult. *J* in Hz)		δ_H (mult. *J* in Hz)	δ_H (mult. *J* in Hz)
L-Thr (1)							
NH	8.10 (d, 8.5)	[8.19]		7.36 (d, 8)	[7.45]	8.09 (d, 8)	7.41 (br)
α-CH	4.41 (dd, 8.5, 2)		56.7	4.43 (dd, 2, 8)		4.42 (dd, 8, 2)	4.45 (br)
β-CH	5.32 (dq, 2, 6)		70.1	5.34 (dq, 2, 6)		5.32 (dq, 2, 7)	~ 5.35 (m)
γ-CH_3	1.15 (d, 6)		16.3	1.17 (d, 6)		1.15 (d, 7)	1.17 (d, 6)
CO			168.0				
Residue (2)		(D-Val)				(D-*allo*-Ile)	
NH	7.15 (d, 9)	[7.29]		7.20 (d, 9)	[7.28]	7.15 (d, 8)	7.25 (br)
α-CH	4.39 (dd, 9, 8)		56.6	4.45 (dd, 9, 7)		4.40 (m)	4.40 (m)
β-CH	1.86 (m)		31.6	1.88 (m)		1.78 (m)	1.92 (m)
γ-CH_3	0.77 (d, 6.5)		19.0	0.75 (d, 7)		0.77 (d, 7)	0.78 (d, 7)
γ-CH_3	0.83 (d, 6.5)		17.7	0.81 (d, 7)	(γ-CH)	———	———
CO			171.0		(γ-CH)	1.46 (m)	1.40 (m)
					(δ-CH_3)	0.83 (t, 6)	0.81 (t, 7)
Residue (3)		(L-Ile)				(L-Val)	
NH	8.27 (d, 7.5)	[8.34]		8.34 (d, 7)	[8.43]	8.27 (d, 5)	8.34 (d, 7)
α-CH	4.22 (t, 7.5)		57.4	4.13 (dd, 7, 8)		4.22 (dd, 5, 7)	4.05 (m)
β-CH	1.78 (m)		35.3	1.80 (m)		1.86 (m)	1.92 (m)
γ-CH_3	0.83 (d, 7)		15.3	0.84 (d, 7)		0.77 (d, 7)	0.76 (d, 7)
γ-CH	1.20 (m)		24.3	1.19 [a]	(γ-CH_3)	0.82 (d, 7)	~ 0.80
γ-CH	1.45 (m)			1.47*			
δ-CH_3	0.81 (t, 7.5)		10.4	0.82 (t, 7)			
CO			172.3				
D-*allo*-Thr (4)							
NH	8.29 (d, 5)	[8.35]		8.28 (d, 7)	[8.37]	8.28 (d, 8)	8.38 (d, 7)
α-CH	3.95 [a]		59.4	4.05 (dd, 7, 6)		3.96 (m)	4.05 (m)
β-CH	3.93 [a]		65.8	3.97 (m)		3.92 (m)	3.93 (m)
β-OH	4.91 (br s)			4.77 (br s) [b]	[4.87]	4.84 (br d, 7)	4.88 (br s)
γ-CH_3	1.11 (d, 6)		19.5	1.12 (d, 6)		1.11 (d, 7)	1.13 (d, 6)
CO			170.3				
Residue (5)	(D-Asn)		(D-Gln)			(D-Asn)	(D-Gln)
NH	7.88 (d, 8)	[8.00]		7.71 (d, 8)	[7.82]	7.89 (d, 8)	7.80 (br)
α-CH	4.29 (m)		52.7	3.98 (m)		4.33 (dd, 8, 7)	3.92 (m)
β-CH	2.56 (dd, 15, 6.5)		26.5	1.97 (m)		2.65 (dd, 7, 14)	1.94 (m)
β-CH	2.74 (dd, 15, 6)			2.22 (m)		2.73 (m)	2.10 (m)
(γ-CH_2)			31.9	2.14 (m)			2.15 (m)
(CO)			170.3				
(δ-CO)			174.2				
NH_2	6.91 (s)	[7.00]		6.75 (s)	[6.84]	6.92 (br)	6.80 (s)
	7.34 (s)	[7.41]		7.21 (s)	[7.26]	7.34 (br)	7.25 (s)
D-Ala (6)							
NH	7.30 (d, 8)	[7.26]		7.44 (d, 7)	[7.45]	7.30 (d, 7)	7.48 (d, 7)
α-CH	3.96 [a]		48.0	4.02 (dq, 7, 7)		3.96 (m)	3.98 (m)
β-CH_3	1.16 (d, 7)		17.0	1.24 [a]		1.20 (d, 7)	———
CO			170.5				

[a] Overlapping with the other signals and assignment with ^{1}H-^{1}H COSY and/or *J*-resolved 2D NMR.

[b] The signal may be exchanged the signal of δ 4.89 assigned as C-3-OH of guanidino side chain.

be determination.

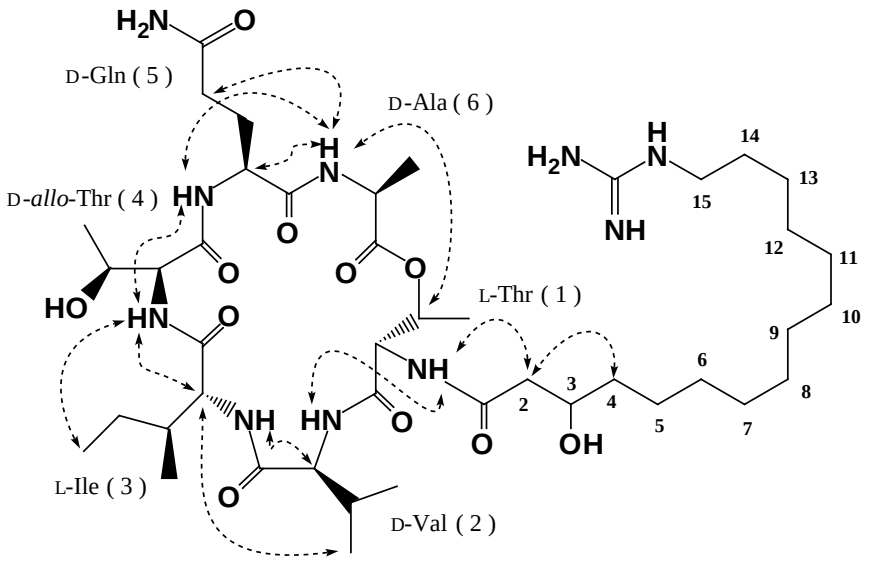


Figure 2. NOE data of LI-F05b in DMSO-d_6 at 30 °C.

On the ^{1}H NMR spectrum of the antibiotic (measured at 50 ° C), a negligible upfiled shift, 0.01 ppm, was observed at the amide proton signal of the Ala (6) when compared with the corresponding signal observed at 30 °C (Table 3). The amide hydrogen may form intramolecular hydrogen-bond.[20, 21]

HR-MALDI-TOF-MS of LI-F05a ($C_{42}H_{76}N_{10}O_{11}$) displayed the protonated molecule $[M + H]^+$ at *m/z* 897.5814 (a CH_2 unit smaller than that of LI-F05b). The amino acid analysis and chiral HPLC disclosed that LI-F05a possessed L-Thr, D-*allo*-Thr, D-Ala, D-Val, L-Ile and D-Asp demonstrating replacement of D-Gln of LI-F05b by D-Asn in LI-F05a. This difference of amino acid reflected the difference of their molecular formulae. The sequence of amino acids and **1a** was unambiguously established by ESI-MS for the ring-opened straight chain peptide of LI-F05a (**5c**). As shown in Table 2, the fragment ions of **5c** and **5d** were completely identical until amino acid residue (4) (b_1 –b_4), but the ions of **5c** were 14 Da smaller than the corresponding ions of **5d** after amino acid residue (5) (b_5), confirming that this amino acid was Asn in LI-F05a while Gln in LI-F05b. The difference of ^{1}H NMR spectra of the LI-F05 pair was similar to that of LI-F04 pair. Nevertheless, the details were different; it might be due to the difference of the volume of the residues (3) and subtle difference of a stable conformation has arisen between them. Combining all above data, the structure of LI-F05a was written as in Figure 1.

LI-F06a and LI-F06b: The constituent amino acids of LI-F06a ($C_{42}H_{76}N_{10}O_{11}$) by amino acid analysis were same as those of LI-F05a. But, the chiralities of Ile (D-*allo*-Ile) and Val (L-form) were different from those of LI-F05a (L-Ile and D-Val). The sequence of **1a** and amino acids of LI-F06a was demonstrated by ESI-MS fragment analysis of its ring-opened peptide (**6c**) produced by alkaline hydrolysis (Table 2). As shown in Figure 1, the sequence is similar to that of LI-F05a, differing in the amino acid residue (2), D-Val, and (3), L-Ile, was replaced by D-*allo*-Ile and L-Val, respectively. CD spectrum of LI-F06a exhibited a positive Cotton effect at 217 nm and a negative Cotton effect at 194 nm indicating a same stereochemical sequence of the amino acids (L- or D-form) as the other components. Thus, the structure of LI-F06a was assigned as shown in Figure 1.

The exact molecular formula of LI-F06b was determined as $C_{43}H_{78}N_{10}O_{11}$ (a CH_2 unit larger than that of LI-F06a) by HR-MALDI-TOF-MS as described above. As expected, the complete hydrolysate of LI-F06b contained D-Glu in place of D-Asp of LI-F06a, and other amino acids and dehydrated GHPD (**1b**) being same for both compounds. The peptide sequence was again confirmed by MS fragmentations of the straight chain compound (**6d**) produced by mild alkaline hydrolysis (Table 2). LI-F06b also showed a positive (219 nm) and a negative Cotton effects (196 nm). From the above data along with ^{1}H NMR data (Table 3), the structure was elucidated as shown in Figure 1.

LI-F07a and LI-F07b: The protonated molecules of LI-F07a ($C_{45}H_{74}N_{10}O_{11}$) and LI-F07b ($C_{46}H_{76}N_{10}O_{11}$) were observed at *m/z* 931.5622 and 945.5817, respectively. This difference again suggested a CH_2 difference of their molecular. The amino acid analysis and the chiral HPLC disclosed that both components contained L-Thr, D-*allo*-Thr, D-Ala, D-Val, and L-Phe in common but D-Asp was observed in LI-F07a while D-Glu in LI-F07b. These results suggested that LI-F07a and LI-F07b were the analogs of LI-F03a and LI-F03b, respectively, having L-Phe in place of L-Tyr of the later two antibiotics. The speculation was substantiated by ESI-MS analysis of their mild alkaline hydrolysis products (**7c** and **7d**). As shown in Table 2, the fragment ions of straight chain peptides were same until amino acid (2), D-Val, but the ions of LI-F07a and LI-F07b peptides were 16 Da lower than those of LI-F03a and LI-F03b peptides, respectively, after amino acid residue (3), indicating Phe at this position for LI-F07 instead of Tyr for LI-F03. From these data, the structures of LI-F07a and -F07b were assigned as shown in Figure 1. On the ^{1}H NMR spectrum of LI-F07b (Table 4), the amide proton of D-Ala (6) was observed at δ 7.40 at 50 °C and at δ 7.35 at 30 °C. The amide proton of D-Val (2) was observed at δ 7.23 measured at both 30 °C and 50 °C. These hydrogens form presumably intramolecular hydrogen-bond as described in LI-F05b.[19,20] The chemical shifts of the protons of the Val (2) appeared at upper field (0.2 – 0.36 ppm) than those of LI-F05b except for the amide proton (Tables 3 and 4). The upfield shifts were presumably caused by the anisotropic effect of aromatic ring of Phe (3), thus, the lipophilic moiety of Val (2) may locate above the aromatic ring of Phe (3). In a molecular model considering the above information, the amide hydrogen of D-Ala (6) locates near the oxygen atom of carbonyl group of D-*allo*-Thr (4) and the NH hydrogen of D-Val (2) exist also near the oxygen atom of 1-carbonyl group of D-Gln (5). The distance between the atoms is less than 3 , respectively. Thus, the intramolecular hydrogen-bonds may exist between these pairs of the group, respectively. A part of molecular model with these hydrogen-bonds was shown in Figure 3: The GHPD group and unshared electron pairs (lone electron pairs) were removed from the final model.

LI-F08a and LI-F08b: The presence of L-Thr, D-*allo*-Thr, D-Ala, D-Ile, L-*allo*-Ile and D-Asn and **1a** was confirmed by acid hydrolysis experiment of LI-F08a ($C_{43}H_{78}N_{10}O_{11}$). As analyzed in Table 2, the sequence of these constituents were determined to be GHPD $\rightarrow$ Thr $\rightarrow$ Ile $\rightarrow$ Ile $\rightarrow$ Thr $\rightarrow$ D-Asn $\rightarrow$ D-AlaOH by the MS fragment ions of its open chain peptide (**8c**) in comparison with those of other components. With consideration of structures of LI-F03a, -F03b, -F04a, -F05a, -F05b, -F06a, -F06b, -F07a and -F07b, LI-F synthetase(s) recognizes exactly the chirality on α-positions of amino acids (2), (3), (5) and (6) and distinguishes probably the chirality of residues (1) and (4). Thus, the residue (2) of LI-F08a was considered to be D-Ile and residue (3) is L-*allo*-Ile. The sequence was supported by the fact

Table 4. ^{1}H NMR Data of Amino Acid Residues of LI-F07a, LI-F07b, LI-F86a and LI-F08b in DMSO-d_6 measured at 50 °C

	LI-F07a		LI-F07b		LI-F08a	LI-F08b
position	δ_C	δ_H (mult. *J* in Hz)	δ_H (mult. *J* in Hz)		δ_H (mult. *J* in Hz)	δ_H (mult. *J* in Hz)
L-Thr (1)						
NH		8.04 (d, 8.5)	7.52 (br d, 8)		8.12 (d, 8)	7.50 (d, 8)
α-CH	56.7	4.42 (dd, 8.5, 2)	4.44 (dd, 7, 2)		4.40 (dd, 8, 2)	4.44 (br d)
β-CH	70.2	5.31 (dq, 2, 6.5)	5.33 (dq, 2, 6)		5.32 (dq, 2, 6)	5.34 (dq, 2, 6.5)
γ-CH_3	16.3	1.13 (d, 6.5)	1.15 (d, 6)		1.14 (d, 6)	1.16 (d, 6.5)
CO	168.6					
Residue (2)		(D-Val)	(D-Val)		(D-Ile)	(D-Ile)
NH		7.22 [a]	7.23 [a]		7.11 (d, 7)	7.16 (d, 7)
α-CH	57.4	4.10 [a]	4.10 [a]		4.49 (dd-like)	4.55 (dd-like)[c]
β-CH	30.8	1.61 (m, dq-like)	1.64 (m, dq-like)		1.79 (m)[c]	1.79 (m)[c]
γ-CH_3	17.9	0.54 (d, 6.5)	0.55 (d, 7)		0.73 (d, 6)	0.71 (d, 6)
γ-CH_3	18.5	0.40 (d, 6.5)	0.45 (d, 7)	(γ-CH)	——— [b]	——— [b]
CO	170.4			(γ-CH)	1.45 (br)[b]	1.39 (br)[c]
				(δ-CH_3)	0.83 (t, 6)	~ 0.82 [b]
Residue (3)	(L-Phe)		(L-Phe)		(L-*allo*-Ile)	(L-*allo*-Ile)
NH		8.40 (d, 8)	8.44 (d, 7)		8.27 (d, 7)	8.37 (d, 7)
α-CH	53.8	4.67 (m)	4.61 (m)		4.21 (t-like)	4.11 (t-like)[c]
β-CH	36.7	2.75 [a]	2.78 (m)		1.66 (m) [c]	1.66 (m)[c]
β-CH		3.01 (dd, 14, 4)	3.01 (dd, 14, 7)	(γ-CH_3)	0.84 (d, 7)	~ 0.83 [b]
H-2',6'	127.9	7.28 (d, 7)	7.28 (d, 7)	(γ-CH)	——— [b]	~ 1.28 (m)[c]
H-3',5'	129.1	7.21 (t, 7)	7.22 (t, 7)	(γ-CH)	1.45 (br)[b]	1.45 (br)[b]
H-4'	126.2	7.14 (t, 7)	7.14 (t, 7)	(δ-CH_3)	0.73 (d, 7)	~ 0.81 [b]
CO	172.1					
D-*allo*-Thr (4)						
NH		8.33 (d, 6)	8.32 (d, 6)		8.30 (d, 7)	8.30 (d, 7)
α-CH	60.2	3.93 (t, 6)	4.00 (t, 6)		3.95 (br)	4.00 (t-like, 7)
β-CH	65.9	3.87 (m)	3.88 (m)		3.95 (br)[b]	3.98 (br)[b]
β-OH		4.91 (br s)	4.93 (br)		4.83 (br s)	4.92 (br s)
γ-CH_3	19.5	1.07 (d, 6.5)	1.05 (d, 6)		1.10 (d, 6)	1.11 (d, 6)
CO	170.4					
Residue (5)		(D-Asn)	(D-Gln)		(D-Asn)	(D-Gln)
NH		8.03 (d, 7)	7.86 (d, 6)		7.90 (d, 8)	7.71 (d, 8)
α-CH	50.4	4.22 (m)	3.93 (m)		4.33 (td-like)	4.06 (t-like)[c]
β-CH	36.1	2.61 (dd, 15, 6)	1.97 (m)		2.61 (dd, 14, 5)	1.98 (m)
β-CH		2.75 [a]	2.15 (m)		2.72 (dd, 14, 5)	2.23 (m)
CO	170.4	(γ-CH_2)	2.15 (m)		(γ-CH_2)	2.15 (m)
γ-CO	172.5					
NH_2		6.88 (s)	6.75 (s)		6.94 (s)	6.75 (s)
		7.35 (s)	~ 7.2 [b]		7.35 (s)	7.21 (s)
D-Ala (6)						
NH		7.35 (d, 7)	7.40 (d, 7)		7.32 (d, 7)	7.38 (d, 7)
α-CH	47.9	4.09 [a]	4.12 [a]		3.95 (br)[b]	3.98 (br)[b]
β-CH_3	17.2	1.18 (d, 7)	1.22 (d, 7)		1.20 (d, 7)	1.21 (d, 7)
CO	170.1					

[a] Overlapping with the other signals and assignment with ^{1}H-^{1}H COSY. [b] Overlapping with the other signals.
[c] The signal may be exchanged with the corresponding proton signal.

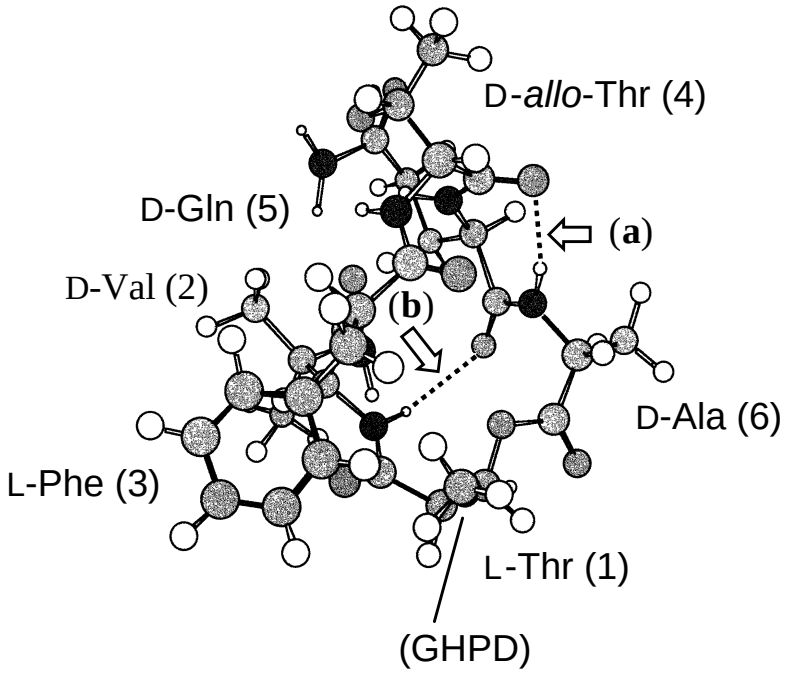


Figure 3. A part of molecular model of a stable conformation of LI-F07b (local minimum calculated with MM2). [Arrows indicate the hydrogen-bonds: (**a**) between D-Ala (6)-NH and D-*allo*-Thr (4)-CO, 1.93 Å, (**b**) between D-Val (2)-NH and D-Gln (5)-CO, 2.28 Å]

that the intact antibiotic LI-F08a exhibited similar Cotton effects as the other components (a positive Cotton effect at 219 nm and a negative Cotton effect at 197 nm). From the data, the structure of LI-F08a was assigned as shown in Figure 1.

HR-MALDI-TOF-MS of LI-F08b ($C_{44}H_{80}N_{10}O_{11}$) indicated a CH_2 unit larger molecule for the antibiotic than that of LI-F08a. Complete acid hydrolysate of LI-F08b contained D-Glu in place of D-Asp of LI-F08a and the other five amino acids and **1b** were common to both antibiotics suggesting the difference of amino acid residue (5), Gln for LI-F08b instead of Asn for LI-F08a, as in all other sub-components of LI-F antibiotics. The sequence was confirmed by the fragment ions of the ring-opened peptide (**8d**) of LI-F08b (Table 2). CD spectrum supported the same stereochemical sequence of amino acids (L- or D-form) of this component to those of other components. From the data the structure of LI-F08b was written as in Figure 1.

Antifungal activity of LI-F antibiotics: Fusaricidins were reported to have strong inhibitory activity against a variety of fungi and Gram-positive bacteria. [11, 12] As described in previous paper,[6] LI-F03, -F04, -F05, -F07 and -F08 complex exhibited a similar activity as fusaricidins. The antifungal activities of isolated single components of LI-F were shown in Table 5. These antibiotics exhibited broad activities against *Candida*, *Cryptococcus* and *Penicillum* strains. Their antifungal activities against three strains of *Aspergillus* were weak. It is interesting that the potency of antifungal activity against *Penicillum chrysogenum* was stronger than that of amphotericin B.

Table 5. Antifungal Spectra of Antibiotics LI-F04a, -F04b, -F05a, -F05b, -F06a, -F06b, -F07a, -F07b, -F08a and -F08b

Organisms tested	MIC (μg/mL)										
	LI-F04a	-F04b	-F05a	-F05b	-F06a	-F06b	-F07a	-F07b	-F08a	-F08b	AMP
Candida albicans ATCC 90028	2.0	2.0	4.0	2.0	8.0	16.0	4.0	2.0	2.0	2.0	0.25
C. albicans ATCC 90029	4.0	4.0	8.0	2.0	4.0	8.0	8.0	4.0	4.0	2.0	0.5
C. albicans ATCC 24433	2.0	2.0	4.0	2.0	2.0	4.0	4.0	2.0	4.0	4.0	0.25
C. tropicalis IFM 40018	2.0	2.0	4.0	4.0	2.0	8.0	2.0	2.0	4.0	2.0	0.25
C. parapsilosis ATCC 90018	2.0	2.0	4.0	2.0	8.0	16.0	8.0	4.0	4.0	4.0	0.5
C. parapsilosis ATCC 22019	1.0	2.0	1.0	1.0	4.0	8.0	4.0	2.0	4.0	2.0	0.5
C. glabrata ATCC 90030	8.0	8.0	4.0	4.0	8.0	16.0	8.0	8.0	8.0	4.0	0.5
C. krusei ATCC 6258	2.0	4.0	8.0	2.0	4.0	16.0	4.0	4.0	2.0	2.0	2.0
Cryptococcus neoformans ATCC 90112	1.0	1.0	1.0	1.0	2.0	8.0	1.0	4.0	1.0	1.0	0.5
C. neoformans ATCC 90113	0.5	1.0	1.0	1.0	1.0	4.0	2.0	2.0	2.0	1.0	1.0
Penicillium expansum IFM 40619	1.0	4.0	4.0	8.0	16.0	8.0	8.0	4.0	4.0	4.0	2.0
Penicillium chrysogenum IFM 40614	4.0	4.0	8.0	8.0	32.0	16.0	16.0	8.0	4.0	8.0	32.0
Aspergillus flavus IFM 41934	16.0	32.0	64.0	32.0	32.0	32.0	64.0	32.0	32.0	32.0	4.0
A. fumigatus IFM 147	32.0	32.0	64.0	32.0	32.0	32.0	64.0	32.0	64.0	64.0	2.0
A. nidulans IFM 41395	16.0	16.0	16.0	16.0	16.0	16.0	32.0	16.0	32.0	16.0	2.0

Micro broth dilution method was according to the method of The National Committee for Clinical Laboratory Standards. Medium: RPMI-1640. Incubation: 2 days (*Candida* and *Cryptococcus*) or 4 days (*Penicillium* and *Aspergillus*) at 35 °C, depending on test strain. Amphotericin B (AMP) was used as a positive control. ATCC: American Type Culture Collection, IFM: collection of former Institute of Food Microbiology, Chiba University.

Discussions

Ten single components have successfully been isolated from the LI-F antibiotic complex by use of extensive preparative HPLC and their structures determined by spectrometric and chemical procedures. They are cyclic depsipeptides constructed of six amino acid-peptide nucleus and a unique 15-guanidino-3-hydroxypentadecanoic acid side chain. Among the six amino acids, amino acid residues (1), (4) and (6) are L-Thr, D-*allo*-Thr and D-Ala, respectively, in common to all the components. The components also share the same side chain fatty acid. These amino acid and guanidino-fatty acid residues, therefore, seem to be essential for their biological activity. The amino acid residue (2) was found to be D-series lipophilic aliphatic amino acid, D-Val, D-Ile or D-*allo*-Ile. Amino acid residue (3) is the most variable, Val, Ile, *allo*-Ile, Phe, or Tyr depending on the components, but their stereochemistry is always L-form. Amino acid (5) is D-Asn in all LI-F a sub-group, the later eluting sub-series of each component in HPLC, while it is D-Gln in all LI-F b sub-group, the faster eluting sub-series. On the LI-F syntheases, the binding sites (pocket) of residue (1), (4) and (6) may be rigid. On the other hand, the binding sites of amino acids (2) and (5) may be flexible and that of (3) may be more flexible than those of (2) and (5). Nevertheless, stereochemical orders of the amino acids are L (amino acid 1), D (2), L (3), D (4), D (5) and D (6) in all LI-F components, which were reflected in their high similarity of CD curves. Upon this structure determination, LI-F03a, LI-F03b, LI-F04a and LI-F04b are considered to be identical with fusaricidins C, D, A and B, respectively, although a direct comparison has not been performed yet. Detail biological evaluation of the isolated single components of LI-F antibiotics is in progress and will be reported in a separate paper.

Their interesting synergistic antifungal effect in combination with the azole group antifungal agents is also under evaluation.

EXPERIMENTAL

Commercially available reagents purchased from Wako Pure Chemical Industries (Osaka, Japan), Tokyo Kasei Kogyo (Tokyo), Merck (Dramstadt, Germany), Sigma (St. Louis, MO, USA), Aldrich (Milwaukee, WI) and J.T. Baker (Phillipsburg, NJ) were used without further purification. Waters 600E System (Waters Co., Milford, MA, USA) equipped Chromatocorder 21 Integrator (System Instrument Co., Hachioji, Japan) were used for HPLC analyses and preparative HPLC. Cosmosil 5C_{18} column (10 × 250 mm) obtained from Nacalai tesque (Kyoto, Japan) and Pegasil ODS (10 × 250 mm) from Senshu Science Co. (Tokyo) were used for preparative HPLC. Sumichiral OA-5000 column (4.6 × 150 mm) purchased from Sumitomo Chem. Anal. Service Ltd. (Osaka) was used for chiral HPLC analyses. Amino acid analyses were performed with a Hitachi L-8500 amino acid auto-analyzer (Hitachi Ltd., Akishima, Japan). All NMR data were recorded on JEOL-alpha-500 or EX-400 NMR spectrometers (Jeol Ltd., Tokyo) at 30 °C or 50 °C in DMSO-d_6. As ^{1}H NMR chemical shift reference, the center signal of DMSO-d_6 at δ 2.50, and as ^{13}C NMR reference, the methyl signal of DMSO-d_6 at δ 39.5 were used. CD spectra were measured on JASCO J-720 instrument (Jasco Co., Hachioji, Japan) in acetonitrile-H_2O (1:1) at 25 °C. The optical rotations were measured on JASCO DIP-370-Digital Polarimeter in MeOH at 25 °C. ESI MS spectra were measured with Finigan LCQ^{TM} LC/MS^n System (ThermoQuest Inst., Riviera, FL, USA), and flow rate was 10 mL/min, capillary temperature; 200 °C, capillary voltage; 7.00 V. All MALDI MS spectra were obtained on a Voyager-DE STR (PerSeptive Biosystems, Framingham, MA, USA) TOF MS spectrometer equipped with a nitrogen laser (337 nm), TDS 540C 500 MHz digitizing oscilloscope (500 MHz bandwidth, 2G samples/s, Tektronix inc., Beaverton, OR), PerSeptive Biospectrometry Workstation (Version 3.09) and PerSeptive GRAMS/386 for Microsoft Windows (Version 3.04 Level III, Driver Version 1.00, Galactic Industries Corp., Salem, NH). α-Cyano-4-hydroxycinnamic acid was used as the matrix. Calibration was performed with two internal standards, des-Arg(9),Leu(8)-bradykinin and des-Pro(2)-bradykinin or angiotensin II ($[M + H]^+$) except in the measurements of LI-F03a and -F03b (the data were already reported[14]). The details of the measurement of HR-MS were reported in our recent paper.[14] In the experiments, absolute MS error of angiotensin II, $[M + H]^+$, was – 2.1 mDa and 95.5% confidence level ($\pm 2\sigma$) of the ion was ± 6.6 mDa. Molecular models were calculated with Chem3D (CambridgeSoft, Cambridge, MA) equipped with MM2 and MOPAC 97. First model of peptide core was built with D- and L-Ala, L-Thr and 3-hydroxybutyric acid and then calculated with the MOPAC. The build-up of molecular model of LI-F07b was made on the above model with additions of each functional group on amino acids (2) – (5) and the side chain. The molecular dynamics of the LI-F07b model was run with MM2 (target temperature, 1000 K) and the suitable conformations fitted with the NMR data were picked up from the calculated models. The molecular dynamics was calculated further on the conformation with MM2 (target temperature, 300 K) and then two hydrogen-bonds were added on the model. The minimize energy of the model (local minimum) was calculated with the software (Figure 3).

Isolation of LI-F complex and primary separation of the components: The crude antibiotic complex (LI-F) was prepared following the method reported previously.[6] The complex was first separated to six fractions by the primary reversed phase preparative HPLC. The chromatography was performed using Cosmosil 5C_{18} with 33% aqueous acetonitrile containing 0.05% trifluoroacetic acid (TFA) as a developing solvent at flow rate of 1 mL/min and UV detection at 215 nm. Nine peaks were observed in the HPLC and they were collected in six fractions, Fraction A (Retention time (t_R) = 10 –13min): mixture of LI-F03a and-F03b(50mg), B (t_R = 13 – 17 min): mixture of LI-F04a and -F04b (120 mg), C (t_R = 20 – 25 min): mixture of

LI-F05a, -F05b, -F06a and -F06b (32 mg), D (t_R = 28 −32 min): mixture of LI-F07a and -F07b (25 mg), E (t_R = 33−38 min): mixture of LI-F08a and -F08b (12 mg) and F (t_R = 45 − 55 min, 15 mg).

Purification of LI-F components: A mixture of four to one in the ratio of LI-F03a and -F03b (ESI-MS analysis) was obtained from the Fraction A by repetition of the HPLC with cutting out of first eluent of the LI-F03 peak. Nevertheless, a mixture consisting mainly of LI-F03b was not obtained. LI-F04a (10 mg) and LI-F04b (7 mg) were isolated from 50 mg of the Fraction B by repeated HPLC on Pegasil ODS eluting with 32% acetonitrile in 0.1% TFA at flow rate of 2.5 mL/min and UV detection at 215 nm. The components in the Fraction C (50 mg) were separated by the same HPLC system with small change of the eluent (addition of 5 mL of MeOH in one liter of the above eluent) to isolate LI-F05a (5 mg), LI-F05b (3 mg), LI-F06a (2.5 mg) and LI-F06b (2 mg). Similarly, the Fraction D (15 mg) was purified by repetition of preparative HPLC using 34% acetonitrile in 0.1% TFA to yield LI-F07a (3 mg) and LI-F07b (2 mg). LI-F08a (1.5 mg) and LI-F08b (1 mg) were obtained from 10 mg of the Fraction E by a similar preparative HPLC using 35% acetonitrile in 0.1% TFA as mobile phase. The Fraction F was shown to contain more than four components by the analytical HPLC but their separation was not carried out in this study because of the small sample amount.

General characteristics of LI-F components: All isolated components of LI-F antibiotics are soluble in aqueous lower alcohols and dimethyl sulfoxide, slightly soluble in water, methanol, ethanol and acetonitrile but insoluble in other organic solvents such as chloroform, EtOAc and acetone. These compounds were positive to the iodine, Sakaguchi and biuret reactions but showed negative response to ninhydrin, Ehrlich s, Fehling s and Molish reagents.

Amino acid analysis: A mixture of each peptide (0.5 mg) and 6 mol/L HCl (0.5 mL) in a sealed tube was heated at 110 °C for 24 h. The reaction solution was then evaporated *in vacuo* and the residue was partitioned between EtOAc and H_2O. The H_2O layer was evaporated, and the residue was dissolved in 0.5 mL of 0.02 mol/L HCl. Ten μL of the solution was used for amino acid analysis. The following solvents were used for the chiral HPLC analysis of the amino acids: A mixture of 2 mmol/L $Cu(AcO)_2$ solution and H_2O (85:15) was used for Phe, Tyr, Asp and Glu, and 1.0 mmol/L $CuSO_4$ solution for Ala, Val, Ile and *allo*-Ile.

Partial hydrolysis of LI-F04a (formation of 2a and 2b): A mixture of LI-F04a (1.5 mg) and 6 mol/L (0.5 mL) was heated at 50 °C for 8 h in a sealed tube. The reaction mixture was treated as usual and the residue was separated with preparative TLC [Kiesel gel 60F-254, developing solvent: 2-BuOH-AcOH-H_2O (3:1:1)] to give 8 peptide fragments. Each peptide fragment was purified by Speedisk® Column, PolarPlus® Octadecyl C_{18} (J.T. Baker), eluent; H_2O-MeOH (1:1), and analyzed by ESI-MS. Further experiments were not done except **2a** and **2b**.

Test organism: The fungal strains (IFM) were derived from the Culture Collection of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan. Yeasts were maintained on modified Sabouraud dextrose agar slant (2% glucose, 1% peptone and 1.5% agar, SDA) or potato dextrose agar (Difco Laboratories, Detroit, MI) slant (PDA). For filamentous fungi were also maintained on Potato dextrose agar slants (Difco Laboratories).

Antifungal activity testing: Antifungal activity were estimated by a broth microdilution method with RPMI 1640 medium (Life Technologies, INC., New York, NY) with L-Gln, without sodium bicarbonate, and buffered with 0.165 mol/L 3-morpholinopropanesulfonic acid (MOPS). The pH of the medium was adjusted to 7.0 with 6 mol/L NaOH.[22] All compounds were dissolved in DMSO. The final concentration of solvents was adjusted to less than 1%. The compounds at a concentration of 12.8 mg/mL in DMSO was diluted with RPMI-1640 medium with a serial two-fold dilution at the range from 0.125 to 128.0 μg/mL. Twenty μL of each dilution was added to 0.16 mL of the RPMI-1640 medium in disposable multiple-well plates (96-well flat-bottom plates, Iwaki Glass Co. Ltd., Tokyo, Japan). Fungi inocula were prepared as followed. The fresh cultures for

yeasts were grown on SDA at 35 °C for 48 h. The yeast cells were suspended in sterile 0.85% saline and were adjusted to suspensions containing 0.5×10^4 to 2.5×10^4 yeast cells/mL. Filamentous fungi were prepared from a mature culture grown at 30 °C for 1 to 2 weeks on potato dextrose agar (Difco Laboratories). Fungal slants were covered with 5 mL of sterile 0.85% saline containing 0.05% Tween 80 and gently probed with a sterile wire loop. Heavy particles were filtrated with funnel with fritted disc (pore size: 40 – 100 μm), and the filter layer were diluted with sterile 0.85% saline and were then adjusted to suspension containing 0.5×10^4 to 2.5×10^4 cells/mL. Each well was inoculated with 20 μL of the homogeneous suspensions (final inoculum, 0.5×10^3 to 2.5×10^3 cells/mL). The final volume in each well was 200 μL. The trays were incubation at 35 °C. Broth microdilution endpoints were determined after 1 or 4 days of incubation with the aid of a plate analyzer (Toyo Sokki Co. Ltd., Sagamihara, Japan). The MIC was the lowest drug concentration which reduce the optical density at 630 nm by 80% compared with the optical density at 630 nm of the drug-free control.

LI-F03a and -F03b (3:2 mixture): $[\alpha]_D$ + 68° (*c* 0.2). CD (*c* 3.0×10^{-5} mol/L) [θ] (nm): ± 0 (192), – 11800 (198), ± 0 (207), + 5700 (229), ± 0 (241).

LI-F04a: HRMS: Found, *m/z* 883.5643 $[M + H]^+$, Calcd for $C_{41}H_{75}N_{10}O_{11}$, *m/z* 883.5617. $[\alpha]_D$ + 24° (*c* 0.3). CD (*c* 6.1×10^{-5} mol/L) [θ] (nm): ± 0 (194), – 21200 (199), ± 0 (208), + 7100 (219), ± 0 (243). ^{1}H NMR: (500 MHz, DMSO-d_6): side chain, δ 2.39 and 2.46 (each 1H, dd, *J* = 7 and 14 Hz, 2-H_2), 3.84 (1H, br, 3-H), 4.83 (1H, d, *J* = 7 Hz, 3-OH), 1.40 (2H, br, 4-H_2), 1.25 (18H, br, 5 ~ 13-H_2), 1.47 (2H, m, 14-H_2) and 3.08 (2H, td, *J* = 7 and 6 Hz, 15-H_2), 7.42 (NH, overlapping with Asn-NH), ^{13}C NMR: δ 171.9 (C-1), 43.0 (C-2), 67.5 (C-3), 36.7 (C-4), 25.2 (C-5), 28.5, 29.8 and 29.0 (C-6 ~ C-12), 26.0 (C-13), 28.3 (C-14), 40.6 (C-15), 156.7 (C-16).

LI-F04b: HRMS: Found, *m/z* 897.5779 $[M + H]^+$, Calcd for $C_{42}H_{77}N_{10}O_{11}$, *m/z* 897.5773. $[\alpha]_D$ + 23° (*c* 0.3). CD (*c* 6.1×10^{-5} mol/L) [θ] (nm): ± 0 (194), – 10000 (199), ± 0 (207), + 4100 (219), ± 0 (245). ^{1}H NMR (500 MHz, DMSO-d_6): side chain, δ 2.39[2H, AMM' -type (q-like), 2-H_2], 3.83 (1H, br s, 3-H), 4.74 (1H, d, *J* = 7 Hz, 3-OH), 1.38 (2H, br s, 4-H_2), 1.25 (18H, br s, 5~13-H_2), 1.46 (2H, t-like, 14-H_2), 3.08 (2H, td, *J* = 7, 6 Hz, 15-H_2), 7.42 (1H, br, -NH); ^{13}C NMR: δ 171.8 (C-1), 43.1 (C-2), 67.4 (C-3), 36.5 (C-4), 25.0 (C-5), 28.5~28.8 (C-6~C-12), 25.8 (C-13), 28.2 (C-14), 40.6 (C-15), 156.7 (C-16).

LI-F05a: HRMS: Found, *m/z* 897.5814 $[M + H]^+$, Calcd for $C_{42}H_{77}N_{10}O_{11}$: 897.5773. $[\alpha]_D$ + 36° (*c* 0.3). CD (*c* 6.1×10^{-5} mol/L) [θ] (nm): ± 0 (193), – 11400 (198), ± 0 (211), + 1500 (219), ± 0 (233). ^{1}H NMR (500 MHz, DMSO-d_6): side chain, δ 2.32 (1H, dd, *J* = 5, 14 Hz, 2-H), 2.38 (1H, dd, *J* = 7, 14 Hz, 2-H), 3.83 (1H, br s, 3-H), 4.90 (1H, br, 3-OH), 1.38 (2H, br s, 4-H_2), 1.23 (18H, br s, 5~13-H_2), 1.45 (2H, t-like, 14-H_2), 3.08 (2H, td, *J* = 7, 6 Hz, 15-H_2), 7.67 (1H, br, -NH).

LI-F05b: HRMS: Found, *m/z* 911.5989 $[M + H]^+$, Calcd for $C_{43}H_{79}N_{10}O_{11}$: *m/z* 911.5930. $[\alpha]_D$ + 31° (*c* 0.4). CD (*c* 6.1×10^{-5} mol/L) [θ] (nm): ± 0 (193), – 9800 (196), ± 0 (206), + 2100 (219), ± 0 (242). ^{1}H NMR (500 MHz, DMSO-d_6): side chain, δ 2.38 (1H, m, 2-H), 2.45 (1H, dd, *J* = 5, 13 Hz, 2-H), 3.80 (1H, br s, 3-H), 4.77 (1H, br, 3-OH), 1.40 (2H, br s, 4-H_2), 1.25 (18H, br s, 5~13-H_2), 1.46 (2H, t-like, 14-H_2), 3.08 (2H, td, *J* = 7, 6 Hz, 15-H_2), 7.38 (-NH, overlapping with Thr -NH); ^{13}C NMR: δ 171.9 (C-1), 43.3 (C-2), 67.5 (C-3), 36.7 (C-4), 25.2 (C-5), 28.5~29.0 (C-6~C-12), 26.0 (C-13), 28.3 (C-14), 40.6 (C-15), 156.7 (C-16).

LI-F06a: HRMS: Found: *m/z* 897.5818 $[M + H]^+$, Calcd for $C_{42}H_{77}N_{10}O_{11}$: *m/z* 897.5773. $[\alpha]_D$ + 52° (*c* 0.3). CD (*c* 6.1×10^{-5} mol/L) [θ] (nm): ± 0 (192), – 6300 (194), ± 0 (210), + 2600 (217), ± 0 (238). ^{1}H NMR (500 MHz, DMSO-d_6): side chain, δ 2.41 (2H, m, 2-H_2), 3.80 (1H, br s, 3-H), 4.79 (1H, d, *J* = 7 Hz, 3-OH), 1.39 (2H, br s, 4-H_2), 1.25 (18H, br s, 5~13-H_2), 1.46 (2H, t-like, 14-H_2), 3.08 (2H, td, *J* = 7, 6 Hz, 15-H_2), 7.15 (1H, br, -NH).

LI-F06b: HRMS: Found*: m/z* 911.5931 $[M + H]^+$, Calcd for $C_{43}H_{79}N_{10}O_{11}$, 911.5930. $[\alpha]_D$ + 50° (*c* 0.3). CD (*c* 6.1×10^{-5} mol/L) [θ] (nm): ± 0 (192), – 11100 (196), ± 0 (205), + 2800 (219), ± 0 (238). ^{1}H NMR (500

MHz, DMSO-d_6): side chain, δ 2.37 (2H, m, 2-H_2), 3.79 (1H, br s, 3-H), 4.82 (1H, br, 3-OH), 1.37 (2H, br s, 4-H_2), 1.24 (18H, br s, 5~13-H_2), 1.46 (2H, t-like, 14-H_2), 3.07 (2H, td, J = 7, 6 Hz, 15-H_2), 7.15 (1H, br, -NH).

LI-F07a: HRMS: Found: *m/z* 931.5622 $[M + H]^+$, Calcd for $C_{45}H_{75}N_{10}O_{11}$: 931.5617. $[\alpha]_D$ + 61° (*c* 0.3). CD (*c* 3.0 × 10^{-5} mol/L) [θ] (nm): ± 0 (192), − 8100 (197), ± 0 (208), + 10900 (224), ± 0 (245). ^{1}H NMR (500 MHz, DMSO-d_6): side chain, δ 2.38 (2H, AMM -type, 2-H_2), 3.80 (1H, br s, 3-H), 1.35 (2H, br s, 4-H_2), 4.81 (1H, br, 3-OH), 1.23 (18H, br s, 5~13-H_2), 1.45 (2H, t-like, 14-H_2), 3.07 (2H, td, J = 7, 6 Hz, 15-H_2), 7.44 (H, br, -NH).

LI-F07b: HRMS: Found: *m/z* 945.5817 $[M + H]^+$, Calcd for $C_{46}H_{77}N_{10}O_{11}$, 945.5773. $[\alpha]_D$ + 61° (*c* 0.2). CD (*c* 6.1 × 10^{-5}mol/L) [θ] (nm): ± 0 (198), — 7200 (201), ± 0 (208), + 8800 (221), ± 0 (241).^{1}H NMR (500 MHz, DMSO-d_6): side chain, δ 2.37 (2H, AMM' -type, 2-H_2), 3.80 (1H, br s, 3-H), 4.82 (1H, br, 3-OH), 1.37 (2H, br s, 4-H_2), 1.24 (18H, br s, 5~13-H_2), 1.45 (2H, t-like, 14-H_2), 3.07 (2H, td, J = 7, 6 Hz, 15-H_2), 7.52 (1H, br, -NH); (500 MHz, DMSO-d_6, 30 °C): amide protons, δ 7.23 (1H, br, D-Val), 7.35 (1H, d, J = 7 Hz, D-Ala), 7.60 (1H, d, J = 8 Hz, L-Thr), 8.02 (1H, br, D-Gln), 8.42 (1H, d, J = 6 Hz, D-*allo*-Thr), 8.53 (1H, d, J = 7 Hz, L-Phe).

LI-F08a: HRMS: Found, *m/z* 911.5931 $[M + H]^+$, Calcd for $C_{43}H_{79}N_{10}O_{11}$, 911.5930. $[\alpha]_D$ + 87° (*c* 0.2). CD (*c* 6.1 × 10^{-5} mol/L) [θ] (nm): ± 0 (193), − 10400 (197), ± 0 (210), + 1300 (219), ± 0 (233). ^{1}H NMR (400 MHz, DMSO-d_6): side chain, δ 2.38 (2H, AMM' -type, 2-H_2), 3.80 (1H, br s, 3-H), 4.82 (1H, br, 3-OH), 1.38 (2H, br s, 4-H_2), 1.24 (18H, br s, 5~13-H_2), 1.45 (2H, t-like, 14-H_2), 3.08 (2H, br, 15-H_2), 7.35 (1H, br, -NH).

LI-F08b: HRMS: Found, *m/z* 925.6103 $[M + H]^+$, Calcd for $C_{44}H_{81}N_{10}O_{11}$, 925.6086. $[\alpha]_D$ + 71° (*c* 0.2). CD (*c* 6.1 × 10^{-5} mol/L) [θ] (nm): ± 0 (193), − 22400 (199), ± 0 (210), + 3700 (218), ± 0 (233). ^{1}H NMR (400 MHz, DMSO-d_6): side chain, δ 2.38 (2H, AMM' -type, 2-H_2), 3.79 (1H, br s, 3-H), 4.77 (1H, br, 3-OH), 1.38 (2H, br s, 4-H_2), 1.25 (18H, br s, 5~13-H_2), 1.45 (2H, t-like, 14-H_2), 3.07 (2H, d-like, J = 6 Hz, 15-H_2), 7.38 (1H, br, -NH).

ACKNOWLEGMENT

This study was partly supported by Cooperative Research Program of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University (93 − 03). We are grateful to Mr. M. Uruno for his technical assistance.

REFERENCES AND NOTES

1. a) H. F. Chambers and M. A. Sande, Goodman & Gilman s The Pharmacological Basis of Therapeutics, 9th ed., ed. by J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, and A. G. Gilman, McGraw-Hill, New York, 1996, pp. 1029 −1056; b) J. E. Bennett, *ibid*., pp. 1175 − 1190.
2. E. Mutschler, H. Derendorf, M. Sch fer-Korting, K. Elrod, and K. S. Ester, Drug Action: Basic Principles and Therapeutic Aspects, medpharm Scientific Publisher, Stuttgart, 1995, pp. 70 −79, 559 −564.
3. G. F. Brooks, J. S. Butel, S. A. Morse, R. Nairm, T. G. Mitchell, and D. Heyneman, Jawetz, Melnick, & Adelberg s Medical Microbiology, 21st ed., Appleton & Lange, Stamford, 1998, pp. 1 − 6, 583 − 616.
4. E. Eberle, Iryo-jujisha no tameno AIDS (in Japanese, The AIDS for the medical Workers) , ed. by J. Tadano, M. Sano, and E. Inoue, Nanzan-do, Tokyo, 1995, pp. 17 − 56.
5. Authority of the United States Pharmacopeial Convention Inc., Advice for the Patient, Drug Information in lay language, Vol. II , U.S. Pharmacopeial Convention Inc., Rockville, 1992, pp. 74 −77.
6. K. Kurusu, K. Ohba, T. Arai, and K. Fukushima, *J. Antibiotics*, 1987, **40**, 1506.

7. J. Uno, unpublished data.
8. J. Uno, M. L. Shigematsu, and T. Arai, *Antimicrob. Agents Chemother.*, 1983, **24**, 552.
9. J. Uno, *Jpn. J. Med. Mycol.*, 1985, **26**, 133.
10. K. Takesako, T. Beppu, T. Nakamura, and A. Obayashi, *J. Antibiotics*, 1985, **38**, 1363.
11. K. Kajimura and M. Kaneda, *J. Antibiotics*, 1996, **49**, 129.
12. K. Kajimura and M. Kaneda, *J. Antibiotics*, 1997, **50**, 220.
13. Y. Takayama, S. Yamada, M. Yamanaka, T. Koyama, T. Mori, M. Ozawa, and H. Asami, (Mitsubishi Petrochemical Co., Ltd.), **Jpn. Kokai Tokkyo Koho 1990**, **JP 02275898 A2**, (*Chem. Abstr.*, 1991, **115**, 66784).
14. T. Fukai, J. Kuroda, and T. Nomura, *J. Am. Soc. Mass Spectrom.*, 2000, **11**, 458.
15. CID of protonated molecule with the ion trap ESI-MS tend to give only a few informative fragment ions such as $[y_\omega]^+$, $[y_{\omega-1}]^+$, $[b_\omega]^+$ and $[b_{\omega-1}]^+$. Nevertheless, weak fragment ion peaks were detected in the measurements here and useful for determination of the sequence of amino acids. Similar informative weak fragment peaks were already reported in the case of MALDI MS.[16] The details of the weak fragment ion peaks of the ESI-MS will be report elsewhere.
16. M. Takayama and A. Tsugita, *Int. J. Mass Spectrom. Ion Processes* 1998, **181**, L1 and references cited therein.
17. Roepstorff, *Biomed. Mass Spectrom.*, 1984, **11**, 601; K. Biemann,, *Biomed. Environ. Mass Spectrom.*, 1988, **16**, 99; R. S. Johnson, S. A. Martin, and K. Biemann, *Int. J. Mass Spectrom. Ion Processes*, 1988, **86**, 137.
18. The intensities of the $[M + H]^+$ and $[M]^+$ peaks were almost same, but the base peak was $[M]^+$.
19. V. Kasche and G. Michaelis, Biochemistry of Peptide Antibiotics: recent advances in the biotechnology of β-lactams and microbial bioactive peptides , ed. by H. Kleinkauf and K. von D hren, de Gruyter, Berlin, 1990, pp. 81 −101; H. GFliri and R. M. Wenger, *ibid.*, pp. 245 −287; Y. Okumura, *ibid.*, pp. 365 −378; E. J.Vandamme, *ibid.*, pp. 379 −410.
20. Y. Kyogoku and K. Kawano, Protein, Nucleic Acid and Enzyme, Separate volume (in Japanese), 76:5", ed. by H. Umezawa, T. Shiba, and T. Takida, Kyoritu-Shuppan, Tokyo, 1976, pp. 281 −298.
21. T. Fukai, J. Kuroda, M. Konishi, T. Nomura, N. Naruse, and R. Isobe, *J. Antibiotics*, 1995, **48**, 1115.
22. National Committee for Clinical Laboratory Standards, 1997. Reference method for broth dilution antifungal susceptibility testing for yeasts: proposed standard. Document M27-A. National Committee for Clinical Laboratory Standards, Wayne, PA.