Fungal Metabolites. XV.[1] Primary Structures of Antibiotic Peptides, Hypelcins B-I, B-II, B-III, B-IV and B-V, from *Hypocrea peltata*. Application of Electrospray Mass Spectrometry and Electrospray Mass Spectrometry/Mass Spectrometry

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Hypelcin B is a mixture of antibiotic peptides produced by *Hypocrea peltata*. Hypelcins B-I, B-II, B-III, B-IV and B-V are components of this mixture purified by reversed-phase high-performance liquid chromatography. The amino acid sequences of these peptides were determined by electrospray mass spectrometry and electrospray mass spectrometry/mass spectrometry. The molecular weights of these peptides were all *ca.* 2000 and the structures were very similar.

Keywords *Hypocrea peltata*; peptaibol; hypelcin; α-aminoisobutyric acid; isovaline

Hypelcins A and B,[2,3] isolated from *Hypocrea peltata* (Jungh) SACC., prevent the growth of *Lentinus edodes*, a Japanese edible mushroom. Hypelcins belong to the class of peptaibols[4,5] having the following structural features: rich in helix-promoting α-aminoisobutyric acid (Aib), having an amino alcohol, leucinol (Lol) or isoleucinol (Iol), at the C-terminal, and acetylated at the N-terminal. Peptaibols, which can also be regarded as alamethicin[6] analogs, show membrane-modifying properties which give rise to various bioactivities such as formation of voltage-gated ion channels,[7,8] hemolysis,[9] and influx of Ca^{2+} into adrenal chromaffin cells.[10] Hypelcins, which inhibit growth of various fungi and bacteria,[2] have been shown to uncouple oxidative phosphorylation in rat liver mitochondria[11] and to induce change of permeability in phosphatidylcholine bilayers.[12,13]

We previously described the isolation and the structural elucidation of hypelcins A-I—IX.[14] Recently we have found that hypelcin B is a mixture of more than five components on HPLC, though it gave only a single spot on silica gel TLC. The structures of these peptides were elucidated by electrospray mass spectrometry (ES-MS) and electrospray mass spectrometry/mass spectrometry (ES-MS/MS).[15] In this paper, we report the isolation and the structural elucidation of hypelcins B-I—V.

Results and Discussion

Separation of Hypelcins B-I, B-II, B-III, B-IV and B-V Figure 1 shows the HPLC analysis of crude hypelcin B,[3] indicating the presence of hypelcins B-I—V along with minor components. Crude hypelcin B was separated by repeated semi-preparative HPLC using a reversed-phase octadecyl silica (ODS) column to afford hypelcins B-I—V, which appeared to be pure. However, hypelcins B-III, B-IV and B-V were contaminated with small amounts of other peptides as judged from the results of amino acid analysis. Nevertheless, these compounds were sufficiently pure that their structures could be elucidated by using the ES-MS/MS technique.

Characterization of Hypelcins B-I, B-II, B-III, B-IV and B-V Amino acid proportions of the peptides were determined by amino acid analysis of the total acid hydrolysates, except for Aib and isovaline (Iva), because of their low sensitivity in the ninhydrin reaction. The presence of Aib and Iva was confirmed by HPLC with a chiral ligand-exchange-phase column. The numbers of these two amino acids were determined by MS/MS, in which Aib and Iva gave mass units of 85 and 99 amu, respectively. The absolute configurations of optically active amino acids were determined by HPLC with the above chiral column. The results showed that Iva has D configuration while the other amino acids have L configuration. On the other hand, for the identification and configuration analysis of the amino alcohols, Lol and Iol,

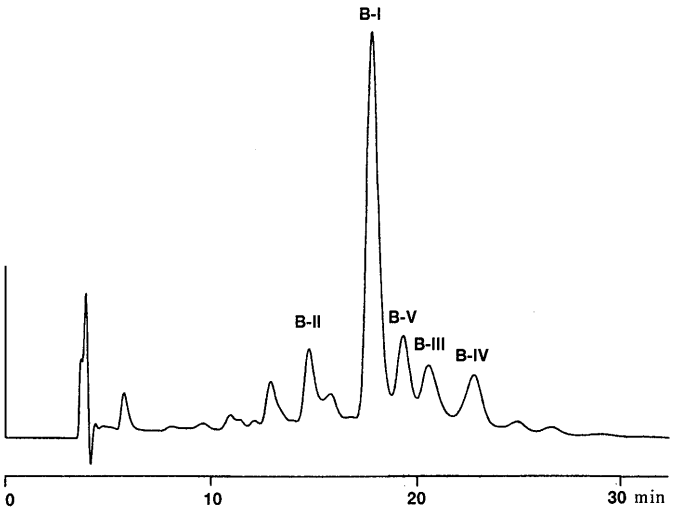

Fig. 1. HPLC Chromatogram of Hypelcin B

Analytical conditions: eluent, MeOH–water–AcOH (85:15:0.1, v/v); column, M & S Pack C18 (4.6 mm i.d. × 150 mm); flow rate, 0.5 ml/min; detector, UV (220 nm).

TABLE I. Amino Acid Proportions for Hypelcin Bs

Hypelcin	Gly	Ala	Aib[a]	Val	Iva[a]	Leu	Ile	Pro	Glu	Lol[b]	Iol[b]
B-I	1.00 (1)	1.07 (1)	10	1.01 (1)		0.94 (1)		1.92 (2)	2.98 (3)	1	
B-II	1.00 (1)	1.89 (2)	9	1.01 (1)		0.98 (1)		2.10 (2)	3.03 (3)	1	
B-III	1.00 (1)	1.01 (1)	9	1.01 (1)	1	0.89 (1)	0.07	1.93 (2)	3.01 (3)	1	
B-IV	1.00 (1)	1.06 (1)	10	1.00 (1)		0.21	0.68 (1)	1.94 (2)	3.00 (3)	1	
B-V	1.00 (1)	1.05 (1)	10	1.03 (1)		0.90 (1)	0.07	2.06 (2)	3.00 (3)		1

a) Determined from the mass spectra and by HPLC. *b*) Determined from the mass spectra and by GC.

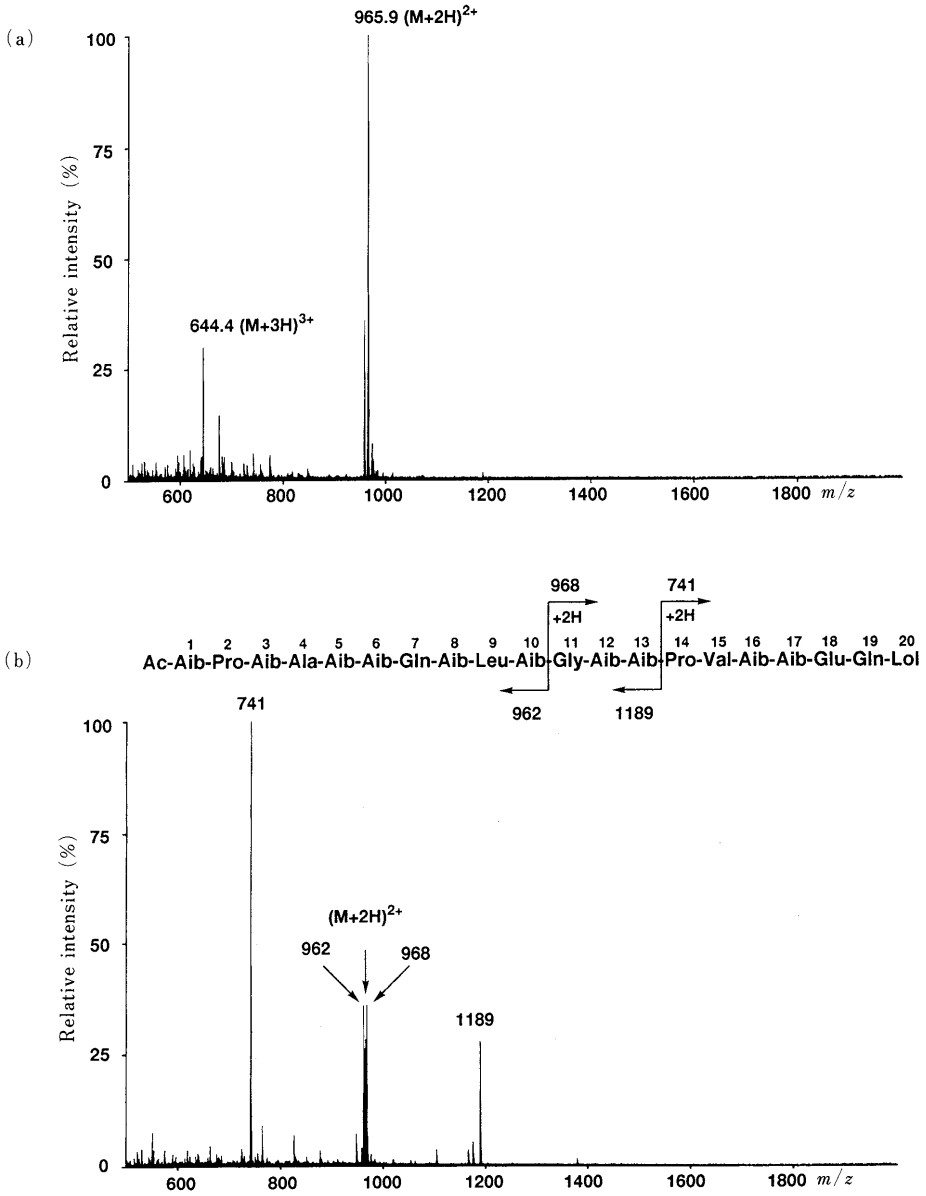

Fig. 2. Electrospray Mass Spectrum of Hypelcin B-I

(a) Orifice voltage, 70 V; (b) orifice voltage, 140 V.

acid hydrolysates were first converted into the *N,O*-ditrifluoroacetyl derivative and then subjected to GC using a chiral capillary column. The identity and configuration (L) of the amino alcohols were determined by comparison with standard samples. The acetyl group (δ_H: *ca.* 2.3 ppm) was identified by ^{1}H-NMR spectroscopy. Furthermore, the methyl esters of hypelcins B-I—V, obtained by diazomethane treatment, showed a methoxyl signal (δ_H: *ca.* 3.6 ppm) in the ^{1}H-NMR spectra, indicating that the peptides each contain one Glu residue in the molecule. The amino acid proportions are summarized in Table I.

Structure of Hypelcin B-I The results mentioned above show that hypelcin B-I is a linear peptide. The ES-MS measured with an orifice voltage of 70 V (Fig. 2a) gave

TABLE II. Molecular Ions Observed in the ES-MS of Hypelcin Bs and Their Methyl Ester Derivatives[a]

Hypelcin	Formula	M.W. (nominal)	Multiply charged molecular ions	
			$[M+2H]^{2+}$	$[M+3H]^{3+}$
B-I	$C_{89}H_{152}N_{22}O_{25}$	1928	965.9	644.4
B-I–Me[a]	$C_{90}H_{154}N_{22}O_{25}$	1942	973.2	649.1
B-II	$C_{88}H_{150}N_{22}O_{25}$	1914	958.5	639.7
B-II–Me[a]	$C_{89}H_{152}N_{22}O_{25}$	1928	966.1	644.4
B-III	$C_{90}H_{154}N_{22}O_{25}$	1942	973.1	649.1
B-III–Me[a]	$C_{91}H_{156}N_{22}O_{25}$	1954	980.2	653.8
B-IV	$C_{89}H_{152}N_{22}O_{25}$	1928	966.1	644.4
B-IV–Me[a]	$C_{90}H_{154}N_{22}O_{25}$	1942	973.1	649.1
B-V	$C_{89}H_{152}N_{22}O_{25}$	1928	965.7	644.5
B-V–Me[a]	$C_{90}H_{154}N_{22}O_{25}$	1942	973.1	649.1

multiply charged molecular ions, $(M+2H)^{2+}$ and $(M+3H)^{3+}$ at *m/z* 965.9 and 644.4, respectively. The molecular mass, 1928, estimated from these ions was in agreement with $C_{89}H_{152}N_{22}O_{25}$, corresponding to the replacement of one Gln in hypelcin A-I by a Glu residue. In this spectrum, no significant peak was observed except for the multiply charged molecular ions. By increasing the orifice voltage to 140 V, four notable fragment ions were generated at *m/z* 741, 962, 968 and 1189 (Fig. 2b). In order to obtain structural information, we applied the ES-MS/MS technique to these ions.

The ES-MS/MS experiments were carried out by collision-induced dissociation (CID). The CID spectra of the N-terminal oligopeptide ions at *m/z* 1189 and 962 showed successive acylium ions assignable as shown in Fig. 3a and b, respectively. The ion at *m/z* 225 in Fig. 3b was consistent with Ac-Aib–Pro. Careful inspection of these spectra revealed the presence of one more acylium ion series, suggesting the preferential cleavage of the Aib^1–Pro^2 peptide bond. The H-Pro^2–Aib^3 fragment at *m/z* 183 in Fig. 3b supported the existence of an Ac-Aib residue in the ion at *m/z* 225. Therefore, the amino acid sequence of the N-terminal oligopeptide (residues 1—13) of hypelcin B-I was assigned as follows: Ac-Aib–Pro–Aib–Ala–Aib–Aib–Glx–Aib–Leu–Aib–Gly–Aib–Aib.

The next problem was to determine the C-terminal sequence. The CID spectrum of the C-terminal oligopeptide ion at *m/z* 741 showed successive acylium ions assignable as shown in Fig. 4a. This spectrum did not give the expected proline acylium ion at *m/z* 98, but showed the a-type ion at *m/z* 70, which originates from loss of CO from proline, as reported before.[16] On the other hand, the CID spectrum of the *m/z* 968 ion (Fig. 4b) revealed successive losses of Lol, Glx, Glx, Aib, Aib, Val, Pro, (Aib–Aib), leaving Gly (Fig. 4b). In addition, the ions beginning at *m/z* 741 were generated by the cleavage of the Aib^{13}–Pro^{14} bond, affording sequence-specific ions. These observations showed that the amino acid sequence of the C-terminal oligopeptide (residues 11—20) was Gly–Aib–Aib–Pro–Val–Aib–Aib–Glx–Glx–Lol, and the ion at *m/z* 228 was consistent with H-Gly–Aib–Aib (Fig. 4b). This Gly–Aib–Aib fragment overlaps in the C-terminal and the N-terminal oligopeptides.

The remaining problem was the location of the Glu residue. This problem was solved through examination of the CID spectra of hypelcin B-I methyl ester (B-I–Me). In the CID spectrum of the C-terminal oligopeptide ion at *m/z* 755 (Fig. 5), successive loss of Lol, Gln, Glu(OMe), Aib, Aib and (Pro + Val) gave ions at *m/z* 639, 510, 367, 282 and 197, respectively, indicating that Glu is present at position 18 in the molecule. On the basis of the findings obtained by mass spectrometry, the entire sequence of hypelcin B-I was determined to be as follows: Ac-Aib–Pro–Aib–Ala–Aib–Aib–Gln–Aib–Leu–Aib–Gly–Aib–Aib–Pro–Val–Aib–Aib–Glu–Gln–Lol.

Structures of Other Hypelcin Bs The structures of the remaining peptides were also determined in the same manner as that of hypelcin B-I. The ES-MS of hypelcins B-II, B-III, B-IV and B-V showed multiply charged molecular ions, and the molecular masses were assigned to be 1914, 1942, 1928 and 1928, respectively (Table II). According to the ES-MS and ES-MS/MS results, the hypelcin Bs had differences in their amino acid sequences at positions 6, 9, 17 and 20, as shown in Tables III and IV. Position 6 was occupied by either Aib or Ala as evidenced by the ion observed at *m/z* 537 for Ala in hypelcin B-II, and *m/z* 551 for Aib in hypelcins B-I, B-III, B-IV and B-V. On the other hand, Leu or Ile at position 9 and Lol or Iol at position 20 could not be differentiated by mass spectroscopy. The ion attributed to Leu or Ile at position 9 in the ES-MS/MS was assigned to be Ile in hypelcin B-IV, and Leu in the others on the basis of the amino acid analysis results. The amino alcohols were identified as Lol or Iol by GC comparison with standard samples, and it was found that hypelcin B-V had Iol at position 20 and that the others had Lol. The CID spectrum of the C-terminal oligopeptide ion (residues 14—20) of hypelcin B-III showed a prominent ion at *m/z* 381 corresponding to position 17, while the other peptides gave corresponding ions at *m/z* 367. Thus, it is indicated that hypelcin B-III has an Iva (or Val) residue at position 17, while the other peptides have Aib. However, Iva or Val in hypelcin B-III could not be differentiated by mass spectroscopy. In surveying the C-terminal sequences of the other peptides, it was found that position 15 is commonly occupied by Val. In addition, replacement of Aib and Iva occurs at position 17 in the C-terminal oligopeptide of hypelcins A-III, A-VII, and A-IX. Therefore, position 17 of hypelcin B-III could be deduced to be occupied by Iva. The CID spectra of the C-terminal oligopeptide ion (residues 14—20) of the hypelcin B methyl esters showed an increase of 14 mass units at position 18, compared with those of hypelcin Bs. Thus, it was shown that all the hypelcin Bs had Glu at position 18. In conclusion, hypelcins B-I—V differ from the corresponding hypelcins A-I—V only by the replacement of Gln at position 18 in the hypelcin As by Glu.

Experimental

General Procedures All NMR experiments were carried out by using a JEOL NMR-FX 200 spectrometer. ES-MS was performed on an API III instrument (Perkin Elmer Sciex). MS/MS experiments were carried out by collision-induced dissociation with argon atoms as the collision gas. Samples were dissolved in acetonitrile–water (1 : 1) containing 1% trifluoroacetic acid (TFA) for measurement of ES-MS and ES-MS/MS.

Reversed-Phase HPLC Separation of Hypelcins B-I, B-II, B-III, B-IV

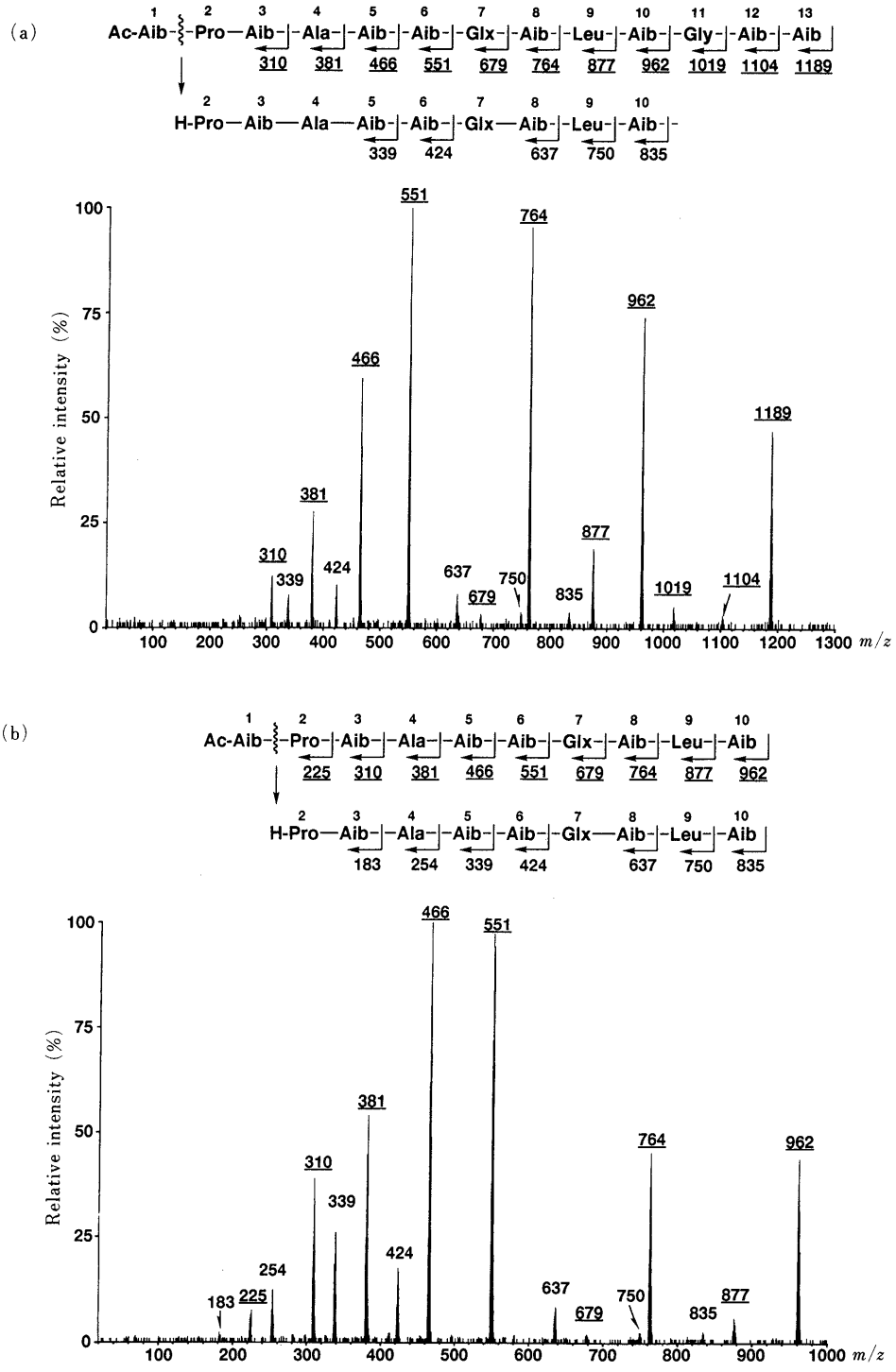

Fig. 3. CID Spectrum of N-Terminal Oligopeptide Ions at *m/z* 1189 (a) and 962 (b)

and B-V For HPLC analysis a Shimadzu LC-4A system was used. Separation of crude hypelcin B (4.5 g) was performed repeatedly with a Toyo Soda ODS-120A column (21.5 mm i.d. × 300 mm) [eluent MeOH–water–AcOH (83 : 17 : 0.1, v/v) for hypelcin B-II, eluent MeOH–water–AcOH (85 : 15 : 0.1, v/v) for hypelcins B-I, B-III—V; flow rate 6 ml/min; detector, UV (220 nm)] to give five main fractions containing hypelcins B-I, B-II, B-III, B-IV and B-V, respectively. The fractions were each purified by gel-filtration on Sephadex LH-20 in MeOH to give hypelcin B-I (145.2 mg), B-II (81.2 mg), B-III (97.2 mg), B-IV (73.5 mg), and B-V (65.7 mg).

Identification and Absolute Configuration of Amino Acids and Amino Alcohols For amino acid analyses, samples (*ca.* 1 mg) were hydrolyzed in 6 N HCl at 110 °C for 24 h. The hydrolysate was analyzed by a Hitachi amino acid analyzer (model 853). For the chiral separation of amino acids, the hydrolysate was analyzed by HPLC with a Sumichiral OA-5000 column (4.6 mm i.d. × 150 mm) [eluent, 2 mM $CuSO_4$ in water; flow rate, 1 ml/min; detector, UV (254 nm); column temperature, 40 °C]. t_R from standard equimolar mixtures of DL isomers (min): Ala, 4.8; D-Ala, 6.7; Aib, 7.8; Pro, 9.4; Iva, 11.2; Val, 13.0; D-Iva, 13.6; D-Pro, 19.3; D-Val, 22.4; Ile, 32.9; Leu, 39.4; Glu, 55.8; D-Ile, 59.6; D-Leu, 67.5; D-Glu, 68.6. The absolute configuration of the amino alcohol of each peptide was established in the following manner. An acid hydrolysate (*ca.* 1 mg) was dissolved in an anhydrous solution of 1.25 N HCl in 2-propanol and heated at 100 °C for 3 h. The reagents were evaporated under reduced pressure, the residue was dissolved in CH_2Cl_2 (2 ml) and 0.5 ml of trifluoroacetic anhydride was added. The mixture was kept in a

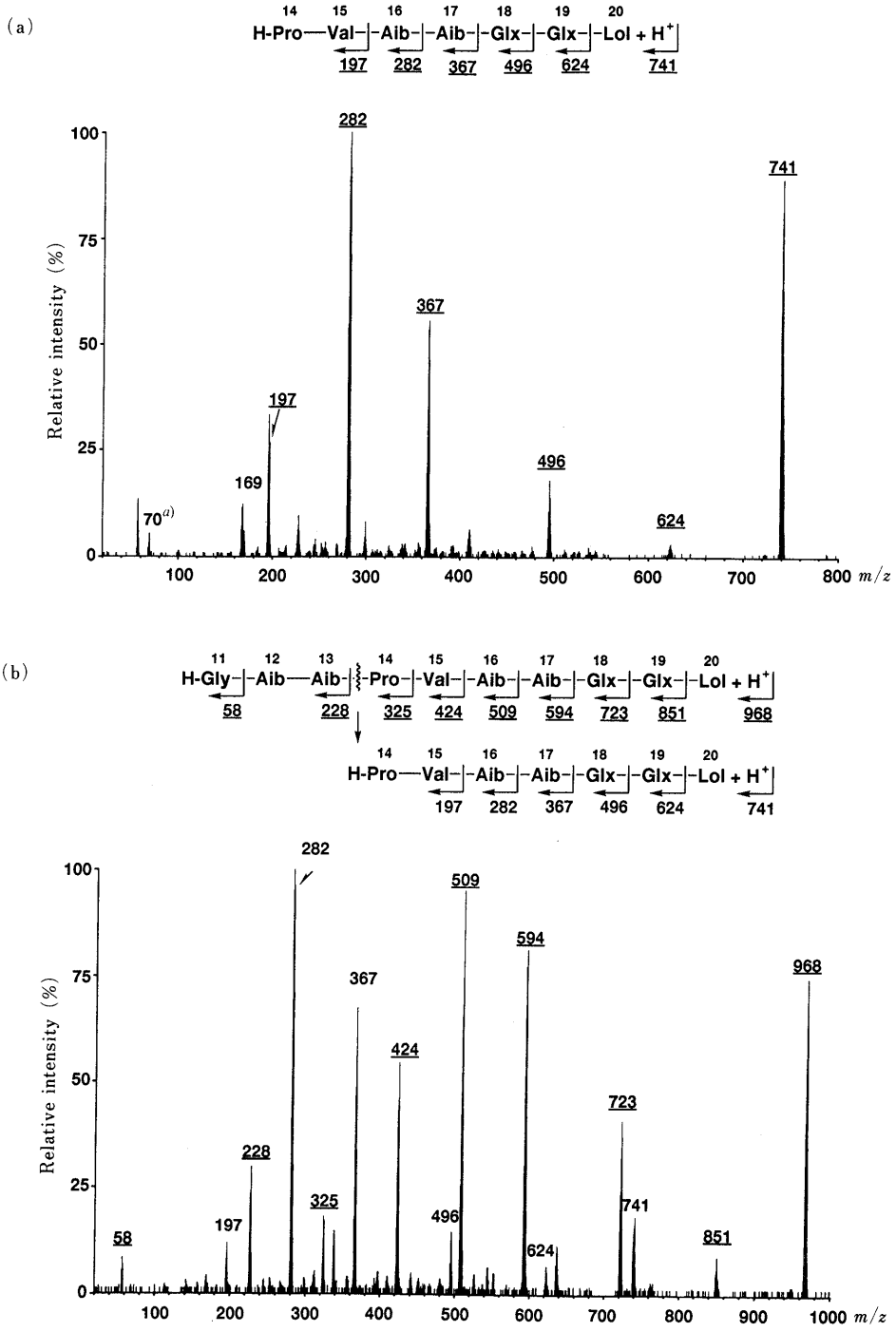

Fig. 4. CID Spectrum of C-Terminal Oligopeptide Ions at *m/z* 741 (a) and 968 (b)

a) a-type ion originated from loss of CO from proline.

screw-capped tube at 110 °C for 1 h. The reagents were removed under N_2, and the *N,O*-ditrifluoroacetyl derivative was analyzed with GC. The GC conditions were: OA-201, 20 m × 0.25 mm glass WCOT column; carrier flow, N_2 0.5 ml/min; temperature program, 0—15 min 100 °C, 15—25 min 5 °C/min and after 25 min 150 °C. t_R from standard equimolar mixture of DL isomers (min): D-Iol, 15.5; Iol, 15.9; D-Lol, 17.3; Lol, 17.5.

Methylation of Hypelcin Bs A solution of each peptide (*ca.* 10 mg) in MeOH (1 ml) was treated with ethereal diazomethane and the reaction mixture was left overnight at room temperature, then evaporated to give a residue. The residue was purified by HPLC using M & S Pack C18-A (20 mm × 250 mm) to give a methyl ester. The HPLC conditions were as follows: eluent, MeOH–water–AcOH (85 : 15 : 0.1, v/v); flow rate, 6 ml/min; detector, UV (220 nm).

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References

1) S. Sasaki, R. Hashimoto, M. Kiuchi, K. Inoue, T. Ikumoto, R. Toyama, K. Chiba, Y. Hoshino, T. Okumoto, T. Fujita, *J. Antibiot.*, **47**, 420 (1994).
2) T. Fujita, Y. Takaishi, T. Shiromoto, *J. Chem. Soc., Chem. Commum.*, **1979**, 413.
3) T. Fujita, Y. Takaishi, H. Moritoki, T. Ogawa, K. Tokimoto, *Chem. Pharm. Bull.*, **32**, 1822 (1984).

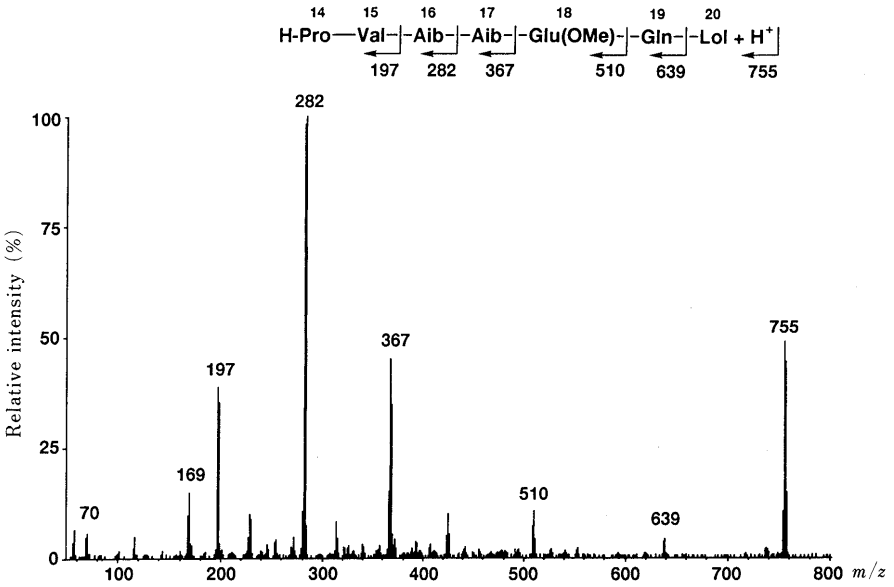

Fig. 5. CID Spectrum of N-Terminal Oligopeptide Ion at *m/z* 755 from Hypelcin B-I Methyl Ester

TABLE III. Diagnostic Ions Observed in the CID Spectra of Hypelcin Bs and Their Methyl Ester Derivatives[a)]

Hypelcin	ES-MS		Sequence-specific ions observed in the CID spectra																			
	N-Terminal oligopeptide ions	C-Terminal oligopeptide ions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
			Ac–Aib	Pro	Aib	Ala	Aib	Aib	Gln	Aib	Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	Aib	Glu	Gln	Lol
B-I	1189				310	381	466	551	679	764	877	962	1019	1104	1189							
	962			225	310	381	466	551	679	764	877	962										
		968											58		228	325	424	509	594	723	852	968
		741															197	282	367	496	625	741
B-I–Me[a)]		755															197	282	367	510	639	755
			----------					Ala														
B-II	1175				310	381	466	537	665	750	863	948	1005	1090	1175							
	948			225	310	381	466	537	665	750	863	948										
		968											58		228	325	424	509	594	723	852	968
		741															197	282	367	496	625	741
B-II–Me[a)]		755															197	282	367	510	639	755
			----------																		Iva	----------
B-III	1189				310	381	466	551	679	764	877	962	1019	1104	1189							
	962			225	310	381	466	551	679	764	877	962										
		981											58		228	325	424	509	608	737	866	981
		755															197	282	381	510	638	755
B-III–Me[a)]		769															197	282	381	524	653	769
			----------								Ile	----------										
B-IV	1189				310	381	466	551	679	764	877	962	1019	1104	1189							
	962			225	310	381	466	551	679	764	877	962										
		968											58		228	325	424	509	594	723	852	968
		741															197	282	367	496	625	741
B-IV–Me[a)]		755															197	282	367	510	639	755
			----------																			Iol
B-V	1189				310	381	466	551	679	764	877	962	1019	1104	1189							
	962			225	310	381	466	551	679	764	877	962										
		968											58		228	325	424	509	594	723	852	968
		741															197	282	367	496	625	741
B-V–Me[a)]		755															197	282	367	510	639	755

TABLE IV. Primary Structures of Hypelcin Bs

Hypelcin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B-I	Ac	Aib	Pro	Aib	Ala	Aib	Aib	Gln	Aib	Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	Aib	Glu	Gln	Lol
B-II	Ac	Aib	Pro	Aib	Ala	Aib	Ala	Gln	Aib	Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	Aib	Glu	Gln	Lol
B-III	Ac	Aib	Pro	Aib	Ala	Aib	Aib	Gln	Aib	Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	Iva	Glu	Gln	Lol
B-IV	Ac	Aib	Pro	Aib	Ala	Aib	Aib	Gln	Aib	Ile	Aib	Gly	Aib	Aib	Pro	Val	Aib	Aib	Glu	Gln	Lol
B-V	Ac	Aib	Pro	Aib	Ala	Aib	Aib	Gln	Aib	Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	Aib	Glu	Gln	Iol

4) R. C. Pandey, H. Meng, J. C. Cook, Jr., K. L. Rinehart, Jr., *J. Am. Chem. Soc.*, **99**, 5203 (1977).
5) H. Brückner, M. Przybylski, *J. Chromatogr.*, **296**, 263 (1984).
6) R. C. Pandey, J. C. Cook, Jr., K. L. Rinehart, Jr., *J. Am. Chem. Soc.*, **99**, 8469 (1977).
7) P. Mueller, D. O. Rudin, *Nature* (London), **217**, 713 (1968).
8) M. S. P. Sansom, *Prog. Biophys. Mol. Biol.*, **55**, 139 (1991).
9) G. Irmscher, G. Jung, *Eur. J. Biochem.*, **80**, 165 (1977).
10) E. Tachikawa, S. Takahashi, K. Furumachi, T. Kashimoto, A. Iida, Y. Nagaoka, T. Fujita, Y. Takaishi, *Mol. Pharmacol.*, **40**, 790 (1991).
11) Y. Takaishi, H. Terada, T. Fujita, *Experientia*, **36**, 550 (1980).
12) K. Matsuzaki, S. Nakai, T. Handa, Y. Takaishi, T. Fujita, K. Miyajima, *Biochemistry*, **28**, 9392 (1989).
13) K. Matsuzaki, Y. Takaishi, T. Fujita, K. Miyajima, *Colloid Polym. Sci.*, **269**, 604 (1991).
14) K. Matsuura, A. Yesilada, A. Iida, Y. Takaishi, M. Kanai, T. Fujita, *J. Chem. Soc., Perkin Trans. 1*, **1993**, 381.
15) A. P. Bruins, T. R. Corey, J. D. Henion, *Anal. Chem.*, **59**, 2642 (1987).
16) A. Iida, M. Okuda, S. Uesato, Y. Takaishi, T. Shingu, M. Morita, T. Fujita, *J. Chem. Soc., Perkin Trans. 1*, **1990**, 3249.