Fungal Metabolites. XIX.[1] Structural Elucidation of Channel-Forming Peptides, Trichorovins-I—XIV, from the Fungus *Trichoderma viride*

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Trichorovins (TV)-I—XIV are new antibiotic peptides obtained from conidia of the fungus *Trichoderma viride*. The peptide mixture of TVs was repeatedly fractionated by preparative HPLC until individual TVs showed a single peak on their analytical HPLC chromatograms. Nevertheless, FAB-MS or NMR indicated that each of TVs-I—XIV was composed of at least two components. We attempted to elucidate their structures within the fractions by electrospray ionization (ESI)-MS, FAB-MS, FAB-MS/MS and NMR. TVs generally have molecular weights of approximately 1100—1200 Da, and are characterized by an acetylated N-terminus, the presence of an aminoalcohol, *e.g.* leucinol, isoleucinol or valinol, at the C-terminus, and eleven residues including three α-aminoisobutyric acids in the molecule. Thus, it was determined that TVs belong to the class of peptaibols.

Key words *Trichoderma viride*; peptaibol; α-aminoisobutyric acid; trichorovin; ion channel

Many peptaibols such as alamethicin[2] and trichosporin[3] have been isolated from *Trichoderma* spp. Peptaibols are antibiotic peptides with up to 20 residues which are characterized by an acylated N-terminus, the presence of an aminoalcohol such as leucinol (Lol), isoleucinol (Iol) or valinol (Vol) at the C-terminus, and the incorporation of α-aminoisobutyric acid (Aib) residues in the molecule. Peptaibols generally form voltage-gated ion channels in black lipid membranes, and, therefore, they could serve as models for elucidation of the general mechanistic principles of biological ion channels.[4] Biological activities of peptaibols related to channel formation include their ability to uncouple oxidative phosphorylation in rat liver mitochondria[5] and to induce catecholamine secretion in bovine adrenal chromaffin cells.[6] These activities are thought to be caused by ion influx into the organelles or the cells through channels formed in the membranes by peptaibols.

In the course of our search for components with antibacterial properties in the methanolic extract of conidia of *T. viride*,[7] three new groups of peptaibols, trichodecenins (TD), trichorovins (TV) and trichocellins (TC), have been isolated. The isolation procedure for the three groups of peptaibols and the structural elucidation of TDs[7] and TCs[8] have been reported. In this paper, we describe the structural elucidation of TVs by electrospray ionization (ESI)-MS, FAB-MS, FAB-MS/MS, and NMR.

Results and Discussion

Characterization of TVs The peptide mixture of TVs showed 14 peaks on the analytical HPLC chromatogram.[7] It was repeatedly fractionated by preparative HPLC to give TVs-I—XIV, each of which yielded a single peak on individual analytical HPLC chromatograms. However, it was found by FAB-MS or NMR that each of TVs-I—XIV was still a mixture consisting of at least two components. Further purification of TVs-I—XIV was unsuccessful, so we attempted to determine the structures of the components within the mixtures.

Each TV fraction exhibited an acetyl signal in the ^{1}H-NMR spectrum (δ_H 2.0—2.1). The proteinic amino acid ratios of TVs were obtained from amino acid analyses of the total acidic hydrolysates (Table 1). The absolute configurations of the amino acids were determined to be all L-form by the HPLC method reported previously.[9] In this step, the presence of Aib residues was confirmed. TVs-I—XIV did not react with diazomethane or ninhydrin reagent but did react with HBr–ninhydrin reagent. These facts suggested that the N-terminal amino acids of TVs were blocked with acetyl groups and the TV molecules do not have free carboxyl groups. Therefore, Glu and Asp in the amino acid analyses were deduced to be Gln and Asn, respectively.

Structure of TV-XII TV-XII was found to be composed of two components in the molar ratio of 7:3, judging from the intensities of NH signals in the ^{1}H-NMR spectrum. HPLC analysis[9] of the 3,5-dinitrobenzoate derivatives of the acid hydrolysate of TV-XII gave L-Iol and L-Lol with relative peak intensities of about 7:3. These observations suggested that the C-terminal amino acids of the major (TV-XIIa) and minor (TV-XIIb) com-

Table 1. Amino Acid Compositions of TVs-I—XIV

TV	Asn	Gln	Ile	Leu	Val	Pro
I	1.2		1.0	2.3	1.4	1.9
II	1.0		0.5	1.8	1.4	2.1
III		1.0	1.0	2.0	1.3	2.5
IV	0.6	1.5	1.0	4.0	2.7	2.5
V	1.2		1.0	3.1	1.0	3.1
VI	1.4		2.1	2.9	1.0	2.5
VII	0.8	0.3	1.0	2.3	1.1	2.4
VIII		1.3	1.0	3.1	1.1	2.3
IX		1.2	1.7	2.5	1.0	2.2
X	0.6	1.0	2.0	3.4	1.0	2.8
XI	1.0		2.0	2.5		2.7
XII	1.0		2.0	2.0		2.3
XIII		1.0	1.8	2.0		2.1
XIV		0.9	2.0	1.9		2.2

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ponents are linked with Iol and Lol, respectively. Considering that TV-XII contain Aib residues, TV-XIIa and -XIIb belong to the class of peptaibols. The FAB-MS of TV-XII showed two ions, *m/z* 1159 and *m/z* 1175, in the molecular ion region (Fig. 1a). On the other hand, ESI-MS,[10] which is softer than FAB ionization, clearly showed $[M+H]^+$ (*m/z* 1175) and doubly charged $[M+2H]^{2+}$ (*m/z* 588) ions but did not give the expected *m/z* 1159 ion (Fig. 1b). Furthermore, when sodium chloride was added to the matrix during FAB-MS analysis of TV-XII, only one sodium adduct ion, $[M+Na]^+$ at *m/z* 1197 was observed (Fig. 1c). These results suggest that both TVs-XIIa and -XIIb have the same molecular weight (1174; $C_{58}H_{102}N_{12}O_{13}$) and that the *m/z* 1159 ion is an unknown ion from TV-XII. The FAB-MS of TV-XII showed several

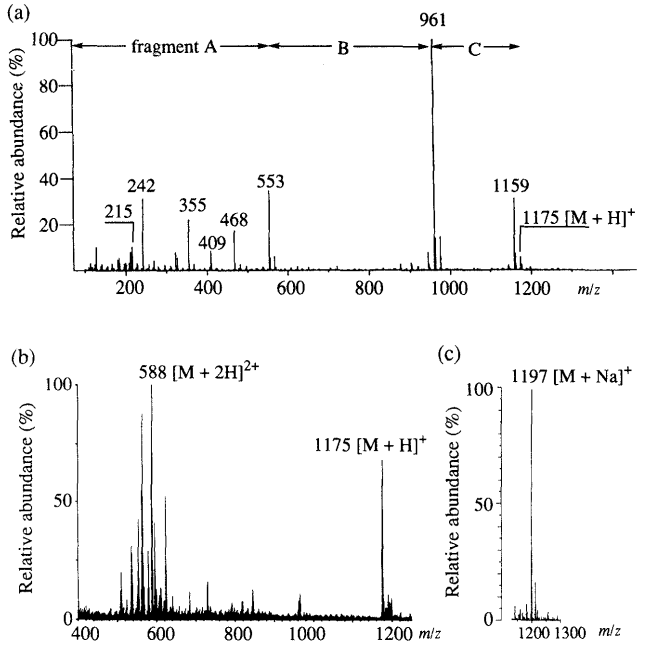


Fig. 1. Positive-Ion FAB-MS (a), ESI-MS (b) and FAB-MS by Adding NaCl to the Matrix of TV-XII

b-type ions[11] at *m/z* 961, 553, 468, 355 and 242 (Fig. 1a), but clear fragment ions were not observed in the mass regions between *m/z* 553 and 961 (408 a.m.u.), and between *m/z* 961 and the molecular ion (214 a.m.u.). This fact suggests that TVs-XIIa and -XIIb have two chemically unstable Aib–Pro peptide bonds in the molecules, as in the case of the hypelcins[9] and the trichorozins.[1] Consequently, it is believed that the three fragment ions A (*m/z* 553), B (*m/z* 409) and C (*m/z* 215) result from cleavages at the two Aib–Pro bonds in the molecules, as depicted in Fig. 2. In fact, a product ion spectrum from the *m/z* 961 ion obtained by low-energy collision-induced dissociation (CID)[12] (−5 to −20 V) gave two ions (*m/z* 553, 409) corresponding to fragments A and B (Fig. 3a). In addition, a consecutive acylium ion (b-type) series was observed at *m/z* 876, 763, 650, 553, 468, 355, 242 and 128, assignable as the amino acid sequence of the N-terminal of the oligopeptide: Ac–Aib[1]–Asn[2]–Lxx[3]–Lxx[4]–Aib[5]–Pro[6]–Lxx[7]–Lxx[8]–Aib[9] (Lxx: Leu or Ile). The product ion spectrum of fragment B, *m/z* 409 ([H-Pro[6]–Lxx[7]–Lxx[8]–Aib[9]]$^+$), confirmed the sequence at positions 5 to 9 (Fig. 3b). Furthermore, the product ion spectrum (not shown here) of fragment C, *m/z* 215, afforded the *m/z* 70 ion, which results from the losses of Iol (or Lol) and the carbonyl group of the Pro acylium ion at *m/z* 98. This result indicated that the amino acid sequence of the C-terminal oligopeptide is Pro–Iol (or Lol). Therefore, the amino acid sequences of both TVs-XIIa and -XIIb are obtained by connecting these two fragments as follows; Ac–Aib[1]–Asn[2]–Lxx[3]–Lxx[4]–Aib[5]–Pro[6]–Lxx[7]–Lxx[8]–Aib[9]–Pro[10]–Iol[11] for TV-XIIa (and Lol[11] for -XIIb).

The differentiation of isomeric amino acids, Leu and Ile, in TV-XIIa was achieved by two-dimensional (2D) NMR and high-energy CID. The proton signals of the major component, TV-XIIa, could be identified by using double-quantum-filtered (DQF) correlated spectroscopy (COSY)[13] and nuclear Overhauser enhancement spectroscopy (NOESY),[14] whereas many small signals of the minor component, TV-XIIb, could not be identified be-

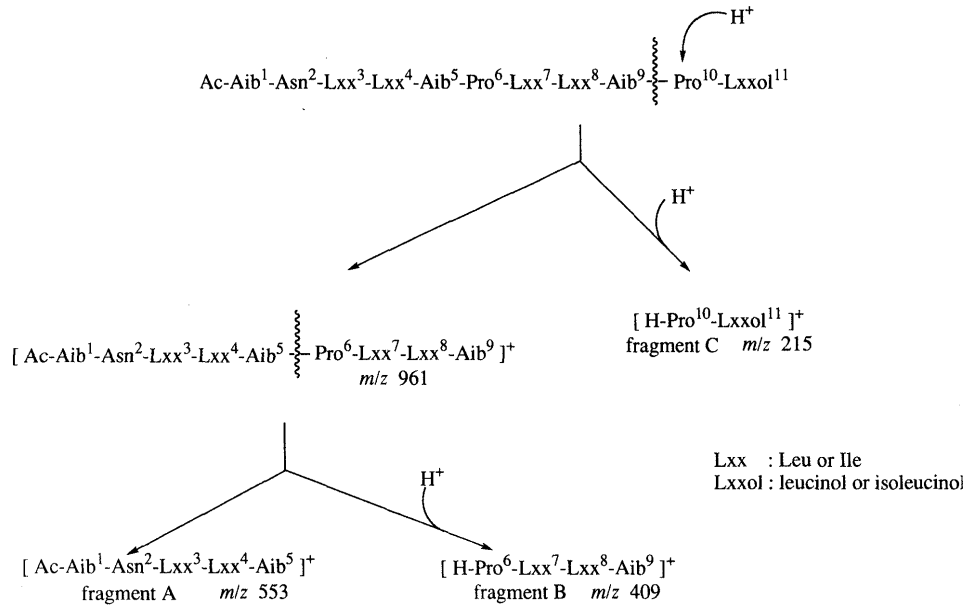


Fig. 2. Proposed Fragmentation for TV-XII in FAB-MS

cause of overlapping with the major signals. The differentiation of Leu and Ile in TV-XIIa could be achieved in the following way. First, the spin systems of amino acids[15] and Iol were identified by DQF-COSY. Next, sequence-specific assignments[16] of the NH protons were carried out using NOESY. Figure 4 shows the NH–NH region of the NOESY spectrum of TV-XII. The singlet signal at the lowest field, which was correlated with the acetyl protons, was assigned to Aib^1–NH. Successive NH–NH cross peaks from Aib^1–NH to Aib^5–NH were observed and both NHs of Ile were included in the sequential connectivities as shown in Fig. 4. This finding, together with the amino acid composition (Table 1), indicates that the two Ile residues occupy positions 3 and 4 and the remaining two Leu residues are necessarily placed at positions 7 and 8. Consequently, the primary structure of the main component, TV-XIIa, was completely elucidated as follows: Ac–Aib^1–Asn^2–Ile^3–Ile^4–

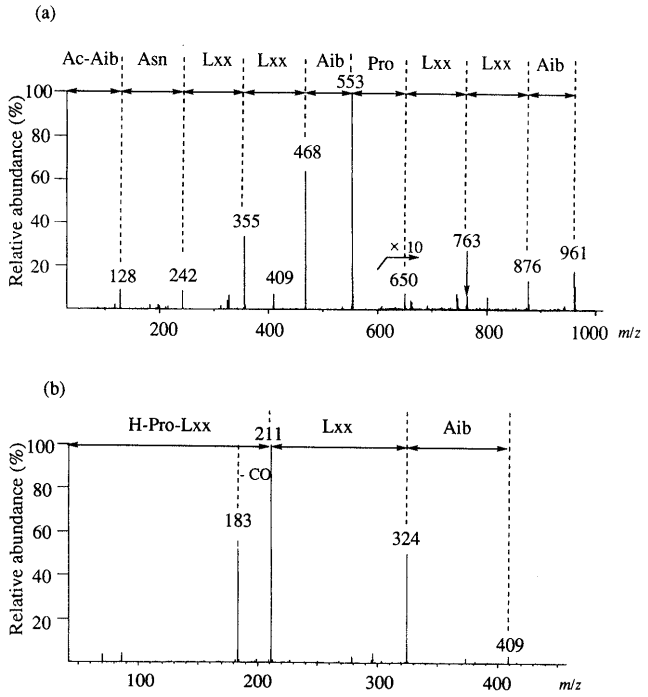


Fig. 3. Product Ion Spectra from the m/z 961 (a) and 409 (b) Ions Observed in TV-XII

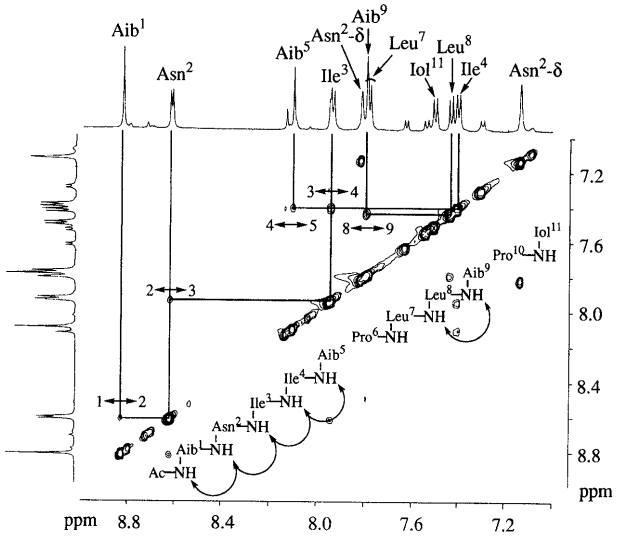
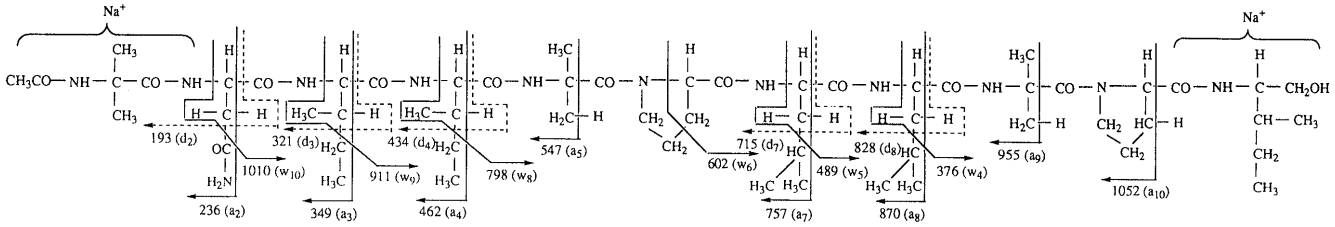


Fig. 4. NH–NH Region of the 600 MHz NOESY Spectrum of TV-XII in CD_3OH at 10 °C

Mixing time: 300 ms, unsymmetrized. The lines reveal the NH–NH connectivities.



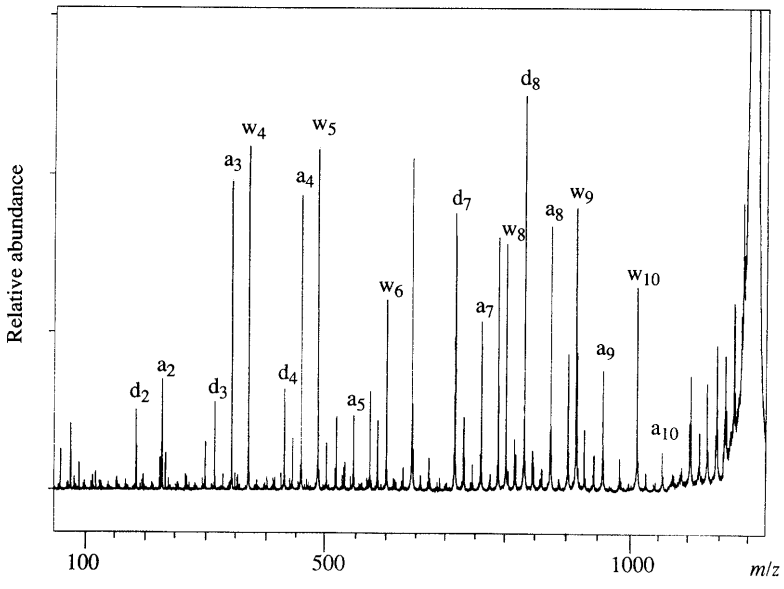


Fig. 5. Product Ion Spectrum of TV-XII from the $[M+Na]^+$ Ion (m/z 1196) Using High-Energy Collision (7 kV)

Aib[5]–Pro[6]–Leu[7]–Leu[8]–Aib[9]–Pro[10]–Iol[11].

Alternatively, high-energy CID was carried out using two double-focusing magnetic instruments. The $[M+Na]^+$ ion (*m/z* 1197) of TV-XII was collided at 7 kV to afford several d-[17] and w-type[18] ions generating side chain-specific fragmentations (Fig. 5). The generation mechanisms of the d- and w-ions were proposed by Johnson *et al.*[17,18] The d- and a-type ions are generated by cleavages of the β–γ (path A) and C_β–H (path B) bonds of the C-terminal amino acid in an a_n+1 ion, respectively, and the w_n ion *via* cleavage of the β–γ bond of the N-terminal amino acid in an z_n+1 ion (path C). Thus, the mechanisms of generation of d- and w-type ions from $[M+Na]^+$ are proposed to be as shown in Chart 1 for Leu as an example. Observation of the *m/z* 321 (d_3) and 434 (d_4) ions suggests that positions 3 and 4 are occupied by Ile because they are 28 a.m.u. lower than *m/z* 349 (a_3) and 462 (a_4), respectively. On the other hand, the *m/z* 715 (d_7) and 828 (d_8) ions are assigned to Leu at positions 7 and 8 because they are 42 a.m.u. lower than *m/z* 757 (a_7) and 870 (a_8). The w-type ions at *m/z* 376 (w_4), 489 (w_5), 798 (w_8) and 911 (w_9) also support the above assignment.

On the other hand, the sequence-specific side chain loss fragment ions arising from TV-XIIb were not observed and we could not determine the complete sequence of TV-XIIb.

The ion channel formation of peptaibols in planar lipid bilayers[4] has been studied. The ion channel model formed

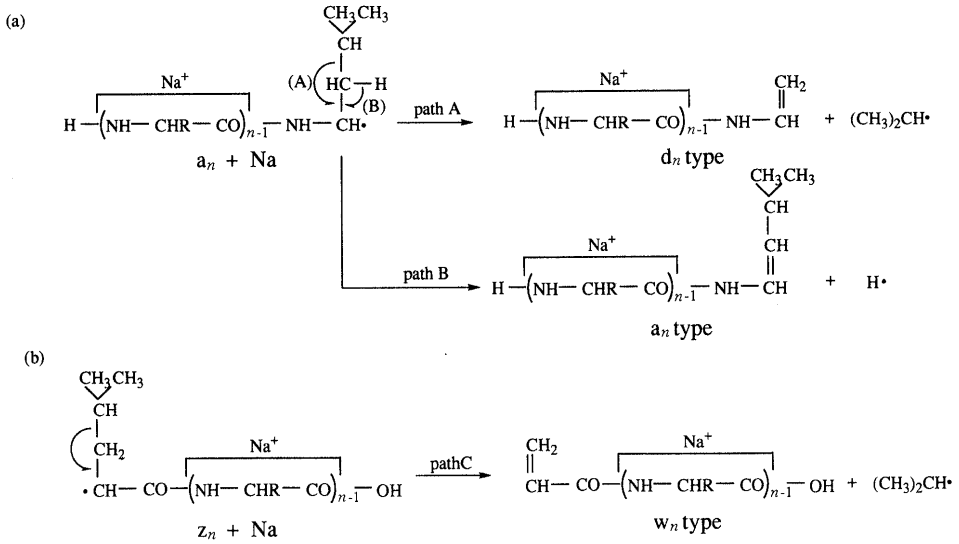


Chart 1. Mechanisms for Generation of d_n, a_n and w_n-Type Ions (for Leu as an Example)

Table 2. Diagnostic Ions Observed in the FAB-MS and the Product Ion Spectra of TVs-I—XIV

Position	Acylium ions, *m/z*												
	1	2	3	4	5	6	7	8	9	10[a)]	11	$(M+H)^+$	Molecular formula
TV-Ia	128	242	341	454	539	636	749	862	947	70	201	1147	$C_{56}H_{98}N_{12}O_{13}$
b	128	242	341	440	525	622	735	848	933	70	215	1147	$C_{56}H_{98}N_{12}O_{13}$
IIa	128	242	341	440	525	622	735	848	933	70	215	1147	$C_{56}H_{98}N_{12}O_{13}$
b	128	242	355	454	539	636	749	862	947	70	201	1147	$C_{56}H_{98}N_{12}O_{13}$
IIIa	128	256	355	454	539	636	749	862	947	70	215	1161	$C_{57}H_{100}N_{12}O_{13}$
b	128	256	355	468	553	650	763	876	961	70	201	1161	$C_{57}H_{100}N_{12}O_{13}$
IVa	128	256	355	454	539	636	749	862	947	70	215	1161	$C_{57}H_{100}N_{12}O_{13}$
b	128	256	369	468	553	650	763	876	961	70	201	1161	$C_{57}H_{100}N_{12}O_{13}$
c	128	242	341	454	539	636	749	862	947	70	215	1161	$C_{57}H_{100}N_{12}O_{13}$
Va	128	242	341	454	539	636	749	862	947	70	215	1161	$C_{57}H_{100}N_{12}O_{13}$
b	128	242	355	468	553	650	763	876	961	70	201	1161	$C_{57}H_{100}N_{12}O_{13}$
VIa	128	242	341	454	539	636	749	862	947	70	215	1161	$C_{57}H_{100}N_{12}O_{13}$
b	128	242	355	468	553	650	763	876	961	70	201	1161	$C_{57}H_{100}N_{12}O_{13}$
VIIa	128	242	355	454	539	636	749	862	947	70	215	1161	$C_{57}H_{100}N_{12}O_{13}$
b	128	256	355	468	553	650	763	876	961	70	201	1161	$C_{57}H_{100}N_{12}O_{13}$
VIII	128	256	355	468	553	650	763	876	961	70	215	1175	$C_{58}H_{102}N_{12}O_{13}$
IXa	128	256	355	468	553	650	763	876	961	70	215	1175	$C_{58}H_{102}N_{12}O_{13}$
b	128	256	369	482	567	664	777	890	975	70	201	1175	$C_{58}H_{102}N_{12}O_{13}$
Xa	128	256	369	468	553	650	763	876	961	70	215	1175	$C_{58}H_{102}N_{12}O_{13}$
b	128	242	355	468	553	650	763	876	961	70	215	1175	$C_{58}H_{102}N_{12}O_{13}$
XI	128	242	355	468	553	650	763	876	961	70	215	1175	$C_{58}H_{102}N_{12}O_{13}$
XII	128	242	355	468	553	650	763	876	961	70	215	1175	$C_{58}H_{102}N_{12}O_{13}$
XIII	128	256	369	482	567	664	777	890	975	70	215	1189	$C_{59}H_{104}N_{12}O_{13}$
XIV	128	256	369	482	567	664	777	890	975	70	215	1189	$C_{59}H_{104}N_{12}O_{13}$

a) Ions at position 10 reveal a-type ions of Pro.

by 20-residue peptaibols seems to be consistent with the barrel–stave model[19] formed by self-assembly of parallel bundles of α-helical rods. To span the *ca.* 3 nm-thick hydrophobic region of the lipid, an α-helix peptide would need to be composed of at least 20 residues. Unexpectedly, the shorter peptide, TV-XII was found to form voltage-dependent ion channels in lipid bilayers. The ion, channel-forming activity of TV-XII will be discussed elsewhere.

Structures of the Other TVs The structures of the other TVs were presumed on the basis of the fragmentation pattern of TV-XII. Aminoalcohols at the C-termini were estimated only from mass values observed in the FAB-MS and product ion spectra. The diagnostic ions observed for TVs are summarized in Table 2.

TVs-VIII, XI, XIII and XIV have one acylium ion series in the FAB-MS, but are not single compounds, being similar to TV-XII. This conclusion is supported by the NMR spectra. These peptides are thought to be mixtures in which isomeric constituents (Leu and Ile, and Lol and Iol) are arranged at positions 3, 4, 7, 8 and 11 as in the case of TV-XII. The exception is position 3 of TV-VIII, which is occupied by Val.

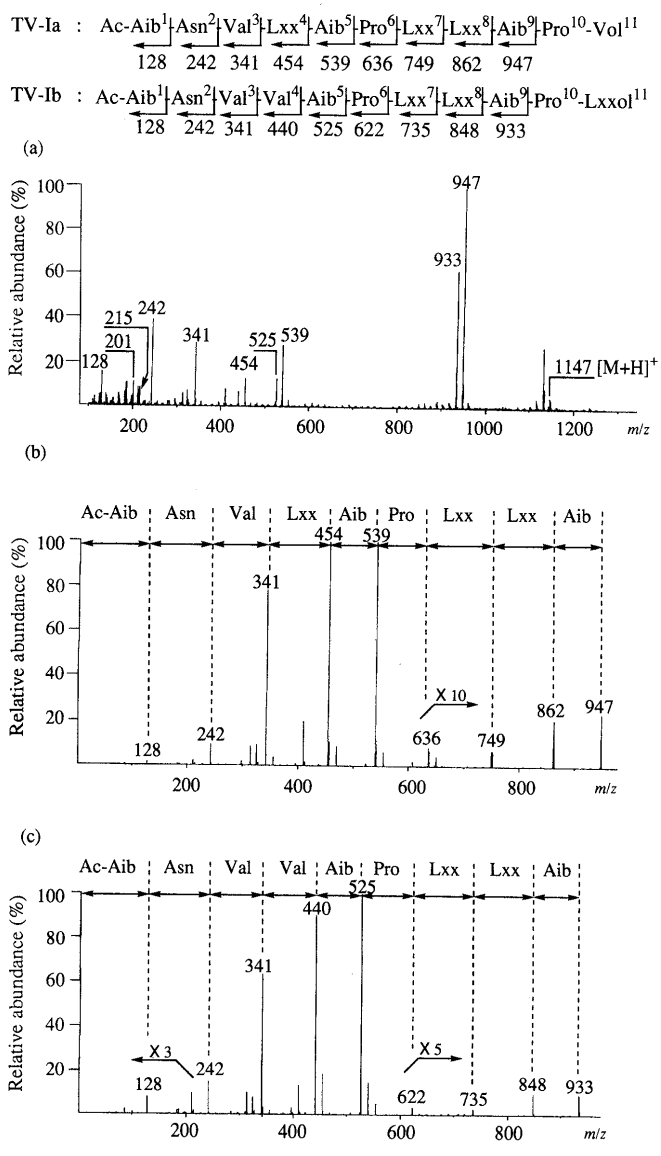


Fig. 6. FAB-MS (a) and Product Ion Spectra from the *m/z* 947 (b) and 933 (c) Ions of TV-I

Two acylium ion series are observed in the spectra of eight TV fractions, TV-I—III, V—VII, IX and X, and three series are observed for TV-IV, suggesting mixtures of two and three components at least, respectively. As an example, the structure of TV-I is discussed. In the FAB-MS of TV-I, there are two acylium ion series as shown in Fig. 6a, indicating that TV-I is composed of two components (major: TV-Ia, minor: TV-Ib) having the same protonated molecular ion, $[M+H]^+$, at *m/z* 1147. One acylium ion series, from TV-Ia, includes the *m/z* 539 and 947 ions, which were generated from the cleavage of two Aib–Pro bonds between positions 6 and 7, and 9 and 10, respectively. The other one, from TV-Ib, includes the *m/z* 525 and 933 ions, which were similarly generated. Collision of the *m/z* 947 (Fig. 6b) and 933 (Fig. 6c) ions indicated that positions 5 to 9 of both TVs-Ia and -Ib have the same sequence, Aib[5]–Pro[6]–Lxx[7]–Lxx[8]–Aib[9]. Below position 4, successive losses of Lxx, Val and Asn for Ia, and Val, Val and Asn for Ib were observed to give the *m/z* 128 ion assignable to Ac–Aib. In addition, collision of the C-terminal dipeptide ions at *m/z* 201 for TV-Ia and *m/z* 215 ion for Ib afforded *m/z* 70 ions which were produced by the losses of Vol and Pro-carbonyl for Ia and Lol (or Iol) and Pro-carbonyl for Ib. Consequently, the sequences of TVs-Ia and -Ib were obtained by connection of these fragments. TV-Ia: Ac–Aib[1]–Asn[2]–Val[3]–Lxx[4]–Aib[5]–Pro[6]–Lxx[7]–Lxx[8]–Aib[9]–Pro[10]–Vol[11] and TV-Ib: Ac–Aib[1]–Asn[2]–Val[3]–Val[4]–Aib[5]–Pro[6]–Lxx[7]–Lxx[8]–Aib[9]–Pro[10]–Lxxol[11] (Lxxol: Lol or Iol). The structures of TVs are summarized in Fig. 7.

Experimental

General Procedures ESI-MS was performed on an API III (Perkin

1 2 3 4 5 6 7 8 9 10 11
Ac - Aib - W - X - Y - Aib - Pro - Lxx - Lxx - Aib - Pro - Z

TV	W	X	Y	Z
Ia	Asn	Val	Lxx	Vol
b	Asn	Val	Val	Lxxol
IIa	Asn	Val	Val	Lxxol
b	Asn	Lxx	Val	Vol
IIIa	Gln	Val	Val	Lxxol
b	Gln	Val	Lxx	Vol
IVa	Gln	Val	Val	Lxxol
b	Gln	Lxx	Val	Vol
c	Asn	Val	Lxx	Lxxol
Va	Asn	Val	Lxx	Lxxol
b	Asn	Lxx	Lxx	Vol
VIa	Asn	Val	Lxx	Lxxol
b	Asn	Lxx	Lxx	Vol
VIIa	Asn	Lxx	Val	Lxxol
b	Gln	Val	Lxx	Vol
VIII	Gln	Val	Lxx	Lxxol
IXa	Gln	Val	Lxx	Lxxol
b	Gln	Lxx	Lxx	Vol
Xa	Gln	Lxx	Val	Lxxol
b	Asn	Lxx	Lxx	Lxxol
XI	Asn	Lxx	Lxx	Lxxol
XIIa	Asn	Ile	Ile	Iol
b	Asn	Lxx	Lxx	Lol
XIII	Gln	Lxx	Lxx	Lxxol
XIV	Gln	Lxx	Lxx	Lxxol

Lxx : Leu or Ile Lxxol : leucinol or isoleucinol Lol : leucinol
Iol : isoleucinol Vol : valinol

Fig. 7. Structures of TVs-I—XIV

Positions 7 and 8 of TV-XIIa are occupied by Leu. In compound names, "a" indicates a major component and "b" or "c" a minor component.

Elmer Sciex). Electrospray voltage was 4.7 kV and the orifice voltage was 50 V. Samples were dissolved in CH_3CN–0.1% trifluoroacetic acid solution (1 : 1, v/v). The solution was introduced into the electrospray source at a constant flow rate of 5 μl/min. For FAB-MS and low-energy CID, a Finnigan MAT 70 triple-stage quadruple mass spectrometer was used. Samples were bombarded with 8 kV xenon atoms. For CID experiments, argon was used as the collision gas and the collision energy was −5 to −20 V. Glycerol–thioglycerol (1 : 1, v/v) was used as a matrix. For high-energy CID, a JEOL JMS-HX/HX 110 A mass spectrometer was used. Samples were bombarded with 6 kV xenon atoms and the $[M+Na]^+$ was collided with argon atoms with a collision energy of 7 kV. Glycerol–thioglycerol (1 : 1, v/v) containing NaCl was also used as a matrix. All NMR experiments were carried out by using Bruker AC-300 and Bruker AM-600 spectrometers. Samples were dissolved in CD_3OH or $(CD_3)_2SO$ containing tetramethylsilane as an internal standard. Amino acid analyses were done with a Hitachi Model 835 amino acid analyzer. HPLC was performed on a Shimadzu LC-6A system or an LC-8A system.

Identification and Absolute Configuration of Amino Acids and Aminoalcohols of TVs The identification and absolute configuration of amino acids were determined according to the procedures described previously.[9] Those of aminoalcohol in TV-XII were determined in the following manner. The acid hydrolysate was refluxed in absolute MeOH–thionyl chloride (10 : 1, v/v; 2 ml) for 3 h. After removal of the solvent and reagent, the residue was treated with a solution of 3,5-dinitrobenzoyl chloride (*ca.* 1 mg) and triethylamine (one drop) in EtOAc (2 ml) and the mixture was stirred for 24 h. The resulting *N*-3,5-dinitrobenzoate of Lol or Iol was analyzed by HPLC with a column having an optically active stationary phase [conditions: mobile phase, hexane–dichloroethane–ethanol (90 : 5 : 5, v/v/v); flow rate, 0.8 ml/min; detector, UV (254 nm); column, Sumipax OA-4100 (4 mm i.d. × 250 mm, Sumika Chemical Analysis Service Ltd.); column temperature, 35°C]. Observed t_Rs (min): 34.1 (L-Lol), 35.4 (L-Iol). t_Rs (min) of standard samples (min): 23.6 (D-Lol), 28.4 (D-Iol), 33.6 (L-Lol), 35.2 (L-Iol). Identification of aminoalcohols in TV-I—XI, XIII and XIV was based on mass values observed in the FAB-MS and product ion spectra.

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