Studies on Metabolites of Mycoparasitic Fungi. II.[1] Metabolites of *Trichoderma koningii*

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Four peptaibols, named trichokonins (TKs) V, VI, VII, and VIII, were isolated from the culture broth of *Trichoderma koningii* OUDEMANS. Primary structures of these peptaibols were elucidated by electrospray ionization mass spectrometry (ESI-MS), FAB-MS, and collision-induced dissociation (CID) techniques along with nuclear Overhauser enhancement spectroscopy (NOESY).

Key words *Trichoderma koningii*; peptaibol; trichokonin; collision-induced dissociation (CID); electrospray ionization mass spectrometry (ESI-MS); NOESY

In a previous paper,[1] we reported the isolation and structure elucidation of antifungal metabolites of *Cladobotryum varium* NEES *et* DUBY (Hyphomycetes), a fungus injurious to the cultivation of a medicinal mushroom, *Ganoderma lucidum* (FR.) KARST (oriental crude drug "Lin-Chi"). In a continuation of our studies on the metabolites of mycoparasitic fungi, we recently isolated a wild strain of *Trichoderma koningii* OUDEMANS from a log material which had been inoculated with mycelia of *G. lucidum* for cultivation, and we examined the metabolites of this fungus.

Trichoderma species are widespread soil fungi characterized by the production of cellulolytic enzymes and unique antibiotic antifungal peptides[2] termed peptaibols. These are linear peptides containing an acetylated N-terminal residue, a C-terminal amino alcohol, and a high content of a hydrophobic amino acid, α-aminoisobutyric acid (Aib), and exhibit a broad range of bioactivities, some of which are related to their membrane-interacting properties. They induce the formation of characteristic voltage-dependent ion-conduction in lipid bilayer membranes.[3] Some of them were shown to induce uncoupling of oxidative phosphorylation in mitochondria,[4] enhancement of catecholamine release from adrenal chromaffin cells,[5] and inhibition of ameba cell multiplication.[6] At high concentrations they cause cell lysis.[6,7]

Auvin-Guette *et al.*[8] have reported the isolation of a nineteen-residue peptaibol, trikoningin KA V, and two eleven-residue peptaibols, trikoningins KB I and KB II, from the culture broth of *T. koningii*. This prompted us to publish our work on the metabolites of the fungus, which led to the isolation of three twenty-residue peptaibols, named trichokonins (TKs) VI, VII, and VIII and a nineteen-residue one, TK-V.

Results and Discussion

Separation and Characterization of TKs Culture broth of *T. koningii* was separated into mycelia and medium by filtration. The filtrate was extracted with BuOH to give a BuOH extract, which showed an inhibitory activity against the mycelial growth of *G. lucidum*. The BuOH extract was separated by a combination of normal-phase (silica gel) and reversed-phase (Cosmosil $75C_{18}$-OPN gel) column chromatography to give a peptide mixture. This peptide mixture was further separated by preparative HPLC with a phenyl-type column and an octadecyl silica (ODS) column to give TK-V, TK-VI, TK-VII, and TK-VIII, together with minor peptaibols (Fig. 1).

Tks V-VIII showed strong IR absorptions at 3340 (NH), 1630 (CO), and 1520 (NH) cm^{-1}, characteristic of peptide linkages.

The proportions of normal amino acids in TKs V-VIII were established from amino acid analyses of the complete acid hydrolysates (Table II). The numbers of Aib and isovaline (Iva) residues, which respond poorly to ninhydrin reagent, were determined based on the numbers and relative intensities of the triplet ^1H-signal due to the γ-methyl group of Iva and the singlet ^1H-signals due to the amide protons of Iva and Aib (Table III), as well as the signals of quaternary carbons observed in the

TABLE I. Primary Structures of Trichokonins

Position[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
TK-V	Ac	Aib	Ala	Aib	Ala	Aib	—	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol
TK-VI (Gliodeliquescin)	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol
TK-VII	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln	Gln	Pheol
TK-VIII (Trichosporin-B-IVc)	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol

a) The squares denote the positions at which heterogeneity of amino acids is observed.

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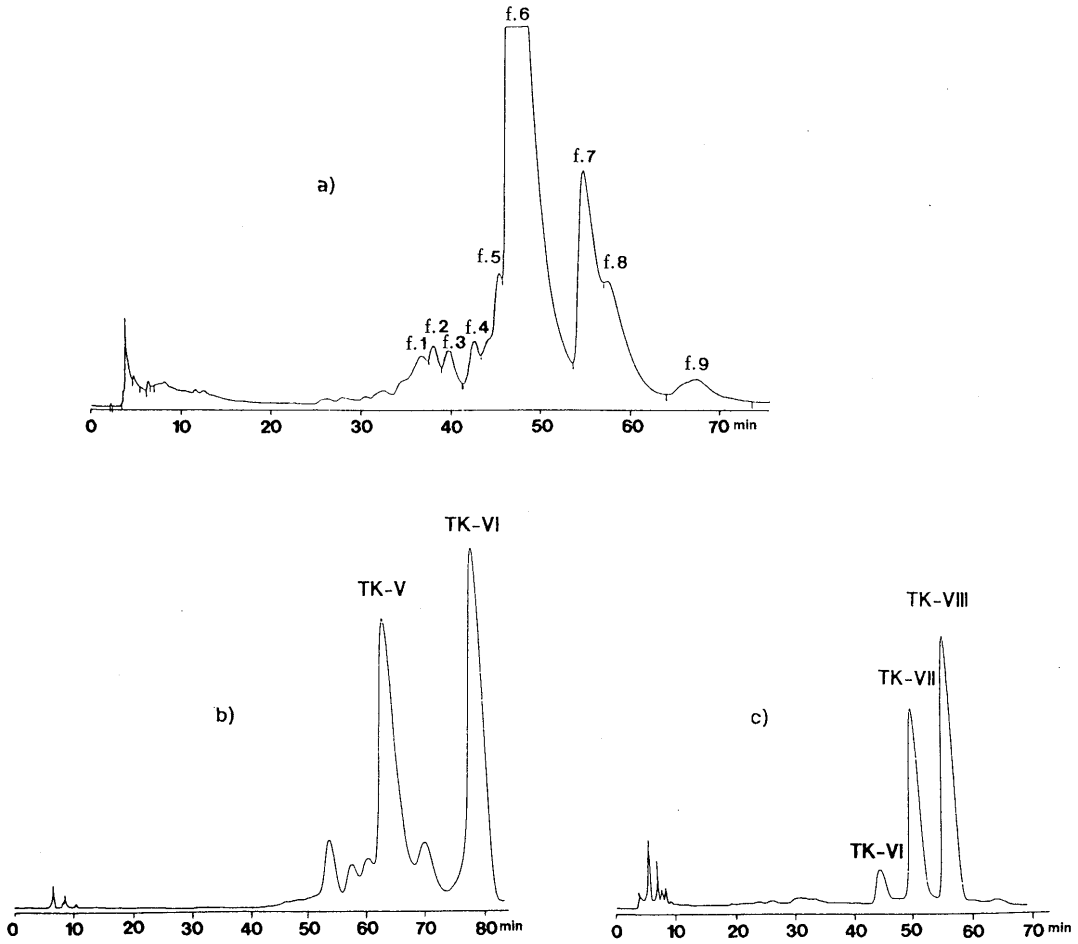

Fig. 1. HPLC Chromatogram of Trichokonins

a) Crude trichokonin mixture obtained from the culture broth of *Trichoderma koningii*. b) Fraction 5 obtained by preparative HPLC. c) Fraction 8 obtained by preparative HPLC. Conditions: a) column, Nacalai Tesque Cosmosil 5Ph (20 mm i.d. × 250 mm); eluate, methanol–water (86 : 14, v/v); flow rate, 9.0 ml/min; detector UV (220 nm). b) column, Shimadzu Prep-ODS (20 mm i.d. × 250 mm); eluate, methanol–water (82 : 18, v/v); flow rate, 8.0 ml/min; detector UV (220 nm). c) column, Shimadzu Prep-ODS (20 mm i.d. × 250 mm); eluate, methanol–water (84 : 16, v/v); flow rate, 8.0 ml/min; detector UV (220 nm).

TABLE II. Characteristic Ions Observed in the ESI-MS and Amino Acid Compositions of Trichokonins

	TK-V	TK-VI	TK-VII	TK-VIII
Characteristic ions observed in ESI-MS	1904.6 $[M+K]^+$ 1888.5 $[M+Na]^+$ 963.9 $[M+K+Na]^{2+}$ 952.8 $[M+K+H]^{2+}$ 944.9 $[M+Na+H]^{2+}$	1959.7 $[M+Na]^+$ 991.5 $[M+2Na]^{2+}$ 980.5 $[M+Na+H]^{2+}$ 668.6 $[M+3Na]^{3+}$	1973.8 $[M+Na]^+$ 998.5 $[M+2Na]^{2+}$ 673.3 $[M+3Na]^{3+}$	1990.1 $[M+K]^+$ 1973.8 $[M+Na]^+$ 1006.6 $[M+K+Na]^{2+}$ 998.5 $[M+2Na]^{2+}$ 995.3 $[M+K+H]^{2+}$ 987.4 $[M+Na+H]^{2+}$ 671.3 $[M+K+Na+H]^{3+}$
Molecular formula (Monoisotopic mass)	$C_{87}H_{144}N_{22}O_{23}$ (1865.1)	$C_{90}H_{149}N_{23}O_{24}$ (1936.1)	$C_{91}H_{151}N_{23}O_{24}$ (1950.1)	$C_{91}H_{151}N_{23}O_{24}$ (1950.1)
Amino acid compositions				
Ala	2.15 (2)	2.94 (3)	2.75 (3)	2.20 (2)
Aib[a)]	8	8	7	9
Gly	1.06 (1)	0.99 (1)	0.99 (1)	0.99 (1)
Gly	2.90 (3)	3.03 (3)	2.99 (3)	3.00 (3)
Iva[a)]			1	
Leu	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Pheol[a)]	1	1	1	1
Pro	1.00 (1)	0.98 (1)	0.97 (1)	0.94 (1)
Val	1.93 (2)	2.00 (2)	1.93 (2)	1.90 (2)

a) The molecular ratios of these amino acids were determined from the NMR spectra.

^{13}C-NMR spectra (Iva: δ 61.3; Aib: δ 57—59).

All of TKs V-VIII showed a negative color reaction to the ninhydrin reagent and their ^{1}H- and ^{13}C-NMR spectra revealed the presence of an acetyl group and a phenylalaninol (Pheol) residue. Thus the TKs were considered to be peptaibols, having an acetyl group at the

N-terminal and a Pheol residue at the C-terminal.

The absolute configuration of the optically active amino acids was determined by HPLC analyses of the complete acid hydrolysates with a chiral ligand-exchange-phase column,[9] while that of Pheol was determined by HPLC analyses of the *N*,*O*-bis(3,5-dinitrobenzoate) derivatives with an optically active stationary-phase column. The results revealed that Iva has the D-configuration and the other amino acids and Pheol have the L-configuration.

Sequence Determination of TK-VII The electrospray ionization mass spectrometry (ESI-MS) spectrum of TK-VII showed the quasi-molecular ion peak at *m/z* 1973.8 $(M+Na)^+$ accompanied with two peaks at *m/z* 998.5 and 673.3 which could be ascribed to multiply charged ions $(M+2Na)^{2+}$ and $(M+3Na)^{3+}$, respectively (Fig. 2). On the basis of the amino acid composition, the molecular weight of TK-VII was determined to be 1950 [calculated monoisotopic mass (H=1.008) for $C_{91}H_{151}N_{23}O_{24}$, 1950.1]. In addition, the ESI-MS showed two complementary fragment ions at *m/z* 1163.9 and 788.5, which were considered to be formed from the entire molecule by a preferential breaking of the very labile Aib–Pro peptide bond.[10] Though the intensities of these fragment ions were very weak, those observed in the FAB-MS were strong enough to be analyzed by collision-induced dissociation (CID). For the C-terminal peptide part, the *m/z* 788 ion was selected and collided to afford the acylium ions at *m/z* 637, 509, 381, 282, and 197, which were interpreted to be generated through successive losses of Pheol, Gln, Gln, Iva (or Val), and Aib (Fig. 3). Since, in the C-terminal peptide fragment, the N-terminal amino acid should be Pro, the *m/z* 197 ion may be ascribed to Pro–Val (or Iva), and thus the C-terminal amino acid sequence was determined as Pro–Val (or Iva)–Aib–Val (or Iva)–Gln–Gln–Pheol. Similarly, the other fragment ion, *m/z* 1163 was subjected to CID to afford the sequential

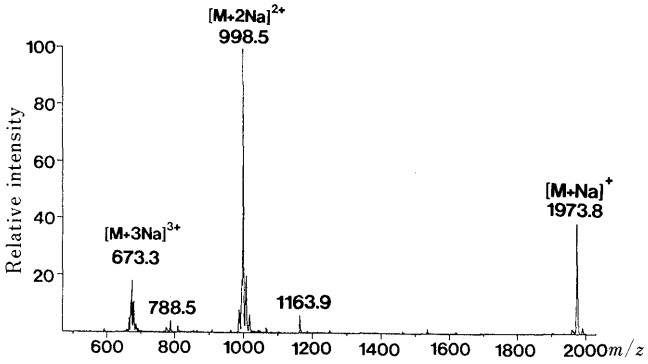

Fig. 2. ESI-MS of TK-VII

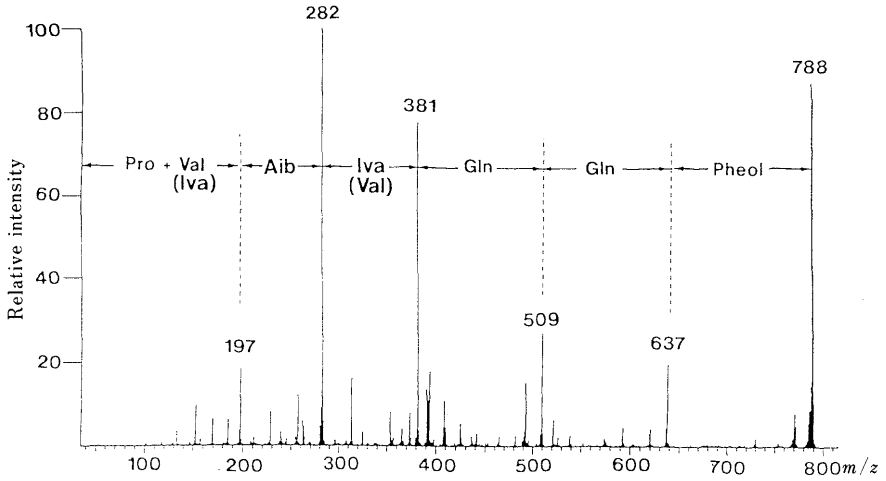

Fig. 3. CID Spectrum of the Fragment Ion at *m/z* 788 (FAB-MS)

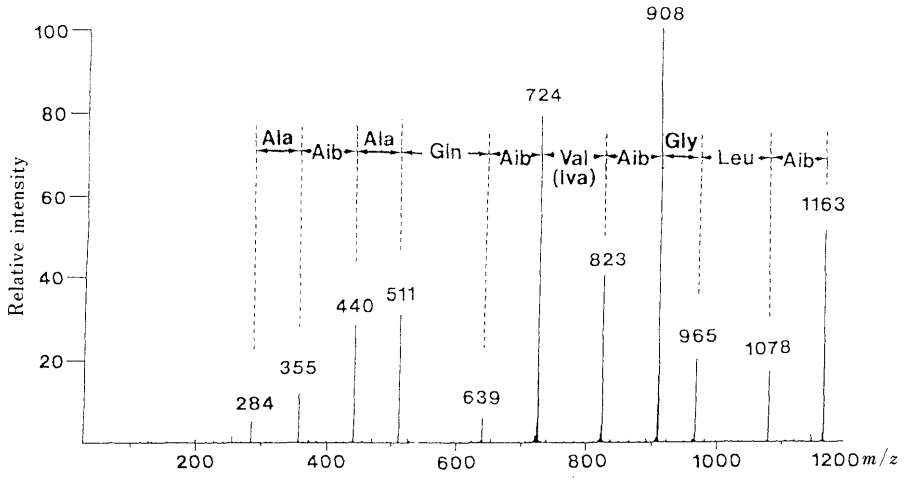

Fig. 4. CID Spectrum of the Fragment Ion at *m/z* 1163 (FAB-MS)

ions generated through successive losses of Aib, Leu, Gly, Aib, Val (or Iva), Aib, Gln, Ala, Aib, and Ala, and gave the *m/z* 284 ion as shown in Fig. 4. The last one at *m/z* 284, on CID, gave the ions at *m/z* 199 (Ac–Aib–Ala) and 128 (Ac–Aib) (Fig. 5). Therefore, the sequence of the N-terminal peptide fragment was determined as Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val (or Iva)–Aib–Gly–Leu–Aib. By connecting the N- and C-terminal oligopeptides, the whole primary structure of TK-VII was obtained as Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val (or Iva)–Aib–Gly–Leu–Aib–Pro–Val (or Iva)–Aib–Val (or Iva)–Gln–Gln–Pheol, except for the locations of the isomeric Val and Iva.

The locations of Iva and Val could be elucidated by the use of two-dimensional (2D) NMR techniques, especially ^1H–^1H shift correlation spectroscopy (COSY) and nuclear

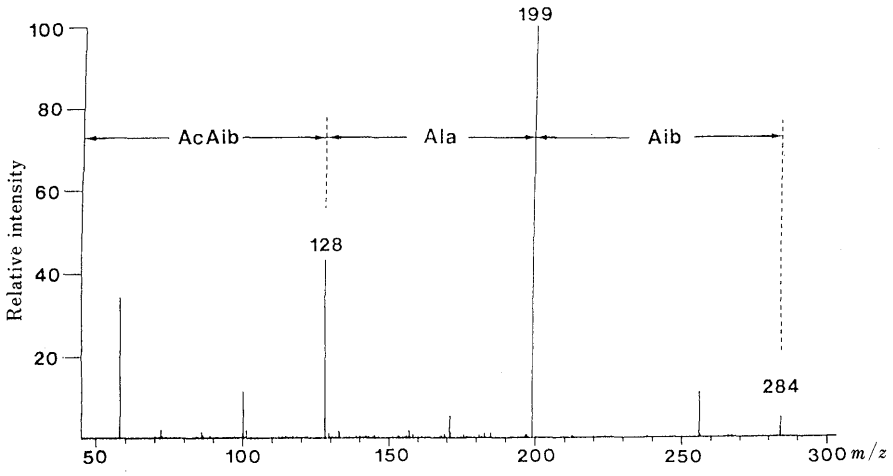

Fig. 5. CID Spectrum of the Fragment Ion at *m/z* 284 (FAB-MS)

TABLE III. ^1H-NMR Assignments (ppm) and Coupling Constants (Hz) of Trichokonin VII (CD_3OH, 20 °C)

Residue	δ_H,[a] *J* (Hz)					
	NH	α-H	β-H	γ-H	δ-H	ε-Carboxamide protons and others
Ac		2.022 s				
Aib[1]	8.419 s[b]		1.476 s, 1.470 s			
Ala[2]	8.269 d (4.0)	4.02 m	1.417 d (7.3)			
Aib[3]	7.636 s		1.516 s, 1.524 s			
Ala[4]	7.693 d (5.2)	4.07 m	1.48[c]			
Aib[5]	7.998 s		1.569 s, 1.530 s			
Ala[6]	7.948 d (4.5)	4.04 m	1.53[c]			
Gln[7]	8.058 d (4.9)	3.92 m	2.32 m, 2.16 m	2.55 m, 2.35 m		7.413 br s, 6.734 br s[d]
Aib[8]	8.113 s		1.593 s, 1.530 s			
Val[9]	7.511 d (5.5)	3.569 dd (9.8, 5.5)	2.24 m	1.142 d (6.7) 1.007 d (6.7)		
Aib[10]	8.234 s		1.565 s, 1.548 s			
Gly[11]	8.343 t (6.0)	3.94 dd (16.4, 6.0) 3.656 dd (16.4, 6.0)				
Leu[12]	8.108 d (6.0)	4.45 m	1.98 m, 1.61 m	1.94 m	0.947 d (6.7) 0.926 d (6.7)	
Aib[13]	8.419 s[b]		1.618 s, 1.548 s			
Pro[14]		4.383 dd (8.5, 5.8)	2.32 m, 1.84 m	2.07 m, 2.00 m	3.88 dt (16.0, 6.0) 3.76 m	
Val[15]	7.602 d (7.9)	3.73 m	2.34 m	1.074 d (6.4) 0.982 d (6.7)		
Aib[16]	7.739 s		1.582 s, 1.489 s			
Iva[17]	7.670 s		2.37 m, 1.80 m 1.489 s	0.857 t (7.5)		
Gln[18]	7.755 d (5.8)	4.04 m	2.27 m	2.642 ddd (14.9, 9.3, 5.3) 2.456 ddd (14.9, 9.3, 7.0)		7.546 br s, 6.779 br s[d]
Gln[19]	7.870 d (7.3)	4.18 m	2.04 m	2.31 m, 2.21 m		7.343 br s, 6.613 br s[d]
Pheol[20]	7.212 d (6.1)	4.15 m	2.947 dd (13.7, 5.5) 2.725 dd (13.7, 8.8)			(α') 3.604 d (5.2) (*o*) 7.253 t (7.2) (*m*) 7.233 d (7.2) (*p*) 7.145 t (7.2)

a) Chemical shifts with three and two figures after the decimal point were obtained from 1D spectrum and 2D spectrum, respectively. *b*) These signals appear separately when measured at −5 °C. *c*) Overlapped with methyl signals of Aib. *d*) Assignment may be interchanged among the three groups.

Overhauser enhancement spectroscopy (NOESY). Actually, the resonances of alkyl and amide protons of Ala, Gln, Val, Gly, Leu, Pro, and Pheol and of the ethyl group of Iva were discriminated by careful analysis of the COSY spectrum (Table III),[11] while the resonances of methyl and amide protons of Aib and Iva were determined based on the result of sequence-specific resonance assignments from the NOESY spectrum (*vide post*).

To suppress the intense signal due to water, we used a selectively excitable 1-$\bar{1}$ pulse (45°_ϕ-τ-$45^\circ_{-\phi}$)[12] instead of the usual 90° pulse in the NOESY experiment and the measurement was carried out at −5 °C in order to improve the signal separation of amide protons. The result is reproduced in Fig. 6, which shows the sequential cross-peaks between the backbone amide protons (NH_i/NH_{i+1}) and between the backbone amide proton and the β- or γ-protons ($C_\beta H_i/NH_{i+1}$, $C_\gamma H_i/NH_{i+1}$).[11] Also in Fig. 6b, a strong cross peak is observed between the acetyl methyl at δ_H 2.02 and the singlet amide proton at δ_H 8.59, indicating that the latter is that of the N-terminal Aib residue. From the series of cross peaks observed in Fig. 6a, coupled with the results of CID experiments, connectivities of amino acid residues Ac–^{1}Aib–^{2}Ala–^{3}Aib–^{4}Ala–^{5}Aib, containing the N-terminal Aib, and ^{18}Gln–^{19}Gln–^{20}Pheol, containing the C-terminal Pheol residue, are readily recognized. Moreover, two series of cross peaks are observed in Fig. 6a, which, on consideration of the CID data, can be assigned to the amino acid sequences ^{6}Aib–^{7}Gln–^{8}Aib–^{9}Val–^{10}Aib–^{11}Gly–^{12}Leu–^{13}Aib and Aib–Iva, respectively. Thus, it is clear that one of the Val residues (δ_{NH} 7.59) occupies the 9-position in TK-VII. On the other hand, as can be seen in Fig. 6b, the γ-protons (δ_H 1.07 and 0.97, each d) of the other Val residue show NOE correlations with a singlet amide proton at δ_H 7.67, due to Aib or Iva. This amide proton is reasonably ascribed to the Aib residue, because the CID data suggested the connectivity of Pro–Val (or Iva)–Aib–Val (or Iva)–Gln, and thus the location of Iva in TK-VII is concluded to be at the 17-position.

Based on the results mentioned above, the complete primary structure of TK-VII was determined to be Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Pro–Val–Aib–Iva–Gln–Gln–Pheol.

Sequence Determination of TK-V, TK-VI, and TK-VIII

The other peptaibols, TKs-V, VI, and VIII, were found to have no Iva residue, and thus their amino acid sequences were elucidated by analyzing their ESI-MS, FAB-MS, and CID spectra (Tables II and IV). The ESI-MS of TK-V showed the ions corresponding to the entire molecule at *m/z* 1904.6 ($M+K^+$), 1888.5 ($M+Na^+$), 963.9 ($M+K^++Na^+$), 952.8 ($M+K^++H^+$), and 944.9 ($M+Na^++H^+$) along with fragment ions at *m/z* 1092.8 (N-terminal oligopeptide) and 774.6 (C-terminal oligopeptide). These data suggested the molecular weight of TK-V to be 1865 (calculated monoisotopic mass for TK-V, 1865.1). From the difference (85 a.m.u.) of the molecular weights of TK-V and TK-VII, together with the results of the amino acid analyses, TK-V was considered to be a nineteen-residue peptide. On the other hand, the FAB-MS of TK-V revealed the protonated molecular ion at *m/z* 1866 and fragment ions at *m/z* 1092 and 774. The CID of the *m/z* 1092 and 774 ions, together with that of the *m/z* 284 ion, revealed the amino acid sequence of TK-V to be Ac–Aib–Ala–Aib–Ala–Aib–Gln–Aib–Val–Aib–Gly–Leu–Aib–

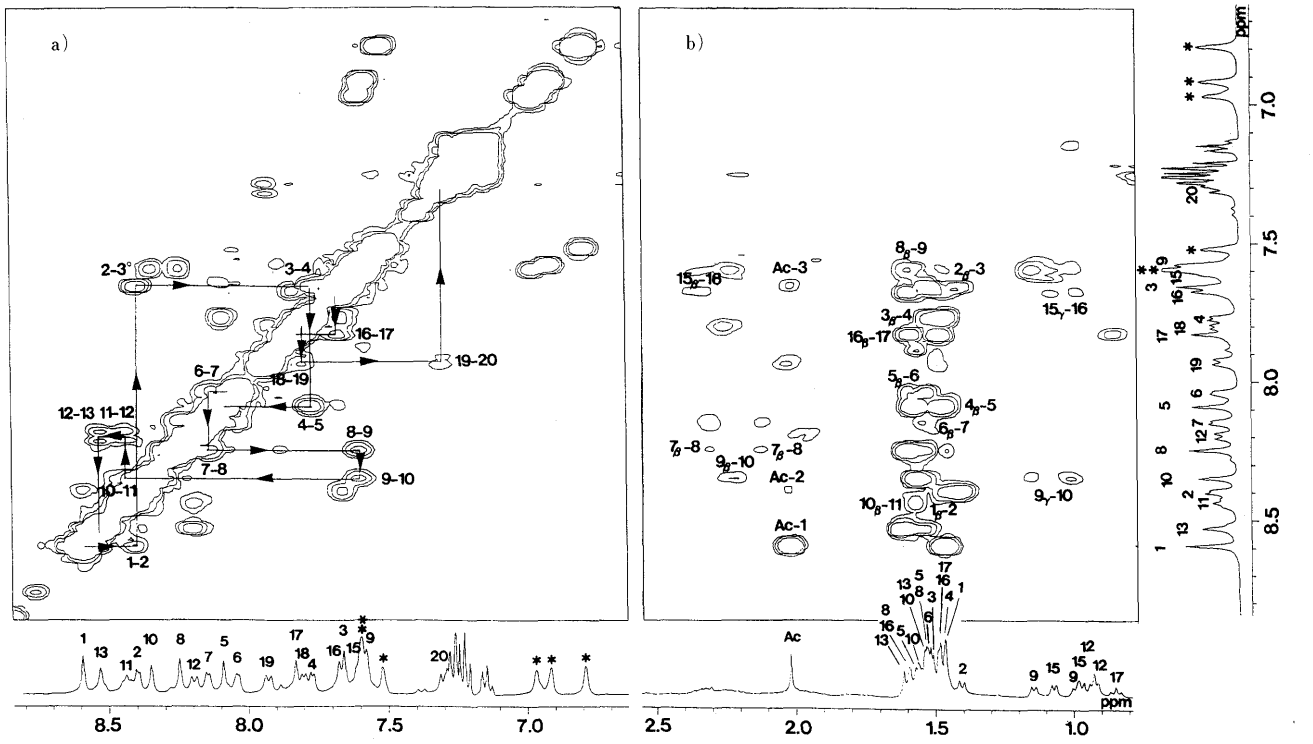

Fig. 6. Part of the NOESY Spectrum of TK-VII in CD_3OH (Temperature, −5 °C; Mixing Time, 250 ms)

a) Cross peaks between the backbone amide protons, labelled with the numbers of the two residues involved. b) Cross peaks between the backbone amide protons and the β- or γ-protons, labelled with the numbers of the two residues involved; intraresidue cross peaks are not labeled. Numbers in the reference spectrum indicate the positions of amino acid residues. Asterisks indicate the ε-carboxamide protons of glutamines.

TABLE IV. Diagnostic Ions[a] Observed in the FAB-MS and CID Spectra of Trichokonins

	Acylium ions, *m/z*																				
	← N-Terminal oligopeptide →												← C-Terminal oligopeptide →								
Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	$[M+H]^+$
TK-V	128	199	**284**	355	**440**	568	**653**	**752**	**837**	894	1007	**1092**		197	282	367	495	623	**774**		1865
TK-VI	128	199	**284**	355	440	**511**	639	**724**	823	**908**	**965**	1078	**1163**		197	282	367	495	623	**774**	1936
TK-VII	128	199	**284**	355	**440**	**511**	639	**724**	823	**908**	965	1978	**1163**		197	282	381	509	637	**788**	1950
TK-VIII	128	199	**284**	**355**	**440**	**525**	653	**738**	837	**922**	979	1092	**1177**		197	282	367	495	623	**774**	1950

a) Mass numbers are indicated by nominal units and bold numbers indicate the ions clearly observed in the FAB-MS.

Pro–Val–Aib–Val–Gln–Gln–Pheol.

Similarly, the primary structures of TK-VI and TK-VIII were examined and they were found to be identical with gliodeliquescin A (Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol)[13] isolated from *Gliocladium deliquescens* and trichosporin B-IVc (Ac–Aib–Ala–Aib–Ala–Aib–Aib–Gln–Aib–Val–Aib–Gly–Leu–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol)[14] obtained from *Trichoderma polysporum*, respectively.

Conclusion

The peptaibols isolated from *T. koningii* were demonstrated to be three twenty-residue peptaibols and a nineteen-residue one. TKs-VI, VII, and VIII varied at positions 6 or 17, and TK-V was a nineteen-residue peptide lacking Ala-6 of TK-VI (gliodeliquescin A) or Aib-6 of TK-VIII (trichosporin B-IVc) (Table I).

TKs V-VIII all showed inhibitory activity against the mycelial growth of *G. lucidum* at 40 μg/disc in the assay system described previously.[1]

Peptaibols exhibit a broad range of bioactivities relating to their membrane-interacting properties, and some of them show antibiotic activities.[6,13,15] It is of interest to note that TKs V-VIII showed a strong Ca^{2+} channel-activating effect. Details of this activity will be reported elsewhere.

Experimental

IR spectra were taken with a Shimadzu IR-408 infrared spectrophotometer in KBr disks. ESI-MS, FAB-MS, and CID spectra were obtained with a Finnigan MAT TSQ-700 mass spectrometer and glycerol–thioglycerol (1 : 1) mixture was used as a matrix for FAB ionization. For CID experiments, argon was used as a collision gas at a pressure of 2 mTorr (collision energy, 10 eV). ^{1}H-NMR and 2D NMR spectra were measured with a JEOL JNM-GX400 spectrometer in CD_3OH solutions. NOESY spectra were measured at -5 °C with a JEOL pulse sequence VNOESS1 using the 1-$\bar{1}$ pulse for the purpose of eliminating the H_2O signal (mixing time, 250 ms; delay time for 1-$\bar{1}$ pulse, 0.25 ms). Amino acid analyses were done with a Hitachi 835 amino acid analyzer using the ninhydrin method. Determination of absolute configurations of amino acids and phenylalaninol was carried out with a Tosoh CCP&8000 system equipped with a Tosoh UV-8000 detector.

Analytical TLC was carried out on precoated Merck Kieselgel 60 F_{254} plates (0.25 mm). HPLC was carried out on a Shimadzu LC-5A system equipped with an SPD-2A UV detector (220 nm). Analytical HPLC was on a Nacalai Tesque Cosmosil 5Ph column (4.6 mm i.d. × 250 mm) or on a Shimadzu STR ODS-H column (4.6 mm i.d. × 250 mm) with MeOH–H_2O (85 : 15) at a flow rate of 0.5 ml/min.

Culture Medium Ordinary yeast-malt medium[16] was modified as follows. Yeast extract (Difco Laboratories, 4.0 g), malt extract (Difco Laboratories, 10.0 g), glucose (4.0 g), and Kagome vegetable juice with no salt added (Kagome Co., Ltd., 100 ml) were dissolved in distilled water (diluted to 1000 ml) and the pH was adjusted to 5.5 with 1 N HCl.

Culture of *Trichoderma koningii* A wild strain of *T. koningii* was isolated from a wood material which had been inoculated with mycelia of *G. lucidum* for cultivation and was identified by one of the authors (Tubaki). Sixty Fernbach flasks (2 l),[17] each containing 600 ml of modified yeast-malt medium were sterilized by autoclaving and inoculated with a piece of mycelial mat of *T. koningii*. The culture was carried out at room temperature for 28 d under a static condition.

Preliminary Bioassay of Culture Broth The culture broth of *T. koningii* (600 ml) was separated into mycelia and medium by filtration, and the medium was extracted with BuOH to give a BuOH extract (265 mg). The water layer was lyophilized to yield the H_2O fraction (2.6 g). On the other hand, the mycelia (wet weight, 7 g) were extracted successively with CH_2Cl_2 and MeOH (each 200 ml × 3) to give the CH_2Cl_2 extract (300 mg) and the MeOH extract (530 mg), respectively. Inhibitory activity of these extracts and fractions against the mycelial growth of *G. lucidum* was investigated in the same manner as described previously,[1] and only the BuOH extract of the medium showed inhibitory activity at 600 μg/disc.

Isolation of Trichokonins V to VIII (TK-V to TK-VIII) The culture broth (36 l) was filtered and the filtrate was extracted with BuOH (36 l × 3). The combined BuOH solution was concentrated *in vacuo* and the residue (24 g) was chromatographed on a silica gel (Merck, Art. 7734, 600 g) column with hexane–$CHCl_3$ (80 : 20, 2 l), $CHCl_3$ (3 l), and $CHCl_3$–MeOH mixtures (95 : 5, 3 l; 90 : 10, 3 l; 75 : 25, 2 l; 50 : 50, 3 l). Eluates were collected in 15 ml portions, monitored by TLC, and combined into eight fractions. Fraction 8 (7.4 g) eluted with $CHCl_3$–MeOH (50 : 50) was subjected to reversed-phase column chromatography (Nacalai Tesque Cosmosil 75 C_{18}–OPN gel, 130 g) with MeOH–H_2O (30 : 70, 2 l; 40 : 60, 1 l; 60 : 40, 500 ml; 80 : 20, 500 ml; 90 : 10, 500 ml). Eluates were collected in 100 ml portions, monitored by analytical HPLC (Nacalai Tesque Cosmosil 5Ph column), and combined into a total of seven fractions (fr. 1 to fr. 7).

Fraction 6 (1.3 g) eluted with MeOH–H_2O (80 : 20) in the above chromatography was then subjected to preparative HPLC with a Nacalai Tesque Cosmosil 5Ph column (20 mm i.d. × 250 mm) repeatedly [solvent, MeOH–H_2O (86 : 14); flow rate, 9.0 ml/min; detector setting, UV 220 nm] to give nine fractions (Fig. 1a). Among these, fr. 5 (156 mg) was further separated by preparative HPLC on a Shimadzu Prep-ODS column (20 mm i.d. × 250 mm) with MeOH–H_2O (82 : 18) at a flow rate of 8.0 ml/min to yield trichokonins V (TK-V, 9 mg) and VI (TK-VI, 126 mg) as amorphous solids (Fig. 1b).

Fraction 6 and fr. 7 gave trichokonin VI (TK-VI, 575 mg, amorphous solid) and trichokonin VII (TK-VII, 59 mg, amorphous solid), respectively. Fraction 8 (73 mg) was also separated by preparative HPLC on a Shimadzu Prep-ODS column with MeOH–H_2O (84 : 16) at a flow rate of 8.0 ml/min to give trichokonins VI (TK-VI, 7 mg), VII (TK-VII, 21 mg), and VIII (TK-VIII, 34 mg) as amorphous solids (Fig. 1c).

Absolute Configuration of Amino Acids and Amino Alcohols Each peptide sample (*ca.* 0.2 mg) was hydrolyzed in 6 N HCl (0.5 ml) at 110 °C for 24 h and the reaction mixture was evaporated to dryness *in vacuo* to give the hydrolysate. For the determination of the absolute configurations of amino acids, a part of the hydrolysate was dissolved in distilled water and subjected to HPLC analysis with a Sumichiral OA-5000 column (4.6 mm i.d. × 150 mm, Sumika Chemical Analysis Service Ltd.; column temperature, 40 °C; solvent, 2 mM $CuSO_4$ in H_2O; flow rate, 1.0 ml/min; detector setting, UV 254 nm).[9] Retention time values (min): L-Ala, 4.26;

D-Ala, 5.68; Aib, 7.03; L-Pro, 8.48; L-Iva, 10.35; L-Val. 12.19; D-Iva, 12.81; D-Pro, 18.37; D-Val, 21.68; L-Leu, 38.23; L-Glu, 51.76; D-Leu, 64.45; D-Glu, 66.92.

The absolute configuration of Pheol in each peptide was established in the following manner. A part of the acid hydrolysate (*ca.* 0.1 mg) was dissolved in absolute MeOH (2 ml). To this solution was added thionyl chloride (0.2 ml) and the mixture was refluxed under an Ar atmosphere for 3 h to give the methyl esters of the amino acids. After removal of the reagent and solvent by evaporation *in vacuo*, the residue was treated with a solution of 3,5-dinitrobenzoyl chloride (*ca.* 1 mg) and triethylamine (one drop) in EtOAc (2 ml) with stirring at room temperature for 12 h. The mixture was then concentrated to dryness *in vacuo* and the resulting *N,O*-bis(3,5-dinitrobenzoate) of Pheol was analyzed by HPLC on a Sumichiral OA-4700 column [4.6 mm i.d. × 150 mm, Sumika Chemical Analysis Service Ltd.; column temperature, 35 °C; solvent, hexane–EtOH (230 : 20); flow rate, 1.0 ml/min; detector setting, UV 254 nm]. Retention time values (min): L-Pheol, 26.20; D-Pheol, 36.23.

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References

1) Y. Tezuka, Q. Huang, T. Kikuchi, A. Nishi, K. Tubaki, *Chem. Pharm. Bull.*, **42**, 2612 (1994).
2) A. Iida, M. Sanekata, T. Fujita, H. Tanaka, A. Enoki, G. Fuse, M. Kanai, P. J. Rudewicz, E. Tachikawa, *Chem. Pharm. Bull.*, **42**, 1070 (1994); S. Rebuffat, L. Conraux, M. Massias, C. Auvin-Guette, B. Bodo, *Int. J. Peptide Protein Res.*, **41**, 74 (1993); H. Brückner, H. Graf, M. Bokel, *Experientia*, **40**, 1189 (1984); G. Boheim, G. Irmscher, G. Jung, *Biochim. Biophys. Acta*, **507**, 485 (1978); R. C. Pandey, J. C. Cook Jr., K. L. Rinehart Jr., *J. Am. Chem. Soc.*, **99**, 8649 (1977); G. Boheim, K. Janko, D. Leibfritz, T. Ooka, W. A. König, G. Jung, *Biochim. Biophys. Acta*, **433**, 182 (1976); and references cited therein.
3) M. S. P. Sansom, *Eur. Biophys. J.*, **22**, 105 (1993); P. Balaram, K. Krishna, M. Sukumar, I. R. Mellor, M. S. P. Sansom, *ibid.*, **21**, 117 (1992); S. Rebuffat, H. Duclohier, C. Auvin-Guette, G. Molle, G. Spach, B. Bodo, *FEMS Microbiol. Immunol.*, **105**, 151 (1992); G. Menestrina, K. P. Voges, G. Jung, G. Boheim, *J. Membrane Biol.*, **93**, 111 (1986); J. E. Hall, I. Vodyanoy, T. M. Balasubramanian, G. R. Marshall, *Biophys. J.*, **45**, 233 (1984); M. K. Mathew, P. Balaram, *Mol. Cell. Biochem.*, **50**, 47 (1983).
4) M. Okuda, A. Iida, S. Uesato, Y. Nagaoka, T. Fujita, Y. Takaishi, H. Terada, *Biol. Pharm. Bull.*, **17**, 482 (1994); M. K. Das, S. Raghothama, P. Balaram, *Biochemistry*, **25**, 7110 (1986); M. K. Mathew, R. Nagaraj, P. Balaram, *Biochem. Biophys. Res. Commun.*, **98**, 548 (1981); Y. Takaishi, Y. Terada, T. Fujita, *Experientia*, **36**, 550 (1980).
5) E. Tachikawa, S. Takahashi, K. Furumachi, T. Kashimoto, A. Iida, Y. Nagaoka, T. Fujita, Y. Takaishi, *Mol. Pharmacol.*, **40**, 790 (1991); R. I. Fonteriz, M. G. Lopez, J. Garcia-Sancho, A. G. Garcia, *FEBS Lett.*, **283**, 89 (1991); A. R. Artalejo, C. Montiel, P. Sanchez-Garcia, G. Uceda, J. M. Guantes, A. G. Garcia, *Biochem. Biophys. Res. Commun.*, **169**, 1204 (1990).
6) M. E. Hajji, S. Rebuffat, T. L. Doan, G. Klein, M. Satre, B. Bodo, *Biochim. Biophys. Acta*, **978**, 97 (1989).
7) W. G. Bessler, B. Ottenbreit, G. Irmscher, G. Jung, *Biochem. Biophys. Res. Commun.*, **87**, 99 (1979); G. Irmscher, G. Jung, *European J. Biochem.*, **80**, 165 (1977).
8) C. Auvin-Guette, S. Rebuffat, I. Vuidepot, M. Massias, B. Bodo, *J. Chem. Soc., Perkin Trans. 1*, **1993**, 249.
9) K. Matsuura, A. Yesilada, A. Iida, Y. Takaishi, M. Kanai, T. Fujita, *J. Chem. Soc., Perkin Trans. 1*, **1993**, 381; N. Oi, H. Kitahara, R. Kira, *J. Chromatogr.*, **592**, 291 (1992).
10) S. Rebuffat, M. E. Hajji, P. Hennig, D. Davoust, B. Bodo, *Int. J. Peptide Protein Res.*, **34**, 200 (1989); M. E. Hajji, S. Rebuffat, D. Lecommandeur, B. Bodo, *ibid.*, **29**, 207 (1987); H. Brückner, H. Graf, M. Bokel, *Experientia*, **40**, 1189 (1984).
11) V. J. Basus, "Proton Nuclear Magnetic Resonance Assignments. Methods in Enzymology," Vol. 177, ed. by N. J. Oppenheimer, T. L. James, Academic Press, Inc., San Diego, 1989, p. 132.
12) P. J. Hore, *J. Magn. Reson.*, **55**, 283 (1983); G. M. Clore, B. J. Kimber, A. M. Gronenborn, *ibid.*, **54**, 170 (1983).
13) H. Brückner, M. Przybylski, *Chromatogr.*, **19**, 188 (1984).
14) A. Iida, M. Okuda, S. Uesato, Y. Takaishi, T. Shingu, M. Morita, T. Fujita, *J. Chem. Soc., Perkin Trans. 1*, **1990**, 3249.
15) W.-C. Jen, G. A. Jones, D. Brewer, V. O. Parkinson, A. Taylor, *J. Appl. Bacteriol.*, **63**, 293 (1987); K. Fuji, E. Fujita, Y. Takaishi, T. Fujita, I. Arita, M. Komatsu, N. Hiratsuka, *Experientia*, **34**, 237 (1978).
16) T. Yanagita (ed.), "Experimental Methods in Microbiology (in Japanese)," Kodansha Scientific, Tokyo, 1975, p. 436.
17) Ref. 16, p. 156.