Studies on Metabolites of Mycoparasitic Fungi. IV.[1] Minor Peptaibols of *Trichoderma koningii*

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Three minor peptaibols, trichokonins (TKs)-Ia, Ib, and IX, were obtained from the culture broth of *Trichoderma koningii* OUDEMANS. Primary structures of these peptaibols were elucidated by ion-spray ionization mass spectrometry (ISI-MS) including the collision-induced dissociation (CID) technique together with two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY).

Key words *Trichoderma koningii*; peptaibol; trichokonin; collision-induced dissociation; ion-spray ionization mass spectrometry; NOESY

In a previous paper,[2] we reported the isolation and structure elucidation of four peptaibols, named trichokonins (TKs) V—VIII, from the culture broth of the fungus *Trichoderma koningii* OUDEMANS, which is harmful to the cultivation of a medicinal mushroom, *Ganoderma lucidum* (FR.) KARST. (oriental crude drug "Lin-Chi"). Trichokonin VI (gliodeliquescin A[3]) is a potent agonist of the L-type Ca^{2+} channel in cardiac membrane.[4] This is the first example of a peptaibol acting on Ca^{2+} channels in a biological membrane.[5] In a further study, we isolated three minor peptaibols TK-Ia, TK-Ib, and TK-IX (Table 1). In this paper, we wish to report the structure elucidation of these minor peptaibols.

Results and Discussion

Separation and Characterization of TKs As reported in a previous paper,[2] the culture broth of *T. koningii* was separated into mycelia and medium by filtration and the medium was extracted with BuOH. 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 TKs-Ia, -Ib, and -IX.

TKs-Ia, Ib, and IX showed a negative color reaction to the ninhydrin reagent and showed IR absorptions at 3340 (NH), 1630 (CO), and 1520 (NH) cm^{-1}, characteristic of peptide linkages. Their ^1H- and ^{13}C-NMR spectra revealed the presence of an acetyl group and a phenylalaninol (Pheol) residue in the molecule, suggesting that they are peptaibols, having an acetyl group at the N-terminal and a Pheol residue at the C-terminal.

The proportions of normal amino acids in TKs-Ia, Ib, and IX were established from amino acid analyses of the complete acid hydrolysates (Table 2). The numbers of Aib and isovaline (Iva) residues, which respond poorly to *ortho*-phthalaldehyde (OPA) reagent, were determined on the basis of the numbers and relative intensities of the triplet ^1H-signal due to the γ-methyl group of Iva (δ 0.85) and the singlet ^1H-signals due to the amide protons of Iva (δ 7.76) and Aib.

The absolute configuration of the optically active amino acids was determined by HPLC analyses of complete acid hydrolysates with a chiral ligand-exchange-phase column, 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.[2,6] The results revealed that Iva has the D-configuration and the other amino acids and Pheol have the L-configuration.

Sequence Determination of TK-Ib The ion-spray ionization MS (ISI-MS) of TK-Ib showed the ions corresponding to the entire molecule at *m/z* 1924.4 $[M+H]^+$, 962.4 $[M+2H]^{2+}$, and 642.0 $[M+3H]^{3+}$ (Fig. 1). From these ions, the molecular weight of TK-Ib was calculated as 1923.1, which was compatible with the amino acid composition as given in Table 2 [average mass[7] for $C_{89}H_{147}N_{23}O_{24}$, 1923.3]. Moreover, the ISI-MS showed two complementary fragment ions at *m/z* 1149 and 774, which were considered to be formed from the entire molecule by a preferential breaking of the labile Aib–Pro peptide bond.[2]

The collision-induced dissociation (CID) spectra of the fragment ion at *m/z* 774 showed acylium ions at *m/z* 623,

Table 1. Primary Structures of Trichokonins

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	M.W.[a]
TK-Ia	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Ala	Pro	Val	Aib	Aib	Gln	Gln	Pheol	1921
TK-Ib	Ac	Aib	Gly	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol	1921
TK-IX	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln	Gln	Pheol	1963
TK-VI (Gliodeliquescin A)	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol	1935

a) Nominal molecular weights.

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Table 2. Characteristic Ions Observed in the ISI-MS and Amino Acid Compositions of Trichokonins

	TK-Ia	TK-Ib	TK-IX
Ions corresponding to the entire molecule in ISI-MS	1946.0 $[M+Na]^+$ 1924.0 $[M+H]^+$ 984.4 $[M+2Na]^+$	1924.4 $[M+H]^+$ 962.4 $[M+2H]^{2+}$ 642.0 $[M+3H]^{3+}$	1966.4 $[M+H]^+$ 983.6 $[M+2H]^{2+}$ 656.0 $[M+3H]^{3+}$
(Molecular weight deduced from the ions)	973.6 $[M+H+Na]^+$ 962.4 $[M+2H]^{2+}$ 664.0 $[M+3Na]^{3+}$ 656.4 $[M+H+2Na]^{3+}$ 649.2 $[M+2H+Na]^{3+}$ (1922.9)	(1923.1)	(1965.3)
Molecular formula	$C_{89}H_{147}N_{23}O_{24}$	$C_{89}H_{147}N_{23}O_{24}$	$C_{92}H_{153}N_{23}O_{24}$
(Monoisotopic mass; average mass)	(1922.1; 1923.3)	(1922.1; 1923.3)	(1964.1; 1965.4)
Amino acid compositions			
Ala	3.79 (4)	2.24 (2)	2.15 (2)
Aib[a)]	7	8	8
Gly	1.38 (1)	1.84 (2)	1.08 (1)
Glu	2.77 (3)	3.41 (3)	2.90 (3)
Iva[a)]			1
Leu	1.00 (1)	1.00 (1)	1.00 (1)
Pheol[a)]	1	1	1
Pro	0.97 (1)	0.98 (1)	1.00 (1)
Val	2.05 (2)	1.89 (2)	1.97 (2)

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

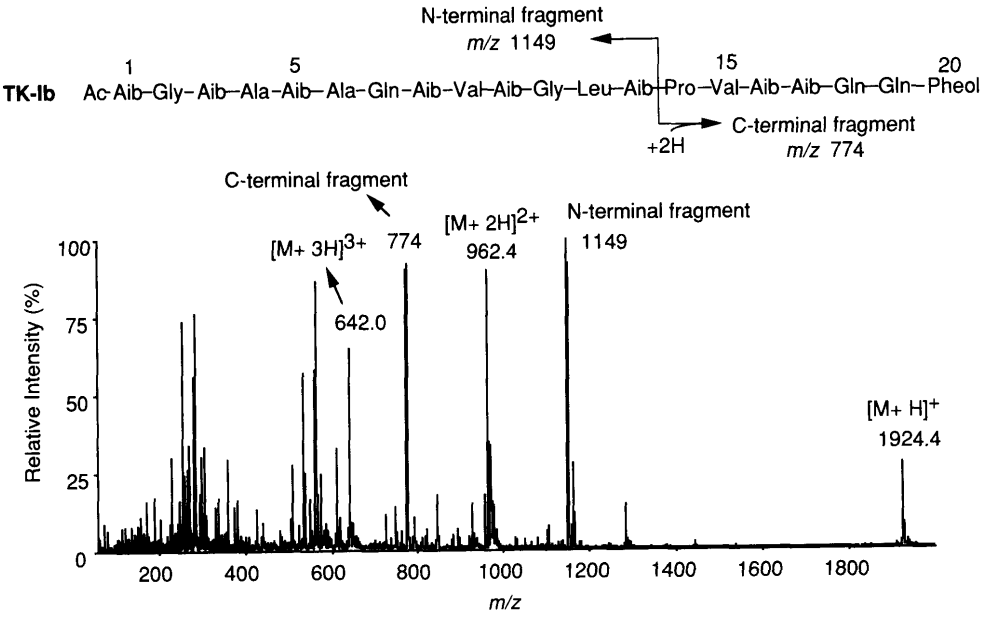

Fig. 1. ISI-MS of TK-Ib

495, 367, 282, and 197, which were interpreted to have been generated through successive losses of Pheol, Gln, Gln, Aib, and Aib (Fig. 2a). Since, in the C-terminal peptide fragment, the N-terminal amino acid was considered to be Pro, the *m/z* 197 ion could be assigned to Pro–Val, and thus the C-terminal amino acid sequence was determined to be Pro–Val–Aib–Aib–Gln–Gln–Pheol. Similarly, the counterpart ion (*m/z* 1149) was subjected to CID to show sequential ions at *m/z* 1064, 951, 894, 809, 710, 625, 497, 426, 341, and 270, generated through successive losses of Aib, Leu, Gly, Aib, Val, Aib, Gln, Ala, Aib, and Ala (Fig. 2b). Though the CID spectrum of the *m/z* 270 ion failed to give significant product ions, that of the *m/z* 426 ion showed fragment ions at *m/z* 270 (Ac–Aib–Gly–Aib), 185 (Ac–Aib–Gly), and 128 (Ac–Aib) (Fig. 2c).[8] Therefore, the sequence of the N-terminal peptide fragment was determined as Ac–Aib–Gly–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib. By connecting the N- and C-terminal oligopeptides, the whole primary structure of TK-Ib was determined to be Ac–Aib–Gly–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol.

Sequence Determination of TK-Ia The ISI-MS of TK-Ia showed the quasi-molecular ions corresponding to the molecular formula $C_{89}H_{147}N_{23}O_{24}$ (Table 2), but it failed to give characteristic bisected ions formed by the fission of the Aib–Pro peptide bond. Instead, it gave three pairs of weak complementary fragment ions at *m/z* 1149 and 774, at *m/z* 1015 and 908, and at *m/z* 1078 and 845 (Fig. 3).

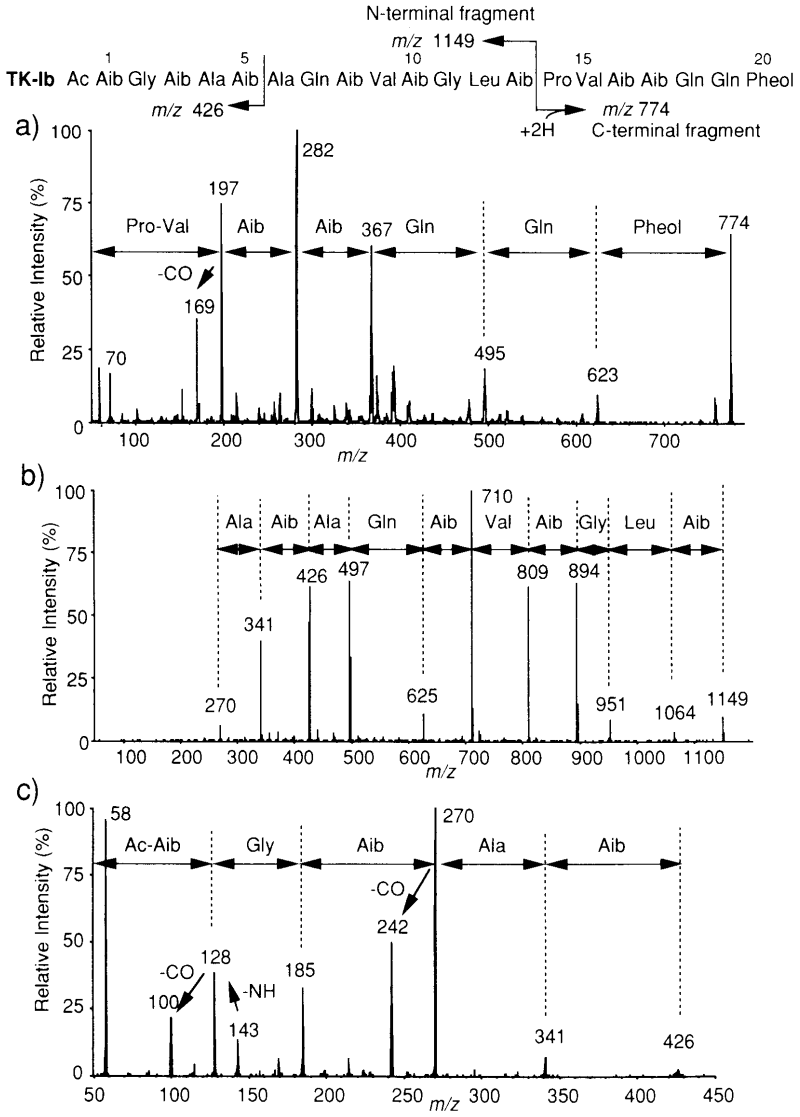

Fig. 2. CID Spectra of the Fragment Ions at *m/z* 774 (a), 1149 (b), and 426 (c) of TK-Ib

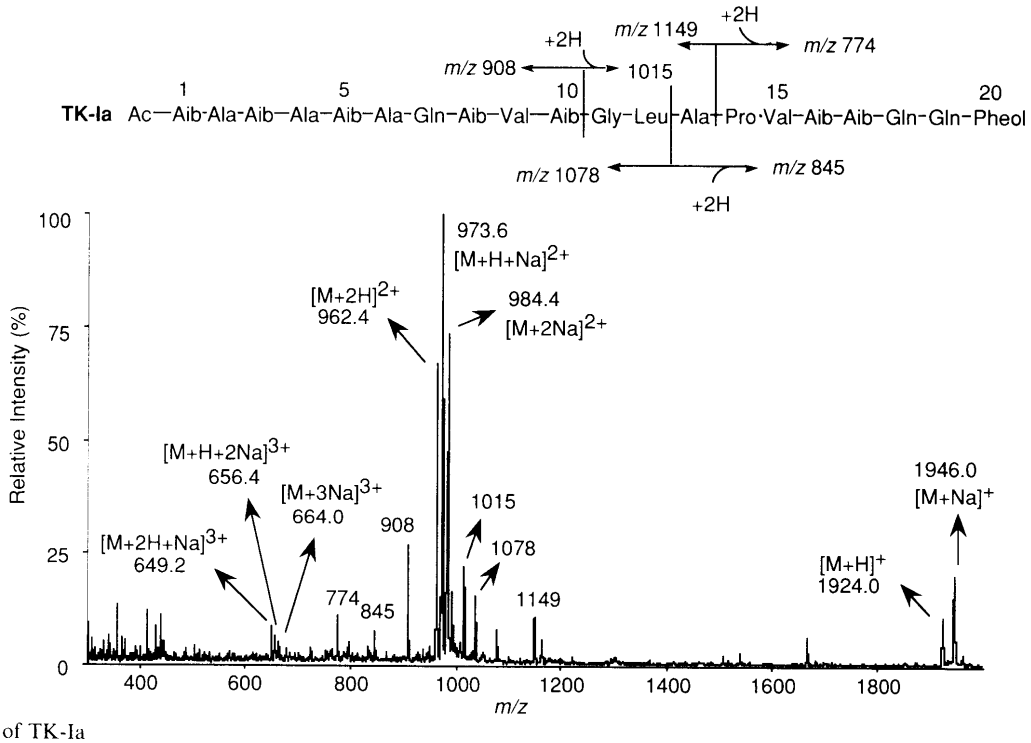

Fig. 3. ISI-MS of TK-Ia

The CID spectra of the m/z 1149 and 774 ions, together with that of the m/z 440 ion,[9] suggested the N- and C-terminal fragments to be Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Ala and (Pro + Val)–Aib–Aib–Gln–Gln–Pheol, respectively (Fig. 4). Similarly, those of the m/z 908 and 1015 ions revealed the sequences Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib and (Gly + Leu + Ala + Pro)–Val–Aib–Aib–Gln–Gln–Pheol (Fig. 4). From these results, the amino acid sequence of TK-Ia was determined to be Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Ala–Pro–Val–Aib–Aib–Gln–Gln–Pheol. This was also supported by the CID spectra of the m/z 1078 and 845 ions.

Sequence Determination of TK-IX The ^{1}H-NMR spectrum of TK-IX exhibited a methyl signal at δ 0.85 (t, $J = 7.5$ Hz) ascribable to the γ-methyl group of Iva. The presence of Iva was confirmed by HPLC analysis of the complete acid hydrolysate with a chiral ligand-exchange-phase column.[6] The ISI-MS of TK-IX showed the ions corresponding to the entire molecule at m/z 1966.4 $[M+H]^+$, 983.6 $[M+2H]^{2+}$, and 656.0 $[M+3H]^{3+}$ (Table 2), along with strong fragment peaks at m/z 1177 (N-terminal fragment) and 788 (C-terminal fragment) arising from the fission of the Aib–Pro peptide bond. Based on the CID spectra of these fragment ions (Fig. 5, upper), the sequences of the N- and C-terminal peptide fragments were deduced to be Ac–Aib–Ala–Aib–Ala–Aib–Aib–Gln–Aib–Val (or Iva)–Aib–Gly–Leu–Aib (N-terminal frag-

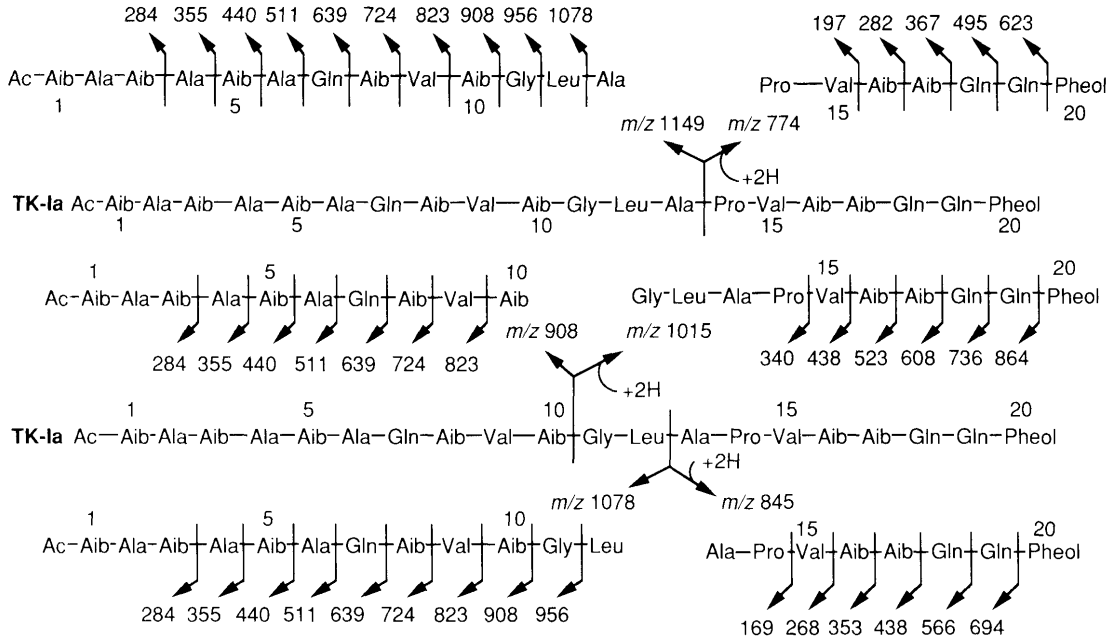

Fig. 4. Acylium Fragment Ions Observed in the CID Spectra of the m/z 1149, 1078, 1015, 908, 845, and 774 Ions of TK-Ia

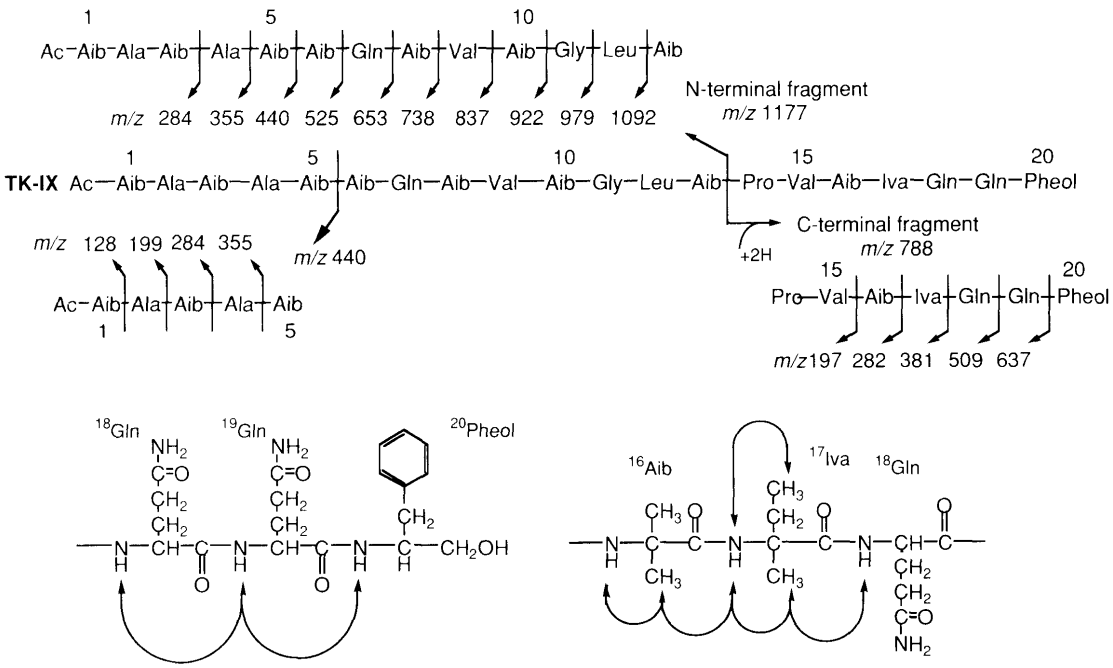

Fig. 5. Fragment Ions Observed in the CID Spectra (Upper) and Diagnostic NOE's for Sequencing Iva (Lower)

ment) and Pro–Val (or Iva)–Aib–Iva (or Val)–Gln–Gln–Pheol (C-terminal fragment), except for the locations of the isomeric Val and Iva.

The locations of Val and Iva were elucidated by the use of ^1H–^1H shift correlation spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY). The sequential cross-peaks between the backbone amide protons (NH_i/NH_{i+1})[10] observed in the NOESY spectrum, coupled with the results of CID experiments, indicated the connectivities of amino acid residues, Ac–^{1}Aib–^{2}Ala–^{3}Aib, ^{4}Ala–^{5}Aib, ^{6}Aib–^{7}Gln–^{8}Aib–^{9}Val–^{10}Aib–^{11}Gly–^{12}Leu–^{13}Aib, Aib–Iva (or Iva–Aib), and ^{18}Gln–^{19}Gln–^{20}Pheol. Thus, one of the Val residues must be located at the 9-position in TK-IX. On the other hand, the amide proton of ^{18}Gln (δ 7.81, d, $J=5.7$ Hz) showed nuclear overhauser effect (NOE) correlation with a singlet methyl signal (δ 1.49), which was assigned to either Aib or Iva. It followed that the location of Iva is at the 17-position, because the CID data suggested the connectivity of ^{14}Pro–^{15}Val (or ^{15}Iva)–^{16}Aib–^{17}Iva (or ^{17}Val)–^{18}Gln (Fig. 5, lower).

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

Conclusion

Three minor peptaibols isolated from *T. koningii* were demonstrated to be twenty-residue peptaibols having an N-terminal acetyl group and a C-terminal Pheol residue. It is of particular interest to note that TK-Ia has the Ala–Pro sequence instead of the Aib–Pro bonding which occurs commonly in a series of peptaibols.[11] The primary structures of TK-Ia, TK-Ib, and TK-IX varied at position 13, position 2, and positions 6 and 17, respectively, from that of TK-VI, which showed a strong Ca^{2+} channel-activating effect. The Ca^{2+} channel-activating effect of TKs-Ia, Ib, and IX are currently under investigation and will be reported elsewhere.

Experimental

IR spectra were taken with a Shimadzu IR-408 infrared spectrophotometer in KBr disks. ISI-MS and CID spectra were obtained with a Perkin–Elmer Sciex API-III mass spectrometer (orifice voltage, 40—100 V), and for CID experiments, argon was used as a collision gas (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 ($45°_{\phi}$–τ–$45°_{-\phi}$)[12] 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 Shimadzu amino acid analyzer using the OPA method. Determination of absolute configurations of amino acids and phenylalaninol was done by HPLC analyses with optically active stationary-phase columns as described previously.[2]

HPLC was carried out on a Shimadzu LC-5A system equipped with an SPD-2A UV detector (220 nm).

Isolation of TKs Extraction and separation of the crude metabolites from the culture broth of *T. koningii* were described in a previous paper[2]; *i.e.* the culture broth (36 l) was extracted with BuOH and the BuOH extract (24 g) was separated by a combination of silica gel and reversed-phase column chromatography and preparative HPLC with a Nacalai Tesque Cosmosil 5Ph column to give nine fractions (f. 1 to f. 9).

Fraction 1 (17 mg) was again subjected to 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 at room temperature. Then the fraction having a retention time (t_R) of 35 min (8 mg) was further separated by preparative HPLC on the same column with MeOH–H_2O (82 : 18) at a flow rate of 8.0 ml/min at 5 °C to yield trichokonins Ia (TK-Ia, 2 mg) and Ib (TK-Ib, 2 mg) as amorphous solids, along with a peptaibol mixture (TK-Ic, 2 mg).

Fraction 9 (40 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 at room temperature to yield trichokonins VI (TK-VI, 2 mg), VII (TK-VII, 6 mg), VIII (TK-VIII, 3 mg), and IX (TK-IX, 9 mg) as amorphous solids.

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