Studies on Metabolites of Mycoparasitic Fungi. V.[1] Ion-Spray Ionization Mass Spectrometric Analysis of Trichokonin-II, a Peptaibol Mixture Obtained from the Culture Broth of *Trichoderma koningii*

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The sequence of a peptide, trichokonin-II (TK-II), obtained from the culture broth of *Trichoderma koningii* OUDEMANS, was examined by ion-spray ionization mass spectrometry (ISI-MS), including the collision-induced dissociation (CID) technique. TK-II was concluded to be a mixture of three peptaibols, TK-IIa, TK-IIb, and TK-IIc.

Key words *Trichoderma koningii*; peptaibol; trichokonin-II (TK-II); collision-induced dissociation (CID); ion-spray ionization mass spectrometry (ISI-MS)

In previous papers,[1,2] we reported the isolation and structure elucidation of seven peptaibols, named trichokonins (TKs)-Ia, Ib, and V—IX, from the culture broth of *Trichoderma koningii* OUDEMANS, which is one of the harmful fungi encountered in the cultivation of a medicinal mushroom, *Ganoderma lucidum* (FR.) KARST. (oriental crude drug "Lin-Chi"). Trichokonins are potent agonists of the L-type Ca^{2+} channel in cardiac membrane, and may be useful for the mechanistic investigation of Ca^{2+} channels in biological membranes.[3] In the course of the isolation of trichokonins, a minor peptaibol, TK-II, was also obtained. TK-II was purified to homogeneity on HPLC, but its ^{1}H-NMR spectrum suggested the existence of at least two components; *e.g.*, acetyl methyls signals at δ 2.06 and 2.02 (intensity ratio, 2:1). Although we tried to isolate each component by HPLC using various conditions, this was unsuccessful. Thus, we examined the structure of each component as a mixture, by ion-spray ionization mass spectrometry (ISI-MS), including the collision-induced dissociation (CID) technique, and found that TK-II contains three peptaibols (TK-IIa, TK-IIb, and TK-IIc, Fig. 2a). In this paper, we wish to report the structure elucidation of the components of TK-II.

The ^{1}H-NMR spectrum of TK-II showed a similar pattern to that of TK-VI,[2] except for the presence of some paired signals as a mixture, and showed acetyl group signals (δ 2.06 and 2.02; intensity ratio, 2:1) and three signals ascribable to the phenyl group (δ 7.14, t; δ 7.22, t; δ 7.28, d; J=7 Hz) of a phenylalaninol (Pheol) residue. Thus, TK-II was supposed to be a peptaibol mixture. Because TK-II was a mixture of peptaibols containing very similar sequences, we did not conduct an analysis of the amino acids composition. However, HPLC analyses of the complete acid hydrolysate with optically active stationary-phase columns[2] revealed peaks due to L-Ala, α-aminoisobutyric acid (Aib), L-Glu, L-Val, L-Gly, L-Leu, L-Pro, and L-Pheol. Although the HPLC analysis showed the peak of L-Glu, the CID spectra of TK-II showed fragment patterns indicating the presence of Gln (difference of mass numbers of fragment ions, 128 a.m.u.) not Glu (difference of mass numbers of fragment ions, 129 a.m.u.) (*vide infra*). Therefore, TK-II was concluded to be a mixture of peptaibols containing L-Ala, Aib, L-Gln (not L-Glu), L-Val, L-Gly, L-Leu, and L-Pro, along with an N-terminal acetyl group and a C-terminal L-Pheol residue.

The ISI-MS of TK-II at an orifice voltage of 40 V showed the primary-charged ions at m/z 1910, 1924, 1932, and 1946 in the absence of trifluoroacetic acid (TFA) (Fig. 1a), while in the presence of TFA it showed the ions only at m/z 1910 and 1924 (Fig. 1b). Thus, the ions at m/z 1932 and 1946 were concluded to be $[M+Na]^+$ ions and those at m/z 1910 and 1924 were $[M+H]^+$ ions. On the other hand, at an orifice voltage of 100 V, the ISI-MS showed two pairs of complementary fragment ions at m/z 1163 and 746, and at m/z 1149 and 774 (Fig. 1c), which were considered to be formed from the entire molecule corresponding to the m/z 1910 and 1924 ions, respectively. In order to confirm this, CID experiments were conducted. On scanning of the product ions, the m/z 1910 and 1924 ions showed fragment ions at m/z 1163 and 746 and at m/z 1149 and 774, respectively (Fig. 2), while on scanning of the parent ions, the m/z 1163 and 746 ions both showed a parent ion at m/z 1910 and both the m/z 1149 and 774 ions revealed one at m/z 1924. A similar fragmentation pattern is observed generally in the MS of peptaibols having an Aib–Pro peptide bond in their molecules.[1,2,4] Thus, TK-II was concluded to be a mixture of at least two peptaibols, corresponding to the m/z 1910 and 1924 ions and containing the Aib–Pro peptide bond in the molecules.

First, in order to determine the sequence corresponding to the ion at m/z 1910, we measured the CID spectra of the fragment ions at m/z 1163 and 746. In the CID spectrum of the m/z 746 ion (Fig. 3a), a series of product ions was observed at m/z 595, 467, 339, 254, and 169, which were interpreted to have been generated through successive losses of Pheol, Gln, Gln, Aib, and Aib. Because the N-terminal amino acid was considered to be Pro in the C-terminal peptide fragment, the m/z 169 ion was ascribed to Pro–Ala, and thus the C-terminal amino acid sequence was determined to be Pro–Ala–Aib–Aib–Gln–Gln–Pheol. On the other hand, in the CID experiment, the counterpart ion (m/z 1163) yielded sequential ions at

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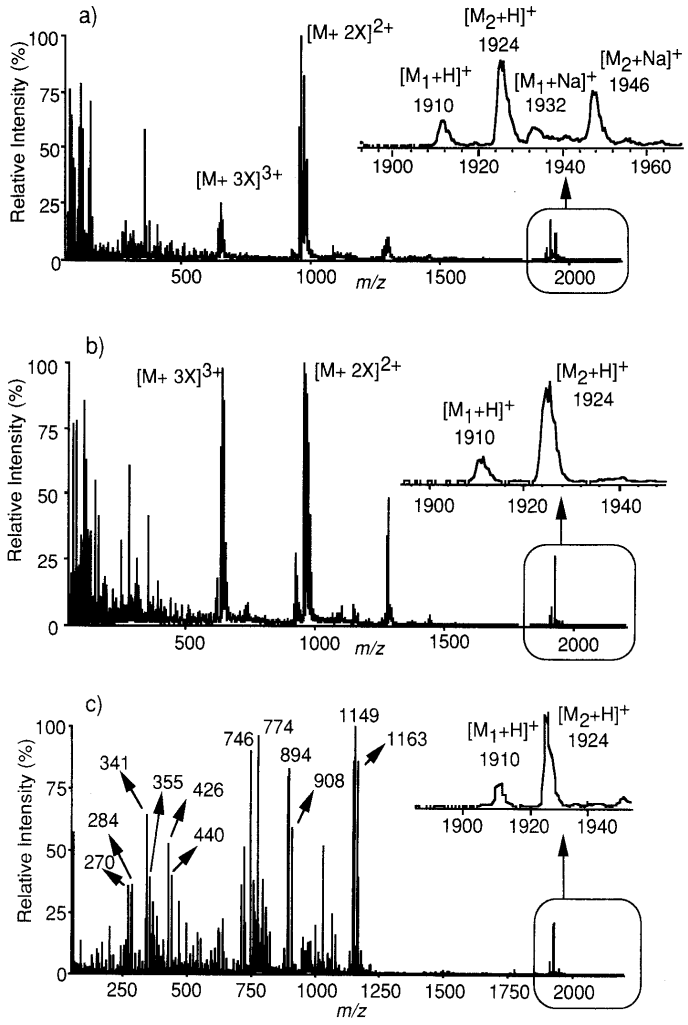

Fig. 1. ISI-MS of TK-II

a) Absence of TFA (orifice voltage, 40 V). b) Presence of TFA (orifice voltage, 40 V). c) Presence of TFA (orifice voltage, 100 V).

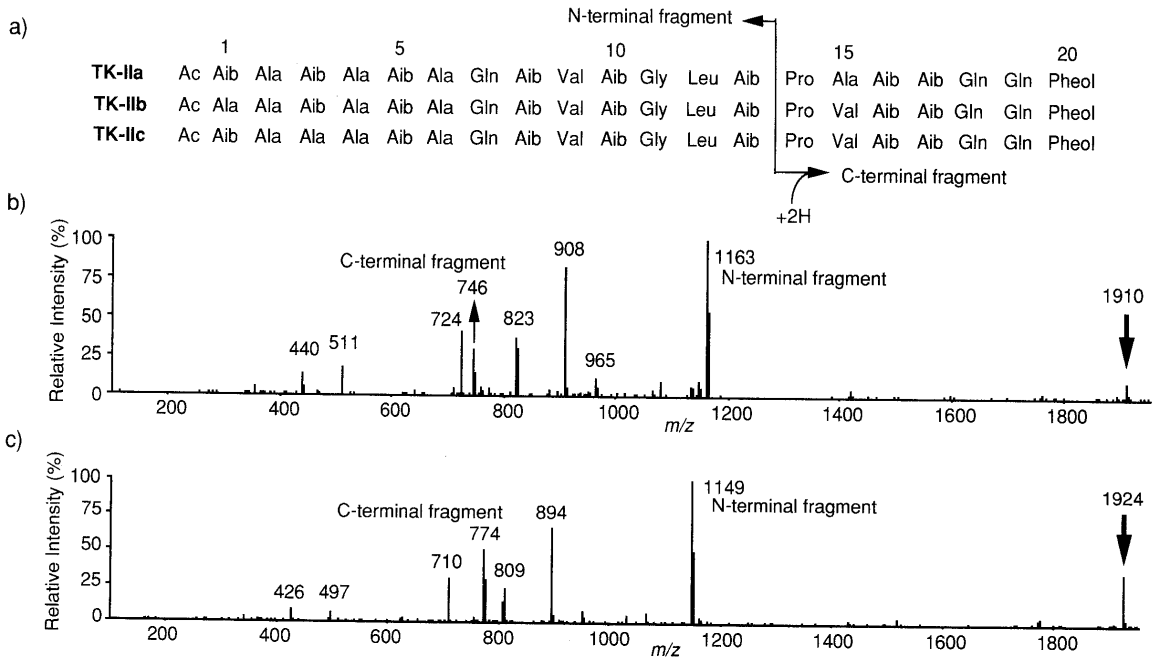

Fig. 2. Structures of TK-IIs (a) and CID Spectra of the *m/z* 1910 (b) and 1924 (c) Ions

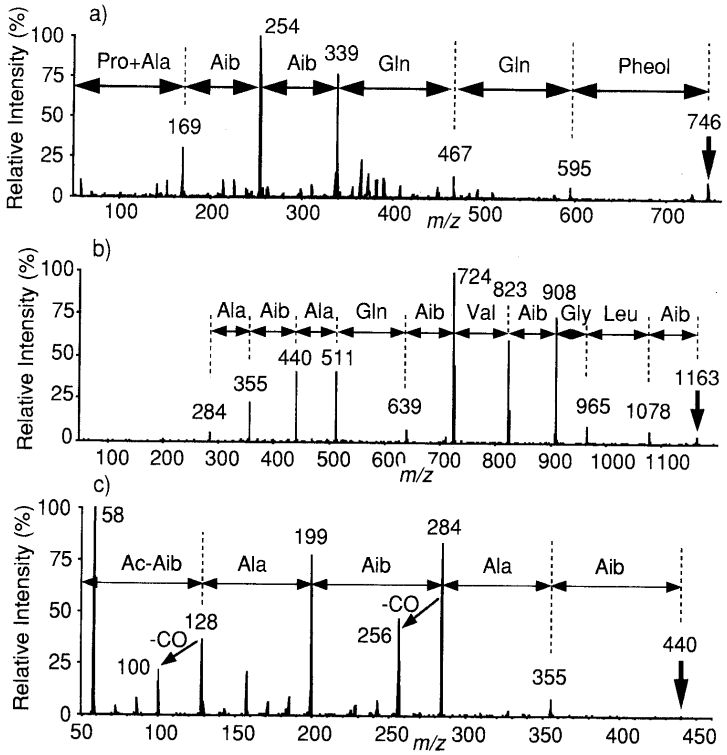

Fig. 3. CID Spectra of the m/z 746 (a), 1163 (b), and 440 (c) Ions

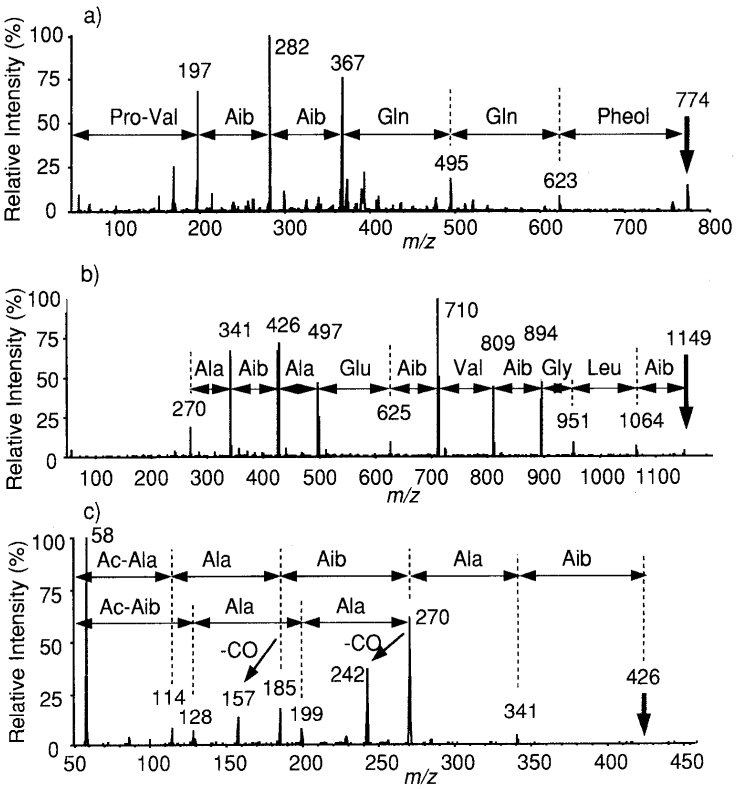

Fig. 4. CID Spectra of the m/z 774 (a), 1149 (b), and 426 (c) Ions

m/z 1078, 965, 908, 823, 724, 639, 511, 440, 355, and 284, generated through successive losses of Aib, Leu, Gly, Aib, Val, Aib, Gln, Ala, Aib, and Ala (Fig. 3b). Then, we measured the CID spectra of the m/z 284 and 355 ions to elucidate the N-terminal amino acid sequence, but these fragment ions failed to give sufficient product ions to allow elucidation of the sequence, as in the case of TKs-Ia, Ib, and IX.[1] However, examination of the m/z 440 ion showed acylium ions at m/z 284, 199, and 128 (Fig. 3c). Therefore, the sequence of the N-terminal peptide was determined to be Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib. By connecting the N- and C-terminal oligopeptides, the whole primary structure of the peptaibol corresponding to the m/z 1910 ion was concluded to be

Ac–Aib–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Pro–Ala–Aib–Aib–Gln–Gln–Pheol (TK-IIa) ($C_{88}H_{145}N_{23}O_{24}$; nominal mass, 1907; monoisotopic mass, 1908.1; average mass, 1909.3).[5]

Next, the structure of the molecule corresponding to the *m/z* 1924 ion was determined by taking the CID spectra of the *m/z* 774 and 1149 ions, along with that of the *m/z* 426 ion. As shown in Fig. 4a, the fragment ions observed in the CID spectrum of the *m/z* 774 ion suggested the amino acid sequence of the C-terminal fragment to be Pro–Val–Aib–Aib–Gln–Gln–Pheol. On the other hand, those of the CID spectrum of the *m/z* 1149 ion indicated the amino acid sequence of the N-terminal fragment to be (*m/z* 270 peptide)-Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib (Fig. 4b). Then, in order to elucidate the structure of the *m/z* 270 ion, the *m/z* 426 ion was subjected to a CID experiment to give fragment ions at *m/z* 270, 199, 185, 128, and 114 (Fig. 4c). Based on the differences of the mass numbers, with consideration of the amino acids present in TK-II, these ions were grouped into two series, *m/z* 270, 185, 114 series and *m/z* 270, 199, 128 series. Therefore, the *m/z* 270 ion was determined to be two fragments, Ac–Ala–Ala–Aib and Ac–Aib–Ala–Ala. Based on the results mentioned above, the *m/z* 1924 ion was determined to be Ac–Ala–Ala–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol (TK-IIb) and Ac–Aib–Ala–Ala–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol (TK-IIc) ($C_{89}H_{147}N_{23}O_{24}$; nominal mass, 1921; monoisotopic mass, 1922.1; average mass, 1923.3).[5]

TK-IIa has Ala^{15} instead of Val^{15}, TK-IIb has Ala^{1} instead of Aib^{1}, and TK-IIa and TK-IIc have Ala^{3} instead of Aib^{3}, in contrast to other trichokonins.[1,2] Thus, the biological activities of these compounds towards Ca^{2+} channels would be of interest from the viewpoint of structure-activity relationships. The syntheses of these minor trichokonins are now under investigation, and will be reported elsewhere, together with their activities.

Experimental

Isolation of Trichokonin-II (TK-II) 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 chromatographies and preparative HPLC with a Nacalai Tesque Cosmosil 5Ph column to give nine fractions (fr. 1 to fr. 9).

Fraction 2 (16 mg) was 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 to give trichokonin-II (TK-II, 5 mg; t_R, 7.7 min) as an amorphous solid.

ISI-MS Measurements of Trichokonin-II (TK-II) ISI-MS and CID spectra were obtained with a Perkin–Elmer Sciex API-III mass spectrometer at an orifice voltage of 40—100 V for ISI-MS and of 100 V for CID spectra. For CID experiments, argon was used as a collision gas (collision energy, 10 eV). Trichokonin-II was dissolved in MeOH (10^{-6} M) and loaded into the mass spectrometer by an automatic injector (Harvard Apparatus 22) at a flow rate of 5 μl/min. For promotion of protonation to the molecule, 1 drop of 0.1% TFA solution was added to the 500 μl of MeOH solution.

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References and Notes

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5) The most abundant mass of large molecules appears near the position of the average mass and differs slightly from nominal mass and monoisotopic mass. Yergey J., Heller D., Hansen G., Cotter R. J., Fenselau C., *Anal. Chem.*, **55**, 353—356 (1983).