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 ¹H-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 ¹H-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 columns²⁾ 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 $[\text{M} + \text{Na}]^+$ ions and those at m/z 1910 and 1924 were $[\text{M} + \text{H}]^+$ 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-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-IIa (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 Ala¹⁵ instead of Val¹⁵, TK-IIb has Ala¹ instead of Aib¹, and TK-IIa and TK-IIc have Ala³ instead of Aib³, 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 paper²⁾; 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. $\times$ 250 mm) with MeOH-H₂O (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 μ l/min. For promotion of protonation to the molecule, 1 drop of 0.1% TFA solution was added to the 500 μ l of MeOH solution.

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References and Notes

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