Fungal Metabolites. Part 5.[1] Rapid Structure Elucidation of Antibiotic Peptides, Minor Components of Trichosporin Bs from *Trichoderma polysporum*. Application of Linked-scan and Continuous-flow Fast-atom Bombardment Mass Spectrometry

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From the minor-components mixture of Trichosporin Bs (TS-Bs) produced by *Trichoderma polysporum*, ten components could be elucidated by means of linked-scan and continuous-flow fast-atom bombardment. The combination of their complementary results made the rapid structure elucidation of the minor components possible without isolation and purification. The identified compounds are antibiotic eicosapeptides and have very similar structures with relative molecular masses of ~2000. Four new peptide groups were found. In their structures, the first group has Gly at the amino acid position 3 in contrast to the known TS-Bs, the second one has Vxx (valine or isovaline) at position 12, and the third and the fourth ones have Ala at position 13 or 17, respectively.

Trichosporin Bs (TS-Bs), which have unique and strong catecholamine-secretion activity in adrenal chromaffin cells,[2] are metabolites from *Trichoderma polysporum*. They are expected to be useful in studies of the impulse transport system of the sympathetic nervous system and have also been reported to be uncouplers for rat liver mitochondria.[3] Until now, eleven kinds of TS-B have been identified.[4,5] They have very similar structures; their *N*-terminal amino acids are protected by an acetyl group and their *C*-terminal residues are linked with phenylalaninol (Pheol). They belong to a class of peptaibols, *e.g.*, alamethicine,[6] hypelcin,[7] trichotoxin,[8,9] and suzukacillin,[10] having unique biological activities[11–14] such as voltage-dependent ion-channel formation. TS-Bs contain a high proportion of an unusual amino acid, α-aminoisobutyric acid (Aib), together with isovaline (Iva). Identification of TS-Bs has been carried out mainly by fast-atom bombardment mass spectrometry (FAB-MS), partially fast-atom bombardment mass spectrometry–mass spectrometry (FAB-MS–MS), and NMR since Edman degradation and enzymatic methods are not applicable due to the compounds' structural characteristics. Another characteristic aspect of their structure is the presence of an unstable peptide bond between the thirteenth amino acid, Aib, and the fourteenth amino acid, Pro. This peptide bond tends to cleave preferentially under FAB-MS and two prominent acylium ions appear in their FAB mass spectra. (These two acylium ions are named as *N*- and *C*-terminal oligopeptide ions.) It has been found in previous investigations[4,5] that partial amino acid sequences of *N*-terminal oligopeptides can be given by some fragment ions, but no fragment ion indicating a *C*-terminal oligopeptide sequence appears in their FAB-MS spectra. It has been reported that in order to confirm amino acid sequences of peptaibols, chemical treatment, such as partial hydrolysis, or NMR measurement must be performed.[15,16] Our group shows the usefulness of low-energy collision-induced dissociation (CID) FAB-MS–MS for peptide sequencing of TS-Bs.[5]

The HPLC chromatograms of crude TS-Bs suggested the existence of other minor components besides the eleven kinds of TS-B which have been identified. However, it is difficult to isolate the necessary amounts of minor components for identification by MS, NMR, and/or other techniques. As a tool to overcome these difficulties, liquid chromatography–mass spectrometry (LC–MS), which has become popular recently, seems very suitable. Among various available LC–MS techniques, continuous-flow fast-atom bombardment mass spectrometry (CF-FAB)[17,18] was applied to analyses of TS-B mixtures. Since FAB-MS spectra of TS-Bs do not give sufficient sequence information as mentioned above, linked scan, a technique of MS–MS, was applied to analyses of minor component mixtures for the purpose of obtaining further information. This report deals with rapid identification of ten minor components of TS-Bs by the combination of LC–MS and MS–MS.

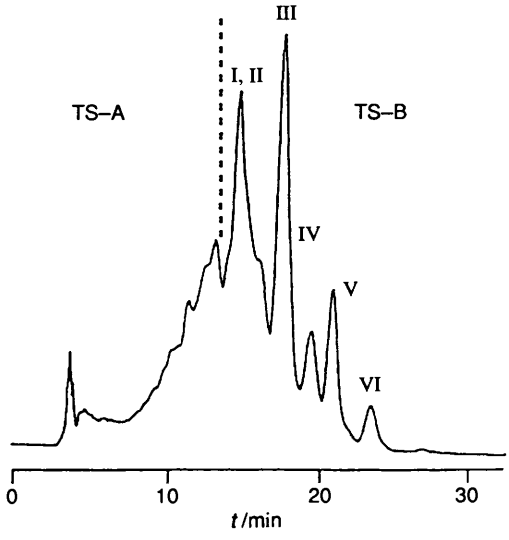


Fig. 1 Analytical HPLC chromatogram of crude trichosporin Bs obtained from the culture broth of *Trichoderma polysporum*. Fractions TS-B-I–VI are shown. Analytical conditions: eluent methanol–water (85:15, v/v); flow rate 0.5 cm^3 min^{-1}; UV detector (220 nm); Nacalai Cosmosil ODS column (4.6 mm i.d. × 150 mm); column temperature 40 °C.

Results and Discussion

After repeated preparative HPLC of the extract from a culture broth of *Trichoderma polysporum*, six main fractions, including

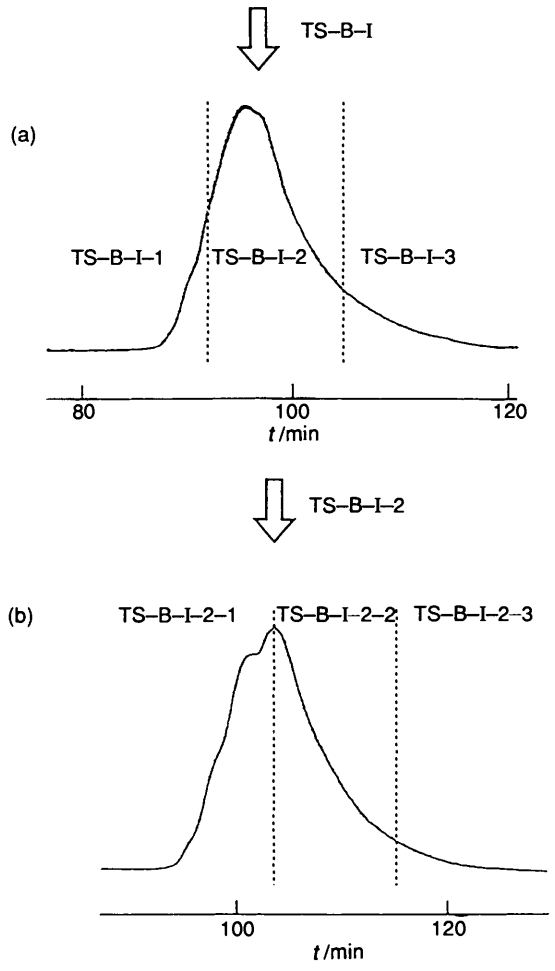


Fig. 2 Preparative HPLC chromatograms of TS-B-I fraction. Analytical conditions: eluent methanol–water (85:15, v/v) for (a), methanol–water (83:17, v/v) for (b); flow rate 2.0 cm^3 min^{-1} for (a), 2.5 cm^3 min^{-1} for (b); UV detector (220 nm); Nacalai Cosmosil Ph column (20 mm i.d. × 250 mm); column temperature 40 °C.

TS-B-I, were obtained (Fig. 1). This fraction was subjected to successive preparative HPLC and divided into five fractions, *i.e.*, TS-B-I-1, TS-B-I-2-1–3, and TS-B-I-3 (Fig. 2). In this study, the fraction named Fr. TS-B-I-2-1 was investigated. From Fr. TS-B-I-2-2, TS-B-Ia (Ac-Aib-Ala-Ser-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol, M 1965; Ac = acetyl) was obtained after further purification. In the TS-B-I-2-1 part of Fig. 2, several shoulder peaks were observed. In order to identify these components, three steps were carried out: (i) Acquiring normal FAB-MS spectra of Fr. TS-B-I-2-1, (ii) analysing the quasimolecular, *N*-, and *C*- terminal oligopeptide ions from Fr. TS-B-I-2-1 by linked scan, (iii) analysing Fr. TS-B-I-2-1 by CF–FAB to confirm the existence of peptaibols suggested by step (ii).

(1) *Acquiring normal FAB-MS Spectra of Fr. TS-B-I*-2-1.—Fig. 3(a) shows the FAB-MS spectrum of Fr. TS-B-I-2-1 obtained when glycerol–thioglycerol (~1:1, v/v) containing ~2% trifluoroacetic acid (TFA) was used as the matrix. In this Figure, *m/z* 1193, which corresponds to the *N*-terminal oligopeptide ion of the known TS-B-Ia, the main component of Fr. TS-B-I-2-2, is observed. In addition to the peak at *m/z* 1193, those at *m/z* 1179, 1163, 1149 and 1135 can be thought of as *N*-terminal oligopeptide ions existing in the target fraction. In the *C*-terminal oligopeptide ion region, besides the peak at *m/z* 774 corresponding to the *C*-terminal oligopeptide ion of TS-B-Ia, ions at *m/z* 788 and 760 appear. In the quasimolecular ion region, many ions are observed. Ions at *m/z* 1967 and 1989 correspond to the species $[M + H]^+$ and $[M + Na]^+$, respectively, of TS-B-Ia. It is not unusual that natural products often give cationized molecular ions such as $[M + Na]^+$ and $[M + K]^+$ even when TFA, which promotes the protonation of molecular ions, is added to matrices. It is difficult to determine relative molecular masses of possibly existing compounds from such a $[M + H]^+ + [M + Na]^+$ mixed mass spectrum. In order to confirm molecular masses, a second matrix, *i.e.*, glycerol-thioglycerol (~1:1, v/v) containing NaCl instead of TFA, was used. This matrix promotes cationization of molecular ions. The acquired mass spectrum is shown in Fig. 3(b). In this figure, $[M + Na]^+$ (*m/z* 1989) is observed as the quasimolecular ion of TS-B-Ia, indicating that only cationization of molecules occurred under these conditions. In Fig. 3(b), peaks at *m/z* 2003, 1989, 1975, 1959, 1945 and 1931 are recognized as $[M + Na]^+$ group ions. These correspond to *m/z* 1981, 1967, 1953, 1937, 1923 and 1909 for $[M + H]^+$, respectively.

(2) *Analysing the Quasimolecular*, N-, *and* C-*Terminal Oligopeptide Ions from Fr. TS-B-I*-2-1 *by Linked Scan.*—Step (i) gave information on quasimolecular ions and characteristic ions, *i.e.*, *N*- and *C*-terminal oligopeptide ions, of compounds contained in Fr. TS-B-I-2-1. In order to obtain structural information, linked-scan (B/E)[19–21] was performed; an ion was chosen as a precursor ion after ionizing Fr. TS-B-I-2-1 (mixture of several components) by FAB, and daughter ions from the precursor ion were obtained by high-energy CID.

It has been reported[22] that high-energy CID can be applied to peptides consisting of 20–25 amino acid residues. Bieman and Martin[23] demonstrated good results for peptide ions up to M_r 3000 with a four-sector instrument. Tomer *et al.*[24] report that the efficiency of the CAD (identical with CID) process is greater for peptide $[M + H]^+$ ions in the 1000–2000 atomic mass range than in the mass range below M_r 1000, although their conclusion is compound-dependent. In the case of TS-Bs, our group has reported the results of the application of low-energy CID to $[M + H]^+$ ion of TS-B-VIb, which did not give sufficient sequence ions because of the unstable and easily cleaved peptide bond between Aib[13] and Pro[14].[5] Similar results were expected for high-energy CID in this case, hence the main purpose of CID linked-scan of quasimolecular ions was focussed on assignment of *N*- and *C*-terminal oligopeptide ions, obtained in step (i), to quasimolecular ions. In addition, the purpose of CID linked-scan of *N*- and *C*-terminal oligopeptide ions was the elucidation of their amino acid sequences.

Since the $[M + Na]^+$ ion intensities in Fig. 3(b) are much higher than $[M + H]^+$ ion intensities in Fig. 3(a), linked scan for the $[M + Na]^+$ group ions under the Fig. 3(b) conditions was performed. However, little significant daughter ion information could be obtained, resulting from the stability of cationized molecular ions. Additionally, the intensity of *N*- and *C*-terminal oligopeptide ions in Fig. 3(b) is very low, presumably due to production of stable quasimolecular ions. In contrast, $[M + H]^+$ ions in Fig. 3(a) gave much better results. Furthermore, when using the matrices containing TFA [Fig. 3(a)], the characteristic ions appeared very clearly. Considering the observed facts described above, linked scan was performed with the matrices containing ~2% TFA.

Fig. 4 shows an example of the CID spectrum of $[M + H]^+$. In this case, the precursor ion chosen was *m/z* 1967, which is identical with the most abundant component of the $[M + H]^+$ isotope cluster of TS-B-Ia. It is not the monoisotopic peak, but 1 mass unit higher due to the contribution of ^{13}C. However, B/E linked-scan has poor precursor-ion resolution, selecting either the monoisotopic ion or the most abundant ion since the precursor ion does not influence results experimentally. Two prominent daughter ions in Fig. 4, *m/z* 1193 and 774, corre-

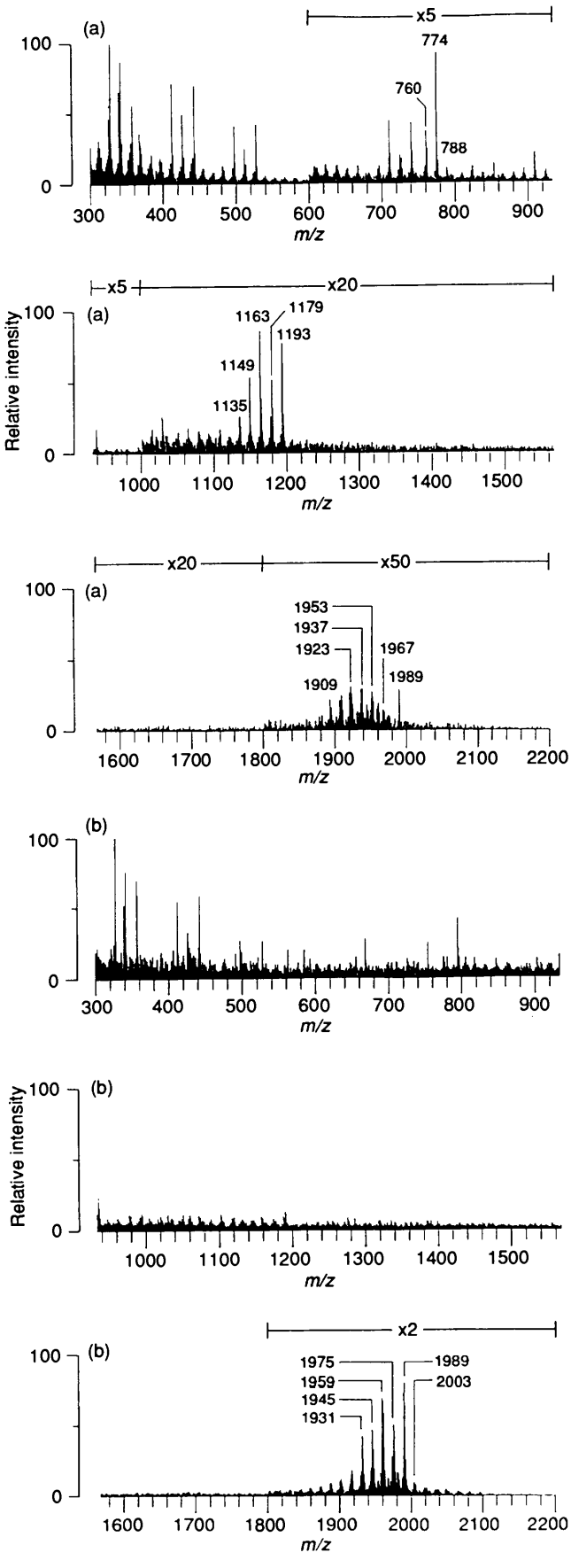


Fig. 3 (a) Positive-ion FAB-MS spectrum of Fr. TS-B-I-2-1. Matrices glycerol–thioglycerol–TFA (50:50:2). (b) Positive-ion FAB-MS spectrum of Fr. TS-B-I-2-1. Matrices glycerol–thioglycerol–saturated aq. NaCl (50:50:2).

Table 1 Main diagnostic daughter ions observed in CID spectra of quasimolecular ions

Precursor ion	Observed prominent diagnostic ions	
	N-Terminal oligopeptide	*C*-Terminal oligopeptide
1967	1193	774
1953	1179	774
1937	1193, 1179, 1177, 1163, 1149	788, 774, 760, 758
1923	1179, 1163, 1149, 1135	788, 774, 760
1909	1163, 1149	774, 760

spond to the *N*- and *C*-terminal oligopeptide ions of TS-B-Ia, respectively. According to the nomenclature of fragment ions of peptides shown below, which was proposed initially by Roepstorff *et al.*[25] and varied by Biemann,[22] the *N*-terminal oligopeptide ion is a b_n-type ion and the *C*-terminal one is a y_n-type ion. Only partial sequence-specific daughter ions (b_n ions) appear. From the CID spectra of $[M + H]^+$ ions, however, the possible combination of *N*- and *C*-terminal oligopeptides and $[M + H]^+$ ions were found. The assignments of *N*- and *C*-terminal oligopeptide ions to $[M + H]^+$ ions are summarized in Table 1.

N-Terminal oligopeptide ions, *m/z* 1193, 1179, 1163, 1149 and 1135, observed in Fig. 3(a) were chosen for CID linked-scan examination. Fig. 5 shows the CID spectrum of the peak at *m/z* 1149. In this Figure, the assignment of the observed daughter ions is given. All diagnostic ions are b_n ions. Among the sequence-specific ions, the intensity of the ion indicating the acetylated Aib (*m/z* 128) is very weak. In addition, the sequence 7–8 could not be determined, as sequence-specific daughter ions were not observed clearly between the ions at *m/z* 497 and *m/z* 710 in the spectrum. In order to examine these points, CID of the ion at *m/z* 710 was carried out. The obtained daughter-ion spectrum clearly gave all the sequence-specific ions from sequences 1 to 8. (The determined sequence is shown in Table 2.) Fig. 5 indicates that the third amino acid is Gly and the thirteenth one is Ala. This is the first time that this type of amino acid sequence has been found in TS-Bs.

The sequences of the *N*-terminal oligopeptide ions suggested from the CID spectra are summarized in Table 2. As shown, ions at *m/z* 1135, 1149 and 1163 have Gly at the position 3, in contrast to the ions at *m/z* 1179 and 1193, which have Ser there. The thirteenth amino acid of the ions at *m/z* 1135, 1149 and 1179 ions is Ala. All TS-Bs identified in previous studies have the specific peptide bond Aib^{13}–Pro^{14} which is thought to be unstable and to be cleaved preferentially.[5] The results obtained here suggest that the thirteenth amino acid could be substituted for Ala and that, even in this case, the peptide bond between positions thirteen and fourteen could be fragmented preferentially, too. This fact is compatible with the report[22] that Pro seems to be particularly prone to eliminate its *N*-terminal peptide as a ketene [–C(R)=C=O] and that the internal fragments often start with Pro. Additionally, as shown in Table 2, the possibility of two isomeric sequences for the ion at *m/z* 1135 was suggested from its CID spectrum. Lxx in Fig. 5 and Table 2 represents either Leu or Ile.

In order to clarify the sequences of *C*-terminal oligopeptides of TS-Bs in Fr. TS-B-I-2-1, CID of *C*-terminal oligopeptide ions observed in Fig. 3(a), *i.e.*, *m/z* 788, 774 and 760, was performed. The CID spectrum of the ion at *m/z* 774 is shown in Fig. 6, as an example. Some TS-Bs, which have already been identified, gave the ion *m/z* 774 as their *C*-terminal oligopeptide ion and the amino acid sequence has been determined as Pro-Val-Aib-Aib-Gln-Gln-Pheol. This ion is a y_n-type and is formed by protonation and cleavage of the CO–NH bond between Aib^{13}–Pro^{14}. In Fig. 6, prominent b_n ions (b_2–b_6) and weak y_n ions (y_1–y_6),

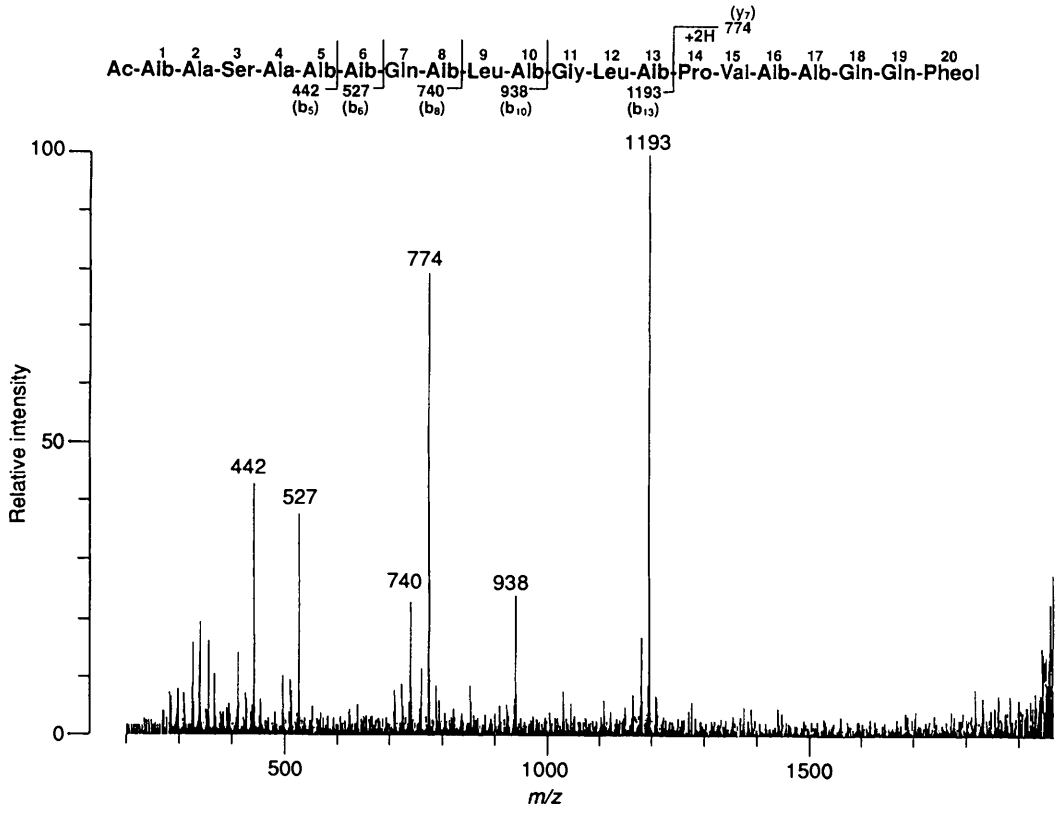


Fig. 4 CID spectrum of *m/z* 1967 ion

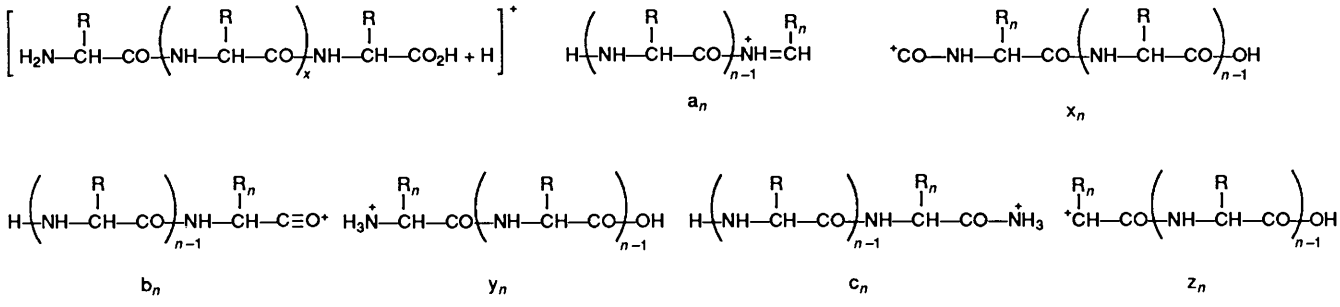


Table 2 Possible primary structures of *N*-terminal oligopeptides in Fr. TS-B-1-2 suggested by CID linked-scan (Lxx = Leu or Ile, Vxx = Val or Iva, Iva = isovaline)

Precursor ion	Suggested structure 1 2 3 4 5 6 7 8 9 10 11 12 13
1193	Ac-Aib-Ala-Ser-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Aib
1179	Ac-Aib-Ala-Ser-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Ala
1163	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Aib
1149	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Ala
1135	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Ala-Ala-Vxx-Ala -Aib-Ala-

appear as sequence-specific daughter ions. The b_n ions at *m/z* 197, 282, 367, 495 and 623 can lead, for the sequence 3–7 (the sequence 16–20 for the whole molecule), to Aib-Aib-Gln-Gln-Pheol. The y_1–y_5 ions support this sequence and the y_6 ion indicates that Pro–Val is located at the *N*-terminus of the *C*-terminal oligopeptide. The ion at *m/z* 169 is identical with a_2. The observation of these daughter ions indicates that the *C*-terminal oligopeptide ion has the structure (b) shown in Scheme 1 and that the subsequent cleavage of peptide bonds after production of the *C*-terminal oligopeptide ion occurred to form y_n and b_n ions. This is the same fragmentation process as proposed by Hunt *et al.*[26] for peptides containing Pro. It is presumed that peptaibols such as TS-Bs obey the fragmentation process proposed for peptides. The structure (a) of Scheme 1 shows that the amide proton of Aib is transferred to produce the *C*-terminal oligopeptide ion [structure (b)]. Johnson *et al.*[27] reported that formation of y_n ions from $[M + H]^+$ ions with localized charge can be rationalized in one of two closely related

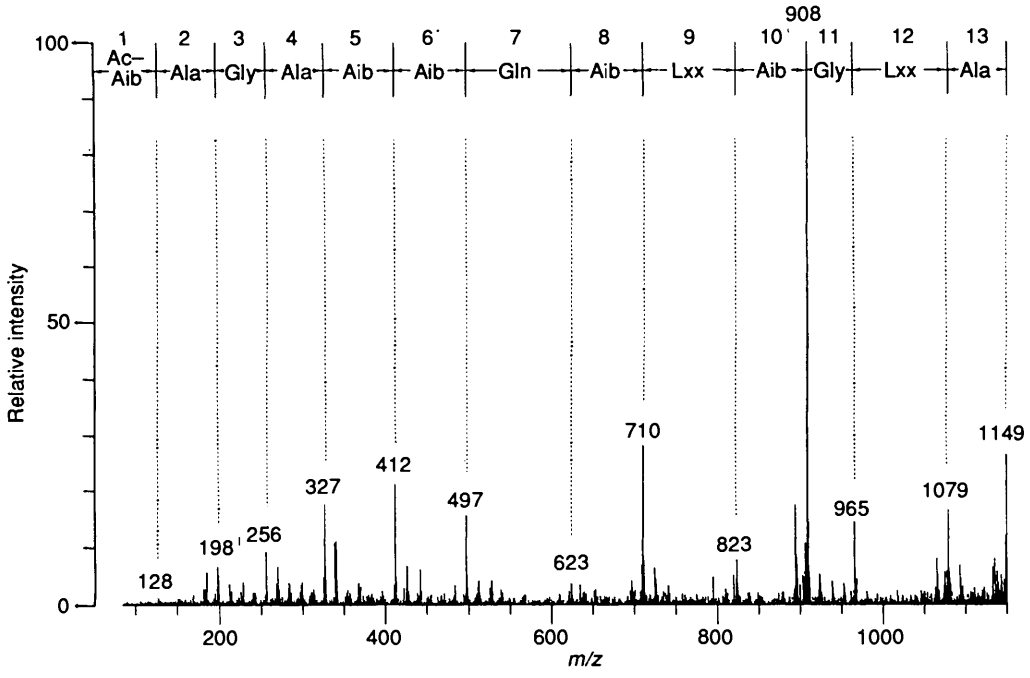


Fig. 5 CID spectrum of *m/z* 1149 ion

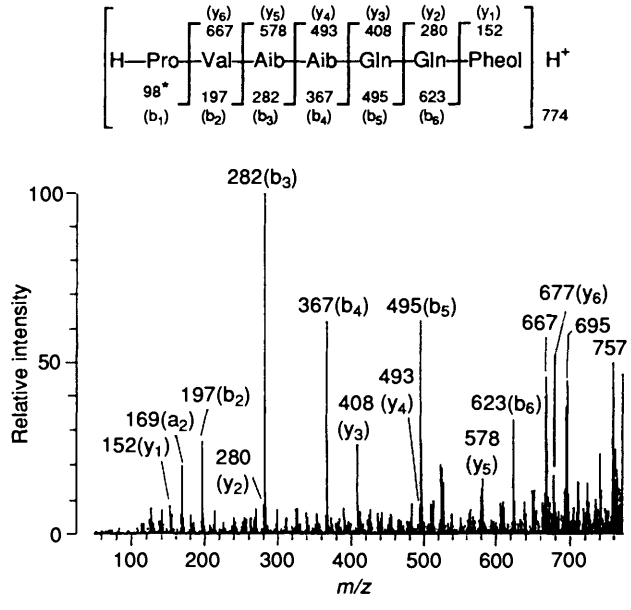


Fig. 6 CID spectrum of *m/z* 774 ion (* Not observed)

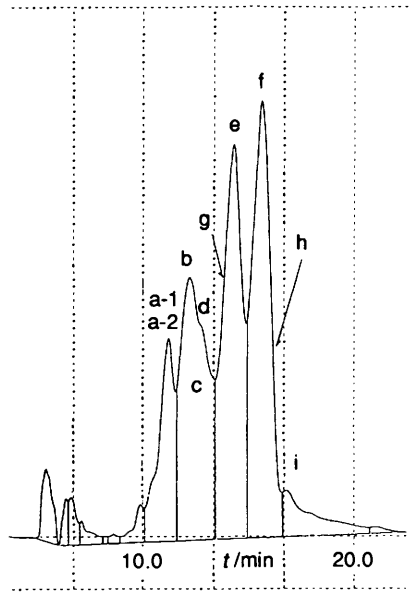


Fig. 7 On-line UV chromatogram of Fr. TS-B-1-2-1. Analytical conditions: eluent acetonitrile–water–glycerol (50:45:5, v/v); flow rate 100 mm^3 min^{-1}; UV detection at 220 nm; Shim-pack SBC ODS column (2.5 mm i.d. × 150 mm); column temperature 40 °C.

Table 3 Possible primary structures of *C*-terminal oligopeptides in Fr. TS-B-1-2 suggested by CID linked-scan (Vxx = Val or Iva, Iva = isovaline)

Precursor ion	Suggested structure 14 15 16 17 18 19 20
788	Pro-Val-Aib-Vxx-Gln-Gln-Pheol
774	Pro-Val-Aib-Aib-Gln-Gln-Pheol
760	Pro-Val-Aib-Ala-Gln-Gln-Pheol

fragmentation pathways (a) and (b) shown in Scheme 2. They also stated that the conventional mechanism (Scheme 3) is probably operative when there is no strongly basic site that competes with the required protonation of amide nitrogen of the peptide bond undergoing cleavage. On the other hand, Mueller *et al.*[28] proposed that the transferred hydrogen is not alpha to the carbonyl group, by using a deuteriated small model peptide. In the two pathways shown in Scheme 2, pathway (a) is not applicable to the case where Aib is located at the *N*-terminus since it does not have a proton alpha to the carbonyl. Appearance of y_4 and y_5 ions in Fig. 6 supports pathway (b). In Fig. 6, along with the sequence-specific ions, ions at *m/z* 757, 695 and 667 are observed as intense peaks. The ion at *m/z* 757 is considered to result from the loss of the side chain of Gln as ammonia (17 mass units) from the ion of *m/z* 774. The ions at *m/z* 695 and 667 seem to be generated by elimination of the side chain from Pheol as shown in Scheme 4.

Table 4 (a) Primary structures of trichosporin Bs (Lxx = Leu or Ile, Vxx = Val or Iva, Iva = isovaline)

Compound	Structure 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	M.W.
a-1	Ac-Aib-Ala-Gly-Aib-Ala-Aib-Gln-Aib-Lxx-Ala-Ala-Vxx-Ala(1135)-Pro(788)-Val-Aib-Vxx-Gln-Gln-Pheol	1921
a-2	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Ala-Ala-Vxx-Ala(1135)-Pro(788)-Val-Aib-Vxx-Gln-Gln-Pheol	1921
b	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Ala(1149)-Pro(760)-Val-Aib-Ala-Gln-Gln-Pheol	1907
d	Ac-Aib-Ala-Ser-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Ala(1179)-Pro(774)-Val-Aib-Aib-Gln-Gln-Pheol	1951
e	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Aib(1163)-Pro(774)-Val-Aib-Aib-Gln-Gln-Pheol	1935
f (TS-B-Ia)	Ac-Aib-Ala-Ser-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Aib(1193)-Pro(774)-Val-Aib-Aib-Gln-Gln-Pheol	1965
g	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Ala(1149)-Pro(774)-Val-Aib-Aib-Gln-Gln-Pheol	1921
h	Ac-Aib-Ala-Gly-Ala-Aib-Aib-Gln-Aib-Lxx-Aib-Gly-Lxx-Aib(1163)-Pro(788)-Val-Aib-Vxx-Gln-Gln-Pheol	1949

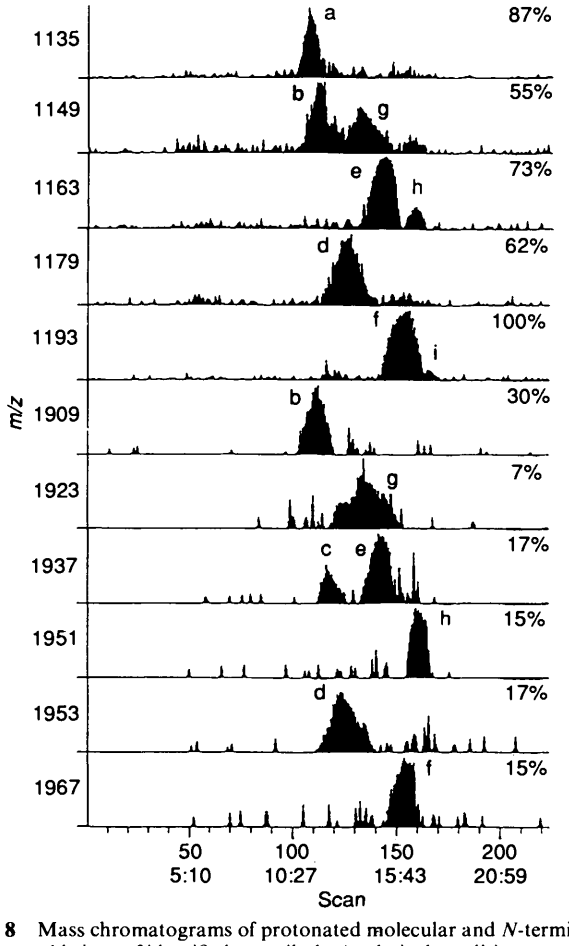


Fig. 8 Mass chromatograms of protonated molecular and *N*-terminal oligopeptide ions of identified peptaibols. Analytical conditions: see the caption to Fig 7.

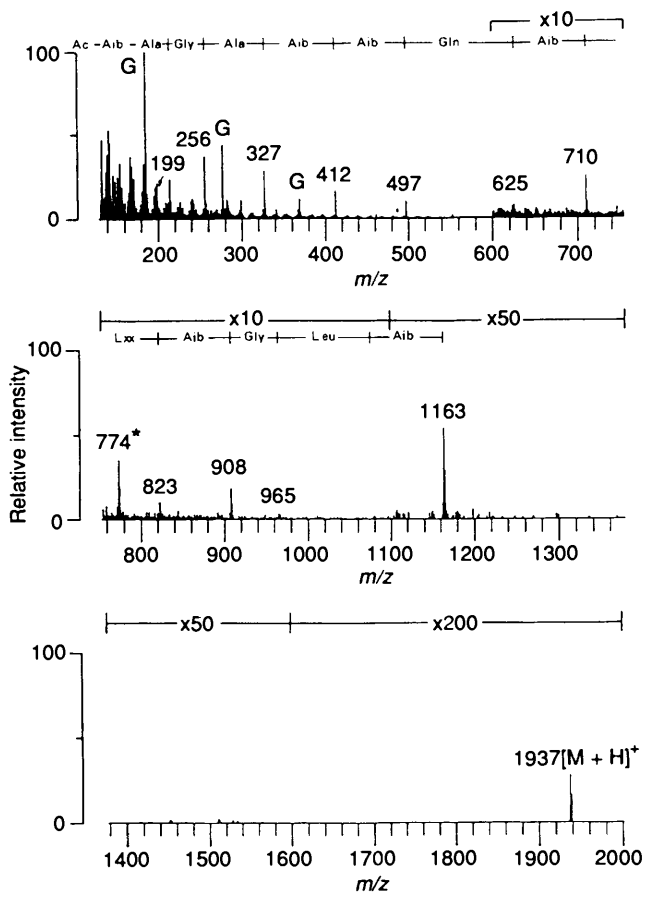
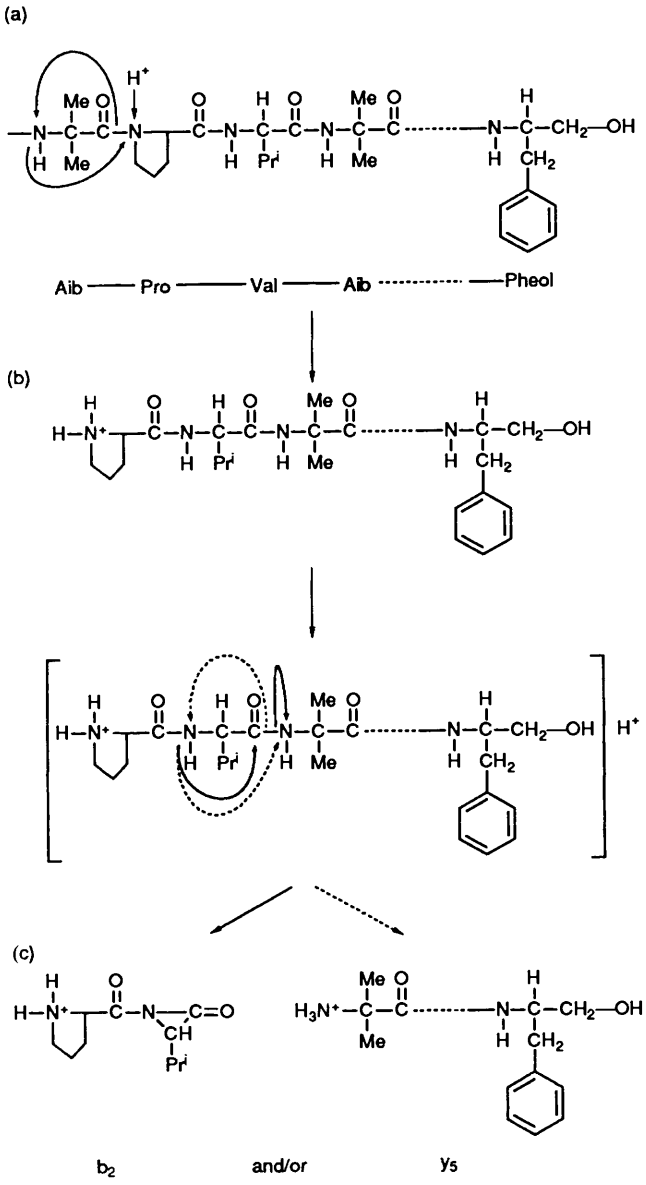
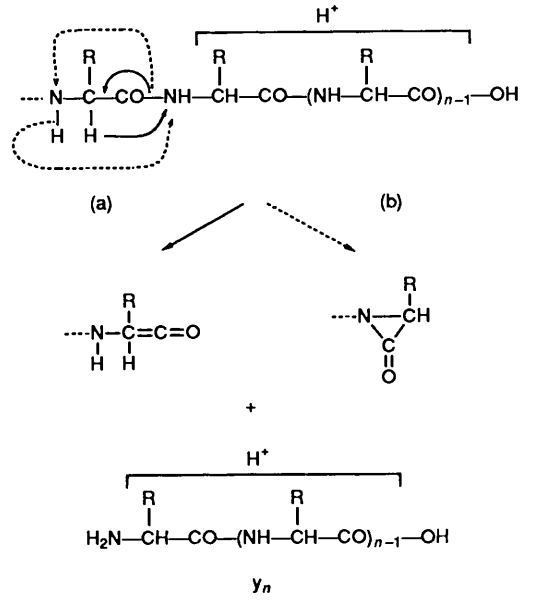


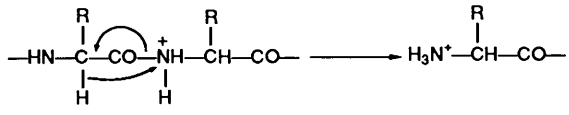
Fig. 9 FAB-MS spectrum of peak **e** in Fig. 8. * *m/z* 774 peak is derived from *C*-terminal oligopeptide.



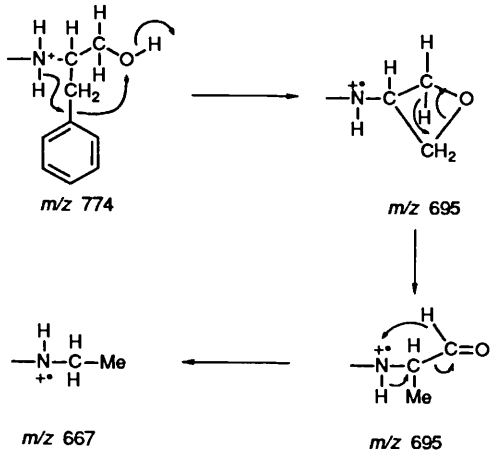
Scheme 1 (a) Possible process of formation of *C*-terminal oligopeptide ion (shown for *m/z* 774 ion as an example), (b) its resulting structure, and (c) mechanism of formation of ions y_n and b_n (shown for ions b_2 and y_5)



Scheme 2 Possible fragmentation pathway to form ions y_n from $[M + H]^+$ with localized charge[27]



Scheme 3 Conventional mechanism of formation of ions y_n[27]



Scheme 4 Presumed process of formation of ions at *m/z* 695 and 667 in Fig. 6

The amino acid sequences suggested from the CID spectra of the *C*-terminal oligopeptide ions are summarized in Table 3. The residue at position 17 of the ion with *m/z* 788 can be Val or Iva. In the case of the ion with *m/z* 760, Ala occupies position 17. This is a new type of sequence for TS-Bs.

(3) *Analysing Fr. TS-B-I-2-1 by CF-FAB.*—The possible combination of *N*- and *C*-terminal oligopeptides and their possible structures were provided up to step (ii). In order to confirm their existence, the fraction was separated by HPLC and the eluate was introduced directly to a CF-FAB interface in order to obtain mass spectra from individual components. Fig. 7 shows the on-line UV detection chromatogram of TS-B-I-2-1, indicating the presence of compounds, **a**-1, **a**-2, . . ., **i**. Fig. 8 shows mass chromatograms of quasimolecular and *N*-terminal oligopeptide ions of a species **a**–**i**. Mass spectra in which characteristic ions appeared could be obtained for species **a**, **b** and **d**–**h**. As an example, the mass spectrum of species **e** is shown in Fig. 9 with the assignment of characteristic ions. Although only partial-sequence-specific ions of the *N*-terminal oligopeptide ion appear in this spectrum, the results of CID of the ions at *m/z* 1163 and 774 enable complete sequence determination [shown in Table 4(a)]. For this analysis, the total sample amount introduced into the mass spectrometer was 1.7 μg per injection (assuming that the average relative molecular mass is 1950, 1.7 μg is ~870 pmol.). Concerning peak **i**, of which the peak area ratio is 0.2% on the basis of its UV response, its *N*- and *C*-terminal oligopeptide ions are suggested by the mass chromatograms (1193 and 788 mass units, respectively). The mass chromatograms and the relevant mass spectrum indicate that the relative molecular mass of species **c** can be 1935 and its *N*- and *C*-terminal oligopeptide ions can be *m/z* 1149 and 788, respectively. For compound **a**, its *N*-terminal oligopeptide ion has *m/z* 1135. The CID spectrum of the ion at *m/z* 1135 suggested the presence of two isomers; their fourth and fifth amino acids are Aib–Ala and *vice versa*. The CF–FAB mass

Table 4 (b) Characteristic ions observed in the CF–FAB–MS spectra of identified trichosporin Bs (Lxx = Leu, or Ile, Vxx = Val, or Iva, Iva = isovaline)

Compound					Characteristic ions[a]												
					Sequence specific ions from *N*-terminal oligopeptide ions												
	M_r (nominal)	$[M + H]^+$ [b]	*N*-Terminal oligopeptide ion	*C*-Terminal oligopeptide ion	1	2	3	4	5	6	7	8	9	10	11	12	13
a-1	1921	**1923**	**1135**	**788**	Ac-Aib-Ala	Gly	Ala	Aib	Aib	Gln	Aib	Lxx	Ala	Ala	Vxx	Ala	
					199	**256**	**327**	**412**	**497**	625	**710**	823	894	965	1064		
a-2	1921	**1923**	**1135**	**788**	Ac-Aib-Ala	Gly	Aib	Ala	Aib	Gln	Aib	Lxx	Ala	Ala	Vxx	Ala	
					199	**256**	**341**	**412**	**497**	625	**710**	823	894	965	1064		
b	1907	**1909**	**1149**	**760**	Ac-Aib-Ala	Gly	Ala	Aib	Aib	Gln	Aib	Lxx	Aib	Gly	Lxx	Ala	
					199	**256**	**327**	**412**	**497**	625	**710**	**823**	**908**	965	1079		
d	1951	**1953**	**1179**	**774**	Ac-Aib-Ala	Ser	Ala	Aib	Aib	Gln	Aib	Lxx	Aib	Gly	Lxx	Ala	
					199	**286**	**357**	**442**	**527**	655	**740**	**853**	**938**	995	1108		
e	1935	**1937**	**1163**	**774**	Ac-Aib-Ala	Gly	Ala	Aib	Aib	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	
					199	**256**	**327**	**412**	**497**	625	**710**	**823**	**908**	965	1078		
f	1965	**1967**	**1193**	**774**	Ac-Aib-Ala	Ser	Ala	Aib	Aib	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	
					199	**286**	**357**	**442**	**527**	655	**740**	**853**	**938**	995	**1108**		
g	1921	**1923**	**1149**	**774**	Ac-Aib-Ala	Gly	Ala	Aib	Aib	Gln	Aib	Lxx	Aib	Gly	Lxx	Ala	
					199	**256**	**327**	**412**	**497**	625	**710**	**823**	**908**	965	1078		
h	1949	**1951**	**1163**	**788**	Ac-Aib-Ala	Gly	Ala	Aib	Aib	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	
					199	**256**	**327**	412	497	625	710	823	908	965	1078		

[a] The sequence-specific ions which were clearly observed in the CF-FAB mass spectra ($S/N > 5$) are indicated by bold characters. [b] The observed, most abundant component of $[M + H]^+$ isotope cluster.

spectrum of species **a** supports this theory. Although they could not be separated on the LC column, they are named **a**-1 and **a**-2. In Table 4(a), the relative molecular masses and amino acid sequences of eight compounds (**a**-1, **a**-2, **b**, **d**–**h**) are summarized. Table 4(b) shows their characteristic ions; $[M + H]^+$, *N*- and *C*-terminal oligopeptide and sequence-specific ions derived from *N*-terminal oligopeptide ions. Among these ions, those which are clearly observed in the CF-FAB mass spectra are indicated with bold characters. Among the eight compounds, **a**-1, **a**-2, **b** and **d**–**h**, all but **f**, which is identical with the known components of TS-B-Ia, are novel and identified. Since isolation and purification of each compound is very difficult, structure elucidation by NMR or other techniques has not been performed. Therefore, the differentiation between Leu and Ile, and between Val and Iva, could not be achieved. Tables 4(a) and (b) show that four types of new TS-Bs could be found; (a) the third amino acid is substituted for Gly, (b) the twelfth is substituted for Vxx (Vxx = Val or Iva), (c) the thirteenth is substituted for Ala and (d) the seventeenth is substituted for Ala, in contrast to the known TS-Bs.

CF-FAB-MS, one technique of LC–MS, allowed FAB-MS data acquisition of minor components without isolation. The target sample investigated in this study could not be elucidated merely by FAB-MS, and CID was indispensable. It is demonstrated in this paper that the combination of CF-FAB and CID is very effective as a rapid and easy method for the structure elucidation of a complex fungal metabolite.

Experimental

Materials.—Since the extraction procedures of trichosporins have been reported,[4] they are outlined here. A cultural broth of *Trichoderma polysporum* was subjected to column chromatography (Amberlite XAD-2) and was successfully extracted with ethyl acetate. The ethyl acetate-insoluble fraction was purified by column chromatography (Sephadex LH-20) to give a trichosporin mixture. Separation of the mixture was performed repeatedly with a Nacalai Cosmosil ODS column (20 mm i.d. × 250 mm) with the following conditions: eluent methanol–water (85:15, v/v); flow rate 5 cm^3 min^{-1}; column temperature 40 °C; UV detection at 220 nm. Further separation was carried out repeatedly with a Nacalai Cosmosil phenyl column (20 mm i.d. × 250 mm). The preparative HPLC conditions to obtain the investigated fraction, TS-B-I-2-1, were as follows: eluent methanol–water (83:17, v/v); flow rate 2.5 cm^3 min^{-1}; column temperature 40 °C; UV detection at 220 nm.

Mass Spectrometry.—A CONCEPT IH forward-geometry mass spectrometer (Shimadzu/Kratos) was used. The accelerating voltage was maintained at 8 kV during all experiments and the primary beam was Xe. The matrix used for normal FAB-MS and B/E linked-scan was glycerol–thioglycerol (~1:1, v/v) containing ~2% TFA. Saturated aq. NaCl was used in place of TFA when FAB-MS measurements were carried out in order to confirm quasimolecular ions. The mass range and the resolution for normal FAB-MS were *m/z* 100–3000 and 3000 (10% valley definition), respectively. The scanning speeds for both normal FAB–MS and linked-scan were 10 s $decade^{-1}$. Helium was used as a collision gas for linked-scan experiments. This was introduced into a collision cell in the first field-free region between the ion source and the electrostatic sector. The optimum pressure of the collision gas was determined by using one of the known trichosporins, TS-B-Ia, to attenuate the precursor ion ($[M + H]^+$ of TS-B-Ia) signal to one-third of its original intensity.

For CF-FAB analysis, the magnet was scanned from *m/z* 3000 to 100, with 3 s $decade^{-1}$ and the resolution was set at 2000 (10% valley definition). The mobile phase used was acetonitrile–water–glycerol (50:45:5, v/v) and it was delivered with an LC-9A pump (Shimadzu) at a flow rate of 100 mm^3 min^{-1}. The fungal metabolite fraction, TS-B-I-2-1, was separated on a Shim-pack SBC-ODS column (2.5 mm i.d. × 150 mm) maintained at 40 °C. The eluate from the column was split (30:1) prior to its introduction through a CF-FAB probe to the mass spectrometer. A UV detector (SPD-6A, Shimadzu) was set between the column and the splitter and separated compounds were monitored at 220 nm. The ion-source temperature was kept at 70 °C.

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