JOURNAL OF MASS SPECTROMETRY, VOL. 33, 154–163 (1998)

Microheterogeneity Characterization of a Trichorzianine-A Mixture from *Trichoderma harzianum*

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The microheterogeneity of trichorzianine-A (TA) from *Trichoderma harzianum* was studied using single-stage mass spectrometry and high-energy collision-induced dissociation (CID) tandem mass spectrometry. Based on the electrospray ionization and the liquid secondary ion single-state mass spectra, the main components with eight different molecular masses were identified in the mixture. Five contain phenylalaninol and three a tryptophanol *C*-terminal amino alcohol residue. The tandem mass spectra taken on the protonated molecular ion of these components (MH^+) show that most of them contain one main and one or more minor sequence analogues. For the microheterogeneity characterization of this mixture, a previously described method was used based on high-energy CID studies of the different MH^+, y_7, b_{12} ions and their satellite ions with ± 14 and ± 28 mass number differences. In addition to the nine known sequence variants, five new trichorzianines were sequenced from the mixture. It was found, furthermore, that amino acid exchange can occur not only at positions 5, 14, 16 and 19 but also at positions 2, 3, 4, 7, 8 and 9. © **1998 John Wiley & Sons, Ltd.**

J. Mass Spectrom. **33**, 154–163 (1998)

KEYWORDS: trichorzianines; peptaibols; high-energy collision-induced dissociation; tandem mass spectrometry; peptide sequencing

INTRODUCTION

Trichorzianines are mixtures of 19-residue long linear hydrophobic peptides isolated from the antagonistic fungus *Trichoderma harzianum*.[1–3] Common structural features of trichorzianines are a high proportion of α-aminoisobutyric acid (Aib) residue, an acetylated *N*-terminal and an amino alcohol C-terminal group, either phenylalaninol (Pheol) or tryptophanol (Trpol). Based on these characteristics, they belong to the peptaibol class.[4]

Trichorzianines are divided into two subgroups: a neutral component, trichorzianine-A (TA), and an acidic component, trichorzianine-B (TB). TA differs from TB by replacement of ^{18}Gln with ^{18}Glu.[5] From the mixture of closely related trichorzianine-A sequence analogues, nine components have been isolated using multi-step high-performance liquid chromatography (HPLC).[6,7] The change at the *C*-terminal residue (position 19) leads to two different series of trichorzianines, one containing Pheol and the other Trpol. Four sequence analogues were previously found to have Pheol (M_r 1908, 1922 and 1936) and five Trpol (M_r 1947, 1967 and 1975) *C*-terminal end groups. The molecular mass and sequence of the isolated components were determined by fast atom bombardment (FAB) mass spectrometry. Amino acid exchanges were found to occur at residues 5, 14 and 16 (counted from the *N*-terminus).

As membrane active peptides, both TA and TB interact with phospholipid bilayers and thus modify their permeability by forming voltage-gated ion channels.[8] TA is considered to have a more important role than TB in the biocontrol and antagonistic activity of *T. harzianum*,[8,9] and acts synergistically with cell wall-degrading enzymes during mycoparasitism.[10] However, because of the multiplicity of peptaibols produced by *T. harzianum* and of the microheterogeneity of the TA preparations available, biological and antibiotic assays have rarely been performed on a single trichorzianine. It is important, therefore, to characterize the complex mixture of TA produced by *Trichoderma*, to improve the knowledge on their biological role and to consider possible applications of TA as an antimicrobial agent.

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Contract/grant sponsor: European Union; *Contract/grant number:* ERB GE1 CT92-0045; *Contract/grant number:* ERB FMGE CT95-0061.

CCC 1076–5174/98/020154–10 $17.50

Received 11 September 1997
Accepted 7 November 1997

The aim of this work was to characterize the microheterogeneity and to find new sequence analogues of trichorzianine-A using a method based on high-energy collision induced dissociation (CID) tandem mass spectrometry (MS/MS). MS/MS is suitable for the sequence determination of peptides even when they are in a mixture. This allows one to leave out the time- and labour-consuming separation and purification steps and to carry out the analysis in a mixture. Recently, this method has been successfully applied in the microheterogeneity characterization of another peptaibol mixture, paracelsin from *Trichoderma reesei*.[11]

EXPERIMENTAL

Materials

Trichoderma harzianum strain P1 is a biocontrol strain isolated from wood chips which is able to control several fungal pathogens. Strain P1 was grown for 20 h on a rotary shaker (250 rpm, 28 °C) in 1 l flasks containing Mandels–Andreotti medium[12] with glycerol (0.5% v/v) as carbon source. The fungal biomass was removed by filtration through a sintered funnel (G1 porosity) without suction to avoid hyphal breakage.

A 1.5 mg amount of trichorzianine-A mixture from P1 strain of *T. harzianum* was dissolved in 1 ml of methanol. Prior to analysis, this stock solution was diluted 10-fold for the liquid secondary ion (LSI) and 100 times for the electrospray ionization (ESI) experiments. 2-Nitrobenzyl alcohol (mNBA) was purchased from Fluka (Buchs, Switzerland). All solvents were of analytical grade.

Instrumentation

For the electrospray experiments a VG Platform single-quadrupole mass spectrometer (Micromass, Altrincham, UK) was used in the positive ion mode. Aliquots of 10 μl were introduced through a Rheodyne external loop injector into the ion source at a flow rate set at 10 μl min^{-1} using a Phoenix 20 CU HPLC pump. As a buffer solution, 1:1 methanol–acetonitrile containing 0.1% v/v acetic acid was applied. Spraying was achieved using nitrogen as the nebulizing gas with a probe voltage of 3.6 kV. The declustering potential (cone voltage) was set at 40 V and the source temperature at 65 °C. Calibration of the mass scale was performed using the multiply charged ions of horse heart myoglobin from a separate sample introduction. Full-scan mass spectra were acquired in the continuous data acquisition mode in the range m/z 400–2100 at a scan rate of 10 s per scan.

The LSI experiments were carried out on a VG-ZAB-T (Micromass, Wythenshawe, Manchester, UK) four-sector mass spectrometer equipped with an inhomogeneous field electrostatic analyser. The configuration of the instrument is $B_1E_1B_2E_2$ (where B refers to the magnetic and E to the electrostatic sectors) with a reversed Mattauch–Herzog geometry of MS2. Ionization was achieved by bombardment of 30 keV caesium ions. Mass spectra were obtained employing 1 μl of mNBA matrix followed by 1 μl of sample solution on the probe. The spectra were recorded in the positive ion mode at 8 kV accelerating potential. The resolution was set to 2500 (static resolution measured at 5% valley definition). Single-stage (MS1) LSI mass spectra were run in the range m/z 200–2500 with a scan rate 8 s per decade; ions were detected with a photomultiplier detector. Data acquisition and processing were performed using OPUS V3.1X software and scans were acquired in the continuum mode. For LSI-MS/MS (high-energy tandem CID) the precursor peak was selected by MS1 and passed to the variable-potential collision cell which was floated at 4 kV. Only the monoisotopic peak (^{12}C isotope) of the precursor ion was selected in MS/MS experiments. Argon was used as the collision gas at a pressure corresponding to 75% attenuation of the precursor ion beam. Product ion spectra were acquired using a 2048 micro-channel photodiode (MCP) array detector placed after E_2. The angle of the face of MCP relative to the incoming ion beam was set to 30°. With this arrangement a product ion resolution around 1000 was achieved (FWHM resolution) with a mass accuracy better than 0.3 u. The exposure time was 0.5 s and the mass range was set from m/z 50 to the mass of the precursor ion selected.

RESULTS AND DISCUSSION

Single-stage mass spectra

Single-stage ESI and LSI mass spectra were acquired to obtain the molecular mass distribution of the mixture. Both ionization methods have been successfully applied in the structural determination of various peptaibols.[14,15] The ESI mass spectrum of trichorzianine-A from *T. harzianum* (strain P1) in the range m/z 400–1200 is shown in Fig. 1(a). Under the experimental conditions applied, no ions of significant abundance were found above m/z 1200. The declustering potential was kept sufficiently low (on 40 V) to obtain molecular mass information of the sample. Under these conditions the spectrum predominantly yields protonated molecular ion peaks in the 3+ and 2+ charge states. Based on the molecular masses and the known sequences of trichorzianines, five of these peaks correspond to Pheol and three to Trpol C-terminal containing trichorzianines. In the following, trichorzianine-A having Pheol and Trpol amino alcohol residues will be designated TAP and TAT, respectively, doubly and triply protonated molecular ions as P^{n+} and T^{n+} ($n = 2, 3$) and sequence analogues at ± 14 and ± 28 mass number differences as $(P + 14)^{n+}$ and $(T + 14)^{n+}$, etc. In the spectrum P^{n+}, $(P \pm 14)^{n+}$ peaks correspond to the TAP components of molecular mass 1908 and 1908 ± 14, while T^{n+}, $(T + 14)^{n+}$ and $(T + 28)^{n+}$ correspond to the TAT components of molecular mass 1947, $1947 + 14$ and $1947 + 28$, respectively (here and throughout this paper monoisotopic nominal masses are used). Molecular ion peaks indicating the presence of new components, such as $(P - 28)^{n+}$ and $(P + 28)^{n+}$, are also observed with lower abundance. The presence of further components at higher masses is difficult to identify

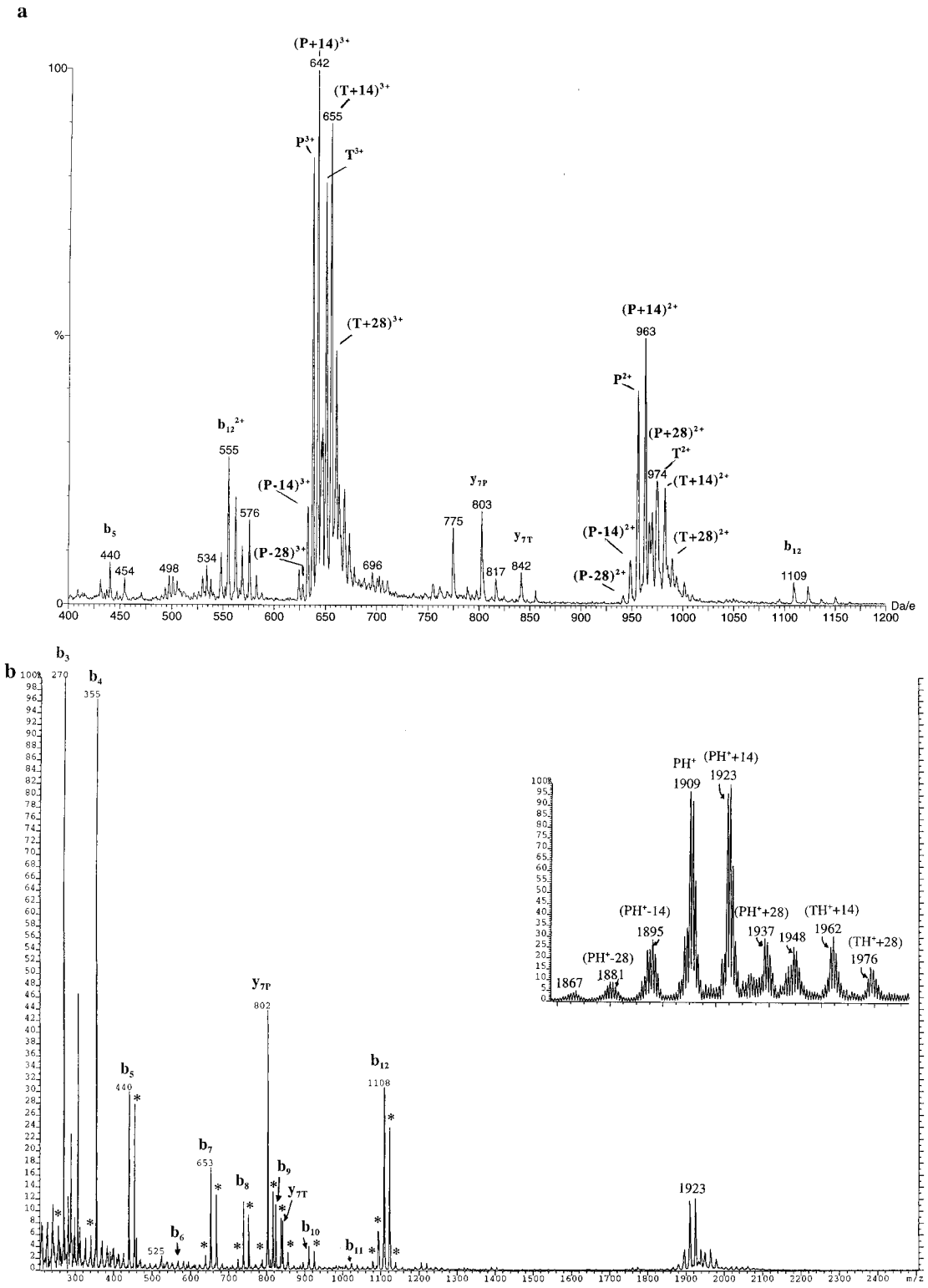

Figure 1. Single-stage positive ion mass spectra of trichorzianine-A mixture. (a) ESI mass spectrum (shown in the range m/z 400–1200). (b) LSI mass spectrum between m/z 200 and 2500. The molecular ion region is shown enlarged.

because they overlap with the sodiated peaks of lower mass components. Besides molecular ion peaks some characteristic fragments are also observed in the ESI mass spectrum. The most abundant and structurally most significant are the y_7 and b_{12} complementary ions. These arise from the cleavage at the proline residue, a characteristic feature of peptaibols.[11,14,15] Throughout this paper peptide fragments are named according to the modified Roepstoff–Biemann notion.[16,17] The mass of the y_7 fragment is different for the TAP and TAT series, and is indicated as y_{7P} and y_{7T}, respectively. The doubly charged form of the b_{12} fragment also appears in the spectrum (b_{12}^{2+}) at m/z 555. (The b ion series is common to TAP- and TAT-type trichorzianines.)

The LSI mass spectrum of trichorzianine-A from *T. harzianum* is shown in Fig. 1(b). The sample was

desalted *in situ* using ion-exchange resin beads.[18] In the molecular ion region (inset of Fig. 1(b)) two groups of protonated molecular ion peaks due to TAP (PH^+, $PH^+ \pm 14$, $PH^+ \pm 28$) and TAT components (TH^+, $TH^+ + 14$, $TH^+ + 28$) can be identified, similarly to the ESI spectrum. Components which were found unequivocally in the LSI and ESI single-stage mass spectra, sorted by *C*-terminal groups and molecular masses, are collected in Table 1. Cleavage at the amide bond of the peptide backbone leads to a series of b-type acylium ions in the LSI mass spectrum. Ions of this series can be found ranging from b_1 to b_{12} with varying abundances. Fragment peaks are often accompanied by peaks at ± 14, ± 28 u distances. These ions are identified as satellite ions and they relate to the microheterogeneity of the mixture (marked with asterisks in the spectra). The $b_n/(b_n \pm 14)$ ion intensity ratio vary along the spectrum, which suggests that there are several positions along the peptide chain where amino acid exchange occur. However, owing to the complex pattern it is not possible to identify sequences of various analogues based on single-stage mass spectra only.

Complete sequence determination of peptaibols has been found to be straightforward by performing high-energy CID experiments on the protonated molecular ion and on the y_7 and b_{12} characteristic complementary fragment ions.[11] The same methodology was followed in the present work.

Sequential fragmentation of y_7 and b_{12} fragments

High-energy CID spectra were measured on y_{7P} and y_{7T} ions and the satellite peaks at a ± 14 u distance (($y_{7P} - 14$), ($y_{7P} + 14$), ($y_{7T} - 14$) and ($y_{7T} + 14$)). As the sequence of y_{7P} and y_{7T} were found to be closely related, only the spectra of the y_{7P} fragment and its satellite ions are discussed here in detail. Spectra of ($y_{7P} - 14$), y_{7P} and ($y_{7P} + 14$) are shown in Fig. 2(a), (b) and (c), respectively. For sequential fragment ions the terminology described previously[11] was used. Briefly, for sequential fragments (e.g. y_7/b_4), the first letter with number indicates the precursor ion according to the Roepstoff–Biemann formalism. The letter following the solidus indicates the type of backbone cleavage, analogously to the Roepstoff–Biemann notion, while the number indicates the amino acid residues retained in this sequential fragment.

In the fragmentation of y_{7P} ions, the full series of b ions appeared (the y_{7P}/b_n series in Fig. 2(b)), based on which sequencing of the y_{7P} fragment is simple and straightforward. There are no satellite peaks in the spectrum, indicating that there is only one sequence corresponding to y_{7P}. Other characteristic fragments such as y_{7P}/a_1, y_{7P}/a_2, y_{7P}/y_1, y_{7P}/y_2, y_{7P}/y_3, y_{7P}/z_2 and y_{7P}/d_6 provide redundant information, making sequence assignment unambiguous. y_{7P}/d_4 side-chain fragmentation leads two peaks at *m/z* 339 and 353 with nearly equal intensities corresponding to a non-symmetrically branched Ile residue at position 4 (position 16 in the intact molecule), and this also exclude the Leu isomer at this position (the corresponding y_{7P}/d_4 fragment would appear at *m/z* 325).[17] Fragmentation of the y_{7P} ion therefore identifies the last seven amino acids in TAP as ^{13}Pro–^{14}Val–^{15}Aib–^{16}Ile–^{17}Gln–^{18}Gln–^{19}Pheol. This corresponds to the known TA-VI-b and TA-VII molecules.[7] There are no satellite ions observed in ($y_{7P} \pm 14$) either (Fig. 2(a) and (c)), showing that there is only one sequence corresponding to ($y_{7P} - 14$) and ($y_{7P} + 14$). The ($y_{7P} - 14$) fragment differs from y_{7P} at position 4 where ^{16}Ile is changed to ^{16}Val/Iva. Exchange at position 16 has not previously been described in Pheol-containing TA.[7] Other peptaibols, such as certain saturnisporins, trichosporins, trichobrachins, tricholongins B-II, suzukacillin, trichotoxin, tricholongin and hypelcin are known to have Iva residues at this position. No peptaibol structure with Val at this position has been

Table 1. Different trichorzianine-A species found unequivocally by the ESI and LSI single-stage mass spectra and sorted by their *C*-terminal types and their protonated molecular masses

Protonated molecular ions (with Pheol)	*m/z*	Main complementary fragment pairs	TAP species[a]	Corresponding TA fraction[b]	Minor complementary fragment pairs[c]	Rel. int. (%)[d]	TAP species	Corresponding TA fraction
$(PH^+ - 28)$	1881	$[(b_{12} - 28), y_{7P}]$	–	–	$[(b_{12} - 14), (y_{7P} - 14)]$	50	TAP − 28b	–
					$[b_{12}, (y_{7P} - 28)]$	40	TAP − 28c	–
$(PH^+ - 14)$	1895	$[(b_{12} - 14), y_{7P}]$	TAP − 14a	–	$[b_{12}, (y_{7P} - 14)]$	10	TAP − 14b	–
PH^+	1909	$[b_{12}, y_{7P}]$	TAPa	TA-VI-b	$[(b_{12} - 14), (y_{7P} + 14)]$	5	–	–
$(PH^+ + 14)$	1923	$[(b_{12} + 14), y_{7P}]$	TAP + 14a	TA-VII	$[b_{12}, (y_{7P} + 14)]$	40	TAP + 14b	TA-V-b
$(PH^+ + 28)$	1937	$[(b_{12} + 14), (y_{7P} + 14)]$	TAP + 28a	TA-VI-a	$[(b_{12} + 14), (y_{7P} + 14)]$	<5	TAP + 28b	–
Protonated molecular ions (with Trpol)	*m/z*	Main complementary fragment pairs	TAT species[a]	Corresponding TA fraction[b]	Minor complementary fragment pairs[c]	Rel. int. (%)[d]	TAT species	Corresponding TA fraction
TH^+	1948	$[b_{12}, y_{7T}]$	TATa	TA-III-b/III-c	$[(b_{12} - 14), (y_{7T} + 14)]$	10	–	–
$(TH^+ + 14)$	1962	$[(b_{12} + 14), y_{7T}]$	TAT + 14a	TA-IV-b	$[b_{12}, (y_{7T} + 14)]$	50	TAT + 14b	TA-II-a
$(TH^+ + 28)$	1976	$[(b_{12} + 14), (y_{7T} + 14)]$	TAT + 28a	TA-III-a	$[b_{12}, (y_{7T} + 28)]$	<5	–	–

[a] Trichorzianine-A species having Pheol and Trpol *C*-terminal end groups are designated TAP and TAT, respectively.
[b] Corresponding TA fractions refer to the HPLC fraction in Ref. 7.
[c] Minor complementary fragment pairs which were observed together with the main complementary fragment pair but having lower abundance.
[d] Intensity values relative to the y_7 ion of the main complementary ion pair.

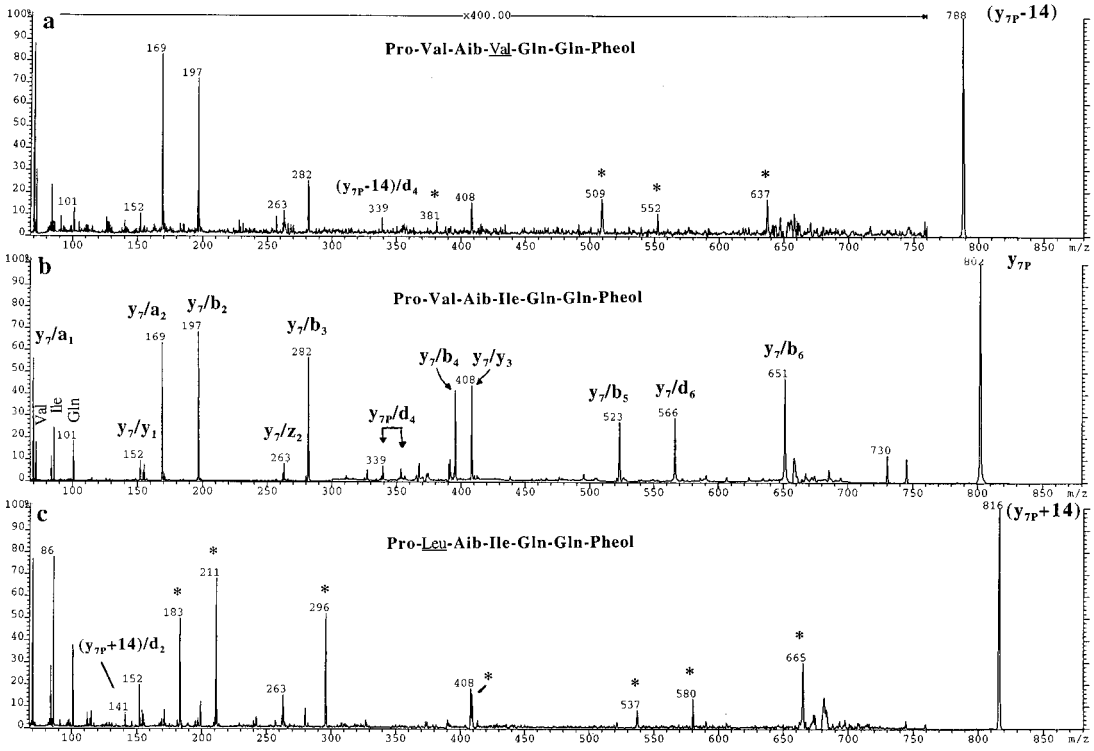

Figure 2. High-energy CID tandem mass spectra of (a) ($y_{7P}-14$), (b) y_{7P} and (c) ($y_{7P}+14$) fragment ions. For sequential ion labelling the terminology described previously[11] is used. Sequential fragments differing by ±14 u from that of y_7 are marked with asterisks.

reported; however, the peak at *m/z* 339 is probably due to $(y_{7P}-14)/d_4$ side-chain loss from the ^{16}Val residue (note that Iva does not have this type of side-chain loss). $(y_{7P}+14)$ differs from y_{7P} at position 2 of the y_7 fragment corresponding to $^{14}\text{Val} \Rightarrow {}^{14}\text{Leu/Ile}$ exchange (sequential fragments different from those of y_7 are marked with asterisks in Fig. 2). A $(y_{7P}+14)/d_2$ fragment is seen at *m/z* 141 corresponds to a ^{14}Leu residue. The sequence of the $(y_{7P}+14)$ ion is therefore the same as the *C*-terminal end of TA-V-b and TA-VI-a compounds.[7] At the low-mass end some immonium ions characteristic of the amino acid composition of the precursor ion, such as Pro, Val/Iva, Leu/Ile and Gln, can also be observed (Fig. 2). Peaks observed in y_{7P} and corresponding to Leu/Ile at *m/z* 86 and to Val/Iva at *m/z* 72 are absent from $(y_{7P}-14)$ and $(y_{7P}+14)$, respectively. This gives further support to the assignments based on sequential fragments. The y_{7P}/b_6 fragment confirms the presence of the *C*-terminal Pheol residue.

The spectra of the y_{7T} fragment and its ± 14 u satellites (not shown) show the same features as the Pheol-containing fragments: amino acid exchange occurs at $^{16}\text{Ile} \Rightarrow {}^{16}\text{Val/Iva}$ in $(y_{7T}-14)$ and at $^{14}\text{Val} \Rightarrow {}^{14}\text{Ile/Leu}$ in $(y_{7T}+14)$ satellites with respect to y_{7T}. In a trichorzianine-A component (TA-III-b), a change from ^{16}Ile to ^{16}Leu was also reported.[7] This isomeric exchange was also confirmed here by the corresponding d_4 side-chain losses which were observed in the spectrum of y_{7T} at *m/z* 325 for Leu and at *m/z* 339 and 353 for Ile.

The tandem mass spectra of $(b_{12}-14)$, b_{12} and $(b_{12}+14)$ fragments are shown in Fig. 3(a), (b) and (c), respectively (these are common for both Pheol- and Trpol-containing trichorzianines). These acylium ions give a complete and abundant series of b_{12}/b_n sequential fragments. Although b_n and b_{12}/b_n fragments are identical, the way of their formation is different: the former is produced from the molecular ion and the latter from the b_{12} fragment. Based on this series, sequencing of the *N*-terminal part of the peptaibol is simple. In other peptaibols (as in paracelsin), in addition to the b_m/b_n ions a b_m/z_n series also appears with high abundance.[11] In the case of trichorzianine-A the b_{12}/z_n series can be unambiguously identified only between $n = 12$ and 10, because at lower masses they coincide with the b_{12}/b_n series. Sequential fragments of b_{12} indicate the *N*-terminal sequence $\text{Ac}^1\text{Aib}{-}^2\text{Ala}{-}^3\text{Ala}{-}^4\text{Aib}{-}^5\text{Aib}{-}^6\text{Gln}{-}^7\text{Aib}{-}^8\text{Aib}{-}^9\text{Aib}{-}^{10}\text{Ser}{-}^{11}\text{Leu}{-}^{12}\text{Aib}$. This is the same as that described for the *N*-terminal part of TA-II-a, TA-III-b, TA-III-c, TA-IV-b and TA-VI-b.[7] The $(b_{12}-14)$ ion was found to be a mixture of more than two sequence analogues (Fig. 3(a)). Compared with b_{12}, in one case the ^{3}Ala residue is changed to ^{3}Gly where sequence alternation with a -14 u difference in the b_{12}/b_n series first appears (b_{12}/b_3 at *m/z* 270); in the other case the ^{8}Aib residue is changed to ^{8}Ala, where sequence alternation in the b_{12}/b_n series almost completely disappears. These possibilities for amino acid exchanges were not noted previously in TAs. The intensity ratio of the two series of ion peaks, the b_{12}/b_n 'normal' and the $(b_{12}-14)/b_n$ 'asterisk marked', varies along the spectrum, indicating that the $(b_{12}-14)$ ion probably contains further sequence variants, but based on these spectra further alterations in the sequence cannot be identified. Fragments of $(b_{12}+14)$, like that of b_{12}, show homogeneity, suggesting one sequence for this fragment. The difference between the two sequences is at position 5 ($^5\text{Aib} \Rightarrow {}^5\text{Iva/Val}$), which corresponds to

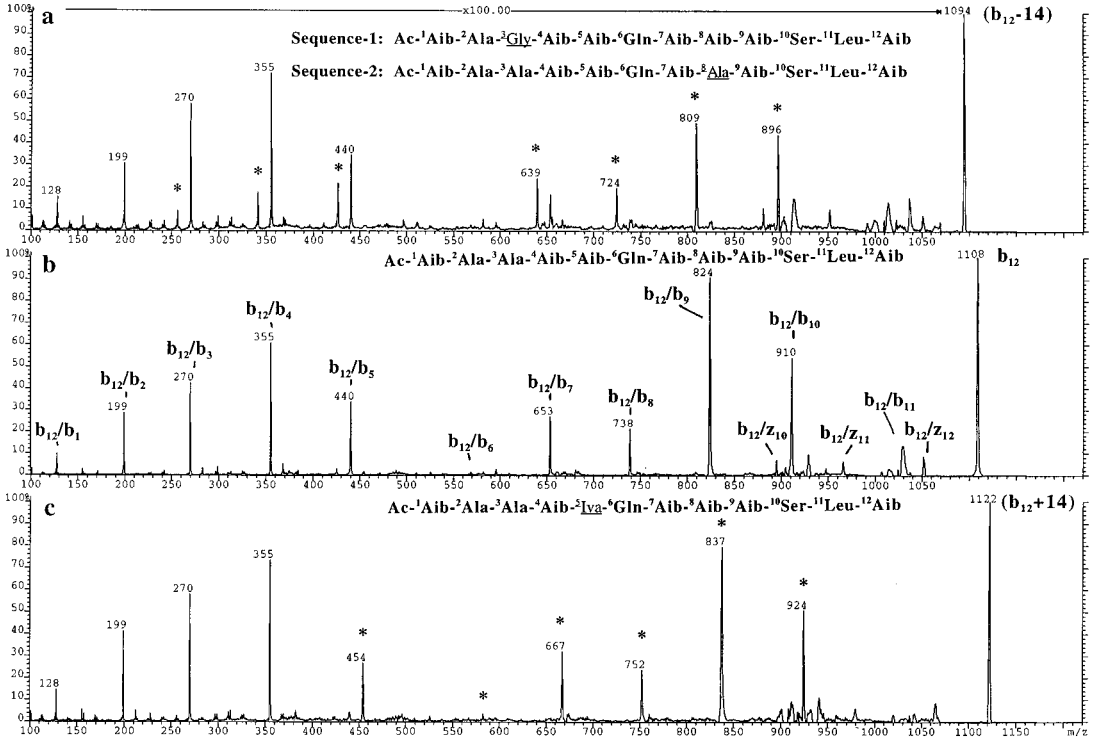

Figure 3. High-energy CID tandem mass spectra of (a) $(b_{12}-14)$, (b) b_{12} and (c) $(b_{12}+14)$ fragment ions. Sequential fragments differing by ±14 u from that of b_{12} are marked with asterisks.

a previously reported *N*-terminal sequence of TA-III-a, TA-IV-b, TA-VI-a and TA-VII.[7]

Determination of the major sequence analogues of Pheol-containing components (TAP, TAP ± 14 and TAP ± 28)

In the high-energy CID tandem mass spectra of MH^+, both b_{12} and y_7 fragments and their satellite ions appear. As these complementary fragments have been sequenced (as discussed above), their combination defines the complete sequence of a TA component.

The sequence of the last seven amino acids (*C*-terminal end) is always unambiguously defined by the mass of the y_7 fragment appearing in the tandem mass spectrum of MH^+, as both y_7 ions and their satellites were found to be homogeneous based on their tandem mass spectra discussed above (Fig. 2). The sequence of the first 12 amino acids (*N*-terminal end) is also unambiguous if the b_{12} or $(b_{12}+14)$ fragment is present in the tandem mass spectrum of MH^+.

The relevant parts of the tandem mass spectra of (PH^+-14), PH^+ and (PH^++14) are shown in Fig. 4(a), (b) and (c), respectively. Peaks which differ from the main fragments of PH^+ by ±14 u are marked with asterisks in the (PH^+-14) and (PH^++14) spectra. The spectrum of the main component (PH^+) is characterized by a complementary fragment ion pair at *m/z* 1108 (b_{12}) and 802 (y_{7P}) (Table 1 and Fig. 4(b)). As both b_{12} and y_{7P} ions were shown to be homogeneous, these data unambiguously identify the full sequence of the main component of TAP (TAPa, M_r 1908, Table 2(a)) and it is identical with the previously reported sequence of the TA-VI-b fraction:[7] Ac^1Aib–^2Ala–^3Ala–^4Aib–^5Aib–^6Gln–^7Aib–^8Aib–^9Aib–^{10}Ser–^{11}Leu–^{12}Aib–^{13}Pro–^{14}Val–^{15}Aib–^{16}Ile–^{17}Gln–^{18}Gln–$^{19}Pheol$. Other fragment ions, notably a nearly full b_n series, are also observed in the spectrum, and supports the sequence determined.

The tandem mass spectrum of the (PH^+-14) peak yields the $[(b_{12}-14), y_{7P}]$ main complementary fragment pair at *m/z* 1094 and 802 (Table 1 and Fig. 4(a)). The sequence of the last seven amino acids is unequivocally identified by y_{7P}. There are two alternative sequences corresponding to the $(b_{12}-14)$ fragment (Fig. 3(a)). The abundant ions at *m/z* 270, 355 and 440 (b_3, b_4, b_5) indicate an *N*-terminal Ac–^1Aib–^2Ala–^3Ala–^4Aib–^5Aib sequence, implying that sequence 2 (Fig. 3(a)) is present in this component. The whole sequence of this component is shown in Table 2(a). It is a new TA component (in fact, there is no known TA of this molecular mass), and is named as TAP − 14a, where TA refers to trichorzianine-A, P refers to the Pheol *C*-terminal end, − 14 shows that its molecular mass is 14 u lower than it is for the major TAP components and a refers to the main component of TAP − 14 (b, c and so on will refer to the minor components). The advantage of sequencing the peptaibol by the b_{12}, y_7 fragment is that the tandem mass spectrum of MH^+ does not give the complete amino acid sequence.

The main component of (PH^++14) is characterized by the $[(b_{12}+14), y_{7P}]$ complementary fragment pair at *m/z* 1122 and 802 (Table 1 and Fig. 4(c)). Since both fragments was found to be homogeneous (Figs 3(c) and 2(b)), sequencing of this component is straightforward. The sequence defined by these fragments is supported by the b_n ion series (b_2–b_{10}) observed in the tandem mass spectrum of MH^+ (Fig. 4(c)). Compared with

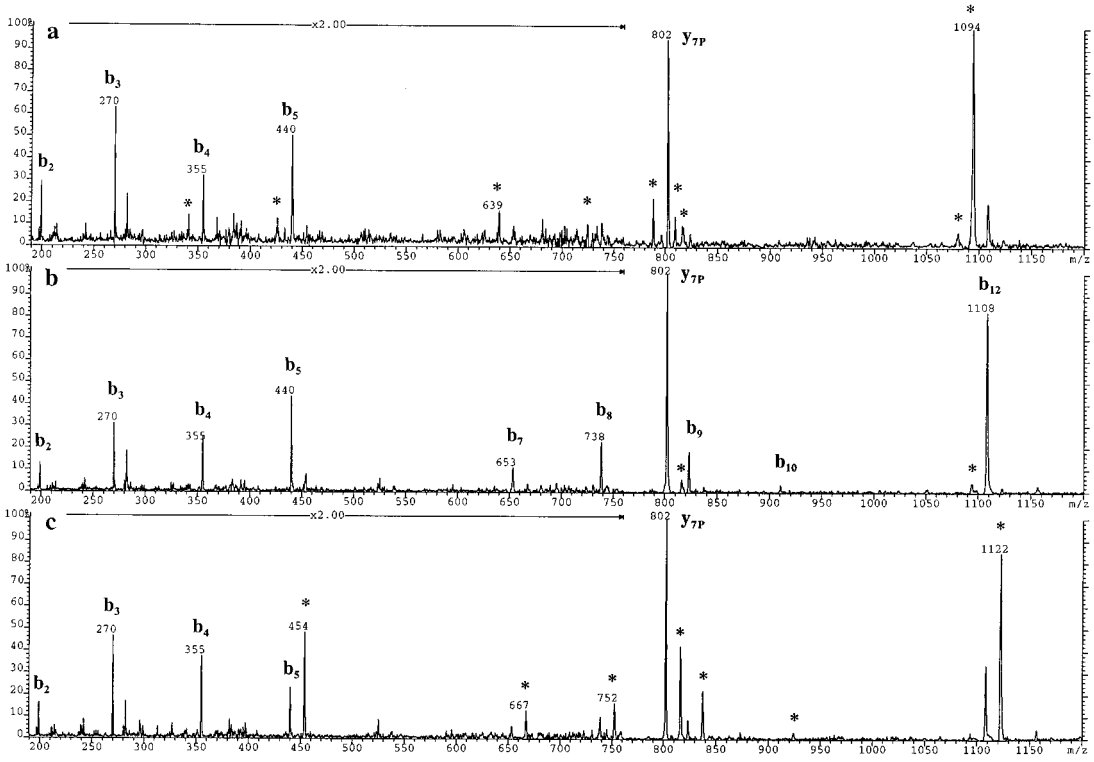

Figure 4. High-energy CID tandem mass spectra of (a) $(PH^+ - 14)$, (b) PH^+ and (c) $(PH^+ + 14)$ Pheol-containing, protonated quasi-molecular ions. Fragment ions differing by ±14 u from the main ion series of PH^+ are marked with asterisks.

TAPa, amino acid exchange is at position 5 where ^{5}Aib changes to ^{5}Iva. The sequence is the same as that of the TA-VII (Table 2(a)).[7] In the spectrum of PH^+ and $(PH^+ \pm 14)$, in addition to the main component, minor components can also be observed, but this will be discussed later in the Microheterogeneity section.

Tandem mass spectra on the $(PH^+ \pm 28)$ peaks at *m/z* 1837 and 1881 were also measured. $(PH^+ + 28)$ is

Table 2. Amino acid sequences and molecular masses of the (a) major and (b) minor Pheol-containing components of trichorzianine-A determined by high-energy CID-MS/MS

		(a)					(b)		
No.	TAPa	TAP-14a	TAP + 14a	TAP + 28a	TAP − 28b	TAP − 28c	TAP − 14b	TAP + 14b	TAP + 28b
1	AcAib	AcAib	AcAib	AcAib	AcAib	AcAib	AcAib	AcAib	AcAib
2	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
3	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
4	Aib	Aib	Aib	Aib	*Ala*	Aib	Aib	Aib	Aib
5	Aib	Aib	*Iva*	*Iva*	Aib	Aib	Aib	Aib	*Val/Iva*
6	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln
7	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib
8	Aib	*Ala*	Aib	Aib	Aib	Aib	Aib	Aib	Aib
9	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib
10	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
11	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
12	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib
13	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro
14	Val	Val	Val	*Leu*	Val	*Ala*	Val	*Leu*	*Leu*
15	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib
16	Ile	Ile	Ile	Ile	*Val*	Ile	*Val*	Ile	Ile
17	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln
18	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln
19	Pheol	Pheol	Pheol	Pheol	Pheol	Pheol	Pheol	Pheol	Pheol
M_r[a]	1908	1894	1922	1936	1880	1880	1894	1922	1936
TA fraction[b]	TA-VI-b	–	TA-VII	TA-VI-a	–	–	–	TA-V-b	–

[a] M_r refers to the calculated nominal molecular masses.
[b] TA fractions refer to the trichorzianine-A sequences as determined and named by Bodo and co-workers.[7]

characterized by [(b_{12} + 14), (y_{7P} + 14)] homogeneous complementary fragment peaks (Table 1). Its sequence corresponds to the previously determined TA-VI-a.[7] The main complementary fragments of (PH^+ − 28) are (b_{12} − 28) and y_{7P}. Since (b_{12} − 28) was found to be a complex mixture of closely related analogues (see later in the Microheterogeneity section), it was not possible to determine the full sequence of the major component of (PH^+ − 28).

Determination of the major sequence analogues of the Trpol-containing components (TAT, TAT + 14 and TAT + 28)

Tandem mass spectra corresponding to TH^+, (TH^+ + 14), and (TH^+ + 28) are shown in Fig. 5(a), (b) and (c), respectively. The y_{7T} part of the main complementary fragment pairs is seen at *m/z* 841 in the case of TH^+ and (TH^+ + 14) (Fig. 5(a) and (b)), but appears with 14 u higher mass, at *m/z* 855 in the case of (TH^+ + 28) (Fig. 5(c)). In the Trpol-containing TA components, the sequences of the first 12 amino acids are similar to those in the Pheol-containing species and can be characterized by the sequential fragments of the homogeneous b_{12} and (b_{12} + 14) fragments. The major sequence analogues, similarly to the Pheol-containing species, were characterized by the main complementary fragment pairs appearing in the tandem mass spectra (Table 1). Since the components of these (that is, y_{7T}, b_{12} and (b_{12} + 14)) were all found to be homogeneous, sequencing was therefore unambiguous in all cases. The sequence of the major analogue of TAT can correspond both to TA-III-b and to TA-III-c (Table 3(a)).[7] The difference between the structures of these two is at position 16, where both Leu (in TA-III-b) and Ile (in TA-III-c) can be located. This isomeric variation was confirmed by the tandem mass spectra of y_{7T}. The main compo-

Table 3. Amino acid sequences and molecular masses of the (a) major and the (b) minor Trpol-containing components of trichorzianine-A determined by high-energy CID-MS/MS

		(a)		(b)
No.	TATa	TAT + 14a	TAT + 28a	TAT + 14b
1	AcAib	AcAib	AcAib	AcAib
2	Ala	Ala	Ala	Ala
3	Ala	Ala	Ala	Ala
4	Aib	Aib	Aib	Aib
5	Aib	*Iva*	*Iva*	Aib
6	Gln	Gln	Gln	Gln
7	Aib	Aib	Aib	Aib
8	Aib	Aib	Aib	Aib
9	Aib	Aib	Aib	Aib
10	Ser	Ser	Ser	Ser
11	Leu	Leu	Leu	Leu
12	Aib	Aib	Aib	Aib
13	Pro	Pro	Pro	Pro
14	Val	Val	*Leu/Ile*	*Leu/Ile*
15	Aib	Aib	Aib	Aib
16	Leu/Ile	Leu/Ile	Leu/Ile	Leu/Ile
17	Gln	Gln	Gln	Gln
18	Gln	Gln	Gln	Gln
19	Trpol	Trpol	Trpol	Trpol
M_r[a]	1947	1961	1975	1961
TA fraction[b]	TA-III-b TA-III-c	TA-IV-b	TA-III-a	TA-II-a

[a] M_r refers to the calculated nominal molecular masses.
[b] TA fractions refer to the trichorzianine-A sequences as determined and named by Bodo and co-workers.[7]

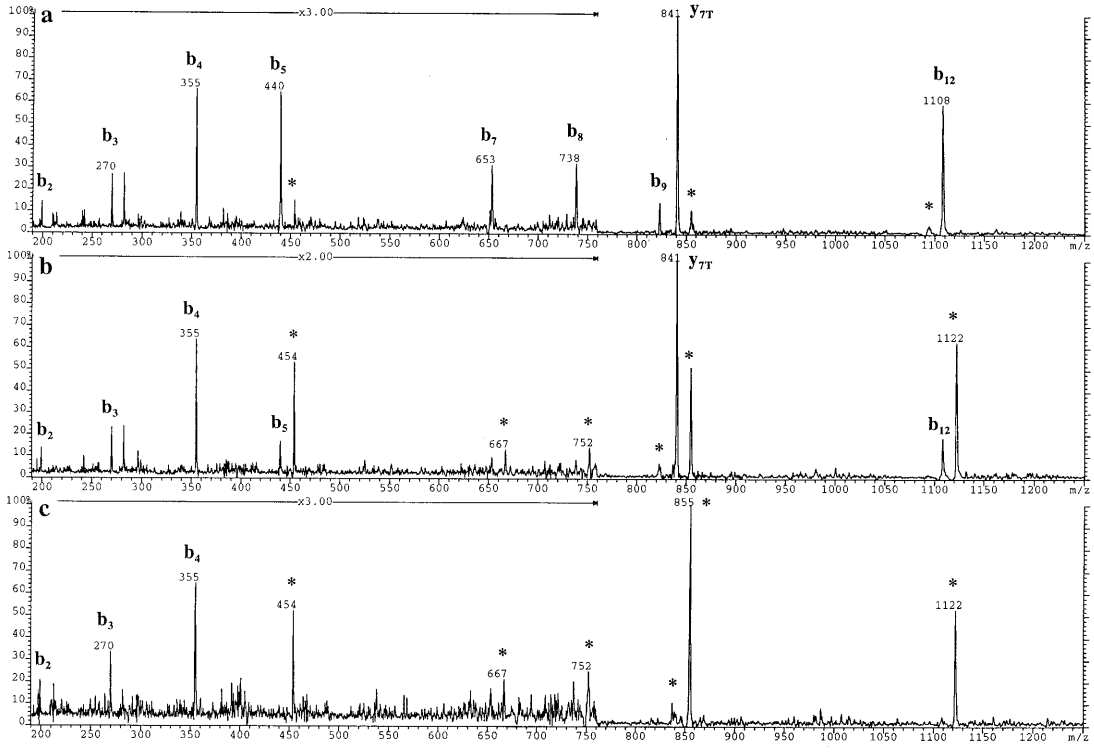

Figure 5. High-energy CID tandem mass spectra of (a) (TH^+ − 14), (b) TH^+ and (c) (TH^+ + 14) Trpol-containing, protonated quasi-molecular ions. Fragment ions differing by ±14 u from the main ion series of TH^+ are marked with asterisks.

Table 4. Positions and types of amino acid exchanges which can occur with respect to the main trichorzianine component TAPa

Position	Sequence (TAPa)		
1	AcAib		
2	Ala	Gly	
3	Ala	Gly	Aib
4	Aib	Ala	
5	Aib	Ala	Val/Iva
6	Gln		
7	Aib	Ala	
8	Aib	Ala	
9	Aib	Ala	
10	Ser		
11	Leu		
12	Aib		
13	Pro		
14	Val	Leu/Ile	Ala
15	Aib		
16	Ile	Leu	
17	Gln		
18	Gln		
19	Pheol	Trpol	

nents of TAT + 14 and TAT + 28 was found to correspond to TA-IV-b and TA-III-a, respectively (Table 3(a)).

Microheterogeneity

In the tandem mass spectra of the protonated molecular ions, in addition to the main complementary fragment ion pair (listed in Table 1), minor, b_{12} and y_7 fragment pair(s) having masses shifted by ± 14 or ± 28 u were also found, reflecting that with the same molecular mass there is more than one component present in the mixture. The abundances of the satellite complementary ion pair relative to that of the main complementary fragment pair is in the range 5–50%, and are referred to as minor complementary fragment pairs in Table 1.

In the TAP series, in addition to the five major components, six minor sequence analogues were found (Table 1). Only one of these, characterized by [b_{12}, (y_{7P} + 14)] and having a molecular mass of 1922, has been reported previously as TA-V-b.[7] Two minor components were found in the highly heterogeneous ($PH^+ - 28$) component, one with [($b_{12} - 14$), (y_{7P} − 14)] and the other with [b_{12}, ($y_{7P} - 28$)] complementary fragment pairs. The first satellite peak observed at m/z 341 in the spectrum is ($b_4 - 14$), and corresponds to a ^{4}Aib ⇒ ^{4}Ala change. This component is called TAP-28b and its sequence is reported in Table 2(b). In the other analogue, [b_{12}, ($y_{7P} - 28$)] fragments, the sequence of the first 12 amino acids is the same as in TA-VI-b; however, there is a −28 u change in the y_{7P} part. A high-energy CID experiment was performed on the ($y_{7P} - 28$) fragment ion in order to establish which residue(s) are involved in the change. The ($y_{7P} - 28$) satellite ion appeared with a very low abundance (1–2%) in the LSI mass spectrum (Fig. 2). The high sensitivity achieved by array detection, however, made it possible to obtain tandem mass spectra even on this ion. The tandem mass spectrum of the ($y_{7P} - 28$) fragment ion shows that ^{14}Val changes to ^{14}Ala (− 28 u). This amino acid exchange is unusual in peptaibols, as Pro is usually followed by a Val/Iva, Aib or Leu residue. This component is called TAP-28c and its full sequence is given in Table 2(b).

The minor component of ($PH^+ - 14$) is characterized by the [b_{12}, ($y_{7P} - 14$)] pair (Fig. 4(a) and Table 1). This gives a new sequence analogue called TAP − 14b (Table 2(b)). The (TAP + 28) ion has a minor component, indicated as TAP + 28b and characterized by the [(b_{12} − 14), ($y_{7P} - 14$)] complementary fragment pair. The complete sequence is given in Table 2(b).

In the TAT series, three major and three minor sequence analogues have been found (Table 1). The minor analogue of TAT (M_r 1947) is characterized by a [($b_{12} - 14$), ($y_{7T} + 14$)] pair. In this case, owing to the low abundances of b_{10} and b_{11} ions, it was possible to determine only the range (position 10–12) and not the exact location of amino acid exchange. TAT + 14 (M_r 1961) is a mixture of two variants. The sequence analogue having a lower relative abundance (about 50%) with respect to the main variant corresponds to TA-II-a (Fig. 4(b) and Table 3(b)).[7] The component TAT + 28 (M_r 1975) appeared to have a minor component, TAT + 28b, with a complementary fragment pair [(b_{12}, ($y_{7P} + 28$)]. It was not possible to determine the sequence of this minor analogue since the tandem mass spectra obtained on the ($y_{7P} + 28$) fragment showed further inhomogeneities.

Previous results obtained on another peptaibol (paracelsin) showed that changes in the amino acid residues combine in a roughly statistical manner and the characterization of microheterogeneity can be made by studying the possible types and positions of amino acid exchanges.[11] To complete the picture of trichorzianine microheterogeneity, several fragment ions such as b_3–b_{12} and y_7 fragments and their ± 14 and ± 28 u satellites were selected for high-energy CID MS/MS experiments. The various types and positions for amino acid exchange in trichorzianines with respect to the TAPa main sequence analogue were determined and are given in Table 4. The results show that the major part of the residues present in the 1–12 segment can alter whereas positions 1, 6, 10–12 (^{1}Ac Aib, ^{6}Gln, ^{10}Ser ^{11}Leu ^{12}Aib, respectively) do not change. In the last seven amino acids (13–19 segment), ^{13}Pro, ^{15}Val and ^{17}Gln ^{18}Gln residues were found to remain unaltered.

CONCLUSIONS

High-energy CID experiments were used to characterize the microheterogeneity of trichorzianine-A in a crude mixture, without separating it into individual components. This approach is highly advantageous, as the time-consuming sample separation steps can be omitted.

Determination of the molecular mass distribution is straightforward both using ESI and LSI ionization. The amino acid sequence corresponding to each molecular

mass is determined by high-energy CID. This approach utilizes a particular mass spectrometric feature common to all peptaibols: the main fragmentation channel of MH^+ is the cleavage at the Pro residue (usually at the 13th position), resulting in complementary b_{12}- and y_7-type ions.[11,15] These two fragments define the masses of the 12 amino- and the 7 carboxy-terminal peptide residues, respectively. Tandem mass spectra taken on these two fragment ions (b_{12} and y_7) give complete sequence information on the *N*- and *C*-terminal amino acids, respectively, so the sequence of the whole peptide can be determined. Note that the tandem mass spectrum of MH^+ gives limited, but not complete, sequence information.

In a peptaibol mixture there are various MH^+, b_{12} and y_7 homologues, usually 14 u apart. The approach described above works perfectly in a mixture provided that each MH^+, b_{12} and y_7 homologue (selected by mass) contains one sequence only. If the selected MH^+ ion fragments into two or more complementary ion pairs (e.g. into $[b_{12}, y_7]$ and $[(b_{12} + 14), (y_7 - 14)]$), but the b_{12} and y_7 homologues (selected by mass) contain a one–one sequence only, the complete mixture can still be analysed perfectly. Luckily, most major trichorzianine (and other peptaibol) components satisfy these criteria. Mixtures containing 8–10 components can be fully characterized this way.

In the cases when a b_{12} or y_7 homologue of given mass contains two main isobaric sequences, both can usually be determined based on the tandem mass spectrum (in the mixture). The tandem mass spectrum of the corresponding MH^+ in many cases contains sufficient fragment information to identify which b_{12} (or y_7) homologue sequence is present in the MH^+ ion of a given mass. In this way, mixtures containing 10–15 components can be sequenced, most sequences being unequivocally and some tentatively identified.

If there are minor components giving isobaric b_{12} or y_7 ions, or if three or more sequences are present in a b_{12} (or y_7) fragment of given mass, the whole peptailbol sequence can not be identified. The position and the type of amino acid exchange (e.g. ^{14}Val to ^{14}Leu), however, can often be determined. Such changes can be combined and these could be (and in peptaibols such as trichorzianines and paracelsins[11]) there are hundreds of sequence alternatives. In such cases these minor sequence variants cannot be completely sequenced in the mixture, but this does not seem to be a real limitation.

The results demonstrate the usefulness of high-energy CID-MS/MS for sequencing peptaibols in mixtures. Several new trichorzianines were identified, which were not found by the 'classical' method of HPLC separation followed by spectroscopic and amino acid analysis. The approach described here is likely to be useful for analysing other peptide and peptaibol mixtures and possibly also other natural compounds showing a large degree of microheterogeniety.

Acknowledgement

This work was supported by EU grants ERB GE1 CT92-0045 and ERB FMGE CT95-0061.

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