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# Detection of new sequences of peptaibol antibiotics trichotoxins A-40 by on-line liquid chromatography–electrospray ionization mass spectrometry

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**Abstract**

Using high-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (ESI-MS) the sequences of the microheterogeneous peptide mixture of the 18-residue ''peptaibol'' antibiotics trichotoxins A-40, isolated from the mold *Trichoderma viride* strain NRRL 5242, were reinvestigated. The structures of two major and one minor component [J. Chromatogr., 296 (1984) 236] could be confirmed and hitherto not known sequences of a further major and two minor peptides could be determined. It is demonstrated that ESI-MS in the positive ionization mode is advantageously completed by applying negative ionization. The methods used make possible the sequence determination of components of peptaibols without previous isolation and allow, in certain cases, sequencing of peptides which are incompletely or not resolved by HPLC. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Mass spectrometry; *Trichoderma viride*; Trichotoxin; Antibiotics; Peptaibiotics; Polypeptide antibiotics; α-Aminoisobutyric acid; D-Isovaline

## 1. Introduction

The isolation and preliminary characterization of a peptide mycotoxin, named trichotoxin (TT) A, from strain NRRL 5242 of the mold *Trichoderma viride* was reported in 1972 [1]. This peptide was found, among other amino acids (AAs), to contain also the non-protein Aib (α-aminoisobutyric acid, 2-amino-2-methyl-propanoic acid), D-Iva (isovaline, 2-amino-2-methyl-butanoic acid) and the amino alcohol Valol (2-amino-3-methyl-1-butanol). The mixture consists of two groups of peptides each of which showed microheterogeneity. These groups were distinguished by an exchange of Glu and Gln as well as some other AAs, thus resulting in an acidic and neutral fraction with different polarity. The peptide mixtures were named TT A-40 and A-50, respectively, according to their different $R_F$ values on thin-layer chromatography (TLC) [2]. Peptide groups TT A-40 and A-50 could also be separated by multiplicative counter-current distribution (CCD) [3–5]. The sequences of TT A-50 could be determined by fast atom bombardment mass spectrometry (FAB-MS) of individual peptides isolated by high-performance liquid chromatography (HPLC) [6].

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Owing to the presence of several Aib-residues, a C-terminal bonded amino alcohol and their bioactivities, the polypeptides were placed in the group of ''peptaibol'' antibiotics [7–9], or, generally spoken, ''peptaibiotics'' [10].

The sequences of some peptides of the TT A-40 peptide mixture, after suitable derivatizations, had been determined by combined gas chromatography–mass spectrometry (GC–MS) of three isolated *N*-acetyl dodecapeptides and two *N*-prolylhexapeptides. These peptide fragments were obtained after selective trifluoroacetolysis of a still microheterogeneous TT A-40 mixture resulting from CCD. The sequences of these fragments, including AA exchange due to their natural microheterogeneity, were reported [3–5,11,12]. Definite arrangements of N- and C-terminal fragments were deduced from the molecular ions of the TT A-40 mixture and their intensities as determined by field desorption MS [13].

Fast screening for [14–16], and rapid sequencing of [6,17], peptaibols/peptaibiotics is of general interest owing to their wide range of bioactivities. They include bactericidal [1,9,10], fungicidal [18], weak antiprotozoal and anthelmintic [19], insecticidal [20,21] and, in part, cytotoxic [22] properties. Recently, antithrombic [23] and neuroleptic (antipsychotic) [24] activities of peptaibiotics were reported as well as uncoupling of oxidative phosphorylation in mitochondria [25]. Some peptaibols attracted much attention as a result of their capability of forming voltage-gated ion channels in lipid bilayer membranes. Thus they might serve as structurally well characterized models for ion transport in excitable cells [26–30].

With the determination of new sequences, and confirmation of reported sequences of the original TT A-40 peptide mixture, in the following we demonstrate advantages and discuss limits of sequencing microheterogeneous peptaibol antibiotics using on-line HPLC–ESI-MS in the positive and negative ionization mode.

## 2. Experimental

### *2.1. Solvents and chemicals*

Methanol (MeOH) and acetonitrile (MeCN) were of the gradient grade and purchased from Merck, trifluoroacetic acid (TFA) and trifluoroacetic acid anhydride (TFAA) were from Fluka (Buchs, Switzerland), dichlormethane (DCM) was from Roth (Karlsruhe, Germany); doubly distilled water from a quartz distill was used for preparing eluents. The synthetic dipeptide Pro–Leu, serving as standard, was from Sigma (Deisenhofen, Germany).

### *2.2. Source of TT A-40 used in this study*

TT was isolated from the mycelium of *Trichoderma viride* NRRL 5242 as described [5] and peptide groups A-40 and A-50 were separated by CCD [5,13]. For HPLC of TT A-40 used for its previous sequencing see Ref. [13] and therein Fig. 3, fraction (C) III (570 mg). After partial trifluoroacetolysis of a 500-mg aliquot from totally 570 mg peptide, unreacted TT A-40 was separated from hydrolyzed material and recovered by silica gel chromatography [5]. This material, representing essentially the peptide mixture used originally for sequencing [5], was investigated in the study presented here. For a TLC of the unreacted material recovered see Ref. [5] and therein Fig. 2, fractions 6–10.

### *2.3. Instruments and chromatography*

For HPLC a HP 1100 instrument (Hewlett-Packard, Waldbronn, Germany) and HP ChemStation with binary pump were used and connected either to a UV detector of the same series or hyphenated to the mass spectrometer.

The stationary phase of the HPLC column was Superspher 100 RP-18, 250 mm×4 mm I.D. with 4 μm particle size (Merck, Darmstadt, Germany). Eluent A: MeOH–water–MeCN–TFA (39:22:39:0.1, v/v); eluent B: MeOH–MeCN–TFA (50:50:0.1, v/v); 0 min 10% B, 10 min 10% B, 30 min 50% B, 35 min 50% B, flow-rate 0.8 ml/min, temperature 40°C; injected amounts were 10 μl of 0.1% peptides in MeOH.

For ESI-MS a LCQ mass spectrometer (ThermoQuest, Finnigan MAT, San Jose, CA, USA) was used. Sheath gas was nitrogen (purity>99.5%) from a nitrogen generator Model 75-72 (Whatman, Balston, Haverhill, MA, USA) and the collision gas was

helium of 5.0 quality (Messer-Griesheim, Krefeld, Germany). Sequence analysis was carried out by positive- and negative-ionization, recorded in the centroid mode, providing $m/z$ values having an accuracy of one decimal. Values of Table 1 are rounded up or down, respectively. Conditions for positive (negative)-ionization mode: spray voltage 4.25 (4.25) kV, heated capillary temperature 200 (200)°C, capillary voltage +44 (−27) V, tube lens offset +55 (−60) V, maximum ion time 1000 ms. For automatic calibration a mixture of caffeine, the tetrapeptide Met–Arg–Phe–Ala, and the perfluorinated mass spectrometric standard Ultramark 1621 was used. In the negative-ionization mode aqueous 4

Table 1
Nominal masses and adducts of molecular ions of trichotoxins A-40 and fragment ions determined by HPLC–ESI-MS performed in positive and negative ionization mode

| Diagnostic ions[a] | Trichotoxin A-40 peptides ($m/z$) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 5a |
| $b_4$[d] | 383 | 383 | 383 | 383 | 383 | 397 |
| $b_5$[d] | 468 | 468 | 468 | 468 | 468 | 482 |
| $b_6$[d] | 596 | 596 | 596 | 596 | 596 | 610 |
| $b_7$[b] | 681 | 681 | 681 | 681 | 681 | 695 |
| $b_8$[b] | 766 | 766 | 766 | 766 | 766 | 780 |
| $b_9$[b] | 837 | 851 | 837 | 851 | 851 | 865 |
| $b_{10}$[b] | 908 | 922 | 908 | 922 | 922 | 936 |
| $b_{11}$[b] | 993 | 993 | 993 | 1007 | 1007 | 1021 |
| $b_{12}$[b] | 1078 | 1078 | 1078 | 1092 | 1092 | 1106 |
| $b_{15}$[b] | 1373 | 1373 | 1373 | 1387 | 1387 | n.d. |
| $b_{16}$[b] | 1458 | 1458 | 1472 | 1472 | 1486 | n.d. |
| $b_{17}$[b] | 1587 | 1587 | 1601 | 1601 | 1615 | n.d. |
| $(M-H_2O)^+$[b] | 1672 | 1672 | 1686 | 1686 | 1700 | 1700 |
| $(M+H)^+$ | 1691 | 1691 | 1705 | 1705 | 1719 | 1719 |
| $(M+Na)^+$ | 1713 | 1713 | 1727 | 1727 | 1741 | 1741 |
| $y'_{6P}$[b] | 613 | 613 | 627 | 613 | 627 | 613 |
| $(y'_{6P}-H_2O)^+$[c] | 595 | 595 | 609 | 595 | 609 | 609 |
| 1 | 510[c] | 510[c] | n.d. | 510[c] | n.d. | 510[c] |
| 2 | n.d. | n.d. | 524[c] | n.d. | 524[c] | n.d. |
| 3 | n.d. | n.d. | 395[c] | n.d. | 395[c] | n.d. |
| 4 | 381[c] | 381[c] | n.d. | 381[c] | n.d. | 381[c] |
| 5[c] | 296 | 296 | 296 | 296 | 296 | 296 |
| 6[c] | 211 | 211 | 211 | 211 | 211 | 211 |
| $y_{12N}$[e] | 1094 | 1094 | 1108 | 1108 | 1122 | 1108 |
| $y_{13N}$[e] | 1222 | 1222 | 1236 | 1236 | 1250 | n.d. |
| $(y_{13N}-NH_3)^-$[e] | 1205 | 1205 | 1219 | 1219 | 1233 | 1219 |
| $y_{14N}$[e] | 1307 | 1307 | 1321 | 1321 | 1335 | 1321 |
| $y_{15N}$[e] | 1420 | 1420 | 1434 | 1434 | 1448 | 1434 |
| $y_{16N}$[e] | 1505 | 1505 | 1519 | 1519 | 1533 | 1519 |
| $y_{17N}$[e] | 1562 | 1562 | 1576 | 1576 | 1590 | 1590 |
| $(M)^-$ | 1690 | 1690 | 1704 | 1704 | 1718 | 1718 |
| $(M-Ac)^-$[e] | 1647 | 1647 | 1661 | 1661 | 1675 | 1675 |
| $(M-H_2O)^-$[e] | 1672 | 1672 | 1686 | 1686 | 1700 | 1700 |

[a] Nomenclature based on Refs. [33,34].
[b] Identified via MS–MS of $(M+H)^+$.
[c] Identified via MS–MS of the $y'_{6P}$ fragment.
[d] Identified via $MS^3$ of $b_{12}$.
[e] Identified via MS–MS from $(M)^-$; 1=(Pro–Leu–Aib–Aib–Glu)′; 2=(Pro–Leu–Aib–D-Iva–Glu)′; 3=(Pro–Leu–Aib–D-Iva)′; 4=(Pro–Leu–Aib–Aib)′; 5=(Pro–Leu–Aib)′; 6=(Pro–Leu)′; n.d.=not detected; masses of AAs ($-H_2O$) are: Ala (71), Aib (85), Gln (128), Glu (129), Gly (57), Iva (99), Leu (113), Pro (97); amino alcohol Valol (103); Ac (43).

*M* ammonia was added to the HPLC eluate by a syringe pump at a flow-rate of 3 μl/min. The $(M+H)^+$ or $(M)^-$ ions and the internal fragments $y'_{6P}$ of compounds were chosen as precursor for multi stage MS, i.e., $(MS^n)$.

For GC–MS the instrument designated ''A'' was a HP 6890 GC (Hewlett-Packard) with a mass-selective detector equipped with a HP-5MS fused-silica column (crosslinked 5% diphenyl–95% dimethyl siloxane), column 30 m×0.25 mm I.D., film thickness 0.25 μm (Hewlett-Packard); instrument ''B'' was a Shimadzu 17A/QP 5000 mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a Chirasil L-Val column, 25 m×0.25 mm I.D. (Chrompack, Middelburg, The Netherlands) and run in selected ion monitoring (SIM) mode.

### *2.4. GC–MS experiments*

#### *2.4.1. Chiral amino acid analysis (AAA) by GC–SIM-MS*

The mixture of TT A-40 peptides was hydrolyzed (6 *M* HCl, 110°C, 24 h). Composition and chirality of AAs and Valol were assigned after derivatization as *N*-pentafluoropropionyl-AA-(2)-propyl esters (*N*-acetyl 2-propyl ester for Iva) and *N*(*O*)-bis(pentafluoropropionyl)valol by GC–SIM-MS (instrument ''B'') similarly to procedures described previously [5,10].

#### *2.4.2. Dipeptide analysis by GC–MS*

The mixture of TT A-40 peptides (ca. 10 μg) in 4 *M* HCl in MeOH was heated for 8 h at 110°C. Reagents were removed in a nitrogen stream and the resulting dipeptide methyl esters were trifluoroacetylated with TFAA (50 μl) in DCM (200 μl). Reagents were removed in a stream of nitrogen, DCM (50 μl) was added and the resulting TFA–dipeptide-methyl esters were analyzed for the presence for TFA–Pro–Leu–OMe by comparison with retention times and fragmentation pattern of the standard dipeptide by GC–MS on instrument ''A'' as described previously [10].

## 3. Results and discussion

The elution profile of TT A-40 peptides resolved on an analytical Superspher column is shown in Fig. 1a. For comparison HPLC of TT A-40 using a semipreparative Nucleosil ODS stationary phase (taken from Ref. [13]) is shown as mirror image in Fig. 1b. Note that new numbering of peaks for Fig. 1a is used for simplicity. TT A-40 peptides were eluted from Nucleosil using a neutral eluent composed of MeOH–water (85:15, v/v) [13]. However, elution of peptides from Superspher using an eluent consisting of MeOH–water–MeCN required addition of 0.1% TFA. Omission of the acid from the eluent resulted in delayed elution of the mixture from the column as a broad peak of almost unresolved peptides. This indicates that the carboxyl group of $Glu^{17}$ is protonated under the acidic conditions. It is assumed that peak 5 in the original chromatogram and not resolved on Nucleosil (see Fig. 1b) is separated into two peaks designated 3 and 4 in Fig. 1a, and that peak 7 in the original chromatogram corresponds to peak 5 in Fig. 1a. The Superspher stationary phase was used for on-line HPLC and ESI-MS in the positive- and negative-ionization mode for sequencing of TT A-40 components.

Chirality and AA ratios of the original TT A-40 mixture [5] were determined as Aib (9.23), Gly (0.93), L-Leu (2.09), L-Glx (2.00), L-Ala (1.82), L-Pro (1.15), D-Iva (0.5, corrected here based on GC–SIM-MS), L-Valol (1.15). Non-stoichiometry of some AAs are the result of AA exchange. Quantification of Aib and Iva suffered from low derivatization yields using the conventional ninhydrin method [31,32]. GC–SIM-MS of the TT A-40 mixture established that D-Iva and Leu were present, but not its isomers Val and Ile, respectively. Since it is not possible to distinguish between amounts of Glu and Gln originally present in peptides after total hydrolysis, the mass difference of 1 Da between Gln (*m*/*z* 128) and Glu (*m*/*z* 129), respectively, was used to determine AA at positions 6 and 17 of peptides. Thus the positions of $Gln^6$ and $Glu^{17}$ residues, as determined previously by GC–MS [5,11], were confirmed.

Positive-ion MS–MS of $(M+H)^+$ precursor ions generated the b and y series of product ions. Notation of the b series, representing acylium ions, is according to Roepstorff and Fohlmann [33], modified by Biemann [34]. The resulting y series, however, are singly protonated internal fragments de-

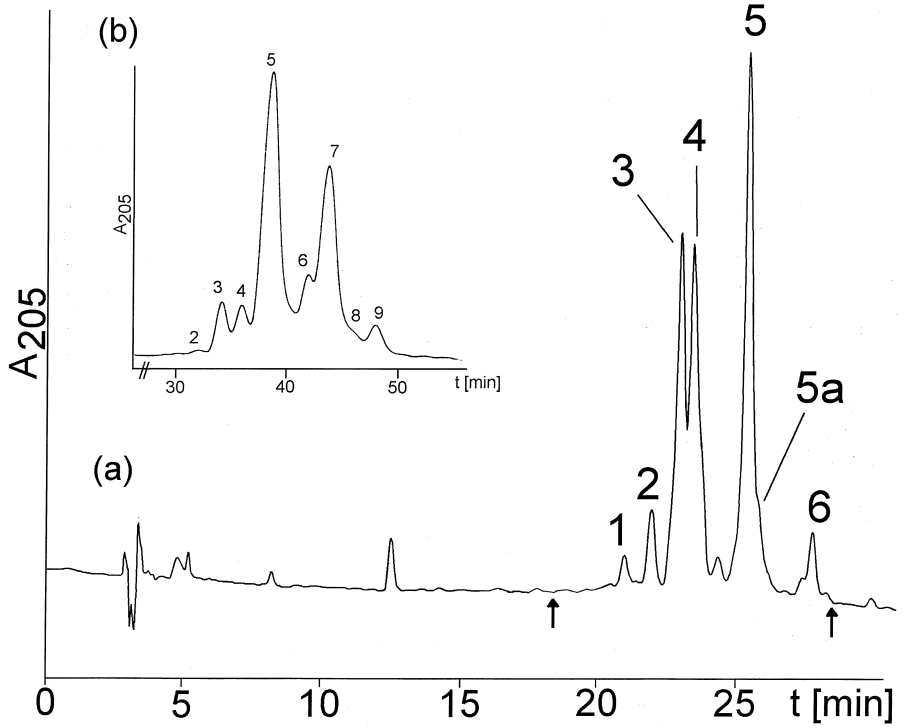


Fig. 1. (a) HPLC of trichotoxin A-40 peptides on Superspher (for chromatographic conditions see Experimental); arrows indicate elution time and sum of peak areas taken as 100%; (b) original elution profile of TT A-40 on a Nucleosil column shown for comparison (mirror image of Fig. 3C in Ref. [13]). Note that numbering of peaks is different for peptides eluting from (a) Superspher (used in this study) and (b) Nucleosil.

rived from Pro and, therefore, are marked $y'_P$ (prime refers to single protonation, P to positive-ion mode). MS–MS of $(M)^-$ in negative-ion mode also formed the y series, but product ions were not protonated and, therefore, designated $y_N$ (N refers to negative-ion mode).

The pseudomolecular ions $(M+H)^+$ and $(M+Na)^+$ as well as a series of characteristic fragment ions of the $b_4$–$b_{17}$ acylium series were obtained using on-line HPLC–ESI-MS in the positive mode, and $MS^n$ of $(M+H)^+$ of each compound. Adducts of molecular ions and diagnostic mass fragments of TT A-40 peptides are presented in Table 1.

The C-terminal sequences of the peptides were determined by MS–MS of the internal $y'_{6P}$ fragments. These characteristic fragment-ions were formed as a result of the particular lability of Aib–Pro bonds [3–6,35]. For the C-terminal internal peptides $y'_{6P}$ the lowest masses detectable by MS–MS were at $m/z$ 211. This is in agreement with positions $Pro^{13}$–$Leu^{14}$. The sequence of this dipeptide, after methanolysis, was also determined as TFA–Pro–Leu–OMe by GC–MS and comparison with synthetic Pro–Leu [TFA–Pro–Leu–OMe; $m/z$ (%) 166 (100), 86 (23), 279 (12), 194 (7)].

For ESI-MS in the negative ionization mode aqueous ammonia was added to the eluent before entering the MS interface in order to increase ionization of peptides. The adducts of $(M+TFA)^-$ (TFA from eluent) were most abundant under conditions of negative ionization. Adjusting the octapole region ion source collision induced energy at 35% (corresponding to +35 V d.c. of octapole offset voltage) resulted in displacement of TFA and formation of increasing amounts of $(M)^-$ ions. MS–MS of $(M)^-$ generated the $y_{12N}$–$y_{17N}$ fragment-ion series, as well as the deacetylated molecular ion $(M-Ac)^-$ and established the sequences of the acetylated N-termini.

Applying these two ionization modes, together

with on-line HPLC–ESI-MS, made possible the assignment of AA positions $Aib^{1}$–$Gln^{6}$ (negative ionization mode) and positions $Aib^{5}$–$Glu^{17}$ (positive ionization mode) of three major and three minor peptides. For determination of C-terminal $Valol^{18}$ see below.

The nominal mass fragments and molecular ions of all peptides discussed in the following are compiled in Table 1 and the structures deduced therefrom are presented in Fig. 2.

As can be seen from the HPLC of TT A-40 (Fig. 1), peak 5 represents a major peptide of the TT A-40 mixture. Its structure was not determined in previous investigations. Therefore, on-line HPLC–ESI-MS and sequencing is demonstrated in detail with TT A-40/5 and the closely eluting minor component TT A-40/5a (Fig. 3a–d).

The positive ion ESI-MS of TT A-40/5 shows an intensive pseudomolecular ion $(M+H)^{+}$ at $m/z$ 1719.1 and $(M+2H)^{2+}$ at $m/z$ 860 of lower abundance (see Fig. 3a and insert upper left). In contrast, negative-ionization ESI-MS provided the molecular ion $(M)^{-}$ at $m/z$ 1718.2 (Fig. 3a, insert upper right). Note that spectra shown in inserts were recorded in ZoomScan, i.e., a high-resolution MS scan mode. Further, an intensive acylium ion of the $b_{12}$ series at $m/z$ 1092 and the internal fragment $y'_{6P}$ at $m/z$ 627 are obtained as a result of the particularly labile $Aib^{12}$–$Pro^{13}$ bond. Notably, this characteristic cleavage [6] was not observed in the negative ionization mode. Positive-ion MS–MS of $(M+H)^{+}$ generated the $b_7$–$b_{12}$ acylium-ion series, as well as $b_{16}$ and $b_{17}$, and established AA positions $Aib^{8}$ to $Aib^{12}$ and C-terminal $Glu^{17}$–$Valol^{18}$ of TT A-40/5 (Fig. 3b). Further, $MS^{3}$ of the $b_{12}$ fragment ion made possible determination of AA positions $Aib^{5}$–$Aib^{12}$ (Fig. 3c). Negative-ion MS–MS of $(M)^{-}$ at $m/z$ 1718 provided the $y_{12N}$–$y_{17N}$ series of fragment ions, thus establishing sequence positions $Aib^{1}$–$Gln^{6}$ of AA (Fig. 3d). Loss of $NH_3$ from $y_{13N}$ lead to a fragment ion at $m/z$ 1235 and also proved presence of Gln in position 6 of TT A-40/5. Negative-ion MS–MS furnished also the deacetylated molecular ion $(M-Ac)^{-}$ at $m/z$ 1675, thus ascertaining Ac–Aib as N-terminal AA in TT A-40/5. It is assumed that an azaradinolate anion is formed accompanied by release of the acetylium ion. Formation of azaradinon fragments has been reported for positive-ion MS of peptaibols trichocellins [36]. The $(M-H_2O)^{-}$ at $m/z$ 1700 is attributed to loss of water from the enolized Ac–Aib N-terminus (see proposed structures in Fig. 3d).

The sequence of the C-terminal prolylhexapeptide $Pro^{13}$–$Valol^{18}$ was deduced from the positive ESI-MS–MS of the $y'_{6P}$ fragment ion at $m/z$ 627. The N-terminal Pro of this fragment is singly protonated and thus neutral. The positive charge of the fragment results from protonation of the C-terminal Valol (see Fig. 3e). The C-terminal position of Valol was deduced from the difference of 103 Da from $y'_{6P}$ ($m/z$ 627) and $Pro^{13}$–$Glu^{17}$ ($m/z$ 524). A series of internal fragment ions at $m/z$ 395 ($Pro^{13}$–$Iva^{16}$), $m/z$ 296 ($Pro^{13}$–$Aib^{15}$), and $m/z$ 211 ($Pro^{13}$–$Leu^{14}$) were obtained (Fig. 3e). An overview is presented in Fig.

| TT A-40 | 1 | **2** | 3 | 4 | 5 | 6 | 7 | 8 | **9** | 10 | **11** | 12 | 13 | 14 | 15 | **16** | 17 | 18 | NMM | (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Ac | **Gly** | Aib | Leu | Aib | Gln | Aib | Aib | **Ala** | Ala | **Aib** | Aib | Pro | Leu | Aib | **Aib** | Glu | Valol | 1690 | 2.1 |
| 2 | Ac | **Gly** | Aib | Leu | Aib | Gln | Aib | Aib | **Aib** | Ala | **Ala** | Aib | Pro | Leu | Aib | **Aib** | Glu | Valol | 1690 | 4.3 |
| 3 * | Ac | **Gly** | Aib | Leu | Aib | Gln | Aib | Aib | **Ala** | Ala | **Aib** | Aib | Pro | Leu | Aib | **D-Iva** | Glu | Valol | 1704 | 22.9 |
| 4 * | Ac | **Gly** | Aib | Leu | Aib | Gln | Aib | Aib | **Aib** | Ala | **Aib** | Aib | Pro | Leu | Aib | **Aib** | Glu | Valol | 1704 | 24.3 |
| 5 | Ac | **Gly** | Aib | Leu | Aib | Gln | Aib | Aib | **Aib** | Ala | **Aib** | Aib | Pro | Leu | Aib | **D-Iva** | Glu | Valol | 1718 | 31.7 |
| 5a * | Ac | **Ala** | Aib | Leu | Aib | Gln | Aib | Aib | **Aib** | Ala | **Aib** | Aib | Pro | Leu | Aib | **Aib** | Glu | Valol | 1718 | 5.7 |

Fig. 2. Sequences of trichotoxins A-40/1–5a; exchanged AA positions in bold letters; NMM, nominal (i.e., monoisotopic) molecular mass; (%), relative amount of peptides in the natural microheterogeneous mixture; Valol and chiral amino acids are of the L-configuration with the exception of D(=*R*)-Iva; sequences denoted with an asterisk were reported previously, but not characterized by numbers [12].

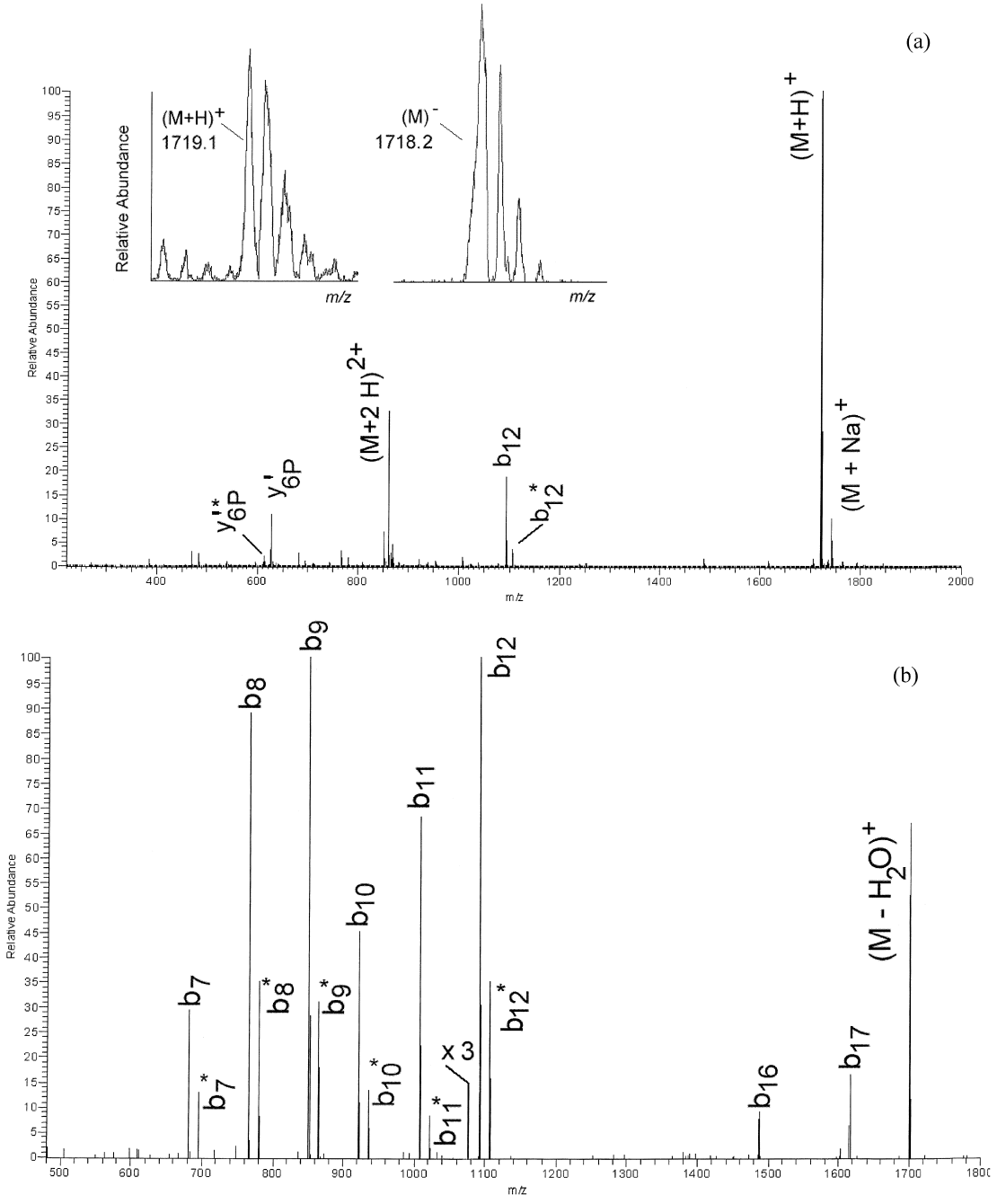


Fig. 3. On-line HPLC–ESI-MS of TT A-40/5; (a) positive-ion MS; insert upper left positive-ion ESI-MS of TT A40/5, insert upper right negative-ion ESI-MS of TT A40/5 (high resolution MS in inserts); (b) positive-ion ESI-MS–MS of $(M+H)^+$ ($m/z$ 1719); (c) positive-ion ESI-$MS^3$ of $b_{12}$ acylium ion ($m/z$ 1092); (d) negative-ion ESI-MS–MS of $(M)^-$ ($m/z$ 1718), inserts show proposed mechanisms for formation of $y_N$ series and release of the acetyl group via azaradinolate anions (left), and the fragment resulting from loss of water via β-elimination from the enolized Ac–Aib bond of the $(M)^-$ precursor ion (right); (e) positive-ion ESI-MS–MS and fragmentation scheme of the $y'_{6P}$ fragment ($m/z$ 627). For chromatography and mass spectrometry see Experimental. For corresponding nominal molecular masses see Table 1. (*Continued on next page*.)

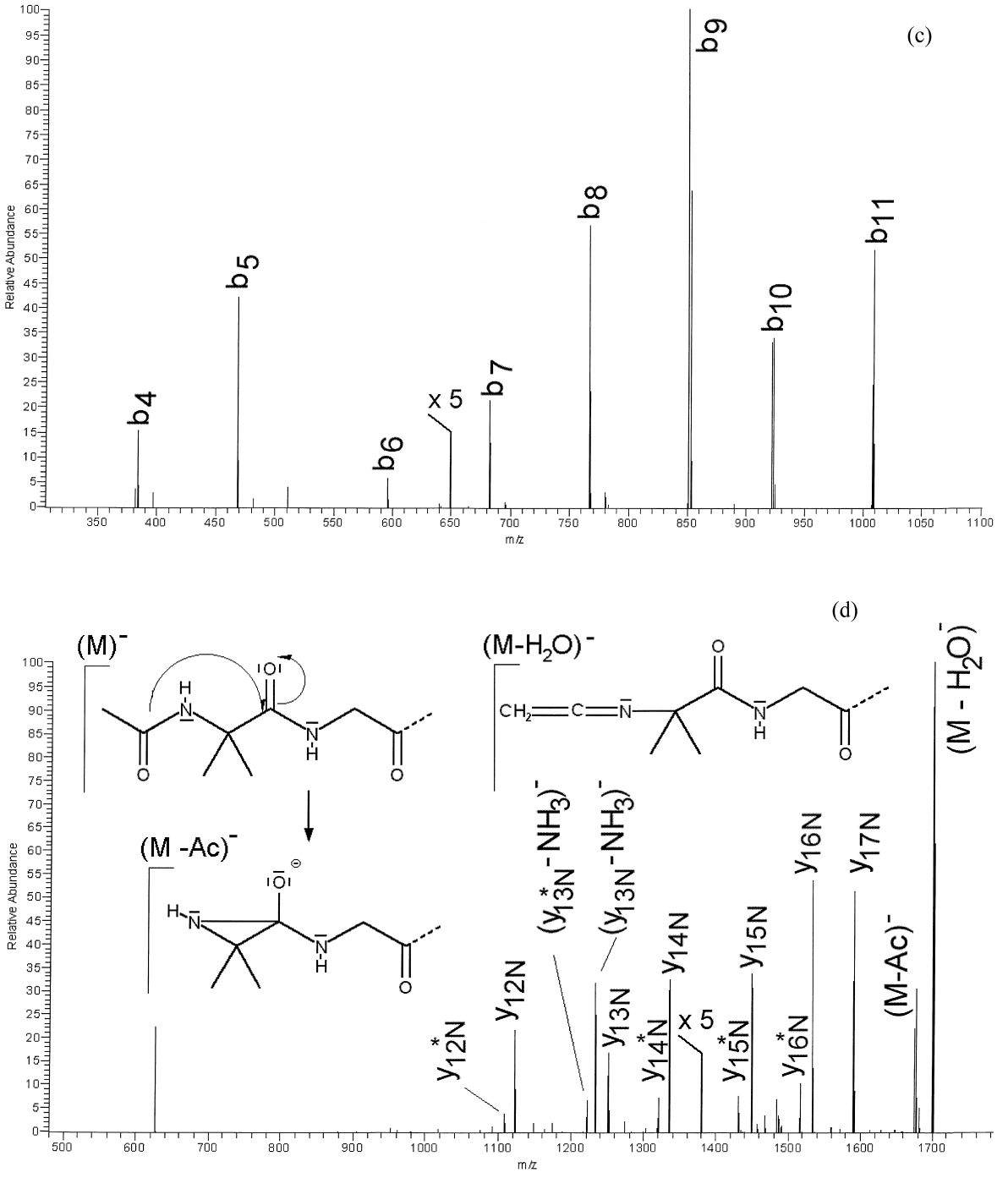


Fig. 3. (*continued*)

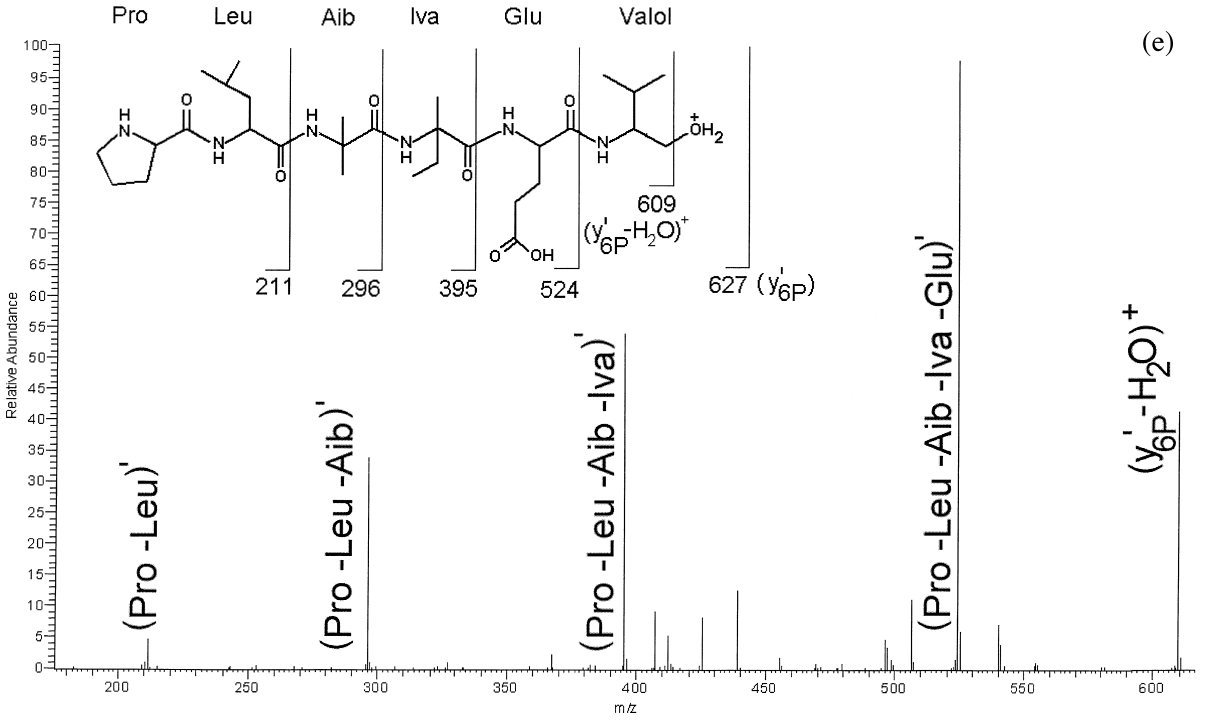


Fig. 3. (*continued*)

4 showing series of diagnostic ions and internal fragments in the positive and negative ionization mode used for the sequence determination of TT A-40/5. Analogously, the sequences of the other TT A-40 peptides presented in Fig. 2 were determined.

Inspection of the HPLC of the TT A-40 mixture (see Fig. 1a) shows a shoulder of peak 5 which is designated peak 5a. The on-line ESI-MS–MS taken from this peak shows a series of satellite acylium fragment-ions of low abundance, distinguished by an asterisk, and designated $b_{12}^{*}$ and $y'^{*}_{6P}$ in Fig. 3a, and $b_{7}^{*}$–$b_{12}^{*}$ in Fig. 3b. Their mass differences to the abundant $b_7$–$b_{12}$ series count for 14 u, thus suggesting AA exchange and presence of a peptide eluting almost together with the major peptide. Indeed, the exchange of $Gly^2$ of the major peptide by an Ala residue in the minor component was deduced from the negative ion ESI-MS–MS of the molecular ion $(M)^-$ resulting in formation of $y_{16N}$ at $m/z$ 1533 for peak 5, and $y_{16N}$ at $m/z$ 1519 for peak 5a.

The sequence of the corresponding C-terminal prolylpeptide of peak 5a was deduced from the $y'_{6P}$ fragment at $m/z$ 613.

Amounts of ca. 10% of this minor peptide (here designated TT A-40/5a) were estimated previously [5]. In this study relative amount of 5.7% were calculated from the intensity of the minor $b_{12}^{*}$ fragment ion relative to $b_{12}$ of TT A40/5 (see Fig. 3b).

The sequences of two minor peptides (peak 1 and 2 of HPLC, Fig. 1a) could be deduced directly from the $b_5$–$b_{17}$ series of acylium ions in the positive ion mode, and from the $y_{12N}$–$y_{17N}$ series in the negative ion mode, together with the internal fragments of the C-terminal prolylpeptide at $m/z$ 613 as demonstrated above. These peptides were designated TT A-40/1 and TT A-40/2, respectively, and differ by an exchange of $Ala^9$/$Aib^9$. These minor sequences, counting for 2% and 4% of the TT A-40 peptides, were not yet known.

The sequence of peak 3 (designated TT A-40/3) could be deduced analogously from the b, $y'_P$ and

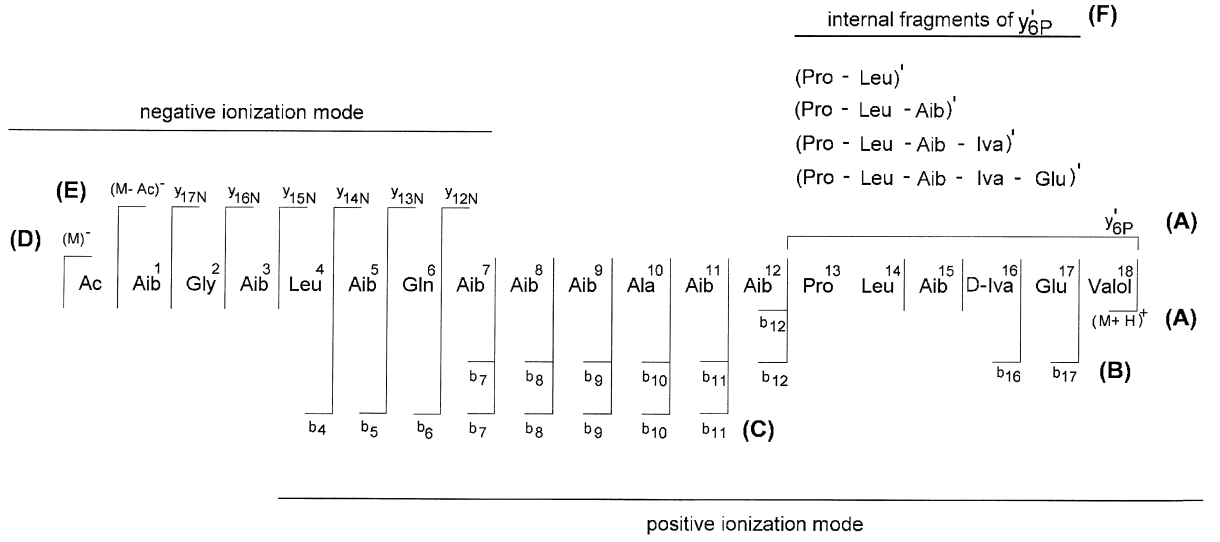


Fig. 4. Overview of sequencing, exemplified with TT A-40/5, using ESI-MS$^n$ in positive- and negative-ionization mode. Mass fragments were generated by (A) positive-ion ESI-MS; (B) MS–MS of $(M+H)^+$; (C) $MS^3$ of $b_{12}$; (D) negative-ion ESI-MS; (E) MS–MS of $(M)^-$; (F) MS–MS of internal fragment $y'_{6P}$. Dipeptide Pro$^{13}$–Leu$^{14}$ was also identified via GC–MS of a TT A-40 methanolysate [9].

$y_N$-series of fragment ions (see Table 1). The sequence of this peptide was reported previously [5] and thus is confirmed by the hyphenated technique described here.

The sequence of peak 4 (designated TT A-40/4) was also deduced from the fragment and molecular ions which are presented in Table 1. This peptide is distinguished from TT A-40/3 by an exchange of Aib$^{16}$/D-Iva$^{16}$. This also confirms a sequence reported previously [5].

Peak 6 of the chromatogram presented in Fig. 1a shows two closely eluting compounds. ESI-MS of the highest peak in this group revealed that actually two peptides at $m/z$ 1719 and 1733 were eluting together. On-line sequencing of this group of peptides was ambiguous according to low abundances and interfering fragment ions. On-line sequencing of these minor peptides (altogether counting for approx. 3.8%) might be possible by use of a stationary phase of different selectivity as shown recently for trichovirins [10].

## 4. Conclusions

On-line HPLC–ESI-MS$^n$ in the positive- and negative-ionization mode enables the fast and reliable sequence determination of peptaibol antibiotics. Ionization and fragmentation of peptaibols under these conditions show some characteristics in comparison to common peptides. From a structural point of view such peculiarities are also observed for the crystal state structures of Aib-peptides and peptaibols [37,38]. In some cases peptides which are not, or incomplete separated, can be sequenced. If isomeric AA Val/Iva and Leu/Ile occur in peptides, complementary methods such as methanolysis of peptides and assignment of characteristic dipeptides released have to be applied [10,35]. HPLC analysis of microheterogeneous mixtures of neutral and acidic peptaibols due to Gln/Glu exchange might require the use of an acidic eluent. Otherwise Glu containing peptides might be not detected.

Further, use of a stationary phase exerting different selectivity towards peptides in comparison to common octadecylsilyl columns might be of advantage for on-line sequencing of peptaibols. Such a phase, for example, is fluorocarbon-coated silica, as recently demonstrated with paracelsins [39] and the sequencing of trichovirins [10] and antiamoebins [40].

As for other peptaibols, the HPLC elution profile

(‘‘fingerprint’’) together with relative amounts of peptaibols in heterogeneous mixtures should be reported. This is required in order to judge the relevance of structural variations of individual peptides in particular when bioactivities are discussed. For example, compound Nos. 3, 4 and 5 in the mixture of TT A-40 represent 84% of the peptides.

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