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ISOLATION AND STRUCTURAL CHARACTERIZATION OF POLYPEPTIDE ANTIBIOTICS OF THE PEPTAIBOL CLASS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FIELD DESORPTION AND FAST ATOM BOMBARDMENT MASS SPECTROMETRY

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SUMMARY

A number of polypeptide antibiotics of the peptaibol class, *i.e.*, trichotoxin, alamethicin, suzukacillin, hypelcin and paracelsin, have been separated into components and isolated by high-performance liquid chromatography on spherical, porous octadecylsilyl bonded phases. All peptaibols were found to reveal a strong microheterogeneity due to single or multiple amino acid exchange. Most of the closely related and partially isobaric sequence analogue could be resolved using mixed alcohol–water eluents. As demonstrated by the structure analysis of the paracelsins and the main component of trichotoxin A-50, high-performance liquid chromatography with field desorption and fast atom bombardment mass spectrometry have been found to be a powerful and universally applicable method for the direct and unequivocal sequence determination of components of this class of polypeptides.

INTRODUCTION

Peptaibols[1,2] are linear, amphiphilic polypeptide antibiotics of fungal origin with molecular masses up to approximately 2000 daltons. In addition to proteinogenic amino acids, a common feature of all peptaibols is that they contain a high proportion (up to 60%) of the unusual α-aminoisobutyric acid (2-methylalanine, Aib) and occasionally isovaline (2-ethylalanine, Iva). The N-termini of these peptides are acetylated and the C-termini linked in a peptide bond with an amino alcohol, *e.g.*, valinol (Valol or Vol), leucinol (Leuol) or phenylalaninol (Pheol or Phol); in the latter case the term peptaibophol has been used[3].

These antibiotics are mainly produced by moulds of the genera *Trichoderma* —or its perfect state *Hypocrea*— and *Emericellopsis*[4]. Because of the non-ribosomal mechanism of enzymatic biosynthesis[5–7], most peptides exhibit an exceptional microheterogeneity, *i.e.*, exchange of amino acids, resulting in a mixture of closely related sequence analogues.

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In addition to these complications, the elucidation of the chemical structures of these peptides by classical methods revealed considerable problems due to their blocked C- and N-termini[8,9], resistance to proteolytic degradation[10] and difficulties in the detection and quantification of the uncommon α,α-dialkyl α-amino acids and amino alcohols[9].

Thus, prerequisites for an unequivocal characterization and sequence determination of peptaibol antibiotics are the isolation of single components from the heterogeneous mixture of the respective peptaibol, and proof of the true homogeneity of the components. Depending on the presence of carboxy group-containing amino acids (see text), partial separations of peptaibol sequence analogues have previously been obtained by thin-layer chromatography (TLC)[4,11,12]. During our investigations on methods for the isolation and structural characterization of peptaibol antibiotics, reversed-phase high-performance liquid chromatography (HPLC) was found to be superior in separating peptaibol components compared to other procedures[1,12–14]. Field desorption (FD) and, more recently, fast atom bombardment (FAB) mass spectrometry (MS) have been successfully applied to the direct structure analysis of synthetic and biological polypeptides[15–18], and of various peptide metabolite conjugates[19,20]. Recently, FD-MS and FAB-MS have been applied directly to prove the structural identity of the peptaibophols samarosporin, stilbellin and emerimicin[12]. Further work, in particular the recent evaluation of highly efficient solvent (matrix) systems for the FAB-MS analysis of hydrophobic peptaibols[16,21], prompted our interest in a systematic and comparative study of several new as well as previously isolated polypeptides of this class. It is shown in this paper that HPLC with FD-MS and both positive and negative ion FAB-MS provides a highly sensitive and universal method for the direct resolution and structural characterization of peptaibol components. Only a single, simple additional acidolytic step for the preferential release of C-terminal prolylpeptides[8,9,22,23] is required to obtain complete sequence determinations[21].

MATERIALS AND METHODS

Instrumentation

MS was performed with a Finnigan MAT 312 double-focusing spectrometer/SS 188 data system equipped with a combined FD–FAB–EI (electron impact) ion source and a self-constructed direct insertion probe for the FD emitter and FAB target described previously[16]. FD mass spectra were obtained with high-temperature activated emitters employing indene (needle length 30 μm) by loading 2 μl of a solution of *ca.* 30 μg peptide in 20 μl methanol spiked with 3 μl tetraethylene glycol (TEG)[21]. The sample preparation for FAB-MS consisted of spotting 1 μl of a 1–2-μg/μl solution in methanol on a stainless-steel FAB target (surface area *ca.* 5 mm^2), followed by careful addition and admixture of 1 μl TEG. FAB mass spectra were obtained with an Ion-Tech (Teddington, U.K.) saddle-field primary atom gun using Xe^0 (*ca.* 7 keV) as ionizing beam. A conversion dynode system between the exit slit and secondary electron multiplier and an automatic switching unit (AMD, Beckeln, F.R.G.) were used for the instantaneous acquisition of positive and negative ion FAB mass spectra[18]. Other instrumental conditions and procedures for mass calibrations and measurements have been described in detail elsewhere[16,18,21].

HPLC was carried out on a Perkin-Elmer liquid chromatograph Series 3B with two dual-head reciprocating pumps, a recorder 561, integrator M3B and a variable-wavelength spectrophotometric detector LC-75, operated at 200 or 205 nm for analytical and 225 or 230 nm for preparative separations. Samples were introduced by a Model 7120 loop injection valve (Rheodyne, Cotati, CA, U.S.A.) or an autosampler ISS-100 (Perkin-Elmer, Überlingen, F.R.G.). In preparative isolations, fractions were collected either manually or by a Model 201 fraction collector (Gilson, Villiers le Bel, France). HPLC columns were 250 mm in length, packed with Nucleosil RP-18, 5 μm (Macherey, Nagel & Co., Düren, F.R.G.), and connected with pre-columns (10–30 mm) packed with the above reversed-phase material. The columns had the following internal diameters (I.D.s): A, 4.6 mm (Perkin-Elmer)[1]; B, 8.0 mm (Hyperchrome, Bischoff Analysentechnik, Leonberg, F.R.G.); C, 16 mm (Knauer, Bad Homburg, F.R.G.).

Organic solvents of chromatographically pure grades and doubly distilled water were used. All solvents were degassed by sonification. All eluents employed for preparative isolations of paracelsin and trichotoxin components were redistilled.

Analytical and preparative separation of polypeptides

Detailed conditions are given in the figure captions. For the preparative separation and isolation of peptaibol components, suitable fractions were collected according to their UV absorption and combined in round-bottom flasks. 1-Butanol was added to prevent foaming during subsequent evaporation to dryness *in vacuo*. The residue was dissolved in a few ml of methanol, filtered through a 0.5-μm PTFE filter (Millex SR; Millipore, Bedford, MA, U.S.A.), evaporated to dryness and subjected again (5 mg peptide in 100 μl methanol) to HPLC as described (for conditions see Fig. 6).

Origin of samples

Trichotoxin was isolated from *Trichoderma viride* NRRL 5242[22,24,25] and separated into components A-40 and A-50 by counter-current distribution[22,23]. Alamethicins F-30 and F-50 were isolated from *Trichoderma viride* NRRL 3199[11,13,26], suzukacillin from *Trichoderma viride* 63Cl[27–29] and paracelsin from *Trichoderma reesei* QM 9414[1,21,30]. Hypelcin from *Hypocrea peltata*[31] was a generous gift from Professor T. Fujita (Tokushima, Japan).

RESULTS AND DISCUSSION

HPLC separation of heterogenic peptaibol antibiotics

HPLC separations of the peptaibol antibiotics alamethicin F-30 and F-50, suzukacillin, paracelsin and hypelcin together with their primary structures and sequence analogues as determined in previous studies and in this work (Fig. 1) are compared in Fig. 2. Evidently, all peptides isolated from strains of *Trichoderma* or its teleomorph *Hypocrea* exhibit a pronounced microheterogeneity leading to a complex pattern of sequence analogues even after extensive purification by silica gel and Sephadex LH-20 column chromatography, followed by counter-current distribution; surprisingly, this microheterogeneity was even observed for compounds isolated in crystalline form[1,4,27]. Thus, alamethicin has been shown to contain neutral (F-50)

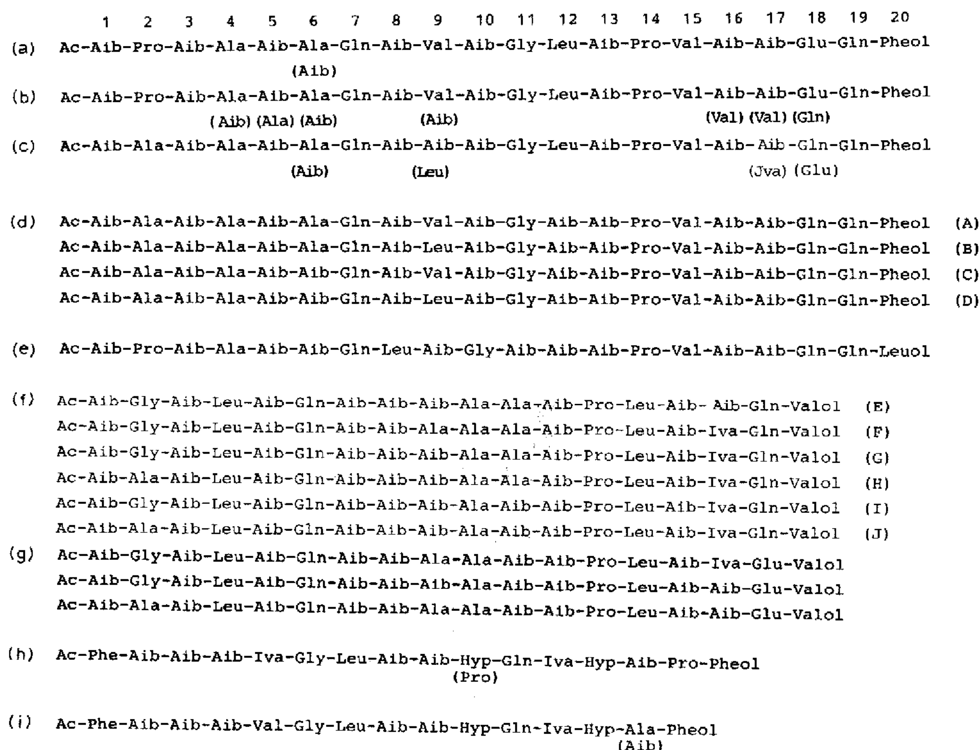

Fig. 1. Sequences of (a) alamethicin I (II)[32], (b) alamethicin F-30[33], (c) suzukacillin[29], (d) paracelsin A, B, C and D, (e) hypelcin A[31], (f) trichotoxin A-50[21] (components E–J, *cf.*, Fig. 4), (g) trichotoxin A-40[9,22] (sequences determined from fraction 3, *cf.*, Fig. 3), (h) antiamoebin[34–36] and (i) emerimicin IV (III)[3] [= samarosporin I (II), = stilbellin I (II)][12].

and acidic (F-30) components due to exchange of Gln and Glu residues[11,13]. Furthermore, alamethicin F-30 contains a major (I) and minor (II) component distinguished by an exchange of Ala and Aib[32] (*cf.*, Fig. 1a). When investigated by HPLC, alamethicin F-30 was separated into five compounds (Fig. 2A), while alamethicin F-50 yielded seven components (Fig. 2B). This microheterogeneity has also been observed in previous HPLC investigations[14], and was also found by gas–liquid chromatographic (GLC)–MS studies of partial alamethicin sequences[33].

Suzukacillin, although isolated in crystalline form[27], exhibits the presence of at least ten components when investigated by HPLC (Fig. 2C). By comparison of the sequences shown in Fig. 1c and d, this peptide most likely contains components identical with, or only slightly different from, those determined for paracelsin (see below). Crystalline paracelsin was separated into three main components by HPLC[1]. In contrast to methanol–water (86:14) as eluent system, which yielded separation of only two compounds (Fig. 2D), the use of a methanol–2-propanol–water eluent enabled resolution of components A and B, while two further analogues (designated herein as component C*) remained unseparated (*cf.*, Fig. 6). However, a microheterogeneity of the latter species was suspected from amino acid analysis, and was established by mass spectral analysis as shown below.

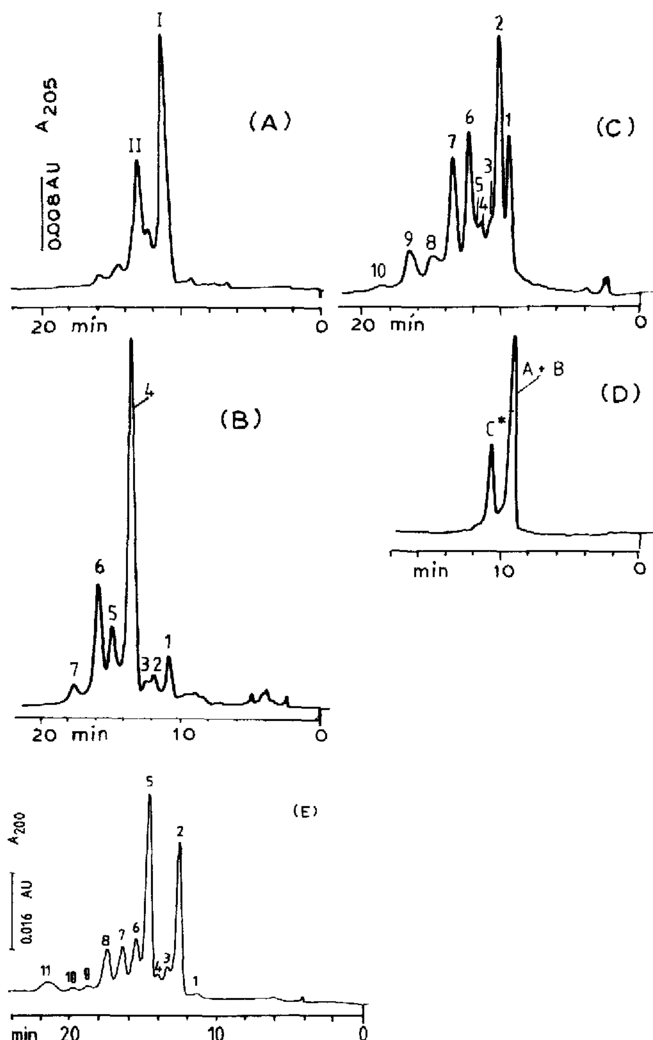

Fig. 2. HPLC of (A) alamethicin (sample: The Upjohn Company, Kalamazoo, MI, U.S.A.), (B) alamethicin F-50, (C) suzukacillin, (D) paracelsin and (E) hypelcin A. Conditions: A–D: column B; eluent, methanol–water (86:14); flow-rate, 2.5 ml min^{-1}; pressure, 13.5 MPa; injection (μg peptide per μl methanol), 150 μg per 3 μl (A and B), 250 μg per 3 μl (C) and 50 μg per 2 μl (D); E: eluent, methanol–water (85:15); flow-rate, 2 ml min^{-1}; pressure, 9 MPa; injection, 280 μg per 5 μl. Common conditions for Figs. 2–4 and 6: isocratic operation; temperature, ambient; sensitivity recorder, 10 mV; chart speed, 0.5 cm min^{-1}.

Consistent with the above results, hypelcin revealed the presence of eleven components by HPLC analysis under the conditions described (Fig. 2E). At least nine minor components were found in addition to the two major compounds 2 and 5, so that the sequence as shown in Fig. 1e can only represent one, uncertain of several homologous sequences. It should be pointed out that several peptaibols isolated from strains of *Emericellopsis* have a considerably lower heterogeneity[3,12,34–36].

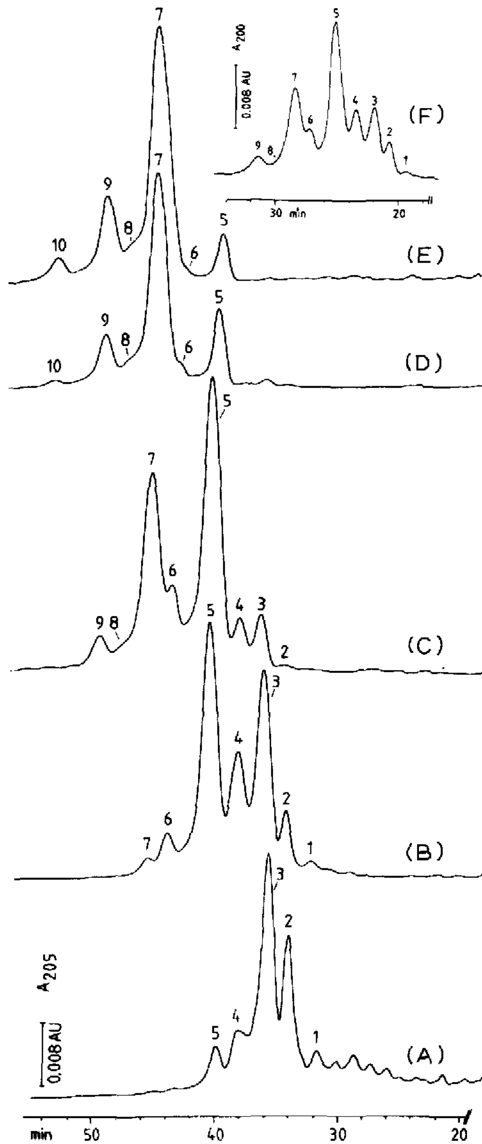

Fig. 3. HPLC of trichotoxin A-40 fractions I–V, obtained by counter-current distribution of 3.6 g of a mixture of trichotoxin components A-40 and A-50, and further subdivision of component A-40 after the separation process. Fractions: (A) I (160 mg); (B) II (310 mg); (C) III (570 mg, main fraction[23], *cf.*, Fig. 1g for sequences); (D) IV (220 mg) and (E) V (40 mg). Injection of 175-μg portions of peptides in 7 μl methanol; conditions for A–E, *cf.*, Fig. 2A–D. For comparison, (F) shows chromatogram of trichotoxin A-40, uniform in TLC and separated from component A-50 by silica gel column chromatography (eluent, chloroform–methanol–17%, aqueous ammonia, 70:35:10). Conditions for HPLC (F): column C; eluent, methanol–water (85:15); flow-rate, 7 ml min^{-1}; pressure, 18 MPa; injection, 250 μg per 20 μl. Relative peak areas (%) of components of F: 1 (0.4); 2 (4.6); 3 (13.0); 4 (12.1); 5 (34.8); 6 (7.3); 7 + 8 (22.4); 9 (5.4).

Thus, the complete structural identity of samarosporin I (II), stilbellin I (II) and emerimicin IV (III) has recently been established by HPLC, FD-MS and FAB-MS[12]. An exception in this class of compounds is zervamicin[37] which was isolated from a strain of *Emericellopsis salmosynnemata* and found have at least eleven components.

The typical microheterogeneity of peptaibols from strains of *Trichoderma* is exemplified by the trichotoxins. Thus, counter-current distribution (CCD) of trichotoxin into its acidic (Glu-containing) and neutral (Gln-containing) components A-40 and A-50 yielded peptides that appeared almost uniform when investigated by TLC[4,12]. However, when fractions (designated I–V, *cf.*, Fig. 3A–E) of trichotoxin A-40 collected from CCD separation were analyzed by HPLC, an enrichment of the more lipophilic components within the upper phase and of the less hydrophobic components within the lower phase of the solvent system was detected (*cf.*, Fig. 3F), and no complete separation of sequence analogues was attained (Fig. 3). Sequential investigations of the major fraction III led to the identification of three trichotoxin A-40 analogues[9,22,23]. FD-MS (data not shown) yielded the most abundant molecular ions, MH^+, at m/z 1705, 1719 and 1733 which are consistent with the molecular masses of the sequences shown in Fig. 1g. The HPLC analyses, however, leave no doubt about the presence of several further sequence analogues. Trichotoxin A-50, obtained by combination of all CCD fractions without further fractionation, could

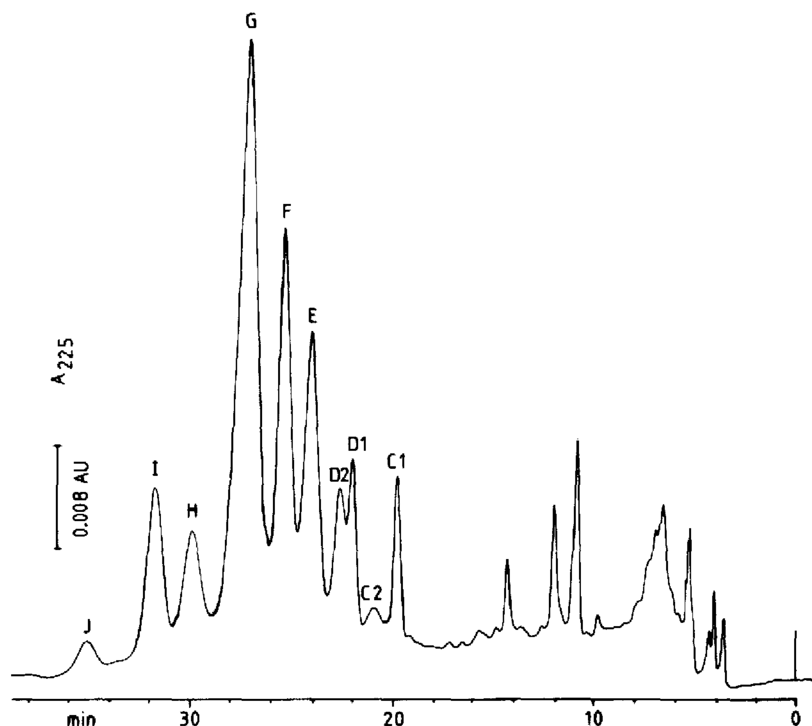

Fig. 4. HPLC of trichotoxin A-50 (combined fractions from a counter-current distribution[23], *cf.*, legend to Fig. 3). Conditions: column C; eluent, methanol–water (85:15); flow-rate, 7 ml min^{-1}; pressure, 17.5 MPa; injection, 10 mg in 200 μl methanol. Relative peak areas (%) of components: C1 (3.7); C2 (1.9); D1 (4.8); D2 (5.2); E (13.7); F (17.5); G (36.2); H (7.4); I (8.3); J (1.3).

be separated by HPLC into ten components C1–J (Fig. 4). These sequence analogues were isolated as single, homogeneous species (see below) and subjected to individual molecular mass and sequence determination by FD-MS and FAB-MS[21].

Structural characterization of the isolated main component of trichotoxin A-50 by FAB-MS and FD-MS

Although the results described above demonstrate analytical HPLC to be a sensitive tool for the investigation of peptaibols, further studies, *e.g.*, of biological activities[38] and spectroscopic properties such as NMR[30] and circular dichroism, required the isolation of larger amounts of highly purified components. The preparative isolation of trichotoxin A-50 components was achieved on a 16 mm I.D. column (C, see Materials and Methods) with methanol–water (85:15) as eluent, by employing the procedures described for the isolation of paracelsin (see below). The HPLC separation of trichotoxin A-50 and isolation of individual components enabled their direct MS structure analysis which, together with quantitative amino acid analyses, gave unequivocal molecular mass and sequence data[21].

Thus, FD mass spectra of the isolated trichotoxin A-50 components C1–J (*cf.*, Fig. 4) yielded in each case a single, most abundant protonated molecular ion which directly confirmed the single or multiple exchange of amino acids to produce various homologous and partly isobaric sequence analogues as indicated from amino acid analysis. Molecular mass determinations were further ascertained by the mass shift in the FD spectra of 22 a.m.u. from each MH^+ to the corresponding, sodiated MNa^+ upon treatment of the peptide with sodium salt[16] and, particularly, by FAB-MS investigation. FAB-MS has already been shown in previous studies to be a powerful technique for the direct structural characterization of peptaibol antibiotics[12,37]. Some difficulties in applying the FAB-MS method to the present, extremely hydrophobic peptaibols were initially encountered due to their poor solubility in glycerol as the liquid matrix that has most often been used for the study of polar peptides[16]. However, further systematic studies revealed oligoethylene glycols, in particular TEG, as highly efficient matrix systems which led to a dramatic enhancement of signal-to-noise ratios in FAB spectra of peptaibols, relative to glycerol; FAB-MS analyses have been routinely obtained with low nanomol to picomol amounts of material dissolved in TEG[21]. The structural information thus obtained is illustrated in Fig. 5 by the positive ion and negative ion FAB mass spectrum of the isolated trichotoxin A-50 main component "G" (*cf.*, Fig. 4). The nominal (monoisotopic) molecular mass of 1702 is immediately established by the MH^+ and MNa^+ at m/z 1703 and 1725 in the cation spectrum, and the most abundant $(M - H)^-$ at m/z 1701 in the negative ion spectrum (the MNa^+ produced by traces of sodium salt present).

In contrast to the negative-ion spectrum, which showed little fragmentation, a series of abundant fragments by peptide bond cleavages (see scheme in Fig. 5) in the positive ion spectrum directly and readily yields sequence data. Notable is that all major, sequence-specific fragments are produced from the N-terminus having acylium-ion end groups (designated as "A" series of sequence ions)[12,16], as opposed to fragments with protonated, amino end groups from the C-terminus which have been typically observed in FAB spectra of polar, hydrophilic peptides ("Z" series of sequence ions)[16]. The series of sequence-specific, N-terminal fragments continues up to, and ends precisely at, the preferential acidolytic cleavage site of the peptaibols,

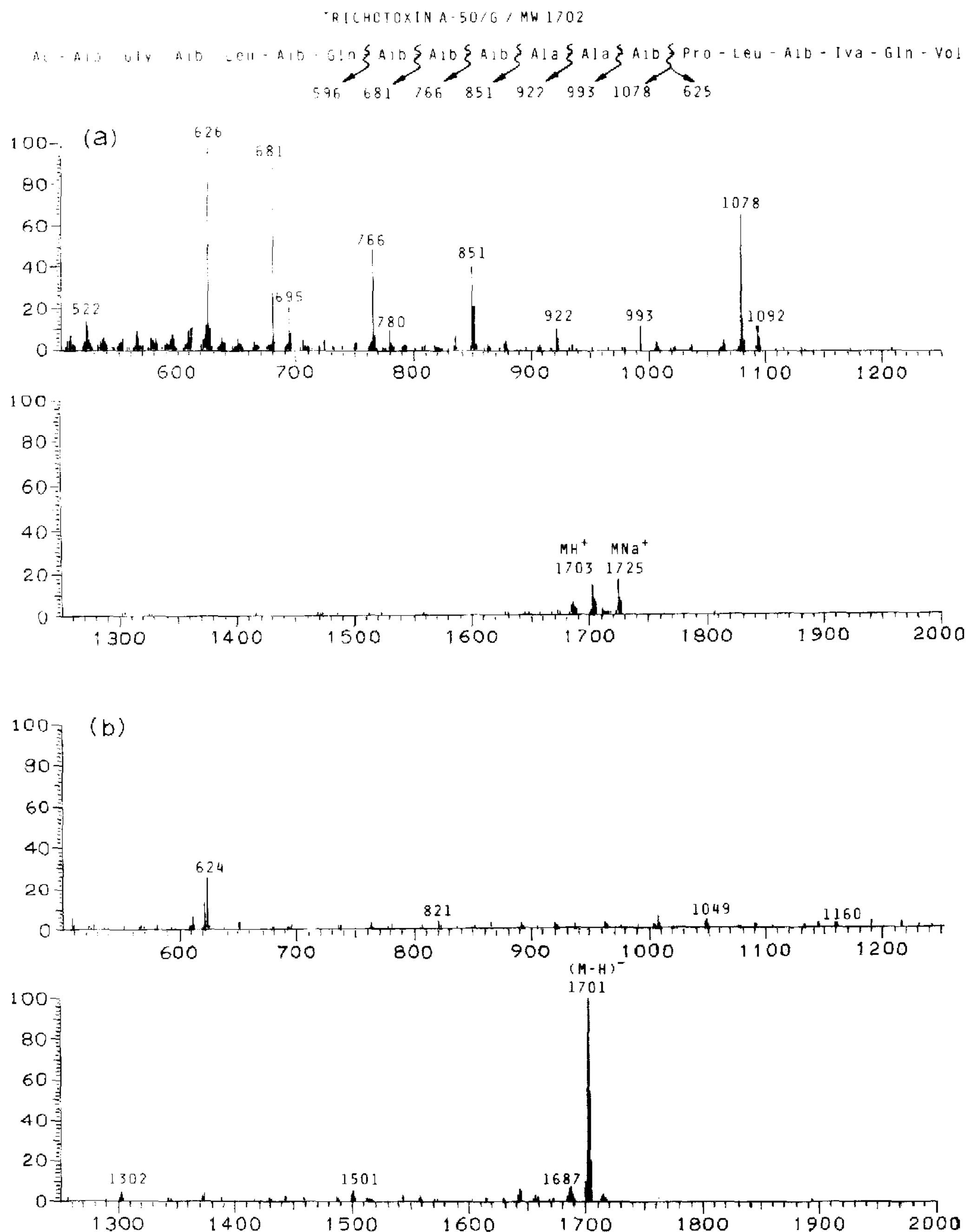

Fig. 5. (a) Positive ion and (b) negative ion FAB mass spectra of the main component of trichotoxin A-50 (G, *cf.*, Fig. 4) isolated by HPLC. The mass range, m/z 500–2000 is shown; sequence-specific fragments by cleavage of peptide bonds are marked with arrows.

i.e., the Pro (or Hyp) residues[12,21,22]. In the spectrum of the trichotoxin A-50 component (Fig. 5a), the only significant "Z" fragment is observed at m/z 626 (protonated Pro-Leu-Aib-Iva-Gln-Valol).

Preparative isolation of paracelsin components by HPLC and structural characterization by FAB-MS and FD-MS

In the case of paracelsin, preparative HPLC on a 16 mm I.D. column was successfully used to isolate mg amounts of components in a single chromatogram, and provided separation of components A, B and C* from up to 10 mg paracelsin originally applied. The best separation was achieved with a mixture of methanol–2-propanol–water as eluent. However, the peaks of components A and B, and to some extent C*, had shoulders after a single chromatogram. Therefore, this procedure was repeated by applying 5-mg aliquots of each component to the column. In this way, components A, B and C* could be isolated with high purity, as demonstrated in Fig. 6. Crystalline paracelsin (400 mg) yielded components A (63 mg), B (99 mg) and C* (70 mg of a mixture of components C and D, not separated by HPLC).

When investigated by amino acid analysis, components A and B yielded stoichiometric ratios of all constituents indicating their homogeneity (paracelsin A = Gln_3 Pro_1 Gly_1 Ala_3 Aib_9 Val_2 $Pheol_1$; paracelsin B = Gln_3 Pro_1 Gly_1 Ala_3 Aib_9

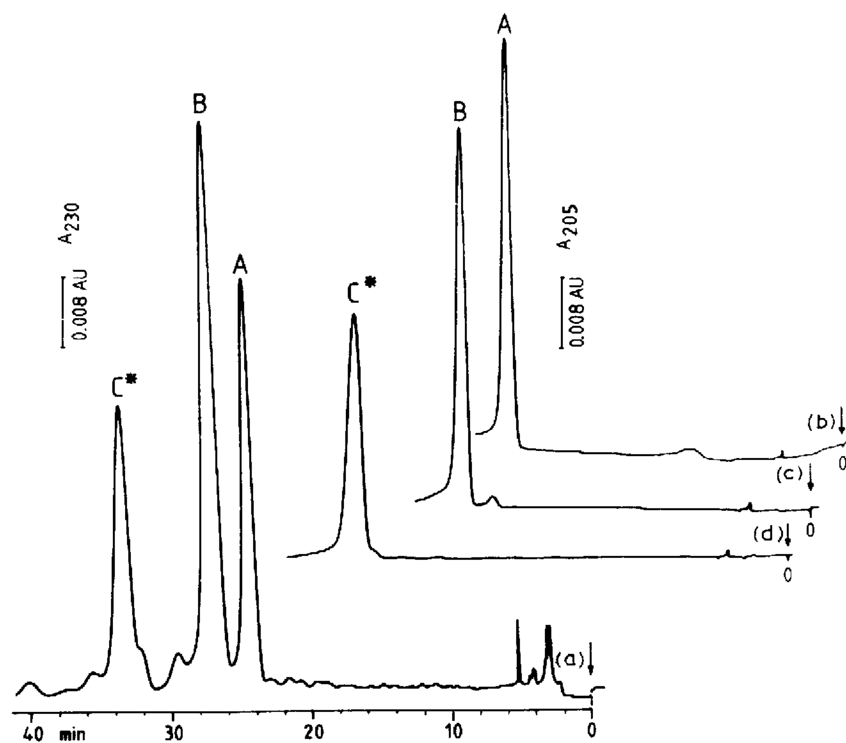

Fig. 6. Preparative separation of paracelsin and analytical investigation after two-fold separation of isolated components A (b), B (c) and C* (d). Conditions: column C; eluent, methanol–2-propanol–water (45:20:35); flow-rate, 8 ml min^{-1}; pressure, 20 MPa; injection, (a) 10 mg peptide in 250 μl methanol, (b–d) 100 μg peptide in 5 μl methanol.

Val_1 Leu_1 $Pheol_1$). By contrast, paracelsin C* revealed non-stoichiometric ratios for Val and Leu (Gln_3 Pro_1 Gly_1 Ala_3 Aib_{10} $Val_{1.5}$ $Leu_{0.5}$ $Pheol_1$), indicating that component C* consists of about equal amounts of two components, unresolved by HPLC and differing only by an exchange of a Val with a Leu residue. This heterogeneity was established by FD-MS and FAB-MS analysis (Fig. 7b and c) each of which revealed two major MH^+ at m/z 1922 and 1936, consistent with the calculated nominal masses of the components now designated C and D (paracelsin C = Gln_3 Pro_1 Gly_1 Ala_2 Aib_{10} Val_2 $Pheol_1$; paracelsin D = Gln_3 Pro_1 Gly_1 Ala_2 Aib_{10} Val_1 Leu_1 $Pheol_1$).

FD mass spectra of paracelsin A and B yielded the most abundant MH^+ at m/z 1908 and 1922 (the latter isobaric with component C) in agreement with the calculated nominal molecular weights. As illustrated in Fig. 7a (component A) and Fig. 7b (component C*), the comparative evaluation of the specific N-terminal fragment-ion series in the positive ion FAB mass spectra enabled direct determination of the sequences of amino acid positions 1–14 (Fig. 7b). Most remarkable is that the sequence analysis was possible even for the heterogeneous component C*, and provided the precise designation of the exchange positions in components C and D by the mass shifts of 14 a.m.u. for each corresponding sequence ion. Since the preferential fragmentation at the Pro residue (C-terminal fragment at m/z 774) in each case is identical with the acidolytic cleavage site, a single-step brief treatment with trifluoroacetic acid[8,21,22] followed by FAB-MS led to the complete sequence determination of the remaining residues, *i.e.*, positions 15–20. Under the conditions used, FAB-MS was found to be a particularly powerful tool for the sequence determination of peptaibols. Details of the mass spectral structural analysis of the paracelsin components (*cf.*, Fig. 1) and the sequence evaluation of the complex pattern of trichotoxin A-50 components (*cf.*, Fig. 4) have been reported elsewhere[21].

CONCLUSIONS

From a chemical point of view, alamethicin, suzukacillin and paracelsin may be regarded as mixtures of very closely related sequence analogues differing by exchange of a few or just one amino acid. Trichotoxin is also microheterogeneous but its components are clearly distinguished from the above peptides by their different primary structures, shorter chain lengths and by valinol as C-terminal constituent. From a microbiological point of view, the separation pattern obtained by HPLC may be representative of, and assignable to, different strains of the corresponding species of the fungi.

The results obtained by application of HPLC, FD-MS and, in particular, FAB-MS in the present study suggest the following generally applicable procedures for the definite structural characterization of peptaibol antibiotics with pronounced microheterogeneity: (i) investigation of the polypeptides by HPLC and quantification of the relative amounts of components; (ii) isolation of uniform components by HPLC and determination of their constituents; (iii) evaluation of the individual molecular masses by FD-MS and FAB-MS and (iv) sequence analysis of the isolated components by FAB mass spectrometry, in combination with single, selective acidolysis at the preferential cleavage sites.

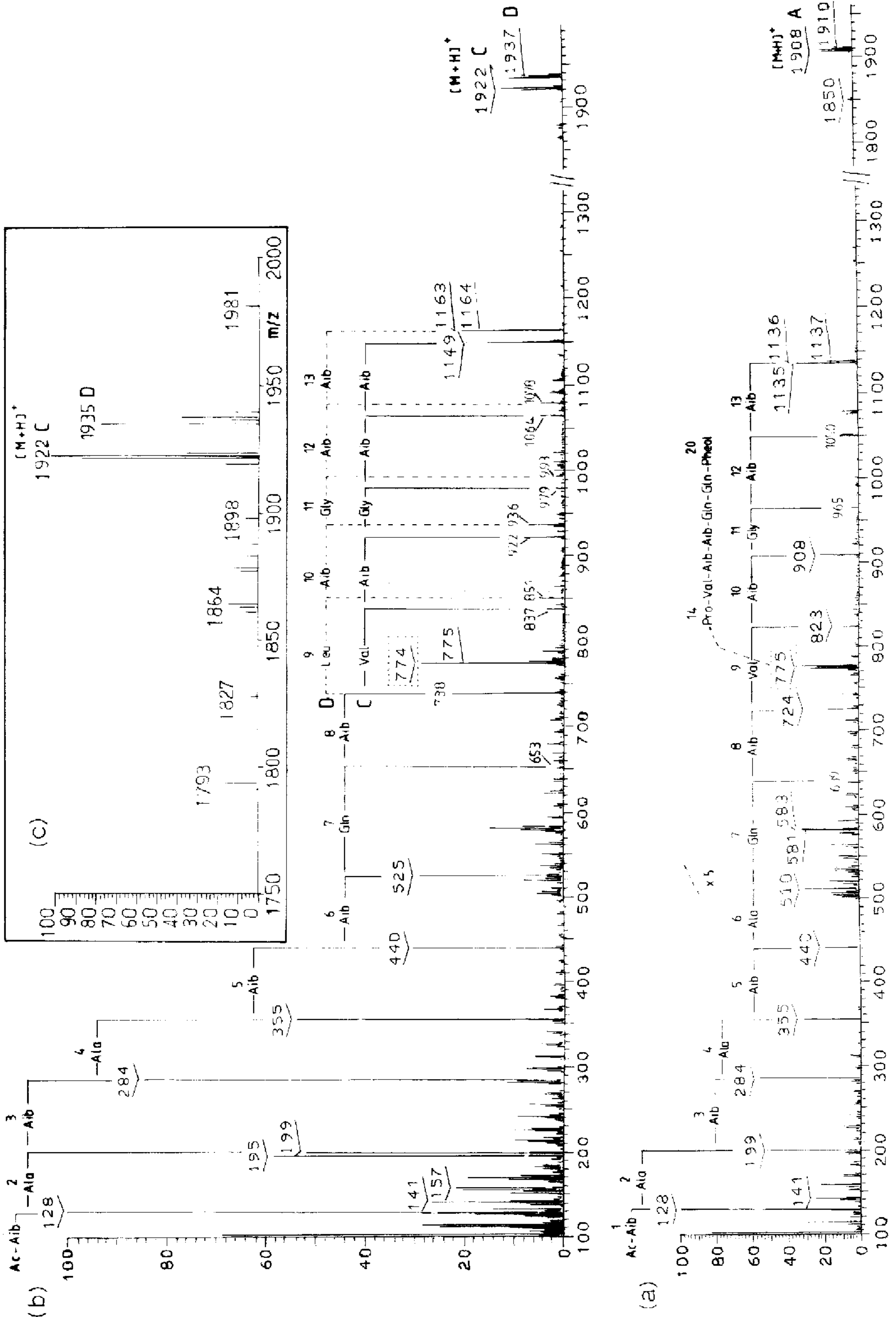

Fig. 7. Positive ion FAB mass spectrum of (a) paracelsin A and (b) paracelsin C* (mixture of components C and D, see text), and (c) molecular ion region of the FD mass spectrum of paracelsin C* showing MH^+ at m/z 1922 (component C) and m/z 1936 (component D).

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