

# Tandem electrospray mass spectrometric studies of proton and sodium ion adducts of neutral peptides with modified N- and C-termini: synthetic model peptides and microheterogeneous peptaibol antibiotics

Varatharajan Sabareesh and Padmanabhan Balaram\*

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

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The fragmentations of  $[M+H]^+$  and  $[M+Na]^+$  adducts of neutral peptides with blocked N- and C-termini have been investigated using electrospray ion trap mass spectrometry. The N-termini of these synthetically designed peptides are blocked with a tertiarybutyloxycarbonyl (Boc) group, and the C-termini are esterified. These peptides do not possess side chains that are capable of complexation and hence the backbone amide units are the sole sites of protonation and metallation. The cleavage patterns of the protonated peptides are strikingly different from those of sodium ion adducts. While the loss of the N-terminal blocking group occurs quite readily in the case of MS/MS of  $[M+Na]^+$ , the cleavage of the C-terminal methoxy group seems to be a facile process in the case of MS/MS of  $[M+H]^+$ . Fragmentation of the protonated adducts yields only  $b_n$  ions, while  $y_n$  and  $a_n$  ions are predominantly formed from the fragmentation of sodium ion adducts. The  $a_n$  ions arising from the fragmentation of  $[M+Na]^+$  lack the N-terminal Boc group (and are here termed  $a_n^*$  ions). MS/MS of  $[M+Na]^+$  species also yields  $b_n$  ions of substantially lower intensities that lack the N-terminal Boc group ( $b_n^*$ ). A similar distinction between the fragmentation patterns of proton and sodium ion adducts is observed in the case of peptides possessing an N-terminal acetyl group. An example of the fragmentation of the  $H^+$  and  $Na^+$  adducts of a naturally occurring peptaibol from a *Trichoderma* species confirms that fragmentation of these two ionized species yields complementary information, useful in sequencing natural peptides. Inspection of the isotopic pattern of  $b_n$  ions derived from  $[M+H]^+$  adducts of peptaibols provided insights into the sequences of microheterogeneous samples. This study reveals that the combined use of protonated and sodium ion adducts should prove useful in *de novo* sequencing of peptides, particularly of naturally occurring neutral peptides with modified N- and C-termini, for example, peptaibols. Copyright © 2006 John Wiley & Sons, Ltd.

Mass spectrometry is rapidly developing as a powerful and convenient means of *de novo* sequencing of peptides and proteins.<sup>1</sup> Mass spectral methods complement the traditional Edman sequencing method.<sup>2</sup> For instance, Chait and co-workers<sup>3</sup> have successfully demonstrated the use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in conjunction with modified Edman degradation in solution (the 'ladder' method). Consequently, mass spectrometry, in particular tandem mass spectrometry (MS/MS), finds major applications in 'bottom-up' proteomics, requiring the use of MS/MS for selection and fragmentation of ions formed from specific species in peptide mixtures, generated by protease digestion.<sup>4</sup> Mass spectrometric sequencing of peptides is most commonly effected by fragmentation

of the  $[M+H]^+$  ions.<sup>5–8</sup> The fragmentation is the result of the inelastic collisions of precursor ions with an inert gas like helium, leading to collision-induced dissociation (CID), sometimes referred to as collisionally activated dissociation (CAD).<sup>6–8</sup> The resulting fragment (product) ions are then analyzed using a tandem mass analyzer. Fragmentation is strongly influenced by the chemical nature of the protein or peptide. The fragmentation efficiency and distribution of product ions are dictated by the amino acid composition, sequence, nature of the peptide backbone (amide vs. N-alkylated), and the size and gas-phase conformation of the peptide.<sup>9–16</sup>

Mass spectral fragmentation patterns are usually interpreted for protonated peptides. The backbone fragmentation is facilitated when protonation occurs at a backbone amide group, resulting in two distinct principal cleavage modes which give rise to  $b_n$  and  $y_n$  ion series.<sup>5–8,17,18</sup> In peptides containing residues with side chains that are capable of complexation, protonation at these sites can influence

\*Correspondence to: P. Balaram, Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India.

E-mail: pb@mbu.iisc.ernet.in

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cleavage patterns.<sup>11,15,16</sup> Consequently, gaps in the series of product ions are often observed, making *de novo* sequencing a difficult task.

A further complicating feature in mass spectrometric studies of peptides is the frequent observation of intense peaks corresponding to Na<sup>+</sup> and K<sup>+</sup> adducts under conventional electrospray and MALDI conditions. Some recent reports have focused on the problem of enhancing the intensity of [M+H]<sup>+</sup> ions of peptides by suppressing alkali cation adduct formation.<sup>19–21</sup> Nevertheless, metal ion/peptide adducts have found use in peptide sequencing. Using linear gramicidin D and cyclic gramicidin S, Cody *et al.* demonstrated the usefulness of CID of laser-desorbed [M+K]<sup>+</sup> species to obtain structural information by tandem Fourier transform mass spectrometry.<sup>22</sup> Subsequently, the gas-phase interactions between metal ions and peptides have been studied in order to probe preferential binding sites of cations and their effect on fragmentation.<sup>23–35</sup> Most such studies have focused on short peptides possessing free N- and C-termini. In these cases, the dominant reaction is the neutral loss of the C-terminal amino acid, leading to the formation of cationized b<sub>n</sub> fragments; this reaction has been studied in particular for sodiated b<sub>n</sub> ions.<sup>25–27,29,31,34,35</sup> This behavior is in contrast to that of the protonated adducts which give rise to both b<sub>n</sub> and y<sub>n</sub> ions, suggesting the possibility that the sodium ion adducts of peptides may prove to be better candidates for complete C-terminal sequencing. Glish and co-workers have clearly demonstrated that multistage mass spectrometric (MS<sup>n</sup>) dissociation of alkali-cationized peptides, in a quadrupole ion trap instrument, gives rise to sequential loss of amino acids from the C-terminus.<sup>34,35</sup> Thus, unlike Edman sequencing, MS/MS of [M+Na]<sup>+</sup> triggers a sequential cleavage of residues from the C-terminus. In principle, mass spectrometric sequencing using C-terminal cation adducts may prove particularly useful for N-terminally blocked peptides.

In order to probe the differences between the fragmentation patterns of protonated and sodiated peptides, we chose to investigate a series of neutral peptides protected at the N-terminus with a *tert*-butyloxycarbonyl (Boc) group and an esterified C-terminus. The peptides examined in this study, listed in Table 1, are hydrophobic and adopt well-defined backbone conformations in solution and in the solid state.<sup>36–40</sup> In these sequences, protonation or metallation can occur only at the backbone amide, urethane or ester functions. The results described in this report establish dramatically

different cleavage patterns, under electrospray MS/MS conditions, for the protonated and sodiated species. While well-developed b<sub>n</sub> ion series are exclusively observed for [M+H]<sup>+</sup> species, y<sub>n</sub> ions are predominantly formed from [M+Na]<sup>+</sup> species. Extension to acetylated peptides is also described. The applicability to sequencing of a microheterogeneous sample of a peptaibol antibiotic is illustrated.

## EXPERIMENTAL

### Materials

The Boc-protected peptide methyl esters were synthesized by conventional solution-phase procedures, and characterized as reported earlier.<sup>38,39</sup> These peptides were shown to be homogeneous; their purity was investigated by high-performance liquid chromatography (HPLC) and by 500 MHz <sup>1</sup>H NMR. The N-acetyl peptide methyl esters were obtained from the Boc-protected peptides by removing the Boc group by dissolving the peptide in methanol/trifluoroacetic acid (6:1, 5 h, room temperature) followed by acetylation using ammonium bicarbonate and acetic anhydride. The peptaibol antibiotics (trichotoxins) were isolated from a *Trichoderma* culture, and fractionated by reversed-phase HPLC as described earlier for related fungal peptides.<sup>41,42</sup>

### Mass spectrometry

The electrospray ionization (ESI)-MS/MS data were obtained using an Esquire 3000-plus mass spectrometer (Bruker Daltonics) consisting of two octapoles followed by an ion trap. Helium was used as the collision gas for CID experiments. The data were analyzed using Esquire data analysis software, version 3.1. The peptides were dissolved in methanol and injected directly into the system using a syringe pump (Cole-Parmer, Vernon Hills, IL, USA) operated at a flow rate of about 200–240 μL h<sup>-1</sup>.

## RESULTS AND DISCUSSION

### Model peptides

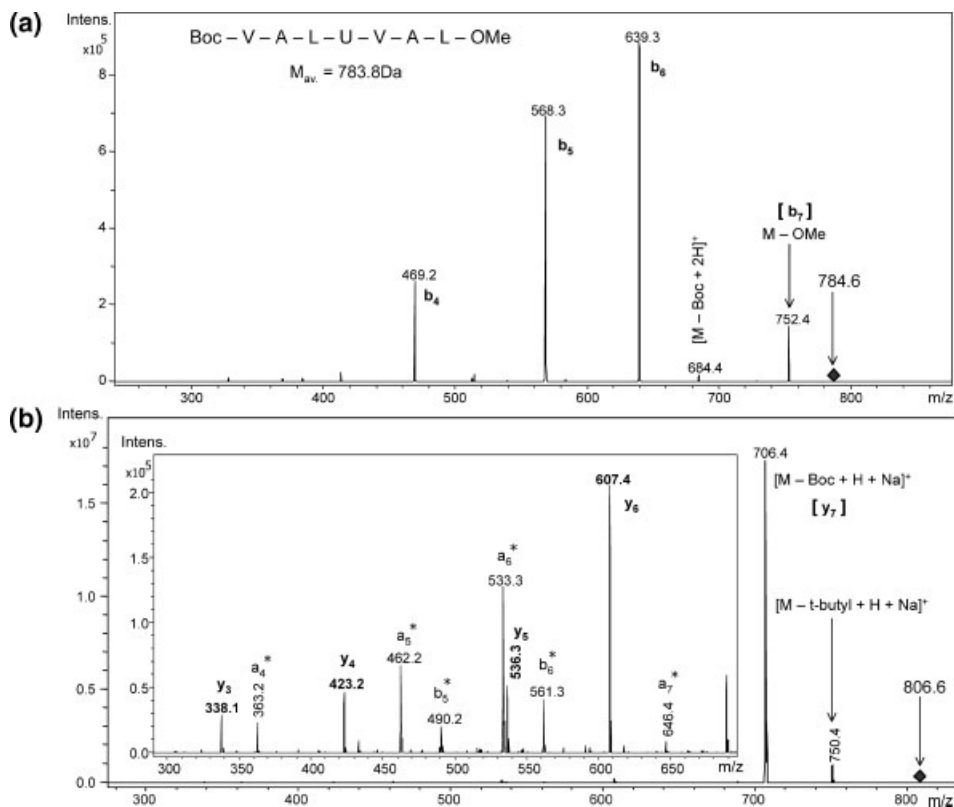
Figure 1(a) shows the ESI-MS/MS spectrum of the protonated heptapeptide Boc-VALUVAL-OMe (1). Peptide 1 is a prototypic hydrophobic helical sequence.<sup>36,37</sup> Inspection of the fragmentation pattern reveals a set of b<sub>n</sub> ions including a peak at *m/z* 752.4, corresponding to loss of a methoxy group, and formally the b<sub>7</sub> ion. In contrast, fragmentation of the [M+Na]<sup>+</sup> ion at *m/z* 806.6 yields a completely different set of

**Table 1.** Peptide sequences and observed *m/z* values of H<sup>+</sup>/Na<sup>+</sup> adducts<sup>a,b</sup>

Peptides		[M+H] <sup>+</sup>		[M+Na] <sup>+</sup>		References
		Calculated	Observed	Calculated	Observed	
Boc-VALUVAL-OMe	1	784.8	784.6	806.8	806.6	36,37
Boc-LFVU <sup>D</sup> ALFV-OMe	2	1008.1	1007.6	1030.1	1029.7	38
Boc-VAFUVAFUVAFU-OMe	3	1340.2	1339.7	1362.2	1361.8	39
Boc-VAFUVAFU-OMe	4	937.8	937.7	959.8	959.6	39
Boc-LUVALUVALU-OMe	5	1068.0	1067.8	1090.0	1089.6	40
Ac-LFVU <sup>D</sup> ALFV-OMe	6	950.2	949.7	972.2	971.4	
Ac-VAFUVAFU-OMe	7	879.8	879.6	901.8	901.6	

<sup>a</sup> The amino acid single letter codes are: A, alanine F, phenylalanine L, leucine U, α-aminoisobutyric acid V, valine.

<sup>b</sup> The molecular masses are calculated using average amino acid residue masses.



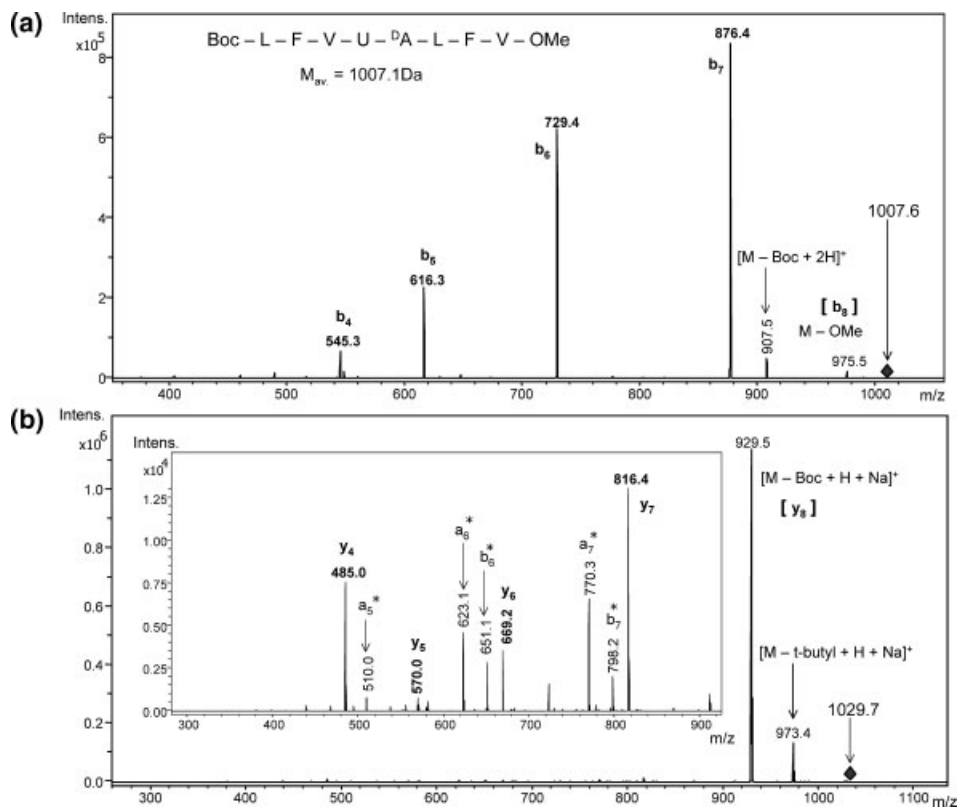
**Figure 1.** ESI-MS/MS spectra of (a)  $[M+H]^+$  of peptide 1, Boc-VALUVAL-OMe, and (b)  $[M+Na]^+$  of peptide 1, Boc-VALUVAL-OMe.

fragments (Fig. 1(b)); here the predominant peak corresponds to loss of the N-terminal Boc group, resulting in a very intense peak at  $m/z$  706.4 corresponding to  $[M-Boc+H+Na]^+$ . Interestingly, loss of the Boc group does not appear to be a favored process in the fragmentation of the  $[M+H]^+$  species. The fragment ions of the  $[M+Na]^+$  species may be assigned as  $y_n$  ions and  $a_n^*$  ions that are formed after loss of the Boc group. A limited number of  $b_n^*$  ions, which also lack the Boc group, are also observed at substantially lower abundances. Figure 2 illustrates fragmentation spectra observed for  $[M+H]^+$  and  $[M+Na]^+$  ions of the peptide Boc-LFVU<sup>D</sup>ALFV-OMe (2). Peptide 2 is a prototypic hydrophobic  $\beta$ -hairpin.<sup>38</sup> Inspection of these spectra (Fig. 2) immediately reveals a striking similarity to those observed in the case of peptide 1. Once again,  $[M+H]^+$  species produce  $b_n$  ions, while the  $[M+Na]^+$  species undergoes extremely facile loss of the Boc group and subsequently yields  $y_n$  and  $a_n^*$  ions. Boc-VAUFUVAUFU-OMe (3) is a long hydrophobic helix which folds in the solid state into a structure encompassing almost three complete helical turns.<sup>39</sup> The cleavage of its  $[M+H]^+$  species gives an almost complete series of  $b_n$  ions, with the predominant species at  $m/z$  1307.7 corresponding to the loss of a methoxy group from the C-terminus, formally a  $b_{12}$  ion (Fig. 3(a)). As in the preceding cases, fragmentation of the  $[M+Na]^+$  species of peptide 3 also results in a distribution of fragments consisting of  $a_n^*$  and  $y_n$  ions with facile loss of the Boc group (Fig. 3(b)). Similar results (data not shown) were obtained for the peptides Boc-VAUFUVAUFU-OMe (4)<sup>39</sup> and Boc-LUVALUVALU-OMe (5).<sup>40</sup> Another notable feature in the case of MS/MS of  $[M+Na]^+$  adducts is

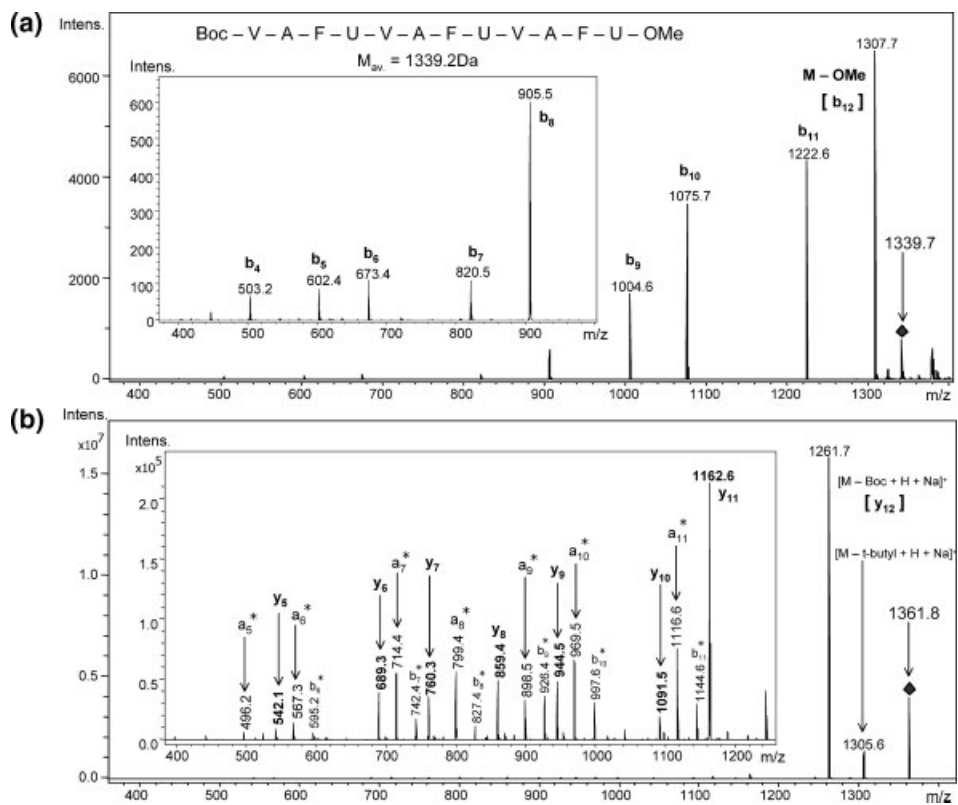
the loss of the tertiary butyl group, identified by a peak corresponding to  $[M+Na-56]^+$ .

Figure 4 summarizes the fragmentation patterns observed for the protonated and sodiated species of the five peptides tested here. Clearly, there is a consistent difference between the two types of fragmentation patterns. In the case of the  $[M+H]^+$  species the almost exclusive formation of  $b_n$  ion series is indicative of protonation of the C-terminus, with successive loss of residues from this end of the molecule. Indeed, the absence of  $y_n$  ions is striking. Figure 5(a) shows a plausible mechanism for the generation of  $b_n$  ions, which involves preferential protonation of the C-terminal ester function. Note that direct spectroscopic evidence for oxazolone formation upon CID of peptides has been presented recently.<sup>43</sup> The behavior of the  $[M+Na]^+$  species is dramatically different. The predominant cleavage is loss of the Boc group followed by formation of  $y_n$  ion series; the mechanism proposed in Fig. 5(b) involves the  $Na^+$  ion preferentially complexed initially to the N-terminal Boc group and subsequent fragmentation with charge retention on the C-terminal fragment with  $Na^+$  complexation to vicinal carbonyl groups.

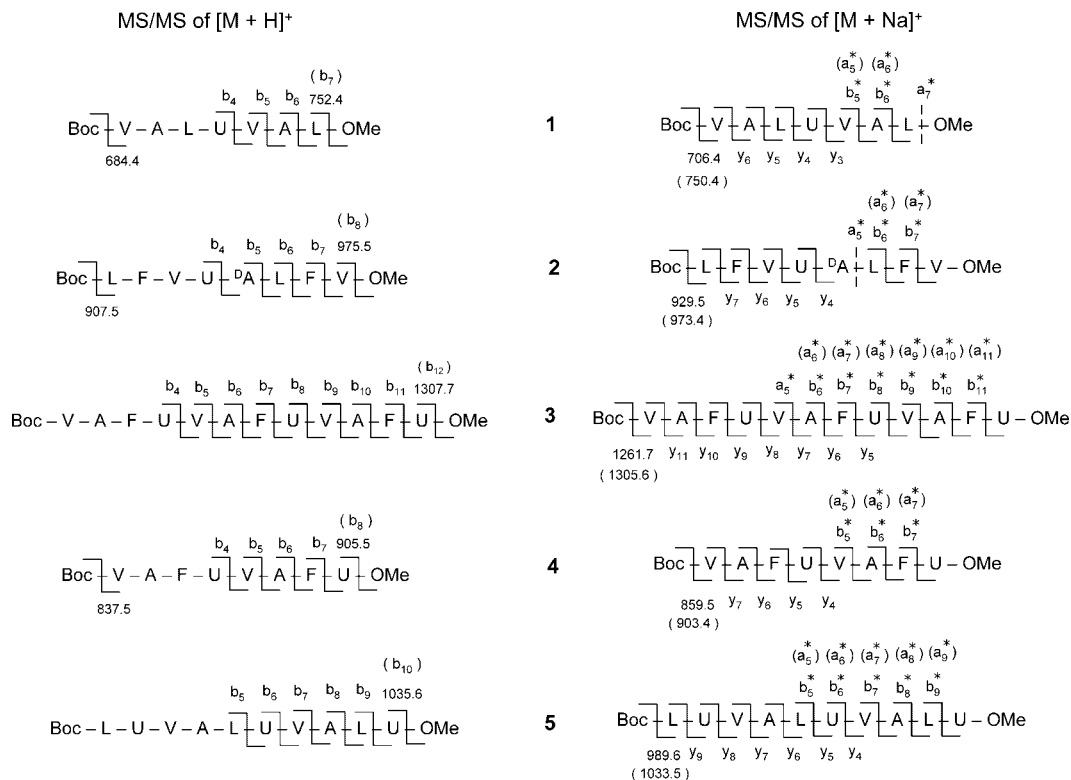
The Boc-protected peptides were used as test cases because of their ready availability as synthetic intermediates. In order to eliminate the possibility of preferential  $Na^+$  complexation by the urethane group, N-acetyl derivatives (that are found in nature) were also examined. Figure 6 compares the ESI-MS/MS spectra of  $H^+$  and  $Na^+$  adducts of a model acetylated peptide ester, Ac-LFVU<sup>D</sup>ALFV-OMe (6). The  $[M+H]^+$  species gives rise almost exclusively to the  $b_n$  ion series, while, in contrast, the  $[M+Na]^+$  ion fragments to yield



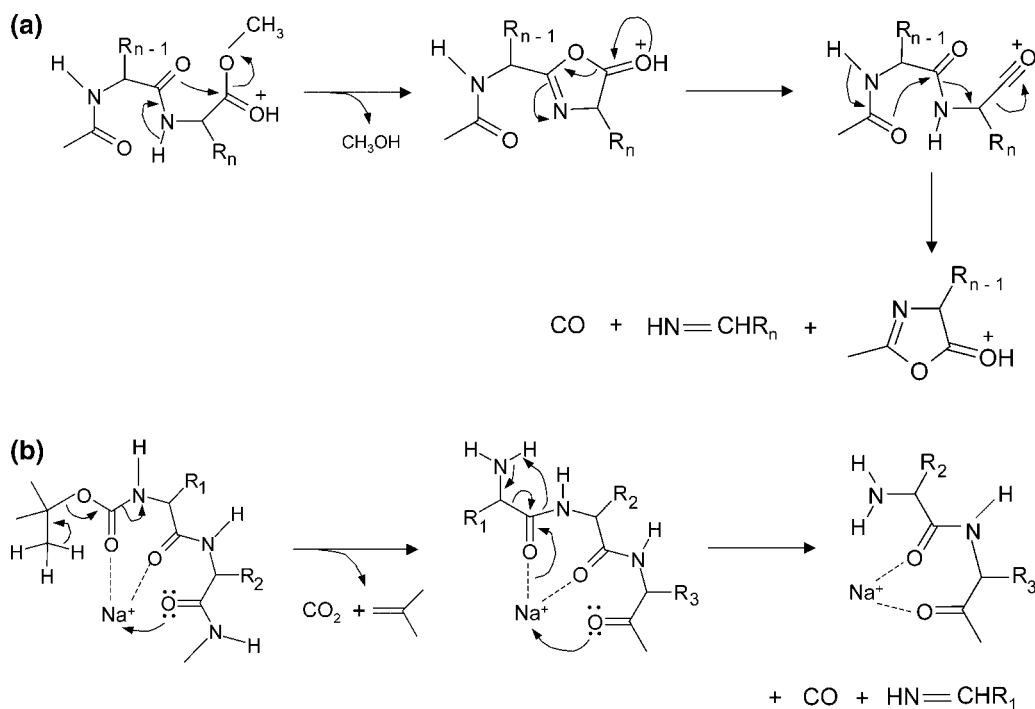
**Figure 2.** ESI-MS/MS spectra of (a)  $[M+H]^+$  of peptide **2**, Boc-LFVU<sup>D</sup>ALFV-OMe, and (b)  $[M+Na]^+$  of peptide **2**, Boc-LFVU<sup>D</sup>ALFV-OMe.



**Figure 3.** ESI-MS/MS spectra of (a)  $[M+H]^+$  of peptide **3**, Boc-VAFUVAFUVAFU-OMe, and (b)  $[M+Na]^+$  of peptide **3**, Boc-VAFUVAFUVAFU-OMe.



**Figure 4.** Summary of fragmentation patterns of  $[M+H]^+$  and  $[M+Na]^+$  ions of neutral peptides under ESI-MS/MS conditions.

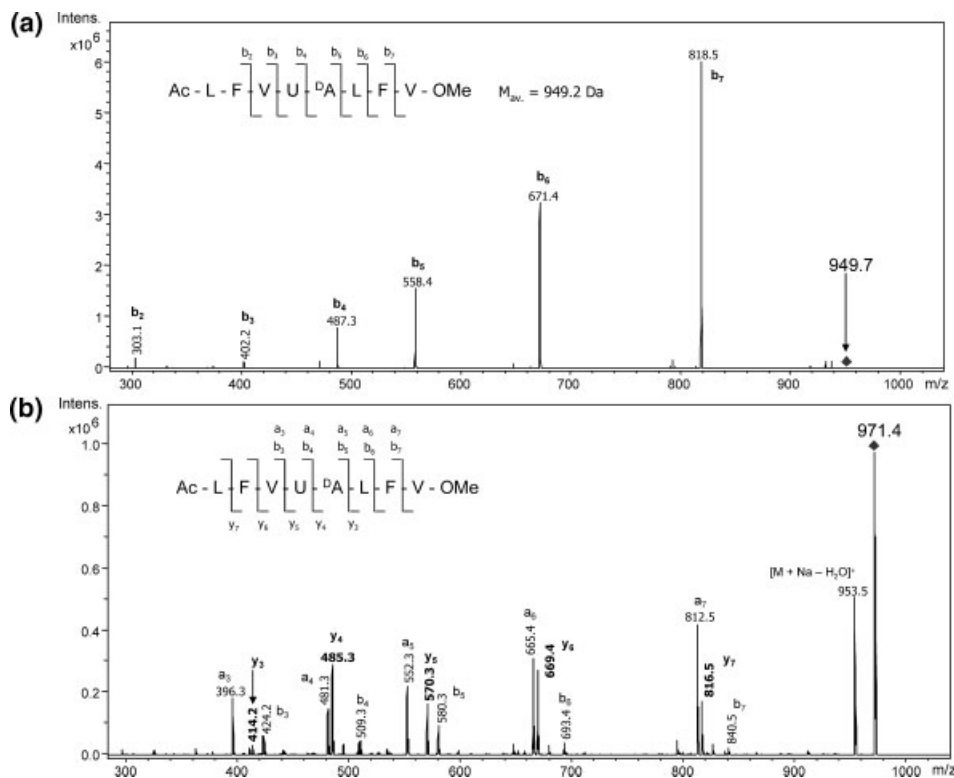


**Figure 5.** Plausible mechanisms proposed for (a) generation of b<sub>n</sub> ions from the C-terminal fragmentation of protonated esters and (b) generation of y<sub>n</sub> ions from the fragmentation of  $[M+Na]^+$  adducts of N-terminal urethanes.

y<sub>n</sub><sup>-</sup> and a<sub>n</sub><sup>-</sup>-type ions of appreciable abundances, although b<sub>n</sub> ions of low abundance are also detectable. Similar results were also observed for another N-acetyl peptide ester, AcVAFUVAFU-OMe (7) (data not shown). While loss of the Boc group is a facile process on fragmenting Na<sup>+</sup> adducts of Boc-

protected peptides (Fig. 5(b)), loss of the acetyl group does not occur in the case of N-acetyl peptides.

A curious feature of the fragmentation of  $[M+Na]^+$  species of these peptides is the observation of a well-developed series of a<sub>n</sub><sup>\*</sup> ions, which correspond to cleavage of residues



**Figure 6.** ESI-MS/MS spectra of (a)  $[M+H]^+$  of peptide 6, Ac-LFVU<sup>D</sup>ALFV-OMe, and (b)  $[M+Na]^+$  of peptide 6, Ac-LFVU<sup>D</sup>ALFV-OMe.

from the C-terminal end. Conventionally,  $a_n$  ions are generated by loss of CO from precursor  $b_n$  ions.<sup>5</sup> However, in the present case, the low abundance or absence of the corresponding  $b_n$  ions suggests the possibility of direct generation of the  $a_n$ -type ions. Early studies of fragmentations of  $[M+Na]^+$  ions, most conducted using fast atom bombardment mass spectrometry (FAB-MS), almost always examined peptides with unprotected N- and C-termini, and, in addition, contained complexing side chains.<sup>23,24,27–31</sup> In the case of peptides with a carboxylic acid group at the C-terminus, binding of  $Na^+$  clearly involves this site, eventually leading to successive loss of residues from the C-terminus.<sup>26,27,29</sup> Many studies of  $[M+Na]^+$  ions have used short free peptides, the enkephalins being notable examples.<sup>25,27,28,30–33,35</sup> One early study using FAB-MS did address the issue of neutral peptides with modified N- and C-termini, in which protonation or metallation is necessarily restricted to the peptide backbone.<sup>31</sup>

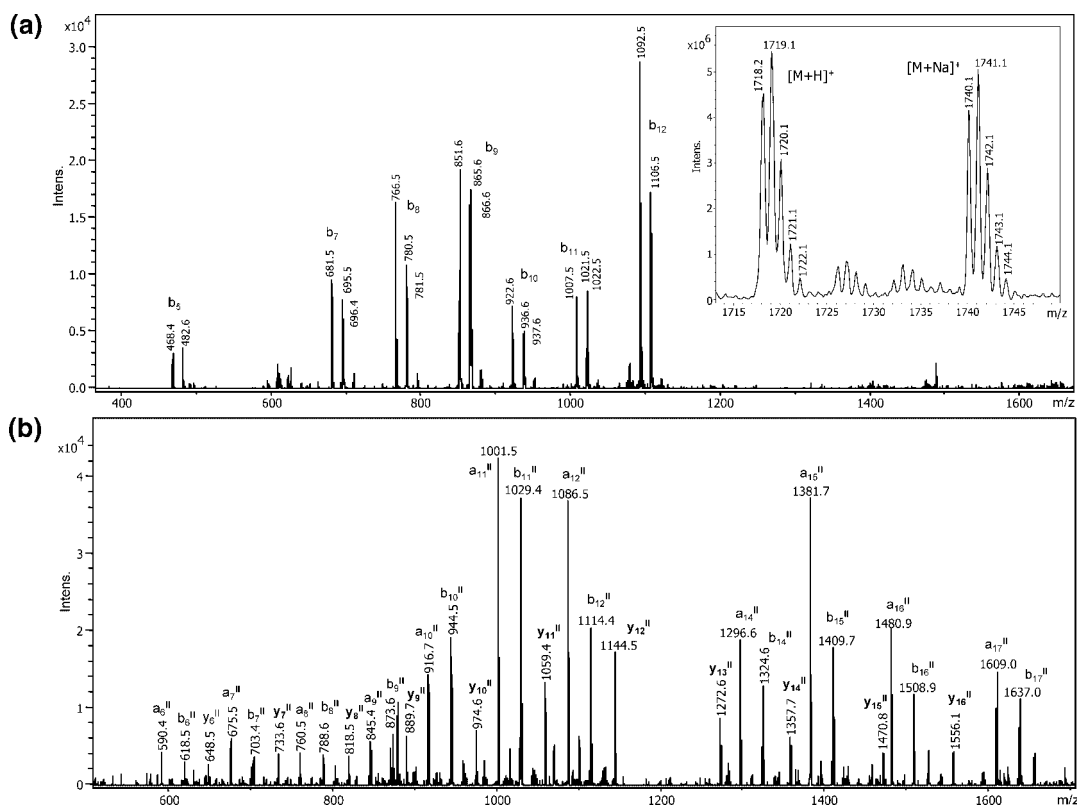
### Application to peptaibols

Peptaibols are linear peptide antibiotics, composed of about 15–20 amino acids, produced by soil fungi. They possess an N-terminal acetyl group, a C-terminal amino alcohol, and are rich in  $\alpha$ -aminoisobutyric acid (Aib: U).<sup>44–47</sup> Several of the longer members have the ability to become accommodated within lipid bilayer membranes, thereby forming ion channels.<sup>44–46</sup> Peptaibol biosynthesis occurs by a non-ribosomal mechanism, involving peptide synthetases resulting in the production of a microheterogeneous family of peptide metabolites.<sup>48–50</sup> Thus far, over 250 sequences have been reported and are available in a database.<sup>51</sup> Based on sequence, length and functional characteristics, peptaibols

are classified into subfamilies (SFs); the longer peptides containing 17–20 amino acids are classified in the SF1 subfamily.<sup>45,47,51</sup> Trichotoxins are 18-residue peptides belonging to the SF1 subfamily that are isolated from *Trichoderma viride* strain NRRL 5242.<sup>52–54</sup> These peptides are so closely related that molecular masses differing by 1 Da, 14 Da and isobars (identical molecular masses) are found, corresponding to exchanges or replacements Gly  $\leftrightarrow$  Ala (G  $\leftrightarrow$  A), Ala  $\leftrightarrow$  Aib (A  $\leftrightarrow$  U), Aib  $\leftrightarrow$  <sup>D</sup>Iva (U  $\leftrightarrow$  J) (J: <sup>D</sup>Iva: R-isovaline), Gln  $\leftrightarrow$  Glu (Q  $\leftrightarrow$  E) within their sequences, resulting in complex microheterogeneous mixtures.

While much of the early sequencing of peptaibols was performed using FAB-MS, more recently ESI-MS methods have been applied to study the peptaibols.<sup>55–58</sup> Also, the utility of liquid chromatography coupled to ESI-MS/MS (both positive and negative ionization modes) has been demonstrated for the analysis of trichotoxins.<sup>54</sup>

In the course of a program to screen for new peptaibol antibiotics from several strains of trichoderma, isolated from a soil sample, we encountered an apparently homogeneous HPLC fraction of apparent molecular mass 1717 Da ( $[M+H]^+$  at  $m/z$  1718.2,  $[M+Na]^+$  at  $m/z$  1740.1, see inset of Fig. 7(a)). Figure 7 compares the ESI-MS/MS spectra of the  $[M+H]^+$  and  $[M+Na]^+$  ions, which clearly reveals the dramatic differences between them. In the case of  $[M+H]^+$ , no peaks are observed between  $m/z$  1150 and 1600, while, in the case of  $[M+Na]^+$  ions, the fragments are more uniformly distributed over the entire mass range. The sequences of trichotoxins with molecular masses about 1717–1718 Da, obtained from the peptaibol database, are listed in Table 2 along with their calculated monoisotopic masses. The microheterogeneity in the trichotoxin sequences,



**Figure 7.** (a) ESI-MS/MS spectrum of  $[M+H]^+$  of peptaibol antibiotic obtained from the fungus *Trichoderma*, isolated from a soil sample, precursor ion centred at  $m/z$  1718.2 (inset, note distribution of isotopic peaks of  $H^+$  and  $Na^+$  adducts). (b) ESI-MS/MS spectrum of  $[M+Na]^+$  adduct of the peptaibol mixture, precursor ion centred at  $m/z$  1740.1.

**Table 2.** Sequences of trichotoxins with their calculated monoisotopic masses<sup>a</sup>

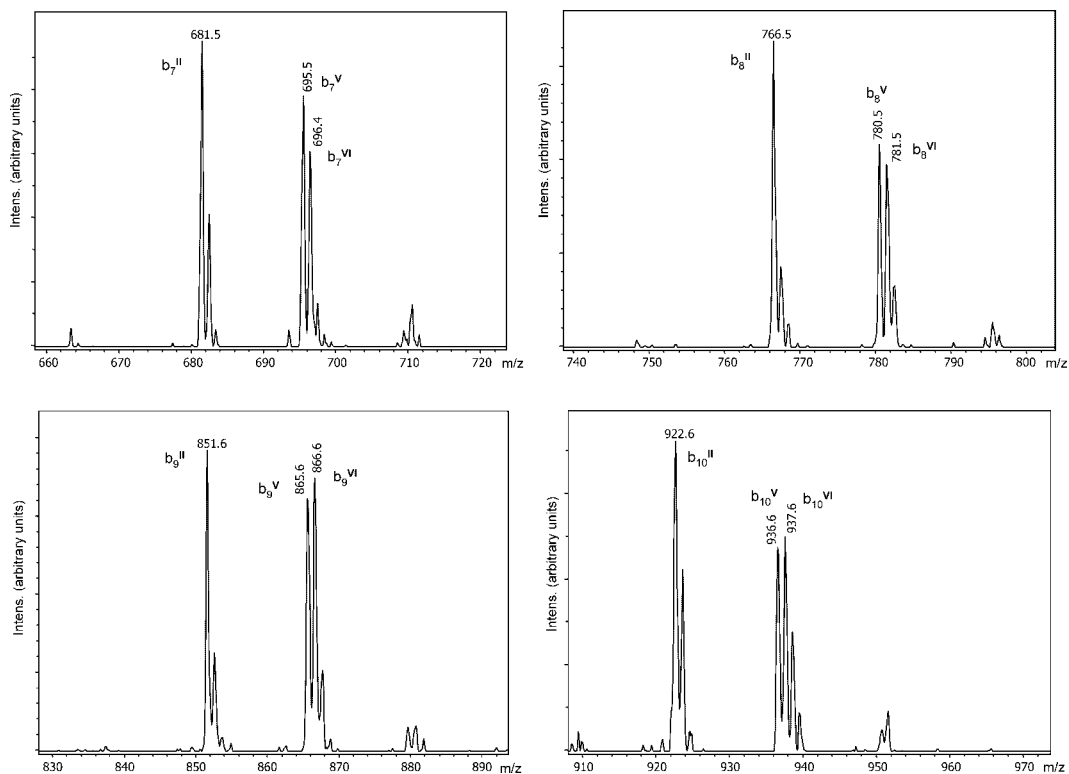
Peptaibols	Sequences	Monoisotopic mass (Da)	References
Trichotoxin_A-50_H	I : Ac-U A U L U Q U U U A A U P L U J Q V-CH <sub>2</sub> -OH	1717.1	50
Trichotoxin_A-50_I	II : Ac-U G U L U Q U U U A A U P L U J Q V-CH <sub>2</sub> -OH	1717.1	50
Trichotoxin_A-40_V	III : Ac-U G U L U Q U U U A U P L U J E V-CH <sub>2</sub> -OH	1718.1	51
Trichotoxin_A-40	IV : Ac-U G U L U E U U U A A U P L U J Q V-CH <sub>2</sub> -OH	1718.1	49
Trichotoxin_A-50_Va	V : Ac-U A U L U Q U U U A A U P L U U E V-CH <sub>2</sub> -OH	1718.1	50

<sup>a</sup>Sequences are from the peptaibol database.<sup>51</sup>

demonstrating the presence of isobars and masses differing by 1 Da, is clearly evident. There are two trichotoxin peptaibols with monoisotopic masses of 1717.1 Da, and three molecules of monoisotopic mass 1718.1 Da.

Mass spectrometric fragmentation (CID) of such highly microheterogeneous peptaibols requires strict unit mass resolution for precise precursor ion selection and detection of the product ions. This would enable appropriate assignment of the product ion spectrum for unambiguous sequencing. However, in practice, with our ion trap it is not possible to precisely select masses that differ by 1 Da. Hence, more than one precursor ion will be selected for fragmentation, and the MS/MS spectra will contain fragment ions obtained from more than one molecule, thereby rendering the assignment of the spectra difficult. Here, the MS/MS spectrum of  $[M+H]^+$  precursor ions selected with  $m/z$  1718.2 as the central value (Fig. 7(a)) shows fragment ions obtained from more than one type of precursor ion; this is evident from the

presence of pairs of fragment peaks separated by 14 and 15 Th, between  $m/z$  650 and 1150. Figure 8 shows an expansion of the region between  $m/z$  650 and 1000 from the MS/MS spectrum of  $[M+H]^+$  (Fig. 7(a)), which clearly depicts fragment peaks separated by 1, 14 and 15 Th. This clearly indicates that the fragment ions are derived from a mixture of microheterogeneous peptaibol molecules. To characterize the mixture it is necessary to establish both the number of peptide components and their precise sequences. Figure 9 shows the calculated  $m/z$  values of the  $b_n$  and  $y_n$  ions formed from  $H^+$  adducts of six trichotoxin peptaibols, together with their monoisotopic masses. Peptides I–V are listed in the database (Table 2), while peptide VI is a new sequence obtained from sequence V by exchanging the E and Q residues in positions 6 and 17. Examination of these six sequences shows variations in amino acids at positions 2, 6, 11, 16 and 17; G(2)/A(2), E(6)/Q(6), A(11)/U(11), J(16)/U(16) and Q(17)/E(17).



**Figure 8.** ESI-MS/MS spectrum of  $[M+H]^+$  of peptaibol antibiotic, showing expansion of the region between  $m/z$  650 and 1000 (from Fig. 7(a)). Peak assignments are indicated.

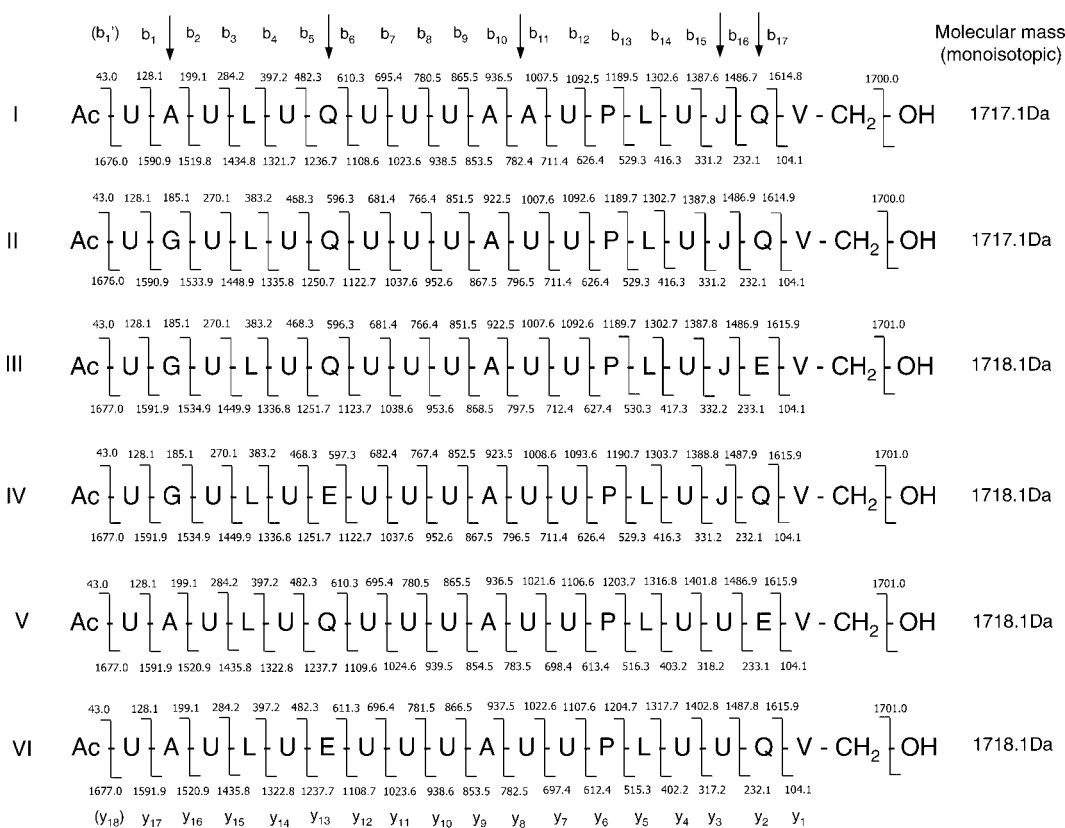


Figure 9

**Figure 9.** Calculated  $m/z$  values of  $b_n$  and  $y_n$  ions of  $[M+H]^+$  adducts of six trichotoxin sequences with their monoisotopic masses. The sequences I–V are available in the database (see Table 2) and sequence VI is new.

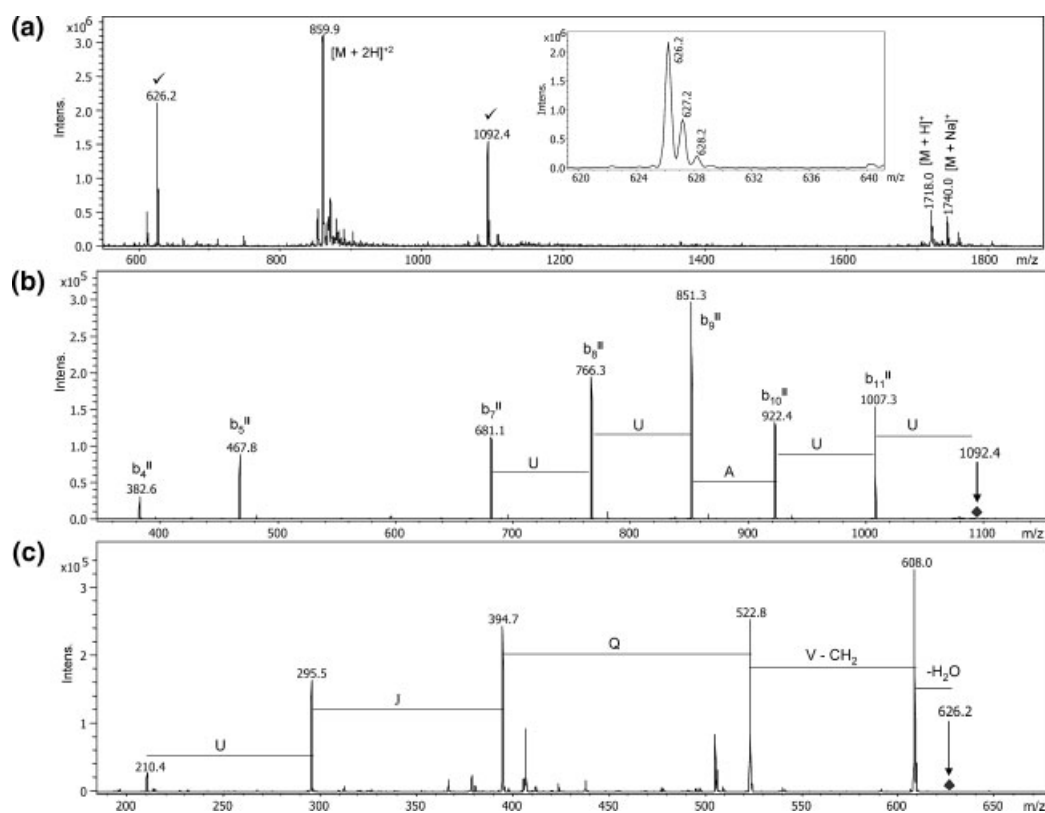


From Figs. 7(a), 8 and 9 it is evident that the fragment ions formed from the  $H^+$  adduct are all  $b_n$ -type ions. Using Fig. 9, the peaks at  $m/z$  468.4, 681.5, 766.5, 851.6, 922.6, 1007.5 and 1092.5 may be interpreted to arise from trichotoxins II and III, while the fragment ions at  $m/z$  482.6, 695.5, 780.5, 865.6, 936.6, 1007.5 and 1092.5 are assigned to trichotoxin I. The product ions at  $m/z$  482.6, 695.4, 780.5, 865.6, 936.6, 1021.5 and 1106.5 all arise from trichotoxin V, while the product ions at  $m/z$  482.6, 696.4, 781.5, 866.6, 937.6, 1022.5 and 1107.6 are assigned to trichotoxin VI. The intense peaks at  $m/z$  1092.5, 1021.5 and 1022.5 ( $b_{12}$ ) are due to the cleavage of the Aib-Pro bond (Figs. 7(a) and 9). Cleavage of the tertiary amide bond is a facile process, usually observed in the fragmentation of  $[M+H]^+$ . Thus, fragmentation of  $[M+H]^+$  corresponds to preferential cleavage of the Aib-Pro bond, with charge retention on the N-terminal fragment, subsequently generating a series of  $b_n$  ions. Thus, the data from the fragmentation of  $[M+H]^+$  is consistent with five of the possible trichotoxin sequences and eliminates the presence of trichotoxin IV. Indeed, the product ions from the  $H^+$  adducts are supportive of a new sequence, trichotoxin VI.

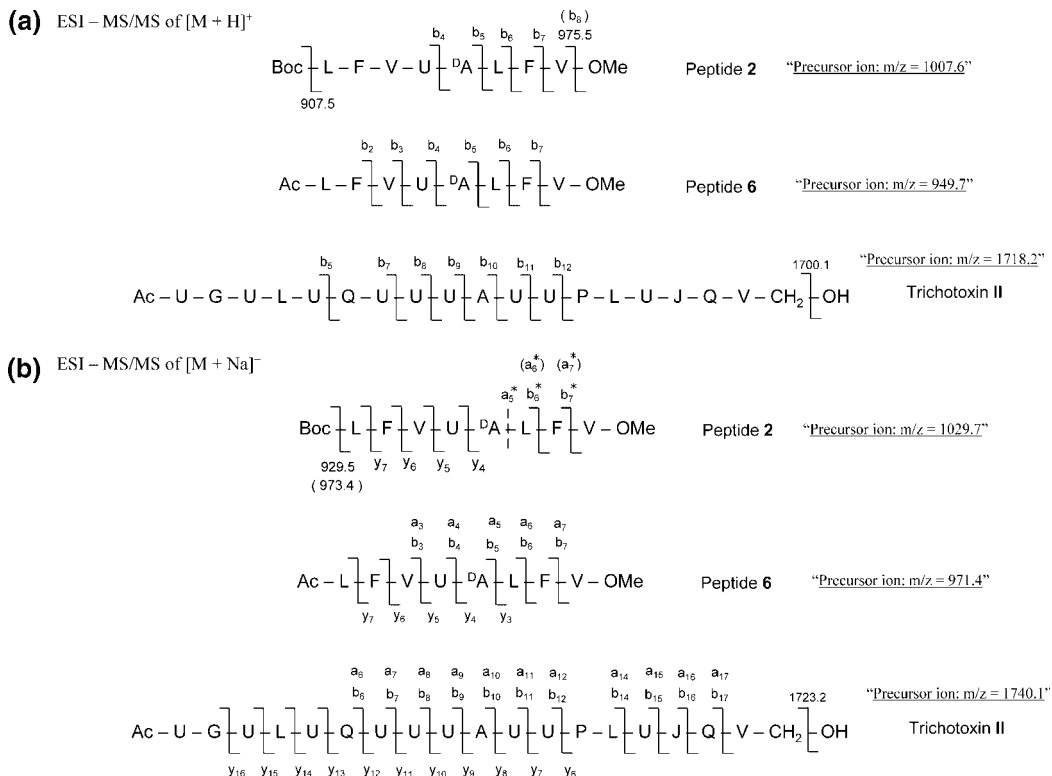
In sharp contrast to the  $H^+$  adduct, fragmentation of  $[M+Na]^+$  yields abundant product ions distributed over the entire mass range between  $m/z$  500 and 1700 (precursor ion centred at  $m/z$  1740.1, Fig. 7(b)). Using Fig. 9, the product ions of the  $Na^+$  adduct are assigned as sodiated  $y_n$ ,  $a_n$  and  $b_n$  ions (note that  $m/z$  values of sodiated  $b_n$  and  $y_n$  ions are 22 Th higher than those of the ions listed in Fig. 9). Notably, the

majority of the fragment ions correspond to those anticipated from trichotoxin II. These observations are striking in that MS/MS of the  $Na^+$  adduct of a microheterogeneous peptide mixture has yielded selective observation of product ions from just one component. Inspection of Fig. 9 reveals that of the six sequences being considered, two are neutral (I and II), while four (III, IV, V, VI) are acidic (contain one E residue each). Figure 7(b) clearly suggests that ionization of the acidic trichotoxin components is suppressed, possibly because of formation of tight ion pairs between the side-chain carboxylic acid and sodium ions. Using Fig. 9 the distinction between trichotoxins I and II can be made by inspecting the  $b_n$  and  $y_n$  ion  $m/z$  values, as they differ by 14 Th over much of the sequence. The observed spectrum in Fig. 7(b) clearly indicates a predominant population of product ions arising from trichotoxin II only, thereby ruling out the presence of trichotoxin I in the sample.

Figure 10(a) shows the ESI-MS spectrum recorded after treating the sample with ammonium acetate, which was intended to enhance the intensity of the protonated species relative to that of sodiated species. The two peaks at  $m/z$  1092.4 and 626.2 arise from the cleavage of the Aib-Pro bond (from Fig. 9,  $b_{12}^I$ ,  $b_{12}^{II}$ , and  $b_{12}^{III}$  at  $m/z$  1092.5;  $y_6^I$  and  $y_6^{II}$  at  $m/z$  626.4, with  $y_6^{III}$  at  $m/z$  627.4). This further confirms the labile nature of this tertiary amide bond, which is cleaved even under the normal electrospray conditions (in-source fragmentation) without resorting to MS/MS; a similar observation has been reported previously.<sup>55</sup> The MS/MS



**Figure 10.** (a) ESI-MS spectra of (a) the peptaibol antibiotic treated with ammonium acetate (inset shows an expansion of the spectrum showing the distribution of isotopic peaks of  $m/z$  626.2). (b) ESI-MS/MS spectrum of  $m/z$  1092.4, indicating sequence assignment. (c) ESI-MS/MS spectrum of  $m/z$  626.2, indicating sequence assignment.



**Figure 11.** Summary of cleavage patterns of (a)  $[M+H]^+$  ions of synthetic peptides (2 and 6) and of the natural peptaibol antibiotic (trichotoxin II) and (b)  $[M+Na]^+$  of synthetic peptides (2 and 6) and of the natural peptaibol antibiotic (trichotoxin II), under ESI-MS/MS conditions.

spectrum of  $m/z$  1092.4 (Fig. 10(b)) is clearly indicative of the presence of the ... UUAUU ... segment and not ... UUAAU ..., thereby confirming the absence of trichotoxin I. Furthermore, the isotopic peak distribution of  $m/z$  626.2 (inset of Fig. 10(a)) does not suggest the possibility of the presence of trichotoxin III in the sample, since  $y_6^{II}$  is at  $m/z$  626.4 and  $y_6^{III}$  at  $m/z$  627.4 (Fig. 9). In addition, the MS/MS spectrum of  $m/z$  626.2 (Fig. 10(c)) is clearly suggestive of ... UJQV-CH<sub>2</sub>-OH.

Thus, the fragmentation studies on  $[M+H]^+$  and  $[M+Na]^+$  adducts has aided in the identification of three peptidic components (trichotoxins II, V and VI) out of six possible sequences of trichotoxins, from an apparently homogeneous HPLC fraction. The fragmentation of the Na<sup>+</sup> adduct did in fact help in removing the ambiguities caused by the microheterogeneity of such peptaibols by suppressing the ionization of acidic peptides. Thus, Na<sup>+</sup> adducts may be of value in selectively reducing the contribution of acidic sequences in peptide mixture analysis. Furthermore, the fragmentations of H<sup>+</sup> and Na<sup>+</sup> adducts of both synthetic neutral peptides (with N- and C-termini modified) and natural peptaibols seem to follow almost identical patterns (Fig. 11).

## CONCLUSIONS

The results presented here suggest that, for non-polar peptide sequences where protonation or metallation is restricted to the peptide backbone, fragmentations of  $[M+H]^+$  and  $[M+Na]^+$  species yield complementary information. The former provides the  $b_n$  ion series, while the latter permits observation of  $y_n$  ions. This may be useful in the sequencing of many natural

peptides that lack amino acid residues with side chains capable of providing complexing sites for H<sup>+</sup> and Na<sup>+</sup> (for instance peptaibols). In fact, the utility of both H<sup>+</sup> and Na<sup>+</sup> adducts in the identification of new sequences from a microheterogeneous mixture from *Trichoderma reesei* has been demonstrated by Pocsfalvi and co-workers using liquid secondary ion mass spectrometry.<sup>59</sup>

In the present study, the fragmentation of H<sup>+</sup> adducts of a peptaibol mixture, which gave rise to only  $b_n$ -type ions, revealed microheterogeneity of an apparently HPLC-pure fraction. Fragmentation of  $[M+H]^+$  ions has indeed aided in identifying a new sequence, trichotoxin VI, which is almost similar to trichotoxin V except that Q and E exchange places between residues 6 and 17.

The results presented here may be applicable in mass spectrometric sequencing of peptide metabolites from cells or cell extracts without extensive fractionation. Also, in the case of microheterogeneous samples, wherein amino acid variations occur only at specific positions in the sequence, complete *de novo* sequencing may not be necessary. In such cases, identification of only the variable regions or positions in the sequence would suffice.

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