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New short peptaibols from a marine Trichoderma strain

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Abstract: The production of peptaibols by a marine-related Trichoderma longibrachiatum strain was studied using electrospray ionisation multiple-stage ion trap mass spectrometry (ESI-MSn-IT) and gas chromatography/electron impact mass spectrometry (GC/EI-MS). Two major groups of peptaibols were identified, those with long sequences (20 amino acids) and others with short sequences (11 amino acids). This paper describes the methodology used to establish the sequences of short peptaibols in a mixture without previous individual separation. Nine peptaibols were identified. Among them, eight are new, namely as trichobrachin A I-IV (Aib9-Pro10 sequence) and as trichobrachin B I-IV (Val9-Pro10 sequence). Original Pro6-Val7 and Val9-Pro10 sequences have to be noted. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: Short peptaibols, electrospray ionisation-multiple-stage mass spectrometry-ion trap (ESI-MSn-IT),marine products, marine fungi, Trichoderma longibrachiatum

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ABSTRACT

The production of peptaibols by a marine-related *Trichoderma longibrachiatum* strain was studied using electrospray ionisation-multiple-stage mass spectrometry-ion trap (ESI-MS^n-IT) and gas chromatography electron impact mass spectrometry (GC/EI-MS). Two major groups of peptaibols were identified, those with long sequences (20 amino-acids) and others with short sequences (11 amino-acids). This paper describes the methodology used to establish the sequences of short peptaibols in a mixture without previous individual separation. Nine peptaibols were identified. Among them, eight are new, namely as trichobrachin A I-IV (Aib_9-Pro_{10} sequence) and as trichobrachin B I-IV (Val_9-Pro_{10} sequence). Original Pro_6-Val_7 and Val_9-Pro_{10} sequences have to be noted.

KEY WORDS

Short peptaibols, electrospray ionisation-multiple-stage mass spectrometry-ion trap (ESI-MS^n-IT), marine products, marine fungi, *Trichoderma longibrachiatum*

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38 **INTRODUCTION**

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40 Peptaibols are an important class of linear peptides specific to fungi. The genus *Trichoderma*
41 (teleomorph *Hypocrea*: Ascomycota) is the most prolific known producer of peptaibols.[1,2]
42 Approximately 200 of more than 300 known peptaibols have been identified from this genus alone.[3]
43 Species of the genus *Trichoderma* are widespread in the marine environment and have been shown to
44 be able to produce peptaibols in marine culture conditions.[4,5] Peptaibols are characterized by a high
45 content of an uncommon amino acid: α-aminoisobutyric acid (Aib), an *N*-terminal acyl (most often
46 acetyl) group, and a *C*-terminal β-amino alcohol. According to the number (7 to 20) and the nature of
47 the amino acid (AA) sequence, they have been classified in 9 subfamilies (SF's).[6]
48 Short peptaibols produced by *Trichoderma* species belong to subfamily SF4. They are generally
49 constituted of 11 residues with 2 Pro at positions 6 and 10. Their sequences are microheterogeneous
50 analogues with different amino acids (AAs) in positions 2, 3, 4 and 11 (Figure 1a). There are about
51 forty short peptaibol sequences listed in the peptaibol database[7] including pseudokonins KL III and KL
52 VI produced by *T. pseudokoningii*, trichorovins by *T. harzianum*[1,8,9] and trikoningins KA and KB by *T.*
53 *koningii*.[10] Trichogin A IV is the unique short peptaibol described from *T. longibrachiatum* characterized
54 by a different elementary composition in AAs and especially by the absence of Pro residues in its
55 sequence (Figure 1b).[8]
56 Short peptaibols have been little studied. They are known to interact with double-layered phospholipid
57 membranes in the same manner as longer peptaibols. They form ion channels probably by using
58 unique insertion and conductance mechanisms. It has been supposed that, due to their short
59 sequences, they can span only the half of the lipid bilayer and two molecules have to be associated by
60 the N-terminal ends at the centre of the membrane.[6] Short peptaibols exhibit antibacterial (Gram$^+$),
61 antifungal and antimycoplasmic activities.[1,8]
62 This paper describes the production, the purification and the sequence identification of new short
63 peptaibols produced by a marine strain of *Trichoderma longibrachiatum* Rifai. Microheterogeneous
64 sequence determination employed an original method with ESI-MS^n-IT based on the establishment of a
65 filiation graph. Among the nine short peptaibols identified in this study, eight sequences are new. The
66 strain studied also produced several different long chain peptaibols with 20 AA residues which will be
67 analysed using the same protocol and reported in a separate communication.

68

69 **MATERIAL AND METHODS**

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Chemicals

Organic solvents were purchased from SDS (Asnières sur Seine, France) and distilled before use. For mass spectrometry analysis, acetonitrile of HPLC quality grade was purchased from Baker (Deventer, Holland). Trifluoroacetic acid (TFA) was purchased from Fluka Chemical (Buchs, Switzerland) and acetic acid from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). Synthetic AAs used as standards were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and Acros Organics (Noisy-Le-Grand, France).

Fungal strain

The studied strain was isolated from a shellfish, *Mytilus edulis*, collected in Tharon (France). This strain was identified as *Trichoderma longibrachiatum* Rifai using metabolic profiles on Biolog FF MicroPlates™ and by sequencing the ITS regions of the ribosomal RNA cluster and the intron-rich region of translation elongation factor 1-α (tef1α).[11,12] The original isolate is deposited in the fungal collection of Université de Nantes as MMS 151. Additional isolates sampling in other marine habitats in French coastal regions, having essentially the same Biolog profile and identical DNA sequences, are deposited in the Canadian Collection of Fungus Cultures, Ottawa (DAOM 234096, 234098, 234099, 234100, 234101, 234102, 234103, 234105). The internal transcribed spacer (ITS) sequence for our isolate was identical to a sequence deposited in Genbank (EMBL Z48935) for *T. longibrachiatum*, and differed by the addition of one thymine (T) in the poly-T region of ITS1 from the ex-type isolate of *T. longibrachiatum* (ATCC 18648, EMBL Z31019). The unique tef1α sequence for our strain has been deposited in Genbank.

Cultures

Cultures were grown on modified Kohlmeyer medium prepared with sea water[13] (glucose 10 g/L; magnesium sulphate 2.4 g/L; ammonium nitrate 2.4 g/L; Tris(hydroxymethyl)aminomethane (Tris) 1.21 g/L, agar-agar 15 g/L adjusted to pH = 6.3 ± 0.2 with 1 M hydrochloric acid). Before solidification, hot liquid medium was poured in 250 mL flasks. After solidification, the medium was inoculated with fungal implants. Then the flasks were incubated for 21 days at 27 °C.

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Extraction and purification of peptaibols

For extraction, agar cultures were melted in a bain-marie at 80 °C, homogenized, cooled to 40 °C to avoid solvent projections and finally extracted 3 times with dichloromethane. Combined dichloromethane phases were evaporated to dryness leading to a crude extract.

The crude extract was fractionated by a vacuum liquid chromatography (VLC) on Nucleoprep 100-30 OH Diol (Macherey-Nagel, Düren, Germany) with dichloromethane/ethanol mixtures (98:2; 90:10; 85:15). The obtained fractions (A, B and C) were evaporated to dryness. Fraction B was subjected to separation through high-performance liquid chromatography (HPLC).

HPLC apparatus included a Constrametric III (LDC/Milton Roy) pump, a Rheodyne inc. injector and spectroMonitor D (LDC) UV modules. HPLC was carried out on an Inertsil ODS 3 column (5 µm, 4.6 x 250 mm), (Interchim, France) with methanol/water/acetic acid (85:15:0.05) as the mobile phase. Acidification of the eluent avoided delayed elution of broad peaks of almost unresolved peptides.[14] The flow rate was 1.0 mL/min. Ultraviolet detection was performed at 220 nm. Fractions eluted were collected for mass spectrometry (MS) analysis.

Mass spectrometry analysis

MS analyses were carried out using a mass spectrometer (LCQ™ Finnigan, Atlanta, GA, USA) equipped with an electrospray ionisation source (ESI) and an ion trap analyser (IT). MS analysis parameters are shown in table 1. All data were acquired and analysed by LCQ Xcalibur software (Finnigan). Charge state and isotopic distribution were analysed by a narrow-scan range mode (Zoomscan mode). Spectra acquisition of the various fractions (0.5 µg/µL) was realised in both neutral medium in a mixture of acetonitrile/water (75:25) and acidic medium with 0.1% of TFA added to the same mixture. These solutions were infused directly into the ESI probe with a 250 µL micrometrically automated syringe (Hamilton, Reno, NV) at a flow-rate of 3 µL/min.

Gas chromatography mass spectrometry analysis (GC/MS)

To separate AAs, HPLC fractions were hydrolysed by 6 N hydrochloric acid for 24 h at 110 °C under vacuum[14,15] on a Pico-Tag Station (Waters, Napa Valley, USA).[16-18] AAs were derivatized as *N*-

trifluoroacetyl isopropyl esters.[8,19] GC/MS was carried out on an Agilent 6890 gas chromatograph equipped with a splitless capillary inlet system and an Optima 5 MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) (Macherey-Nagel, Düren, Germany). The carrier gas was helium at an inlet pressure of 2.48 psi. A Hewlett-Packard 7673A liquid autosampler, operated in the fast mode for splitless injection, was used in conjunction with the gas chromatograph. Volume of sample injections was 2 µL. A Hewlett-Packard 5973 mass spectrometer, used as detector, was operated in electron impact (EI) mode at 70 eV in the full-scan mode and directly interfaced with the chromatograph by the capillary column. The oven temperature was started at 50 °C (held for 3.0 min), followed by a 3 °C/min ramp to 130 °C and a second 10 °C/min ramp to 240 °C. The injector and transfer line temperatures were maintained at 250 °C.

RESULTS

Obtaining the peptaibols mixture

A mass of 606 mg of crude extract was obtained by dichloromethane extraction from 25 flasks (150 mL) of the culture medium. Submitted to VLC, it provided 520 mg of fraction A, 32 mg of B and 11 mg of C. ESI-MS analysis of those fractions revealed that peptaibols could be found only in fraction B. HPLC purification of fraction B gave 8 sub-fractions. Short peptaibols were located in the second one.

Sequence determination

Mass spectrometry

Analysed under neutral conditions by ESI-MS infusion mode, short peptaibols of fraction 2 appeared as a single sodium adduct ion, singly charged $[M+Na]^+$ at m/z 1169.8 and doubly charged $[M+2Na]^{2+}$ at m/z 596.8 (Figure 2). MS^2 of this ion generated a complex spectrum containing ions of the (*a*), (*b*) and (*y*) series[20,21] but did not allow complete sequence identification as observed by Kanai.[22]

In acidic medium, several singly charged ions were generated (Figure 3a), mainly the pseudomolecular ion $[M+H]^+$ at m/z 1146.9 and fragments resulting from the classically observed cleavage at Pro residues.[23] Generally, short peptaibols from *Trichoderma* sp. contain two Pro residues constituting two main cleavage sites. This was observed for the studied peptaibols as shown in Figure 3a. These

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4 166 cleavages produced ion series with a difference of mass 14 (thus 525-539 and 933-947), indicating that
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6 167 the studied pseudomolecular ion at 1146.9 Th was not corresponding to a single sequence but to a
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8 168 microheterogeneous mixture. MS^n fragmentation of these different ions allowed to resolve the
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10 169 composition of this mixture without total separation of their constituents.
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13 171 Microheterogeneity resolution: filiations graph
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17 173 The first cleavage of m/z 1146.9 ion gave two product ions at m/z 933 and 947. A cascade of MS^n
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19 174 fragmentations of these ions and of their product ions (Figure 3) allowed us to construct a filiations
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21 175 graph with the obtained MS^n spectra. When appearing alone in the spectrum, product ions were
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23 176 definitively considered in the sequence. But when they were accompanied by satellite peaks with
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25 177 difference of mass 14 between them, the coupled ions were fragmented separately in order to obtain
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27 178 their filiations. This methodology and the filiations graph of ion 1146 are illustrated in Figure 4. For
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29 179 example, the fragmentation of the m/z 862 ion produced ions at 749, 553 and 539 (Figure 3, b2). The
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31 180 two last ions differed from 14 u, so they have been separately selected, isolated and fragmented in the
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33 181 ion trap (Figure 3 c2 and f2). Exploring the branches of the total sequence filiations graph (Figure 4) led
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35 182 to the establishment of 9 peptaibol sequences.
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37 183 ESI-MS^n-IT does not allow the differentiation of Val/Iva; Valol/Ivaol; Leu/Ile; Leuol/Ileol. GC/EI-MS
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39 184 analysis of AAs as *N*-trifluoroacetyl isopropyl esters allowed this differentiation. In the case of the
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41 185 studied mixture, only Val, Valol, Leu and Leuol were detected, allowing the establishment of final
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43 186 sequences as given in table 2.
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45 187 Among the nine identified structures, eight sequences are new. We named them trichobrachin A I-IV
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47 188 when the AA in position 9 was an Aib and trichobrachin B I-IV when it was a Val. Original Pro_6-Val_7
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49 189 (trichobrachin A-II and trichobrachin B-I) and Val_9-Pro_{10} (trichobrachin B I-IV) sequences have to be
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51 190 noted. It was also the first time that an Aib_8 residue was found in short peptaibols (trichobrachin A-I and
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53 191 trichobrachin B I-IV).
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55 192 Trichobrachin A-III and A-IV would be similar to trichorovin TV-Ia or trichorovin TV-IIb[9] if the unresolved
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57 193 Leu/Ile residues of these last compounds were exclusively Leu.
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59 194 The major component of the studied microheterogeneous mixture of peptaibols seems to be
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 195 trichobrachin A-IV since it corresponds to the cascade of most abundant MS^n fragments (Fig. 3).

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197 **DISCUSSION.**

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The nonribosomal biosynthesis mechanism of peptaibols uses peptide synthetases, enzymes constituted of successive subunits or "fields", responsible for connecting AA pairs.[24] Enzyme fields can have variable or more specific affinities for AA's. Some are restricted to a unique AA.[2,25] Other fields have less restricted specificities and allow sequence diversifications with multiple possible combinations generating the previously recognised peptaibol microheterogeneity phenomenon.[26] This peptaibol characteristic[15], even in crystalline form[26], results by single or multiple spontaneous exchanges of AA residues during biosynthesis within non-specific fields. Thereby, peptaibols with the same chemical characteristics and the same molecular masses are eluted together in HPLC. Wada[9] explained the spectra analysis of such mixed peptides. The complete separation of peptaibol components is usually difficult, so that a method to study their microheterogeneity without the need of absolute purification is very useful. Pocsfalvi[27,28] used high energy collision, collision-induced dissociation mass spectrometry, CID-MS experiments, to characterize peptaibols in a crude extract, but this method was limited to MS/MS grade. The use of ESI-MS^n-IT technology seems to represent a simpler method for peptaibol sequencing, allowing the creation of a filiations graph and leading to the establishment of peptaibol sequences from a mixture of short peptaibols.

CONCLUSION

Application of ESI-MS^n-IT method for sequencing short microheterogeneous peptaibols demonstrated the production of new peptaibols by a marine isolate of *T. longibrachiatum*. Among the 9 described sequences in this study, trichorovin TV-Ib ou IIa was already described but incompletely; the 8 others are new: trichobrakin AI-IV and trichobrachin BI-IV. For the first time, an Aib_8 residue and Pro_6-Val_7 and Val_9-Pro_{10} sequences were found in short peptaibols.

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262 **Table 1: Experimental and instrumental conditions of ESI-MS-IT**

ESI Source	**Ion trap analyser**
Ion Polarity Mode: positive	Full-MS/Full MS^2/ Full MS^3/ Full MS^4
Sheath Gas Flow Rate (N_2): 90 AU (arbitrary units)	Full MS Scan Range (m/z): 150-2000
Auxiliary Gas (N_2): 0 AU	Total Microscans: 3
Spray Voltage: 3 kV	Maximum Injection Time: 50 ms
Spray Current: 3,76 µA	Collision Energy: 22%
Capillary Temperature: 157 °C	Activation Time: 30 ms
Capillary Voltage: 9,07 V	Isolation Width: 3-5 u
	Full MS Target: 5.10^7
Ion optic transmission	Full MS^n Target: 2.10
Lens Voltage: -16 V	Electron Multiplier Voltage (set point): -1200
Octapole 1 offset: -3 V	
Octapole 2 offset: -16 V	
Octapole RF Amp: 400 V (peak to peak)	

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Table 2: Sequences of the short peptaibols produced by marine *T. longibrachiatum* strain

						Cleavage 2					Cleavage 1			
AAs position	1	2	3	4	5		6	7	8	9		10	11	
Ac	Aib	Asn	Leu	Leu	Aib	-	Pro	Leu	Aib	Aib	-	Pro	Leuol	Trichobrachin A-I
Ac	Aib	Asn	Leu	Leu	Aib	-	Pro	Val	Leu	Aib	-	Pro	Valol	Trichobrachin A-II
Ac	Aib	Asn	Val	Leu	Aib	-	Pro	Leu	Leu	Aib	-	Pro	Valol	Trichobrachin A-III
Ac	Aib	Asn	Leu	Val	Aib	-	Pro	Leu	Leu	Aib	-	Pro	Valol	Trichobrachin A-IV*
Ac	Aib	Asn	Leu	Leu	Aib	-	Pro	Val	Aib	Val	-	Pro	Leuol	Trichobrachin B-I
Ac	Aib	Asn	Val	Leu	Aib	-	Pro	Leu	Aib	Val	-	Pro	Leuol	Trichobrachin B-II
Ac	Aib	Asn	Leu	Val	Aib	-	Pro	Leu	Aib	Val	-	Pro	Leuol	Trichobrachin B-III
Ac	Aib	Asn	Leu	Leu	Aib	-	Pro	Leu	Aib	Val	-	Pro	Valol	Trichobrachin B-IV
Ac	Aib	Asn	Val	Val	Aib	-	Pro	Leu	Leu	Aib	-	Pro	Leuol	Trichorovin TV-Ib or IIa

* = major compound

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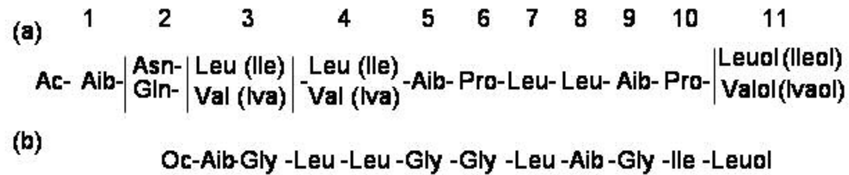

Figure 1. Sequences of short peptaibols produced by Trichoderma sp. (a) Classical sequences with 2 Pro residues (b) Trichogin A IV sequence (Oc =: octanoyl group)

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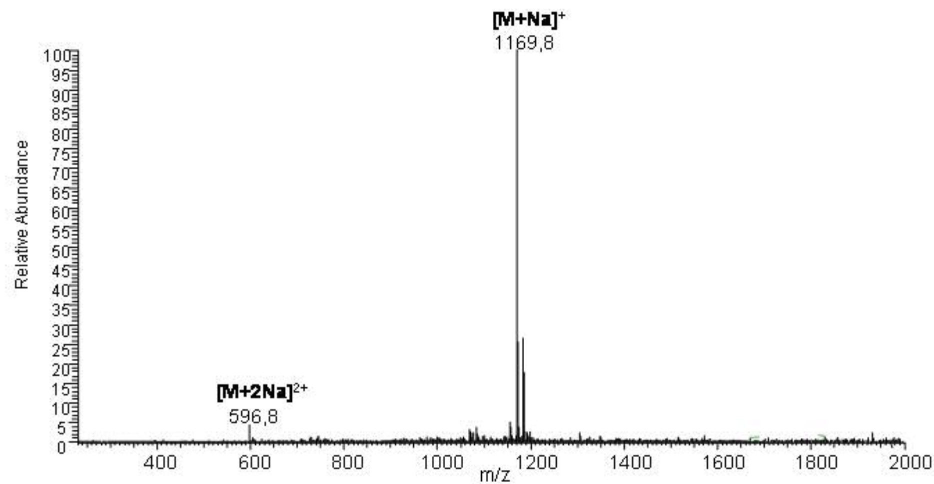

Figure 2. ESI mass spectra of short peptaibols (fraction 2) in neutral medium

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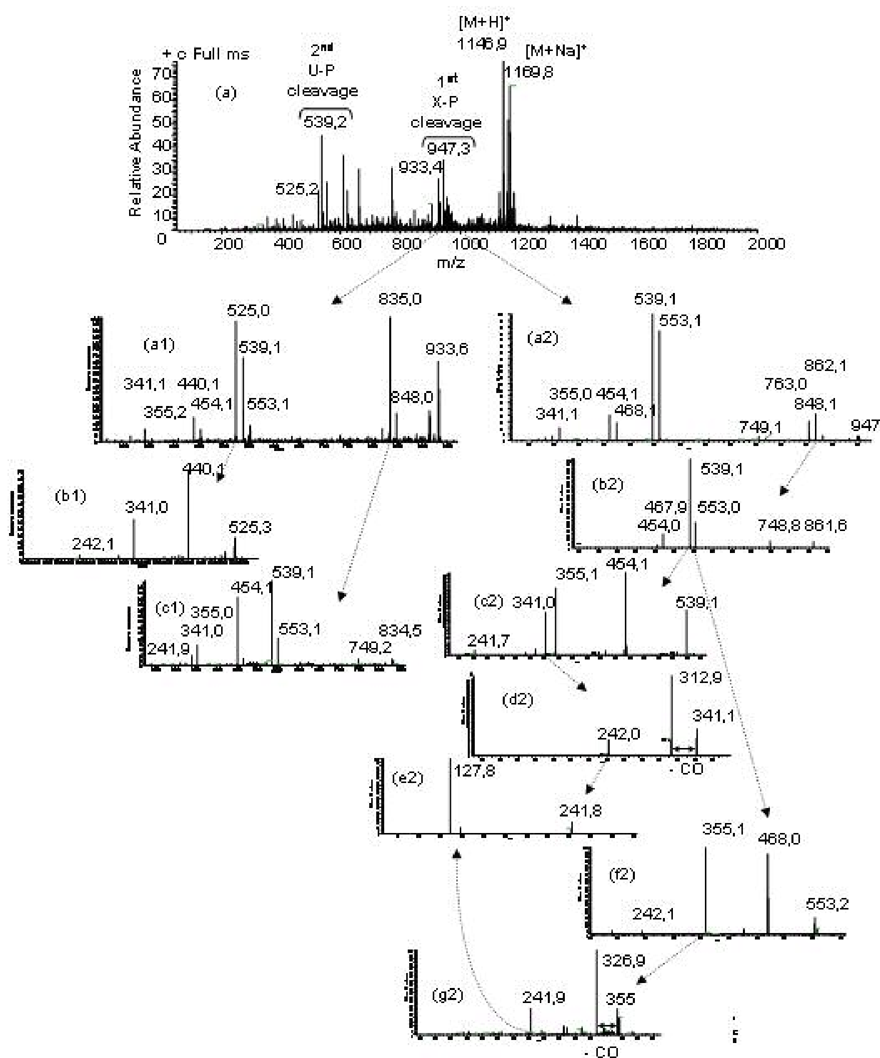

Figure 3. MSn filiations of m/z 1147 ion in acidic medium. (a): Full MS spectrum (x = U or V, U=Aib, V=Val, P=Pro). (a1): MS2 spectrum of m/z 933. (b1) and (c1) respectively MS2 spectra of m/z 525 and 835. (a2), (b2), (c2), (d2) and (e2) respectively MS2 to MS6 spectra of m/z 947, 862, 539, 341 and 242. (f2) and (g2) respectively MS4 and MS5 spectra of m/z 553 and 355.

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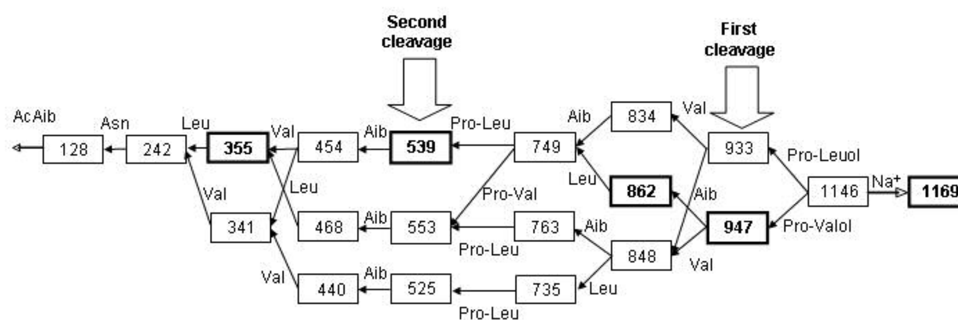

Figure 4. Total filiations graph m/z ions of the microheterogenous peptaibol (MW = 1146 Da) in mixture produced by strain MMS 151. N-terminal acylium ions (bn acylium ions): b1-b5; b7-b9. Bold characters and lines indicate major fragments for the determination of the principal component.