

# **Characterisation of Major Peptides in ‘Jack Jumper’ Ant Venom by Mass Spectrometry**

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## **Abstract:**

The jack jumper ant, *Myrmecia pilosula*, is endemic to South-Eastern Australia, where around 2.7% of the population has a history of systemic allergic reactions (anaphylaxis) to its venom. Previous work had indicated that there were several allergenic peptides derived from the cDNA Myr p 1, the major expressed allergenic product being a 56-residue peptide (Myr p 1 57→112, "pilosulin 1", ~6052 Da). Another major allergen had been described as a 27 residue peptide derived from the cDNA Myr p 2 (Myr p 2 49→75, "pilosulin 2", ~3212 Da), possibly existing as part of a disulfide complex. As a preliminary step in detailed stability studies of a pharmaceutical product used for venom immunotherapy, LC-MS and Edman sequencing analysis of venom collected from various locations by both electrical stimulation and venom sac dissection was undertaken. More than 50 peptides in the 4kDa to 9kDa range were detected in LC-MS analyses. A subsequence of Myr p 2 was found as part of the major peptide present in all samples; this was a bis-disulphide linked, antiparallel aligned heterodimer consisting of Myr p 2 49→74, (des-Gly<sup>27</sup>-pilosulin 2, ~3155 Da) and a previously unreported peptide of ~2457 Da. Pilosulin 1 was found by a combination of tandem mass spectrometry and Edman sequencing to exist mainly, and sometimes exclusively, as a previously unreported ~6067 Da variant, in which the valine at residue 5 is replaced by isoleucine. A range of hydrolysis products of [Ile<sup>5</sup>]pilosulin 1 and pilosulin 1 were also detected in partially degraded venom. Further IgE-binding studies using these peptides are warranted and a revision of the nomenclature of allergenic components of *M. pilosula* venom may be required to conform with established IUIS guidelines.

## **Keywords:**

Jumper ant venom, *Myrmecia pilosula*, pilosulins, Myr p 1, Myr p 2, heterodimer, LC-MS

## **Introduction:**

The “Jack Jumper” ant (*Myrmecia pilosula* species complex), is a commonly encountered member of the genus *Myrmecia* (“bulldog” and “jumper” ants, Hymenoptera: Formicidae: Myrmeciinae) found in southeastern Australia including Tasmania and the southernmost tip of Western Australia (Ogata and Taylor, 1991). In Tasmania, 2.7% of the population has a history of systemic allergic reactions to jack jumper venom (Brown et al., 2003a) and around half of allergic people experience life-threatening reactions (Brown et al., 2003a; Clarke 1986; Douglas et al., 1998). Deaths have been reported (Brown et al., 2001), and the venom appears to be particularly allergenic in comparison to other venoms; in people with a history of wasp and bee sting allergy, only 25-50% react to subsequent deliberate sting challenges (van Haltern et al., 1996), whereas re-sting reaction rates in people with jack jumper sting allergy are 70% for field stings (Brown et al., 2003a) and 72% for deliberate sting challenges (Brown et al., 2003b). *M. pilosula* venom has been shown to contain histamine-like, haemolytic, and eicosanoid releasing factors, and to have a broad range of enzymatic activity including phospholipase A2, phospholipase B, hyaluronidase, acid phosphatase and alkaline phosphatase (Matuszek et al., 1992; Matuszek et al., 1994).

Previously thought to be a single species, *M. pilosula* is in fact a karyologically diverse species complex, consisting of several sibling species (Crozier et al., 1995; Crosland et al., 1988) that may even coexist at a single site (Taylor, 1988). It has nevertheless been claimed that IgE binding profiles of electrophoretically separated venoms from different regions of Australia, and thus “presumably” from different sibling species, are identical (Ford et al., 1991).

Two major protein allergens sharing a common leader sequence have been identified via cDNA sequencing (Donovan et al., 1993; Donovan et al., 1994; Donovan et al., 1995; Street et al., 1996), and found to encode 112 and 75 amino acid residues. The cDNA has been named Myr p 1 and Myr p 2 respectively (Figures 1a and 1b). These encode precursor peptides that differ by only three amino acids in the first 47 residues. However, both appear to undergo extensive post-translational modification. Donovan et al. (1996) have proposed (using SDS-PAGE, immunoblotting and 8 cycles of N-terminal amino acid sequencing) that whole venom contains six

subsequences of Myr p 1 (figure 1c), the major IgE binding band being Myr p 1 57→112. Two other predicted peptide cleavage products of Myr p 1 27→112 and Myr p 1 37→112 were not identified in whole venom.

IgE binding bands at 8.5kDa and a broad band at 2-4kDa have been identified as being due to the presence of Myr p 2 derived peptides (Donovan et al., 1995). Reduction and alkylation of whole venom revealed a single band, which was presumed (on the basis of 8 cycles of N-terminal amino acid sequencing) to contain Myr p 2 49→75 (Street et al., 1996). The dual recognition of Myr p 2 is thought to be due to the presence of multimeric forms of Myr p 2 49→75 within whole venom (Street et al., 1996) and given that Myr p 2 49→75 contains 2 cysteine residues, the 8.5kDa peptide was proposed to contain Myr p 2 49→75 in a disulfide linked complex - either with itself or with another non-allergenic peptide(s) (Donovan et al., 1996). Myr p 2 27→75 was previously proposed as a product but was subsequently not identified in whole venom via SDS-PAGE (Donovan et al., 1996). Myr p 1 57→112 and Myr p 2 49→75 are proposed allergenic products (Donovan et al., 1996) which have been named “pilosulin 1” (Wu et al., 1998; Donovan and Baldo, 1999) and “pilosulin 2” respectively (Donovan and Baldo, 1997). From here on the pilosulin nomenclature will be used when referring to these peptides.

Pilosulin 1 and pilosulin 2 are extremely basic peptides, with 20% and 33% of their residues respectively being lysines or arginines – their theoretical isoelectric points are 9.94 and 10.45 respectively ([http://ca.expasy.org/tools/pi\\_tool.html](http://ca.expasy.org/tools/pi_tool.html)). Pilosulin 1 has been reported to have significant cytotoxic effects (King et al., 1998; Wu et al., 1998), and pilosulin 2 has been reported to have antihypertensive properties (Donovan and Baldo, 1997).

We recently published a double-blind placebo-controlled trial of jack jumper ant venom immunotherapy showing this treatment to be highly efficacious in preventing sting anaphylaxis (Brown et al., 2003b). With the aim of establishing a quality control regime for our venom extracts, we undertook HPLC-MS analysis of the venom, detailed tandem mass spectrometry (MS/MS) sequencing on key peptides and limited Edman sequencing to confirm previous reports of venom composition, and to directly

observe the presence of disulfide-bridged dimers suggested by previous investigators (Street et al., 1996).

## **Materials and Methods:**

### **Venom collection and storage**

*M. pilosula* were harvested from a variety of locations around Tasmania for venom extraction by either electrostimulation or venom sac dissection. Electrostimulation was performed in the field; live ants were harvested and transferred to a customised perspex box. An alternating current was applied across a steel wire grid, inducing them to sting a glass plate below, on which the venom rapidly dried. The plates were stored on dry ice until return to the laboratory where venom was washed from the plates, centrifuged to remove particulate matter, sterilised by filtration through a 0.22 micron low protein binding filter, lyophilised and weighed to ascertain total peptide/protein content. Venom was redissolved in water for injection to produce a 10mg/ml solution. Aliquots of an amount necessary to formulate pharmaceutical batches were prepared and the solutions stored at  $-80^{\circ}\text{C}$  until required.

Ants destined for venom sac dissection were transferred live to the laboratory where they were snap frozen and stored at  $-80^{\circ}\text{C}$ . Later the ants were defrosted and under aseptic conditions their venom sacs were removed using a dissecting microscope and placed in sterile pyrogen-free polypropylene tubes containing water for injection BP. Collections of dissected sacs were kept at  $0-4^{\circ}\text{C}$  for no more than 1 hour before being refrozen to  $-80^{\circ}\text{C}$ . When at least 3000 sacs had been dissected, the collections were defrosted; sacs ruptured using a tissue homogeniser and then processed in the same way as electrostimulation venom. Venom collected by electrostimulation was not mixed with venom collected via sac dissection at any time.

Fresh samples prepared by both electrostimulation and sac dissection were used for HPLC-MS analyses, as were lyophilised samples prepared for therapeutic use. For the latter, 10mg/ml venom aliquots were removed from the  $-80^{\circ}\text{C}$  freezer, mixed with mannitol and dispensed into 50 or 120  $\mu\text{g}$  aliquots (each containing 20mg mannitol). These aliquots were then lyophilised, vacuum-sealed and stored at  $-8^{\circ}\text{C}$ .

## **HPLC-MS**

HPLC analyses were carried out on a Waters Alliance 2690 HPLC, using an Agilent Zorbax 300SB-C3 5 $\mu$ m column (2.1mm x 150mm). The solvent system was; Solvent A: acetonitrile, Solvent B: 1% trifluoroacetic acid, Solvent C: water. The flow rate was 0.3ml/min. A linear gradient from 10% A, 8% B, 82% C to 70% A, 8% B, 22% C at 30 min was used in most cases. A slower gradient from 15% A, 8% B, 77% C to 50% A, 8% B 43% C at 35 min was used to facilitate specific separations. This was followed by a linear gradient to 92% A, 8% B at 45 minutes to flush the column.

The effluent from the HPLC was directly coupled to a Finnigan LCQ equipped with a standard electrospray source. The needle Voltage was 5KV, the capillary temperature was 250°C, sheath gas was 70psi and auxiliary gas was 20psi. The range from  $m/z$  100 to 2000 was typically monitored.

The LC effluent was routinely ‘supercharged’ by post-column addition of 5 ul per minute of a 20% glycerol solution (Iavarone et al., 2001). This dramatically increased the abundance of higher charge states, making it less likely that larger peptides might fall outside the  $m/z$  range of the LCQ, and also significantly increased overall sensitivity. Charge states of all key ions were confirmed by higher resolution “zoom scans”, using peptides collected by HPLC and subsequent direct infusion still in the collection solvent into the electrospray source at 5 ul per minute.

## **HPLC-UV and Peptide Purification**

HPLC-UV profiles of venom samples were obtained using a Waters 996 diode array UV-Vis detector, monitoring wavelengths from 210 to 290 nm. Chromatograms at 220nm and 280nm were generated from this data. Venom peptides were collected from the HPLC effluent (while monitoring the absorbance at 220nm) for subsequent reduction, Edman sequencing, trypsin digestion and Q-TOF MS analyses. These fractions were stored at -50°C immediately after collection, and evaporated to dryness at 30°C under a stream of pure dry nitrogen before use.

## **Reduction and alkylation protocol**

Reduction and alkylation protocols were carried as per Street *et al*, 1996. Whole venom and collected HPLC fractions were reduced by incubation for 30 min at 37°C

in a solution of ~100µl of reduction reagent (10mM Tris-HCL pH 8.0, 1mM di-Na EDTA, 10mM dithiothreitol, 8M urea). Reduced venom fractions were then carboxyamidomethylated with iodoacetamide to protect the cysteine thiol groups and to readily identify the number of cysteines per peptide chain by mass difference. This was done by mixing an aliquot of the reduced venom fraction with 0.25 volumes of 0.25M iodoacetamide in 0.25M Tris-HCl at pH 8.0.

### **Edman Sequencing**

Full and partial Edman sequencing of isolated peptides was undertaken at the Australian Proteome Analysis Facility.

### **Trypsin Digestion**

Venom fractions from HPLC were evaporated to dryness and reconstituted in 25mM ammonium bicarbonate. Trypsin (sequencing grade, Sigma Chemicals) was added at approximately one fiftieth of the amount of the venom peptide, and the samples were incubated at 37°C for 4 hours with occasional shaking, and either immediately analysed by HPLC-MS or stored at -50°C until analysis.

### **Tandem Mass Spectrometry**

Structural information on the peptides was obtained by ion trap tandem mass spectrometry, using a default collision energy of 45%. Molecular clusters of various charge states for the main peptides were targeted to gain maximum structural information. Microsoft Excel spreadsheets of  $m/z$  values of the various charge states of predicted key peptide ions based on published structures were generated to facilitate interpretation of the data. Additional data was obtained by Q-TOF MS analyses undertaken on a Micromass instrument at the Australian Proteome Analysis Facility.

## Results:

Venom samples from several different batches (either different batches of venom to be used therapeutically or samples that were taken immediately following venom collection) were analysed, and all gave broadly similar peptide profiles. More than fifty peptides were readily detected, all in the 4kDa to 9kDa range. The venom samples were dominated by just a few peptides, and a typical chromatogram is shown in Figure 2. Good correlation between LC-UV data and LC-MS data indicated that the majority of the peptides were apparently detected in the LC-MS runs.

From deconvolution of electrospray spectra both with and without ‘supercharging’, the main peptide in both fresh and stored samples (Peak B in Fig. 2) was found to have a molecular weight of ~5608 Da (unless otherwise specified, molecular weights listed refer to the averaged rather than monoisotopic weights); from here on for convenience this peptide is referred to as ‘pilosulin 3’. Direct MS/MS data of pilosulin 3 was beyond direct sequencing due to its expected complexity, and no immediate correlation with the published major peptides was apparent. However, on the theory that this major peptide might contain the previously reported pilosulin 2 as a disulfide complex, the  $y$  ions (Roepstorff and Fohlman, 1984) were calculated for the neutral losses from the N-terminal end of pilosulin 2 from such a complex. These ions were observed for the first 10 residues from the N-terminus of pilosulin 2 (Fig 3), initially supporting the presence of this known peptide. Reduction of pilosulin 3 resulted in peptides of ~3155 and ~2457 Da, confirming it as a heterodimeric peptide. However, the predicted peptides were ~3212 Da (pilosulin 2) and either ~2400 or ~2398 Da (depending on the number of disulfide bonds) if pilosulin 3 consisted of pilosulin 2 disulfide bonded to a smaller peptide. The 4 Da difference between the sum of the molecular weights of the reduced peptides and the molecular weight of the original dimer nevertheless indicated the presence of two disulfide bonds.

Carboxyamidomethylation of the reduced peptides also supported this interpretation, with two cysteines indicated on each peptide by the shift of 114 Da from ~3155 to ~3269 and from ~2457 to ~2571 Da. Detailed MS/MS investigation of the ~3155 Da peptide, which by mass was approximately one glycine residue short of pilosulin 2, indicated that it was indeed consistent by direct MS sequence data to pilosulin 2 minus the C-terminal glycine (‘des-Gly<sup>27</sup>-pilosulin 2’, Myr p 2 49→74, see Fig. 4).

MS/MS data from several charge states of carboxyamidomethylated des-Gly<sup>27</sup>-pilosulin 2 at ~3269 Da also strongly supported this assignment.

The 2457 Da peptide (hereafter referred to as 'pilosulin 3b') was subsequently isolated by HPLC as its carboxyamidomethylated derivative and fully sequenced by N-terminal Edman degradation. This resulted in the unequivocal 23 amino acid sequence,

L I G L V S K G T C V L V K T V C K K V L K Q

which was unrelated to either Myr p 1 or Myr p 2. Evidence from both Q-TOF and ion trap tandem MS of this peptide strongly supported this sequence, and also indicated that the C-terminus was amidated, resulting in a calculated mass of 2457.3 Da. Fig 5 shows the ion trap MS/MS data from the 2+ ion of carboxyamidomethylated pilosulin 3b, with assignments consistent with the Edman data. The expected mass shifts were also observed for appropriate product ions between raw pilosulin 3b and its carboxyamidomethylated derivative.

The unique disulfide bridged peptides that would result from trypsin digestion were calculated for pilosulin 3 for both parallel and antiparallel chain alignment; predicted peptides for the former were 767.4 and 1066.5 Da (monoisotopic weights) and for the latter 797.4 and 1036.5 Da (monoisotopic weights). A trypsin digest of pilosulin 3 resulted in a very prominent peptide with an  $[M+2H]^{2+}$  ion at  $m/z$  519.2 with an isotope pattern that strongly supported the presence of two cysteine residues. This was consistent with the 1036.5 Da disulfide bridged dimer;



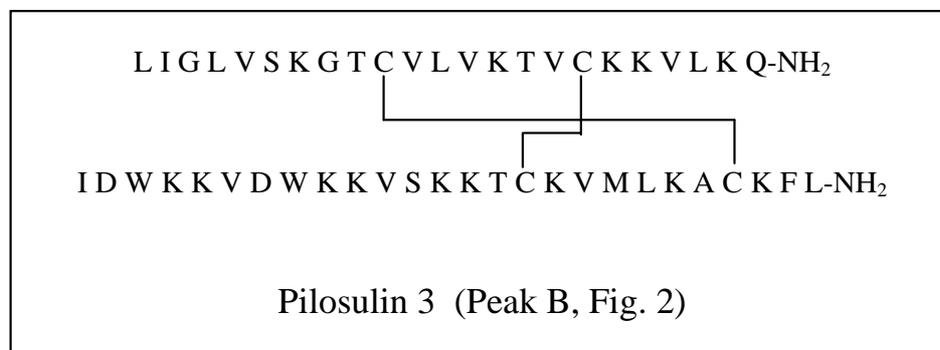
and indicated that pilosulin 3 consisted of an antiparallel alignment of the des-Gly<sup>27</sup>-pilosulin 2 and pilosulin 3b chains. MS/MS data from  $m/z$  519.2 was consistent with the expected peptide, with many ions directly assignable to this trypsin fragment diagnostic for antiparallel alignment (Fig 6).

Trypsin cleavage products of native pilosulin 3 also supported the des-Gly<sup>27</sup>-pilosulin 2 sequence, with strong singly charged ions (monoisotopic values) at  $m/z$  561

(residues 1→4), 490 (residues 18→21) and  $m/z$  278 (putative residues 25→26). The latter was supported by residue specific MS/MS product ions at  $m/z$  120 (from phenylalanine) and  $m/z$  86 (leucine immonium ion); however, this implied C-terminal amidation of the des-Gly<sup>27</sup>-pilosulin 2 sequence since a Phe-Leu dipeptide would produce an  $[M+H]^+$  ion at  $m/z$  279. There were also weak  $y_3$ ,  $y_4$ ,  $y_5$  and  $y_6$  ions at  $m/z$  406, 509, 580 and 708 (monoisotopic values) from the non-alkylated des-Gly<sup>27</sup>-pilosulin 2 obtained by reduction, and  $y_4$  and  $y_5$  ions for its alkylated version at  $m/z$  566 and 637 (monoisotopic values). The predicted monoisotopic  $m/z$  values for these without C-terminal amidation of the leucine at position 26 are 407, 510, 581 and 709 for the non-alkylated form and 567 and 638 for the two observed ions from the alkylated form. The calculated mass of des-Gly<sup>27</sup>-pilosulin 2 with C-terminal amidation is 3153.9 Da, and the calculated mass of pilosulin 3 is then 5607.2 Da.

Peak C in Figure 2 gave MS data indistinguishable from pilosulin 3 by ion trap MS, with the same molecular weight and all directly observable sequence data was identical. Reduction of whole venom resulted in two peaks at ~2457 Da in the same proportions as peaks B and C, but only one peak at ~3155 Da, indicating that peak C contains an isomass variant of pilosulin 3b.

Another variant of pilosulin 3 had a molecular weight of ~5667 Da. This coeluted with pilosulin 3 and from reduction was found to be comprised of des-Gly<sup>27</sup>-pilosulin 2 and a ~ 2514 Da variant of pilosulin 3b which also coeluted with it. Tandem MS data indicated this to have an additional C-terminal glycine. This variant was typically about 20% of the abundance of pilosulin 3.



A peak consistent in mass with pilosulin 1 was directly detected in most venom samples as a relatively minor component. Peak D in Figure 2 indicated a peptide of ~6052 Da, and while well beyond full direct sequencing by MS, sequence data observed from several charge states was consistent with the published sequence of pilosulin 1.

There was also strong supporting evidence for Myr p 1 65→112 as a minor component in all venom samples analysed, and Myr p 1 subsequences starting from position 63 right through to starting from position 72 (all terminating at position 112) were detected in partially degraded venom samples. Several of these corresponded to previously reported peptides (Donovan et al., 1996).

However, pilosulin 1 was observed in all samples mainly as a ~6067 Da variant (Peak E in Figure 2). MS/MS sequence data indicated a very high degree of homology with pilosulin 1, for which all 'b' type ions from  $b_{23}$  to  $b_{54}$  were observed. Given the relative lack of mass accuracy at these values on the ion trap instrument used, initially oxidation of a methionine (+16 Da) or post-translational methylation of a lysine (+14 Da) were considered as possibilities for this peptide, although the significantly later retention time made the former explanation less likely. Direct MS/MS data was only sufficient to indicate that any differences occurred between residues 1 and 22, as all  $b$  ions from  $b_{23}$  to  $b_{54}$  were shifted by ~14 Da relative to pilosulin 1. The presence of three arginine residues in this region may have inhibited the formation of suitable diagnostic ions (Tang et al., 1993; Dongre, 1996). Subsequences starting from position 7 of pilosulin 1, as described above, were also observed in a slightly degraded stored sample that had initially had no pilosulin 1. If these were derived from the 6067 Da peptide, this indicated the difference of the variant from pilosulin 1 was in the first 6 residues. This made post-translational modification a less likely explanation due to the reported residues in these positions (Gly-Leu-Gly-Ser-Val-Phe), so this peptide was investigated further as a genuine variant of pilosulin 1. Trypsin digestion resulted in a prominent peptide at 805.4 Da (monoisotopic weight) rather than at 791.4 (monoisotopic weight) as required for the pilosulin 1 trypsin digest fragment [residues 1-8], also confirming the difference was in the first 8 residues. First principles MS/MS interpretation on this indicated a leucine or isoleucine at position 5 in place of the valine in pilosulin 1, since all  $a$ ,  $b$

and *c* type ions (Roepstorff and Fohlman, 1984) numbered from 5 onwards were 14 Da higher than for the analogous pilosulin 1 trypsin digest peptide. Similarly *y* and *z* ions numbered from 4 onwards were 14 Da higher.

Partial Edman sequencing (10 cycles) of the purified 6067 Da pilosulin 1 variant confirmed isoleucine in place of valine at position 5 ('[Ile<sup>5</sup>]pilosulin 1'), consistent with the ~14 Da difference in molecular weights and with the MS/MS data. Neither variant of pilosulin 1 showed any significant absorbance at 280nm by LC-UV analysis, consistent with an absence of aromatic amino acid residues.

A minor homologous peptide at ~6082 Da eluting just before [Ile<sup>5</sup>]pilosulin 1 was assigned as an oxidized form of [Ile<sup>5</sup>]pilosulin 1, due to its increased abundance in some stored samples.

Peak A in Figure 2 had a molecular weight of ~ 5545 Da and was unaltered by reduction, indicating it to be a monomer. It showed some absorbance at 280nm, indicating it contained more aromatic amino acid residues than pilosulin 1. No peptide of this weight could be derived from subsequences of Myr p 2 and MS/MS data did not show any relationship between it and any of the hypothetical subsequences of Myr p 1 or its [Ile<sup>5</sup>] pilosulin 1 variant that were near this weight. This remains to be confirmed as amino acid sequences of this and all other minor peptides remain to be determined.

To investigate the possibility of genetic variation contributing to our findings using Tasmanian venoms, a lyophilised internal reference sample produced by the Commonwealth Serum Laboratories from Victorian specimens by venom sac dissection in the early 1990s (provided courtesy of Dr Brian Baldo) was also analysed. This was found to contain the same range of major peptides, with the [Ile<sup>5</sup>] variant also more abundant than pilosulin 1.

## **Discussion:**

This work has confirmed previous theories that the major peptide in jack jumper venom is a disulfide complex. We have found this complex to be comprised of a previously unreported subsequence from Myr p 2 and a novel smaller peptide with C-terminal amidation. N-terminal sequencing has previously been used to identify Myr p 1 and Myr p 2 derived polypeptides (Donovan et al., 1996). The 8 cycles used would have been insufficient to detect the lack of a glycine residue, as predicted from the known cDNA sequence, at the C-terminal of des-Gly<sup>27</sup>-pilosulin 2.

Although most allergic sera appear to bind to peptides derived from Myr p 1 and Myr p 2, Radioallergosorbent Test (RAST) studies using these synthetic peptides have indicated that a proportion of sera that recognise native venom do not recognise synthetically prepared peptides (Dr. Brian Baldo, personal communication). These findings might be explained by inaccuracies in our current understanding of peptide structure in the native venom. We initially postulated that IgE reacting to synthetic Myr p 2 subsequences recognised parts “visible” to IgE from within pilosulin 3, as Myr p 2 49→74 is the only form to date that we have recognized any Myr p 2 subsequence in the native venom (existing in pilosulin 3 and its variants). However, since completing our study, we have been made aware that the heterodimer that we describe here as pilosulin 3 had been previously identified in unpublished work (Dr Brian Baldo and Professor Paul Alewood, personal communication) which also found relatively low IgE recognition of a synthetic version of the heterodimer, despite recognition of Myr p 2 (Dr Brian Baldo, personal communication; Dr Qi Xuan Wu, 2001, including original RAST data from 67 of our Tasmanian patients). This presents an interesting problem, given that we have so far been unable to identify Myr p 2 in any form other than these closely related heterodimers. Amino acid sequence data from the majority of minor peptides present in venom is still lacking, and the possibility that other subsequences of Myr p 2 are present in venom as minor abundance peptides cannot be excluded. Therefore we believe that further IgE-binding studies are required, and one method would be to use gel-separated native venom components for which MS has confirmed the identity of the peptides comprising each band.

We also found that pilosulin 1 is a relatively minor constituent of the venom as its published sequence and that a minor variant of it exists in much greater abundance. This is not surprising, as the presence of numerous isoforms of venom peptides has been reported before in ants. The tropical ant *Pseudomyrmex triplarinus* has a complex venom that includes at least 6 myrmexin isoforms, ranging from 6998 to 7142 Da. (Pan and Hink, 2000). These all appeared within one band on SDS-PAGE (Hink et al., 1994). Ponericins from *Pachycondyla goeldii* venom (Orivel et al., 2001) have also been shown to exist as one or more closely related isoforms. Pilosulin 1 and its [Ile<sup>5</sup>] variant would migrate as a single band on SDS-PAGE due to their similar molecular weights. In addition, their calculated isoelectric points are identical (pI=10.45). A similar scenario, where the molecular weights are similar and the isoelectric points identical (pI=9.94), exists for pilosulin 2 and des-Gly<sup>27</sup>-pilosulin 2. Neither isoelectric focusing and/or 2D SDS-PAGE would be likely to be useful in these separations, and this underlines the importance of HPLC in the separation of such closely related peptides.

The possibility of genetic variation explaining the apparent difference from published data was considered. We analysed venom samples from several different sources within Tasmania and an earlier reference sample from Victoria that was used by previous investigators. All gave very similar peptide profiles, with the exception that in some samples, pilosulin 1 was only detected as the [Ile<sup>5</sup>] variant. There was no obvious difference between venom samples obtained by electrostimulation and those obtained by venom sac dissection. Donovan *et al* (1994) have determined that residues 93 →106 of Myr p 1 contain the IgE binding determinant(s). Given this, and the subtle differences between [Ile<sup>5</sup>]pilosulin 1 and pilosulin 1 with respect to primary amino acid structure, it is unlikely that the IgE binding capacity of these two variants of pilosulin 1 will differ greatly. Nevertheless this must be confirmed by further research.

Direct examination of *M. pilosula* venom peptides by HPLC-MS has resulted in the identification of the major expressed forms of allergenic peptides. Detailed tandem mass spectrometry analysis has confirmed the presence of Myr p 1 and Myr p 2 subsequences within the venom, and has also enabled the identification of post-translational modifications of the allergenic peptides. Detailed stability studies of *M.*

*pilosula* venom extract produced for human immunotherapy can now be performed with a clearer understanding of the peptides involved. However, further research into the binding affinity of venom-specific IgE for the various native venom components, including pilosulin 3 and its variants, is required. This will enable firm “pass fail” criteria to be drawn up for diagnostic and therapeutic venom extracts. The possibility of co-existing subspecies of *M. pilosula* being responsible for the various isoforms also requires further exploration. If significant differences in IgE binding are identified, this knowledge will ensure that a consistent mix of venom allergens is produced from representative sub-species. It is now apparent that to conform to established IUIS guidelines (King et al., 1995) a revision of the nomenclature of allergenic components of *M. pilosula* venom may be required.

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## Figure Captions:

### Figure 1

Published Myr p 1 and Myr p 2 (cDNA-derived) sequences, and previously proposed subsequences in expressed jack jumper venom. Pilosulin 1 and pilosulin 2 sequences are in bold.

### Figure 2

Typical *Myrmecia pilosula* venom HPLC-MS Total Ion Chromatogram

**A** = ~5545 Da monomeric peptide (unidentified)

**B** = 'pilosulin 3', ~5608 Da bis-disulfide bonded, antiparallel aligned heterodimer, consisting of des-Gly<sup>27</sup>-pilosulin 2 plus a novel 2457 Da peptide ('pilosulin 3b'), each with C-terminal amidation.

**C** = variant of pilosulin 3

**D** = pilosulin 1 (~6052 Da)

**E** = [Ile<sup>5</sup>]pilosulin 1 (~6067 Da)

### Figure 3

Expanded regions of ion trap MS/MS product ions of the  $[M+4H]^{4+}$  ion at  $m/z$  1403 from the ~5608 Da dimer. *Y* and *B* represent ions from the putative pilosulin 2 chain,  $y_{23}$  represents the intact smaller peptide. Final assignments of amino acid number for the  $Y_{m}y_n$  ions were based on data from reduced venom (see Results).

### Figure 4

Ion trap product ion spectrum from the  $[M+3H]^{3+}$  ion at  $m/z$  1053 of the larger peptide (~3155 Da) obtained after reduction of peak B ('pilosulin 3'). This was ~57 Da lower than expected for pilosulin 2; interpretation of the tandem MS data from first principles and trypsin digest peptides indicated that it was consistent with des-Gly<sup>27</sup>-pilosulin 2 with C-terminal amidation, for which some assignments are shown.

### Figure 5

Ion trap MS/MS product ions from the  $[M+2H]^{2+}$  ion at  $m/z$  1286 of the carboxymethylated ~2457 Da novel peptide ('pilosulin 3b') obtained from reduction of pilosulin 3. Edman sequencing and Q-TOF MS/MS indicated this to be;

L I G L V S K G T C V L V K T V C K K V L K Q-NH<sub>2</sub>.

Individual ion assignments are derived from this.

### Figure 6

Ion trap product ions and their assignments from the diagnostic trypsin cleavage product:



from pilosulin 3 giving unequivocal data on the alignment of the two chains . Capital letters represent peptide sequences originating from **ACK** derived from des-Gly<sup>27</sup>-pilosulin 2, lower case represents sequences originating from **GTCVLVK** derived from pilosulin 3b.

## Figure 1

### A

#### Myr p 1

				5					10					15					20
Met	Lys	Leu	Ser	Cys	Leu	Leu	Leu	Thr	Leu	Ala	Ile	Ile	Phe	Val	Leu	Thr	Ile	Val	His
				25					30					35					40
Ala	Pro	Asn	Val	Glu	Ala	Lys	Asp	Leu	Ala	Asp	Pro	Glu	Ser	Glu	Ala	Val	Gly	Phe	Ala
				45					50					55					60
Asp	Ala	Phe	Gly	Glu	Ala	Asp	Ala	Val	Gly	Glu	Ala	Asp	Pro	Asn	Ala	<b>Gly</b>	<b>Leu</b>	<b>Gly</b>	<b>Ser</b>
				65					70					75					
<b>Val</b>	<b>Phe</b>	<b>Gly</b>	<b>Arg</b>	<b>Leu</b>	<b>Ala</b>	<b>Arg</b>	<b>Ile</b>	<b>Leu</b>	<b>Gly</b>	<b>Arg</b>	<b>Val</b>	<b>Ile</b>	<b>Pro</b>	<b>Lys</b>	<b>Val</b>	<b>Ala</b>	<b>Lys</b>	<b>Lys</b>	<b>Leu</b>
				85					90					95					100
<b>Gly</b>	<b>Pro</b>	<b>Lys</b>	<b>Val</b>	<b>Ala</b>	<b>Lys</b>	<b>Val</b>	<b>Leu</b>	<b>Pro</b>	<b>Lys</b>	<b>Val</b>	<b>Met</b>	<b>Lys</b>	<b>Glu</b>	<b>Ala</b>	<b>Ile</b>	<b>Pro</b>	<b>Met</b>	<b>Ala</b>	<b>Val</b>
				105					110										
<b>Glu</b>	<b>Met</b>	<b>Ala</b>	<b>Lys</b>	<b>Ser</b>	<b>Gln</b>	<b>Glu</b>	<b>Glu</b>	<b>Gln</b>	<b>Gln</b>	<b>Pro</b>	<b>Gln</b>								

### B

#### Myr p 2

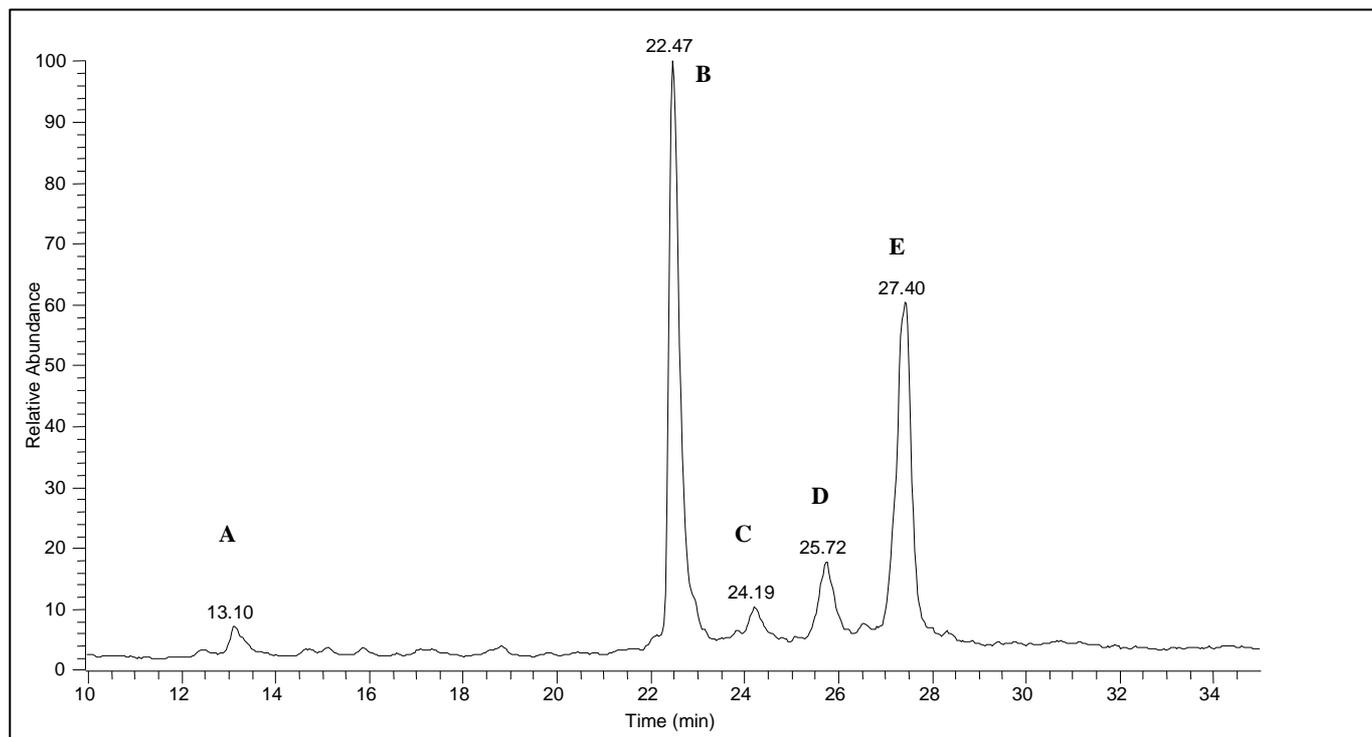
				5					10					15					20
Met	Lys	Leu	Ser	Cys	Leu	Leu	Leu	Thr	Leu	Ala	Ile	Ile	Phe	Val	Leu	Thr	Ile	Val	His
				25					30					35					40
Ala	Pro	Asn	Val	Glu	Ala	Lys	Ala	Leu	Ala	Asp	Pro	Glu	Ser	Asp	Ala	Val	Gly	Phe	Ala
				45					50					55					60
Asp	Ala	Val	Gly	Glu	Ala	Asp	Pro	<b>Ile</b>	<b>Asp</b>	<b>Trp</b>	<b>Lys</b>	<b>Lys</b>	<b>Val</b>	<b>Asp</b>	<b>Trp</b>	<b>Lys</b>	<b>Lys</b>	<b>Val</b>	<b>Ser</b>
				65					70					75					
<b>Lys</b>	<b>Lys</b>	<b>Thr</b>	<b>Cys</b>	<b>Lys</b>	<b>Val</b>	<b>Met</b>	<b>Leu</b>	<b>Lys</b>	<b>Ala</b>	<b>Cys</b>	<b>Lys</b>	<b>Phe</b>	<b>Leu</b>	<b>Gly</b>					

### C

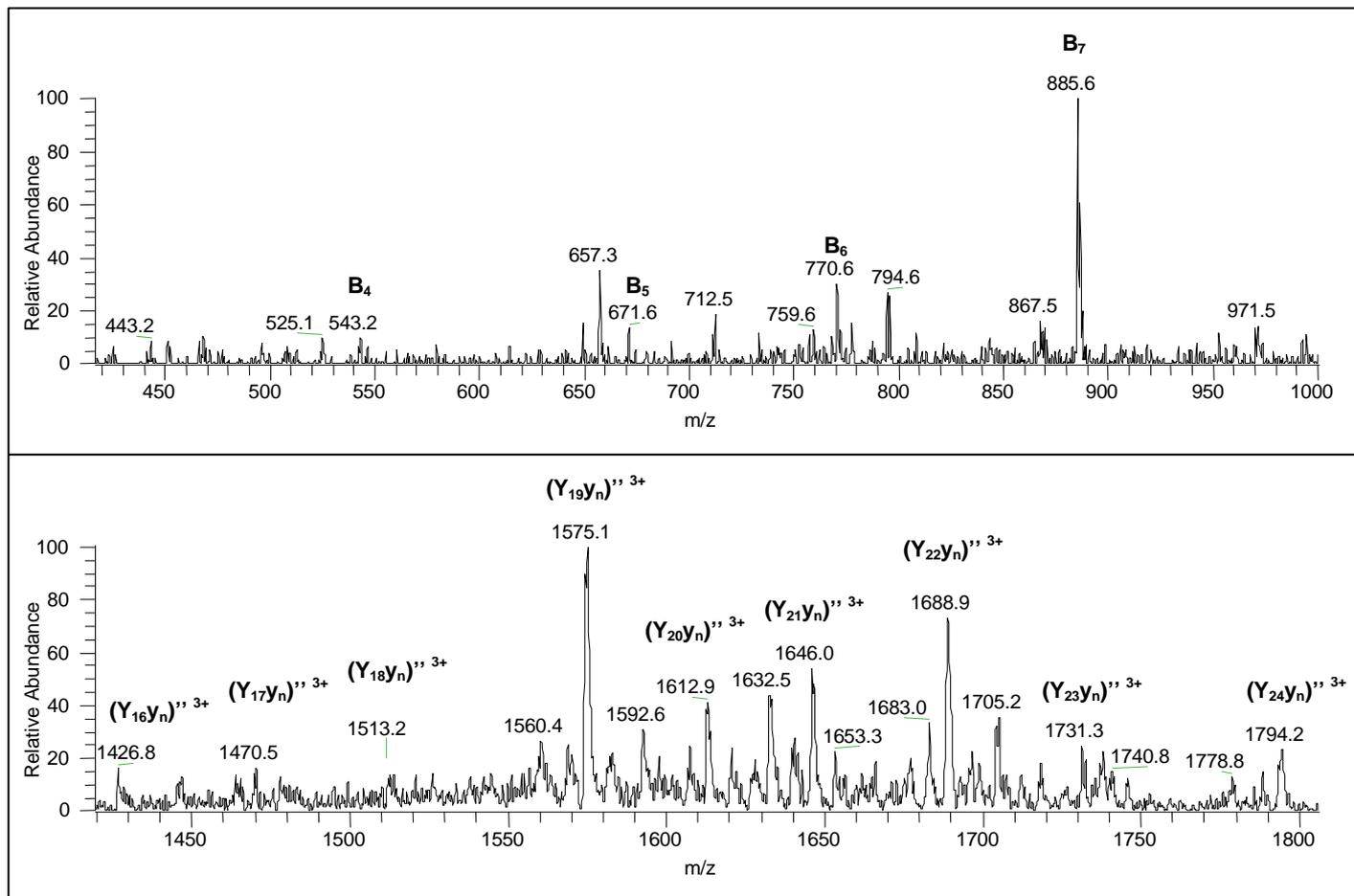
#### Isotopic Average Mol Wt

<b>Myr p 1 57 → 112 (pilosulin 1)</b>	<b>6052</b>
<b>Myr p 2 49 → 75 (pilosulin 2)</b>	<b>3212</b>
Myr p 1 68 → 112	4938
Myr p 1 65 → 112	5279
Myr p 1 71 → 112	4655
Myr p 1 86 → 112	3069

**Figure 2**



**Figure 3**



**Figure 4**

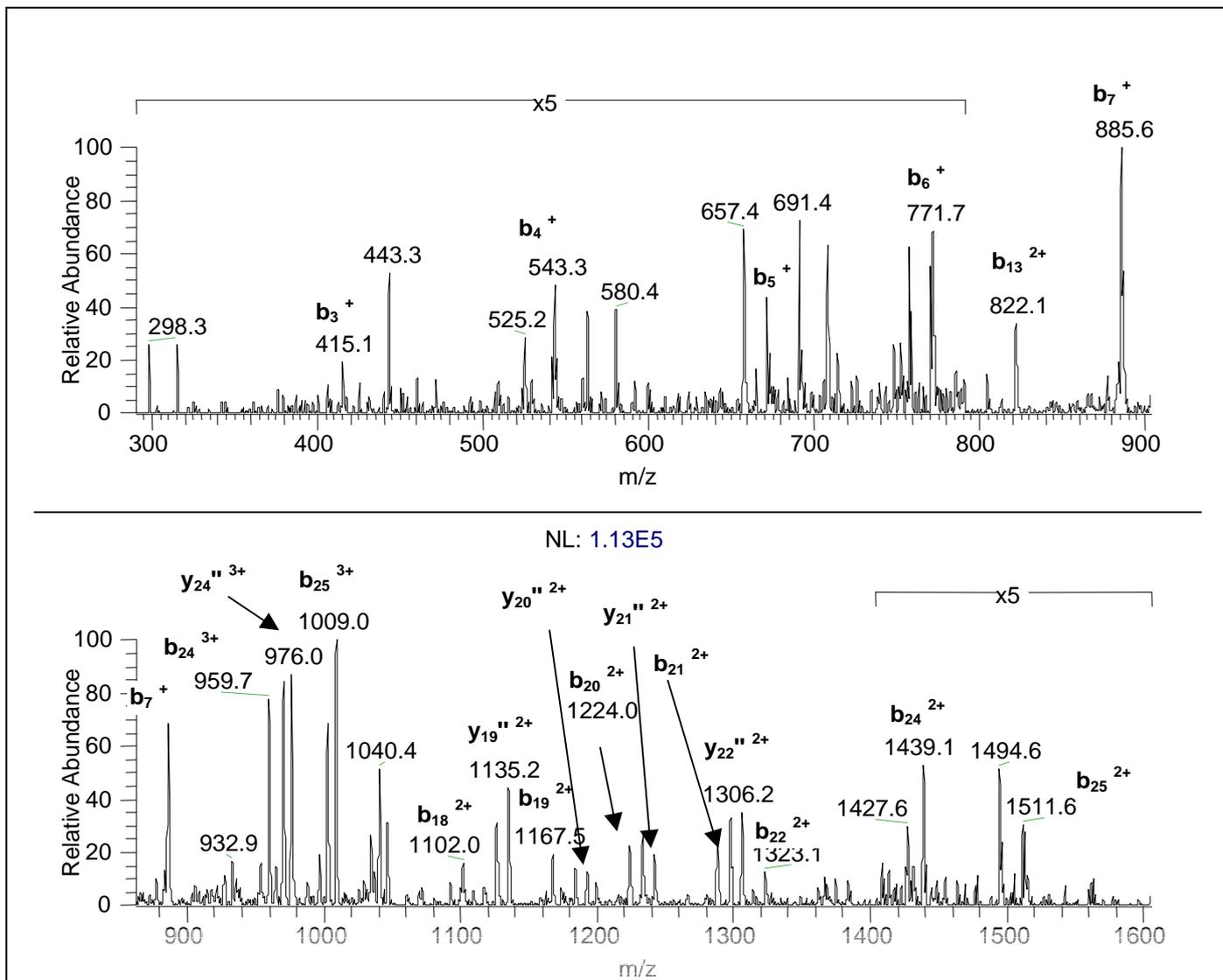
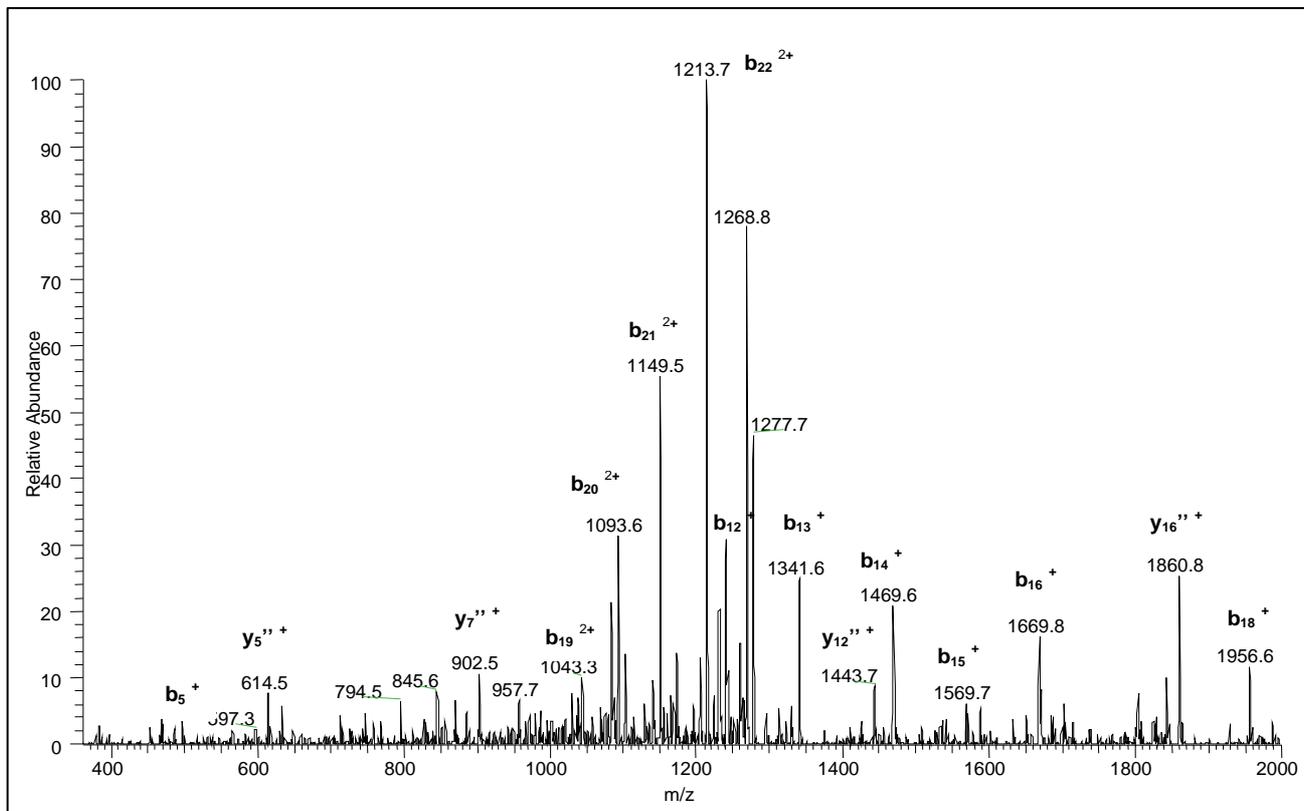
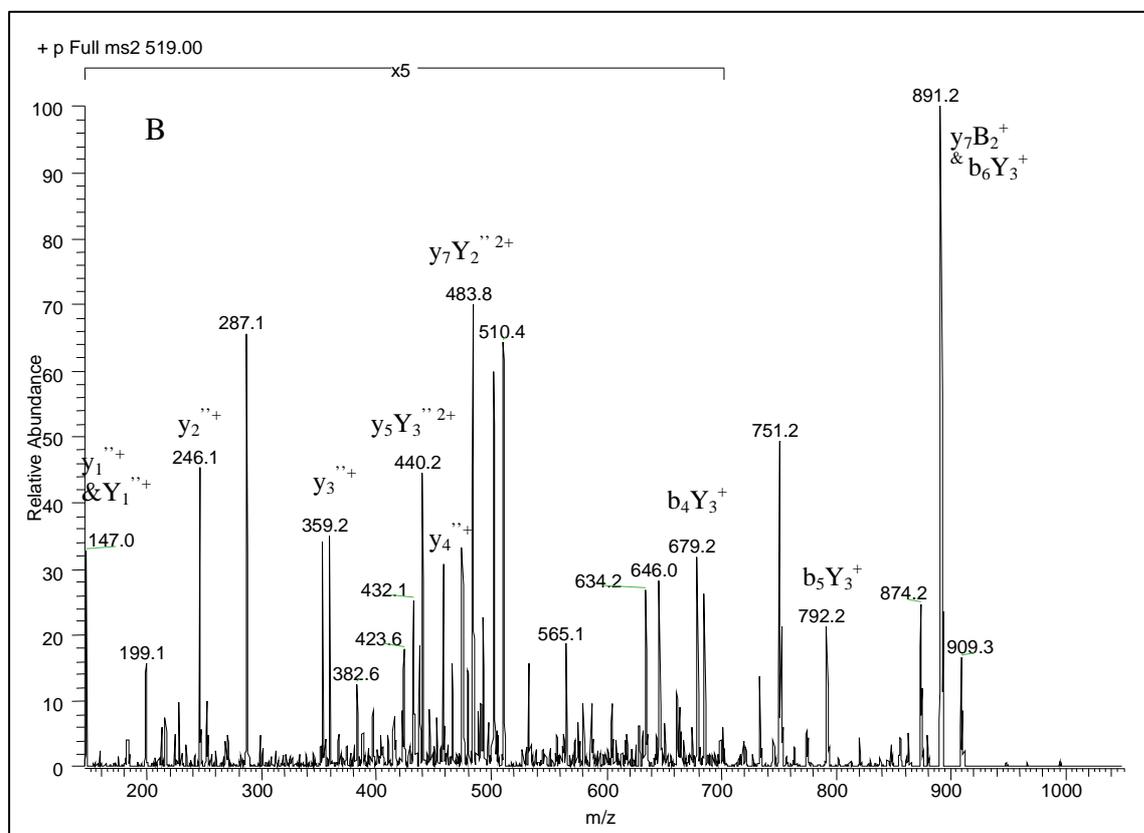


Figure 5



**Figure 6**



**Table 1**

Molecular weights of peptides easily observed in *Myrmecia pilosula* venom by LC-MS. Based on observations from dozens of venom samples, the more abundant peptides are in bold, with these typically accounting for at least 80% of the peptide total ion chromatogram.

<b>Molecular Weight (daltons)</b>	<b>Retention Time</b>	<b>Identity</b>
~4938	18.5	Myr p 1 68→112
~5089	13.1	
~5104	12.5	
~5166	18.5	
~5279	19.0	Myr p 1 65→112
~5467	22.0	
<b>~5545 (Peak A, Fig 2)</b>	13.1	Unidentified monomer
<b>~5608 (Peak B, Fig 2)</b>	22.5	'pilosulin 3' - antiparallel aligned heterodimer of des-Gly <sup>27</sup> -pilosulin 2 (Myr p 2 49 → 74) and a novel 2457 Da peptide ('pilosulin 3b'). Both peptides are C-terminally amidated
<b>~5608 (Peak C, Fig 2)</b>	24.2	variant of pilosulin 3
<b>~5667</b>	22.5	dimer of of des-Gly <sup>27</sup> -pilosulin 2 and a novel ~2514 Da peptide; coelutes with peak B
~5725	22.2	
~5835	16.6	
~5925	15.5	
~6016	17.4	
~6032	14.7	
<b>~6052 (Peak D, Fig.2)</b>	25.7	pilosulin 1 (Myr p 1 57 → 112)
<b>~6067 (Peak E, Fig. 2)</b>	27.4	[Ile <sup>5</sup> ]pilosulin 1
~6082	26.7	oxidized [Ile <sup>5</sup> ]pilosulin 1
~6305	21.5	
~6639	12.5	
~8198	23.4	
~8546	28.4	