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A biochemical characterization of the major peptides from the Venom of the giant Neotropical hunting ant *Dinoponera australis*

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ABSTRACT

Venom from the "false tocandira" *Dinoponera australis*, a giant Neotropical hunting ant, paralyzes small invertebrate prey and induces a myriad of systemic effects in large vertebrates. HPLC/DAD/MS analyses revealed that the venom has over 75 unique proteinaceous components with a large diversity of properties ranging in size, hydrophobicity, and overall abundance. The six most abundant peptides, demonstrative of this diversity and hereafter referred to as *Dinoponeratoxins*, were *de novo* sequenced by exact mass precursor ion selection and Edman degradation. The smallest peptide characterized, Da-1039, is hydrophilic and has similarities to vasoactive peptides like kinin and bombesin. The two largest and most abundant peptides, Da-3105 and Da-3177, have a 92.9% identity in a 28 residue overlap and share ~50 of their sequence with ponericin G2 (an antimicrobial from another ponerine ant *Pachycondyla goeldii*). One peptide, Da-1585, is a hydrophilic cleavage product of an amphipathic peptide, Da-2501. The most hydrophobic peptide, Da-1837, is amidated (a PTM observed in one half of the major peptides) and shares homology with poneratoxin, a sodium channel modifier found in the bullet ant *Paraponera clavata*. This study is the first examination of potential pharmacophores from venom of the genus *Dinoponera* (Order: Hymenoptera).

1. Introduction

The stinging insects of the order Hymenoptera include a number of medically important families: *Apoidea* (bees with ~20,000 species), *Vespoidea* (wasps, hornets and yellow jackets with ~15,000 species) and *Formicidae* (ants with ~15,000 species) (Fitzgerald and Flood, 2006). Utilizing a modified ovipositor (sting) at the terminal end of the abdomen, hymenoptera envenomation produces a variety of clinical and histological manifestations from mild irritation to life-threatening anaphylaxis (Steen et al., 2005). Although venom from members of the family *Formicidae* are understudied relative to bees and wasps, work on a few ant species has yielded many compounds with a variety of novel and potent activities. Fifteen novel peptides have been discovered in the arboreal ponerine ant *Pachycondyla goeldii* (subfamily Ponerinae) that show diverse and selective antibacterial spectrum (Orivel et al., 2001). Antimicrobial peptides from the genus *Myrmecia* (subfamily Myrmeciinae), known as pilosulins, are currently under investigation as a model for the "intelligent" optimization of an antimicrobial peptide through the synthesis of computer based analogs (Zelezetsky et al.,

Abbreviations: PTM, post-translational modification; HPLC, high performance liquid chromatography; MS, mass spectrometry; FMOC, 9-fluorenylmethoxycarbonyl; DAD, diode array detector; BLAST, basic local assignment search tool; APD, antimicrobial peptide database.

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2005). In addition to their potential as antimicrobials, ion channel modification has been observed in two peptides from ant venom: poneratoxin (from *Paraponera clavata*, *subfamily Paraponerinae*) and ectatomin (from *Ectatomma tuberculatum*, *subfamily Ectatomminae*). Poneratoxin, a 25 residue linear peptide, is neurotoxic and interacts with voltage-gated sodium channels (Piek et al., 1991). Ectatomin is a potent inhibitor of calcium currents in rat ventricular myocytes (Pluzhnikov et al., 1999). It is apparent that ant venoms contain many compounds with diverse biological activity as demonstrated for the genus *Pogonomyrmex* (subfamily Myrmicinae) (Schmidt and Blum, 1978a,b,c; Schmidt et al., 1986).

Species in the genus *Dinoponera* (subfamily Ponerinae) include the world's largest ants and occur in tropical and subtropical climates of South America. They are solitary foraging, predatory ants that prey on a diverse group of animals, ranging from insects to small mammals (Haddad Junior et al., 2005). Envenomation from these ants is not commonly reported, but a single sting produces dramatic effects; acute and violent pain, cold sweating, nausea, vomiting, malaise, tachycardia and left axillary's lymphadenopathy (Haddad Junior et al., 2005). Two reports provide some insight into the venom of one false tocandira *Dinoponera grandis*. In the first, several enzyme activities for a variety of Hymenopterans were compared: *D. grandis* exhibited phospholipase A, hyaluronidase, lipase and hemolytic activity (Schmidt et al., 1986). While these activities are pervasive in most aculeate Hymenoptera envenomation, it does not fully explain the variance of effects observed. In the second investigation, comparative studies of the protein composition were performed by SDS-PAGE on a variety of Hymenoptera venom; venom of *D. grandis* was profiled yielding three distinct protein bands with *M*r's ranging from 24,000 to 75,000 (Leluk et al., 1989). Based on the information from these previous studies and in order to further elucidate the venom composition to help explain the mechanism of its toxic action in humans, we have performed a systematic biochemical evaluation of the major peptide components from the venom of one member of these reclusive large ants: *Dinoponera australis*. The striking effect of this venom on humans suggests that these peptides may be bioactive and provide a valuable source of pilot candidates for the generation of novel therapeutics.

2. Materials and methods

2.1. Reagents and chemicals

All the reagents utilized were purchased from ThermoFisher Scientific (Waltham, MA) or Sigma–Aldrich (Saint Louis, MO) as ACS reagent grade or better; LC/MS grade acetonitrile was used for high resolution mass spectrometric determinations. All FMOC-amino-acids, Wang (DVB) and RINK resins used were purchased from AAPPTEC (Louisville, KY).

2.2. Venom collection and preparation

We collected whole colonies of *D. australis* from Parque Nacional Iguazu in the province of Misiones, Argentina and maintained them a humidity range of 70–85%, temperature range of 23–26 °C and fed twice weekly on crickets (*Gryllus abbreviatus* or *Acheta domesticus*) or cockroaches (*Periplaneta americana*) supplemented with both water and sugar water (5% sucrose). Venom sacs were dissected and disrupted into LoBind® centrifuge tubes containing a mixture of 0.10% formic acid:acetonitrile (v/v 9:1), centrifuged at 5000*g* for 3 min. The resultant supernatant was passed through a Microcon® 10,000 NMWL molecular weight filter as per the manufacturer's instructions; the subsequent filtrate was considered fresh samples and the remainder stored at −80 °C until required.

2.3. Peptide modification

2.3.1. Reduction and alkylation

To determine the cysteine content and/or disulphide bridges, solutions of purified peptides were lyophilized to near dryness (<10 μL) and re-constituted in 150 μL of reduction buffer (0.2 M Tris–HCL (pH 8.5)) containing 2.5 mM EDTA and 6 M guainidine-HCl. DTT was added to a final concentration of 15 mM and incubated at 40 °C for 5 h. 20 μl of a 0.5 M iodoacetamide solution was added to give a final concentration of ~0.05 M and incubated for 30 min at 25 °C in the dark.

2.3.2. Acetylation

To discriminate glutamine and lysine, approximately 1 nmol of peptide was reconstituted in 20 μL of 50 mM ammonium bicarbonate. 50 μL of acetylation reagent (20 μL acetic anhydride + 60 μl of methanol) was added and allowed to stand for one hour followed by lyophilization to near dryness. Since this procedure may acetylate cysteine residues, reduction and alkylation was done first.

2.4. Preliminary HPLC–MS analysis

To examine general features of the venom and its components, a 1 μL aliquot of diluted venom was injected into a Waters 2795 Alliance HT High Performance Liquid Chromatograph (HPLC) equipped with a combination Thermo 10 × 4.0 mm 3 μM Hypersil® GOLD guard cartridge coupled with Thermo 150 × 2.1 mm 3 μM Hypersil® GOLD column at a flow rate of 0.150 mL/min; a 2.00 min hold at 90:10 A:B (Mobil Phase A = 0.10% Formic Acid; Mobil Phase B = acetonitrile) followed by a binary mobile phase gradient to 10:90 A:B in 30.00 min. The effluent steam from the chromatographic separation was split 1:100 and coupled to a Waters 996 Photo DAD and a Waters Quattro Ultima Triple Stage Quadrupole (TSQ) mass spectrometer equipped with an electrospray ionization source (ESI) calibrated in (+) ion mode with Met-Arg-Phe-Ala & Apomyoglobin (from horse skeletal muscle) to provide a 0.1 amu mass accuracy for each $[M + nH]^{n+}$ parent ion (m_p^+)

2.5. Accurate mass determinations

An aliquot (50–250 nL) of purified fresh venom was injected into a Waters nanoACQUITY Ultra Performance Liquid Chromatograph (UPLC)® equipped with a Waters

100 mm × 100 μm BEH C18 1.7 μm trap column coupled to a Waters 100 mm × 75 μm BEH130 C_{18} 1.7 μm analytical column at a flow rate of 0.4 μL/min; a 1.00 min hold at 90:10 A:B (Mobil Phase A = 0.10% Formic Acid; Mobil Phase B = acetonitrile) followed by a binary mobile phase gradient to 60:40 A:B in 40.00 min then 15:85 A:B in 2.00 min and hold for 7.00 min providing proper peak shape, separation and reduction of interferences; trapping loading time was 3.00 min at 5.00 μL/min of eluent A:B 95:5. The effluent stream from the chromatographic separation was coupled to a Waters API-US Quadrupole-Time of Flight (Q-ToF) high resolution mass spectrometer (MS) equipped with a nanoLOCK® *z*-spray atmospheric pressure ionization source: all acquisitions were made with lock mass correction using [Glu^1]-Fibrinopeptide B human as a reference. In general, the MS was equipped with a quadrupole analyzer, 200 mm hexapole collision cell, orthogonal acceleration time of flight analyzer (oa-TOF) and chevron microchannel plate 4.0 GHz time to digital converter. To ensure efficient acquisition of co-eluting compounds, all analyses were performed in data directed analysis (DDA™) focused upon only those precursor ions that meet strict selection criteria, i.e. signal intensity, charge state and exact mass, allowing them to be selected preferentially for MS/MS. To maximize ion resolution, all samples were processed for *de novo* sequencing and molecular weight characterization in W-mode (Woptics™); taking advantage of an additional ion mirror that extends the flight path, the resolution (R) was >17,500. The collision energy was mass dependent and was set by algorithms to values between 10 and 40 with a collision cell pressure of $3.9e^{-3}$ mBar. The following MS conditions were routinely employed: Polarity = ES+, Capillary = 3.50 kV, Cone = 45, TOF = 10.15 kV, Pusher Cycle Time 124 μs and Pusher Frequency = 8065 Hz, Source = 125 °C.

2.6. Peptide sequencing by Edman chemistry

To discriminate leucine and isoleucine, manual Edman degradation was performed by modification of the method established byAddona and Clauser (2002). Solutions of purified peptides were lyophilized to near dryness (<5 μL) and re-constituted in 10 μL of a 5% solution of phenylisothiocyanate (PITC) in *n*-heptane and allowed to react for 45 min at 45 °C forming a phenylthiocarbamyl-derivative (PTC-derivative). After the heptane was removed by vacuum centrifugation, cleavage was performed by the addition of 20 μL of TFA and incubation for 10 min at 37 °C producing an anilinothiazolinone (ATZ) derivative. Two extractions with 30 μL of a 1:1 mixture of water and butyl acetate were transferred to the conversion flask that contained 20% acetonitrile in water. The resultant residue was heated and a 25% TFA solution was added to form the more stable phenylthiohydantoin (PTH) derivative. For PTH amino acid determination, a 5 μL aliquot of resultant residue (a 10–100 pmole amount of derivative) was injected into a ThermoFinnigan Surveyor HPLC equipped with a 150 × 2.1 mm NovaPak® phenyl column at a flow rate of 0.300 mL/min; a 2.00 min hold at 95:5 A:B (Mobil Phase A = 70 mM ammonium acetate, pH = 5.1; Mobil Phase B = acetonitrile) followed by a binary mobile phase gradient to 20:80 A:B in 25.00 min. The effluent steam from the chromatographic separation was coupled to a Thermo-Finnigan Surveyor PDA monitoring at two wavelengths: 254 and 269 nM. All 20 PTH amino acids exhibited baseline separation and R_t was used for qualitative identification.

2.7. Peptide synthesis

The primary structure of a peptide was authenticated by solid phase peptide synthesis methods using Fmoc chemistry. A Wang resin (0.3 mM/g; 1% DVB) preloaded with the C-termini Fmoc amino acid was used as a support; if the terminal amino acid was amidated, a Rink resin (0.63 mM/g) was used. Deprotection of the resin was accomplished by pretreating with ~20% piperidine to remove the base labile N-alpha protection (Fmoc). The amino acid to be coupled was activated with 2 M DIEA to form an active ester that allows coupling with 0.5 M HCTU. After these steps were reiterated, the final product was cleaved from the resin in a cocktail of TFA:TIS:DDI:Thionaisole:Phenol (84:1:5:5:5). The following cocktail did not require as many scavengers if the peptide to be synthesized did not contain any of the following residues: His, Cys, Arg, Met or Trp. The synthesis was adjusted for purity and content as needed, but the above outline worked with all peptides synthesized for this investigation.

2.8. Data processing

Spectra were mass measured and the following processing was performed for mass accuracy. All continuum spectra were lock mass calibrated with a mass tolerance of 0.05 Da, for noise reduction all collected spectra were smoothed (Savitzky Golay) and background subtracted (Adaptive; 35% Threshold), and all final molecular weight determinations as well as all Biemann ion series assignments spectra were centered (Top 80%; TOF Resolution 17,000 FWHM). To ensure that spectra acquired were not in dead-time, any 1 s scan spectra greater than 250 counts was discarded. For peptides with a molecular weight greater than 2000 Da, the MaxEnt® algorithm was applied using an auto peak width determination to provide a "zero-charge" spectrum.

3. Results

3.1. General features of the Venom

Venom was harvested from multiple members of a colony and subjected to purification and chromatographic separation. A cursory examination by UV-DAD revealed a complex mixture of compounds with a large diversity of properties ranging in size, hydrophobicity, extinction coefficients and overall abundance although no significant compositional difference was found among individuals (data not shown). A split fraction of effluent from the HPLC was infused into an electrospray ion source coupled to a TSQ–MS; a representative total ion chromatogram (TIC) is provided (Fig. 1). The six most abundant peptides were chosen for complete *de novo* sequencing by high resolution mass spectrometry and were given a preliminary laboratory

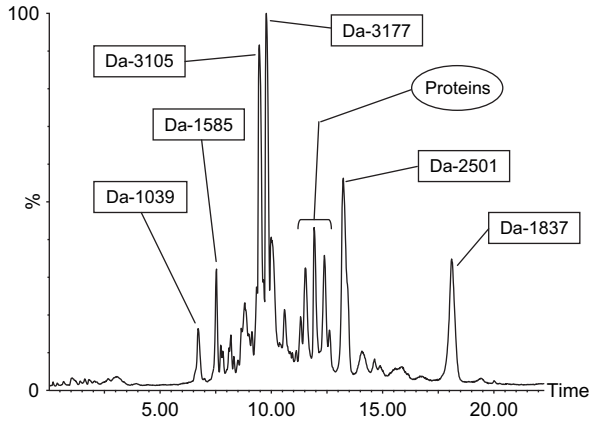

Fig. 1. Representative TIC of *Dinoponera australis* Venom. A reverse-phase liquid chromatographic separation of venom from *D. australis* reveals a complex mixture of compounds with a large diversity of properties ranging in size, hydrophobicity and overall abundance. The six major peptides selected for *de novo* sequencing have been identified and referenced based on their molecular weight.

identifier with the following format: Xx-YYYY, where Xx represent the initials of the genus and species, YYYY is the peptide's molecular weight, e.g. Da-1838. In future studies, the major bioactivity of each sequenced molecule will be assayed and each peptide will be prefixed with an appropriate descriptor as proposed by King et al. (2008).

The two early eluting major peptides, Da-1039 and Da-1585, are hydrophilic and have the two lowest molecular weights. The two most abundant peptides, Da-3105 and Da-3177, are amphipathic and share very similar charge envelopes suggesting the possibility of sequence homogeneity. The late eluting two peptides, Da-2501 and Da-1837, were medium sized peptides in relation to the six examined; the latter being the most hydrophobic of all peptides detected in the venom.

Prior to any *de novo* sequencing, an aliquot of venom was chemically reduced and alkylated to determine if any of the six major peptides under investigation contained cysteines. A comparison of the venom before and after processing shows the appearance of many derivative peaks, none of the six peptides under investigation showed a delta mass of +57 Da. Five of the six peaks did show at least one +58 Da mass shift due to the *S*-carbamidomethylation of methionine (Fig. 2).

Three small proteins were identified during preliminary HPLC/DAD/MS analyses of venom fractions that had not been mass filtered. They are identified as the three peaks in Fig. 1 that precede the major peptide Da-2501 in the elution profile and have approximate molecular weights of 12.6, 13.7 and 22.8 kDa. Although an accurate mass determination was not made for these small proteins at this time, their presence and that of other proteins along with their possible function will be the subject of future investigations.

3.2. Accurate determinations

To determine the monoisotopic molecular weight for any peptide, each averaged spectra was mass measured

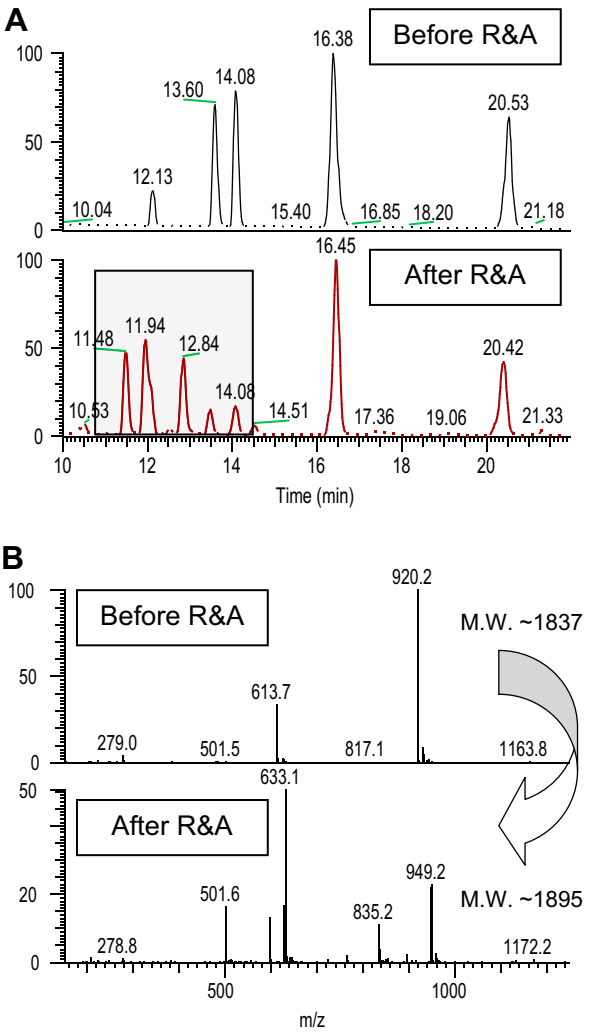

Fig. 2. Reduction and alkylation of Dinoponeratoxins. A) The TIC of six major peptides before and after chemical reduction and alkylation (R&A) demonstrates the formation of derivative peaks (darkened inset). B) A comparison of the mass spectrum of Da-1837 shows a +58 Da shift in molecular weight. An examination of all derivative homologues reveals a similar +58 Da mass defect suggesting the S-carbamidomethylation of methionine(s).

(see Data processing) and the charge cluster with the greatest ion intensity was used for both the calculation of the monoisotopic molecular mass of peptide as well as the set mass for product ion scans. A representative high resolution mass spectrum of Da-3105 is provided (Fig. 3). The $[M+5H]^{5+}$ ion cluster is the most abundant charge state in the spectrum. A closer look at this cluster has been provided to show the mass assignment before (Fig. 3B) and after (Fig. 3C) data processing; the mass error is reduced by a factor of twenty-five with a calculated 56.3 ppm before and 2.28 ppm after. The calculated mass after processing is 3104.700 ± 0.007 Da. Exact mass determinations were made for 75 unique peptides and are listed in Table 1.

The purpose of this investigation was to characterize the primary structure of the major peptides from the venom of *D. australis*. To *de novo* sequence the amino acid composition of the six major peptides, data dependent product ion

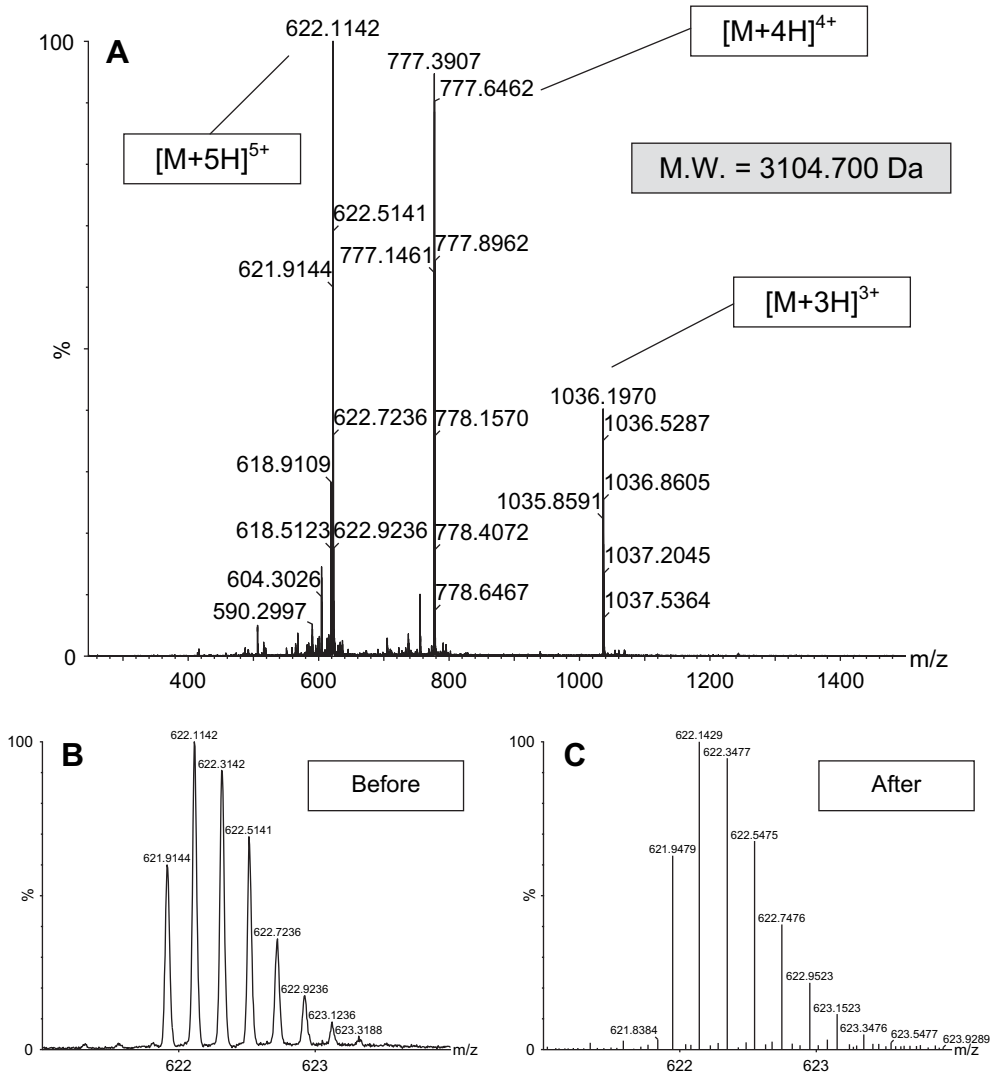

Fig. 3. Representative high resolution mass spectrum. A) A multiply charged electrospray spectrum of Da-3105 shows a $[M + 5H]^{5+}$ ion cluster that was used for monoisotopic molecular weight determinations as well as the parent ion for product ion scans. A closer examination of the cluster B) before and C) after data processing demonstrates the advantage of lock mass correction in high resolution MS; a 25 fold reduction in mass error for the calculated mass was observed (see text).

scans were acquired. After data processing the MS–MS data was manually assessed and iterations of Biemann ion series applied. An example of the MS/MS daughter spectrum of the doubly charged parent ion 520.3 *m*/*z* (CE 21) has been provided as an example of this *de novo* process (Fig. 4). The primary sequence for Da-1039 was determined to be:

$$(H) - G - V - V - P - H - D - F - R - I - (OH)$$

The discrimination between leucine and isoleucine was performed by Edman sequencing (data not shown).

An examination of the two largest peptides, Da-3105 and Da-3177, required further MaxEnt processing to effectively remove background and center the clusters into a single "zero charge" spectrum. An example of a MS/MS spectrum (20 *m*/*z* section) before and after MaxEnt processing has been provided for Da-3177 (Fig. 5). Multiply charged electrospray spectra (Fig. 5A) are converted to a spectrum of maximum entropy (Fig. 5B) that demonstrates the enhanced resolution and signal-to-noise ratio needed to produce true molecular mass spectra. The primary sequence of Da-3177 was determined to be:

$$(H) - G - L - K - D - W - W - N - K - H - K - D - K - I - I - D - V - V - K - E - M - G - K - A - G - L - Q - A - A - (NH_2)$$

Da-3105 had a very similar fragmentation pattern and subsequently a very close sequence homology.

Amidation, a commonly observed post-translational modification (PTM) in venom peptides, was seen in three of the six peptides characterized. An example of this PTM's identification has been provided for Da-1837, i.e. a C-terminal

Table 1
Mass list of peptides from the venom of *Dinoponera australis*.

429.101	*525.366*	668.302	686.307	*749.486*	*772.451*
783.318	797.318	882.411	*885.554*	*934.528*	981.426
987.505	**1038.562**	1084.505	*1087.648*	*1118.645*	*1349.904*
1462.921	1519.707	1566.841	**1584.908**	*1600.891*	1616.848
1665.986	1743.763	1768.701	*1797.041*	1805.941	*1820.042*
1837.123	*1853.208*	*1875.024*	*1881.027*	*1890.989*	*1895.121*
1897.107	1913.847	1936.984	1943.742	1954.907	1986.519
2000.882	2001.112	*2003.113*	2045.891	2113.119	2303.156
2304.099	*2331.675*	2450.024	2483.406	2492.079	**2501.427**
2502.445	*2517.418*	2541.786	2610.078	2611.283	2675.461
2702.308	2813.85	2847.158	3017.138	3087.655	**3104.699**
3120.666	*3159.721*	**3176.729**	*3192.703*	3194.492	*3214.693*
3230.653	*3234.733*	3321.717			

Peptide molecular weights (MW) are shown in Daltons $\pm \leq 2$ ppm mass error.
Italicized molecular weights represent peptides that were present at 10–25 higher concentrations.
The six major peptides were fully sequenced in Table 2 and are indicated with an *.

peptidyl-lysine amide (Fig. 6). The C-terminal Lysine was determined to be amidated due to the large presence of the *y*1 ion at 146.1301 *m*/*z*. If the lysine had been carboxylated or unmodified, the theoretical *y*1 ion would have appeared as a 147.1133 *m*/*z*. The Δmass of −0.983 Da is the difference between the hydroxyl group and the amino group ($OH_{17.0027} - NH2_{16.01872} = 0.9840$). The resultant sequence was determined to be:

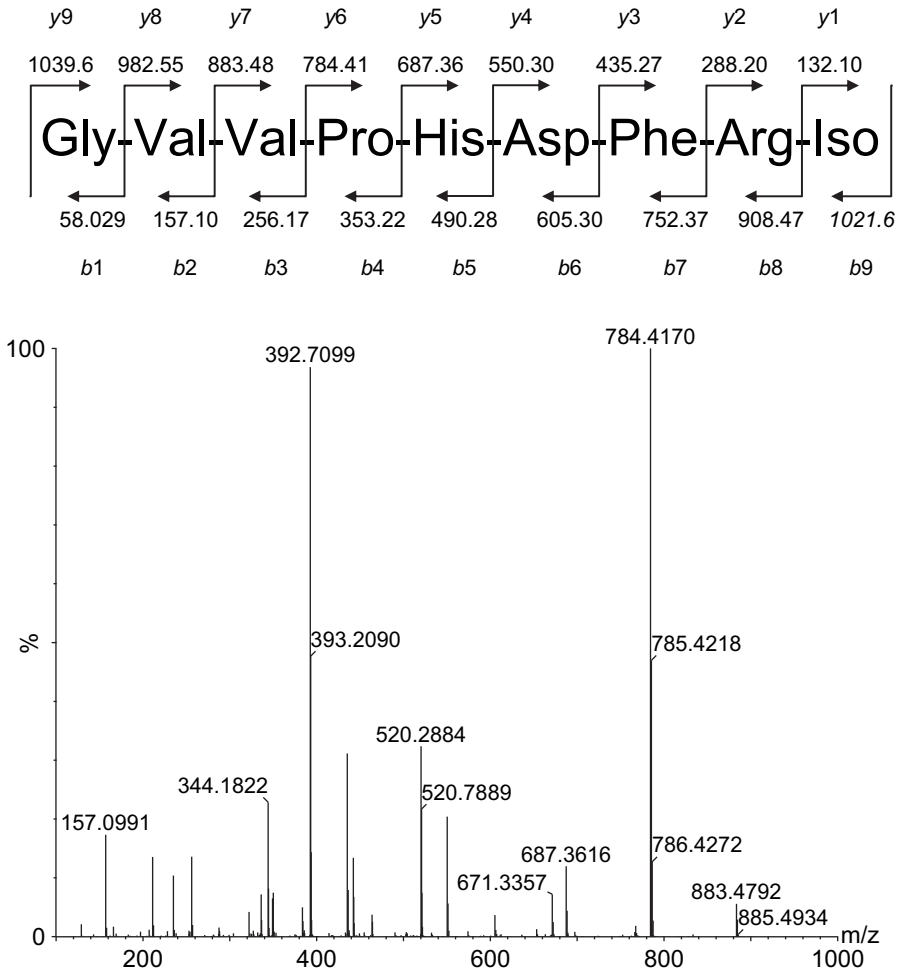

Fig. 4. Data dependent product ion scan. The doubly charged parent ion (m_p^+) of Da-1039 was passed through a charged collision camber filled with argon resulting in high energy collisions. This collision induced dissociated MS/MS spectra (CE = 21) provides unique and characteristic daughter ions (m_d^+) that are used for *de novo* sequencing. The Biemann ion assignments are provided for complete characterization.

Fig. 5. Comparison of original electrospray data and MaxEnt survey spectrum. A 20 Da *m*/*z* section of the MS/MS electrospray data for Da-3177 is shown to elucidate the advantage of MaxEnt processing. A) An original electrospray spectrum has many peaks that are not needed for *de novo* sequencing, e.g. the triple charge state of the parent ion at ~1039. B) MaxEnt processing enhances the signal-to-noise ratio and produces a "zero charge" spectrum. The spectral line at 1028.5662 is the *b*8 fragment in a more recognizable form.

$$(H)-F-L-G-G-L-I-G-P-L-M-S-L-I-P-G-L-L-K-(NH_2)$$

The primary structures for all six major peptides were *de novo* sequenced in a similar fashion and are provided in Table 2. The primary structures of all six major peptides were authenticated by the solid phase synthesis of analogs and each analog was assayed to compare chromatographic properties as well as mass characteristics (data not shown).

4. Discussion

This study is the first biochemical characterization of the peptide components from venom of *D. australis* (Order: Hymenoptera). The major peptides from the venom have been examined by high resolution mass spectrometry and their sequences authenticated by solid phase syntheses.

A protein BLAST search was performed for each of the six primary structures and no putative conserved domains were detected. Due to the high probability that venom from a member of Hymenoptera contains antimicrobial peptides (Davies et al., 2004; Orivel and Dejean, 2001; Orivel et al., 2001), a subsequent alignment was performed in APD, but also yielded no matches. Protein queries were complex and suggest homology with domains in various integral membrane proteins. They also provide a hint at the possible biological actions of these peptides by detecting homologies with known peptides (Table 3).

Da-1039, a small hydrophilic nonapeptide, shares homology with both the antibiotic and tachykinin family of uperins (Chia et al., 1999; Giacometti et al., 2005) as well as

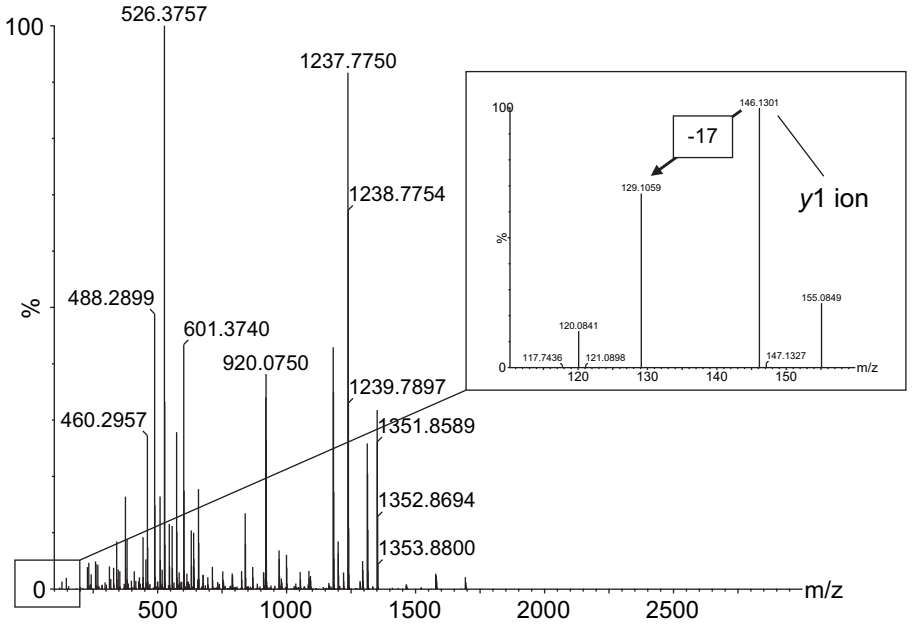

Fig. 6. Identification of a post-translational modification in Da-1837. Amidation is a very common PTM in venom peptides and is not detected by classic Edman chemistry techniques. High resolution mass spectrometry reveals a *y*1 at 146.1301 *m*/*z*, instead of the theoretical 147.1133 *m*/*z*; the Δmass of −0.9832 Da is the difference between the hydroxyl group and the substituted amino group indicative of the presence of a C-terminal peptidyl-lysine. The apparent loss of NH_3 (inset) is also observed and confirms the presence of a terminal amide.

the bombesins (Simmaco et al., 1990), although shares no high sequence homology with any compound published.

The two largest peptides, Da-3105 and Da-3177, have a 92.9% identity in 28 residue overlap. The residue substitutions in positions 15 and 17 (Table 2) change the overall properties of the peptides; Da-3105 is more hydrophobic than Da-3177 and has more positive net charge. However, both peptides would predictably form an alpha helix and both have an overall net positive charge greater than +4. Da-3105 and Da-3177 share ~50 of their sequence with a family of antimicrobials classified as ponericin G from the ponerine ant *P. goeldii* (Orivel et al., 2001). Taken together, these data suggest that both of these peptides will form amphipathic α helical cationic antimicrobial peptides.

Da-1585 has the same sequence as the last 16 residues of the C-terminus of Da-2501 suggesting that it is a cleavage product from the latter. The smaller product is more hydrophilic, carries a smaller net charge and shares homology with antimicrobials like those found in frogs: gaegurin-5 and brevinin-1PTa (Park et al., 1994; Wang et al., 2009a,b). The larger peptide, Da-2502, is more hydrophobic and shares >50% homology with another antimicrobial ponericin W3 (Orivel et al., 2001).

The last peptide sequenced, Da-1837, is the most hydrophobic and shares close homology with regions of poneratoxin from *P. clavata* (Piek et al., 1991). The two peptides share the same net charge, similar hydrophobicity and are both amidated. A closer examination with sequence alignments also shows 52.3% homology with mucroporin, a known antimicrobial peptide from the venom of the Chinese swimming scorpion *Lychas mucronatus*.

As the number of "miracle drugs" introduced each year into human therapy continues to steadily decline, it is apparent that a more intense review of the structural diversity of natural compounds is required. Today, nearly one half of the 100 most prescribed medications in the United States derive directly or indirectly from natural products (Bernstein and Ludwig, 2008; Myles, 2003). A reason for this re-examination, in part, is due to the tremendous technical advances in analytical and separative

Table 2
Primary structure of the major Dinoponeratoxins.

Dinoponeratoxin	MMW[a]	TMW[b]	Error[c]	Sequence
Da-1039	1038.562	1038.5611	−0.39	(H) GVVPHDFRI (OH)
Da-1585	1584.908	1584.9096	0.95	(H) ALKAVPAVMGMIKKE (OH)
Da-3105	3104.700	3104.7070	2.28	(H) GLKDWWNKHKDKIIAVAKEMGKAGLQAA (NH_2)
Da-3177	3176.729	3176.7282	−0.25	(H) GLKDWWNKHKDKIIDVVKEMGKAGLQAA (NH_2)
Da-2501	2501.427	2501.4267	−0.20	(H) FWGTLAKLALKAVPAVMGMIKKE (OH)
Da-1837	1837.123	1837.1264	1.80	(H) FLGGLIGPLMSLIPGLLK (NH_2)

[a] MMW = Measured Molecular Weight.
[b] TMW = Theoretical Molecular Weight.
[c] Error was reported as ppm.

Table 3
Sequence homology of Dinoponeratoxins with other bioactive peptides.

Peptide	Amino acid sequence	% Homology
Da-1039	GVVPHDFRI	–
Uperin 3.1	GVLDAFRKIATVVKNVV	31.6
Kinin	GWFDVVKHIASAV	30.8
Da-3105	GLKDWWNKHKDKIIAVAKEMGKAGLQAA	–
Da-3177	GLKDWWNKHKDKIIDVVKEMGKAGLQAA	92.9
Ponericin G2	GWKDWLKKGKEWLKAKGPGIVKAALQAATQ	50.0
Ponericin G3	GWKDWLNKGKEWLKKKGPGIMKAALKAATQ	47.1
Dermaseptin-H6	GLWSTIKQKGKEAAIAAAKAAGKAVLNAASEAL	45.7
Da-2501	FWGTLAKLALKAVPAVMGMIKKE	–
Ponericin W3	GIWGTLAKIGIKAVPRVISMLKKKKQ	53.8
Gaegurin-5	FLGALFKVASKVLPSVKCAITKKC	42.3
Brevinin-1PTa	FMGGLIKAATKIVPAAYCAITKKC	50.0
Da-1837	FLGGLIGPLMSLIPGLLK	–
Poneratoxin	FLPLLILGSLLMTPPVIQAIHDAQR	30.0
Mucroporin	LFGLIPSLIGGLVSAFK	52.3

techniques which make the study of natural products much easier and much richer than before. This investigation has revealed six new peptides from the genus *D. australis* that could have important implications in understanding the functionality of the venom in the species that may mediate a significant part of the local and systemic effects observed in clinical presentations of envenomation. Further characterization of this ant venom will require tests for specific biological activity, but the striking effects of human envenomation suggest that the peptide components of ant venom may be a valuable source of pilot candidates that have potential application for the generation of novel therapeutics.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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