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ORIGINAL ARTICLE

Novel mastoparan and protonectin analogs isolated from a solitary wasp, *Orancistrocerus drewseni drewseni*

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Abstract Two novel biologically active peptides, Eumenine mastoparan-OD and Orancis-Protonectin, were isolated from a solitary wasp, *Orancistrocerus drewseni drewseni* (Eumeninae, Vespidae). MALDI-TOF MS analysis of a small amount of the crude venom gave two intensive molecular-related ion peaks at *m/z* 1269.9 and 1552.9 that were expected to be novel based on a peptide database search. Purification of the crude venom by HPLC gave two peptide fractions, P-1 and P-2. The amino acid sequence of P-1 (GRILSFIKAGLAEHL-NH_2) and P-2 (ILGIITSLLKSL-NH_2) were determined by ESI-MS/MS, automated Edman degradation, and amino acid analysis. According to the high sequence homology with those of mastoparan and protonectin, P-1 and P-2 were labeled Eumenine mastoparan-OD and Orancis-Protonectin, respectively. Orancis-Protonectin is the first example of a protonectin analog isolated from the venom of a solitary wasp. The hemolytic activities of these new peptides were more potent than that of mastoparan.

Keywords Solitary wasp · Mass spectrometry · Mastoparan · Protonectin · Hemolytic activity

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Introduction

The venom of Hymenoptera has attracted much attention as a rich source of pharmacologically active peptides (Habermann 1972; Tu 1984; Piek 1986; Nakajima 1993). Mast cell degranulating peptides (mastoparans) (Haux 1969; Hirai et al. 1979), kinins (vespakinins and polistes-kinins) (Nakajima et al. 1984; Piek 1999), hemolytic peptide (melittin) (Habermann 1972), neurotoxic peptide (apamin) (Habermann 1972), protonectins (Dohtsu et al. 1993; Mendes et al. 2004; Souza et al. 2005), and chemotactic peptides (Nakajima et al. 1984; Piek 1986; Nakajima 1993) are representative of the venom peptides. To facilitate the venom peptide research, we have recently installed an analysis-guided approach using a highly sensitive Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS or Electrospray ionization-time of flight (ESI-TOF) MS method for a rapid screening and mapping of the crude venom (Nakajima et al. 2003; Nakajima 2006). Along this line, we found four novel mastoparan and protonectin analogs from the venom of the Asian social wasp subfamily, Polistinae, using a few venoms (Murata et al. 2006).

In this study, we now report novel pharmacologically active peptides in the venom of a Vespid solitary wasp, *Orancistrocerus drewseni drewseni* (*O. drewseni*, Eumeninae, Vespidae). *O. drewseni* (Eumeninae, Vespidae) is a close relation to eusocial wasps, Polistinae and Vespinae (Vespidae), in phylogeny (Carpenter 1982; Gullan and Cranston 2005; Grimaldi and Engel 2005). Based on the current hypothesis, these wasps have separated and evolved into the solitary and social wasps from a common ancestor (Hines et al. 2007). We are intrigued by the venom of *O. drewseni* from the following viewpoints: (1) the comparison of the venom peptide distribution of the Vespid

solitary and social wasps would provide an insight into the phylogeny and evolution of Vespidae, (2) the venom of the solitary wasp represents a challenging material for testing the scope of the analysis-guided approach because only a limited amount of the venom from the solitary wasps is available in contrast to those of the social wasps, and (3) the pharmacological evaluation of the peptide components of Vespid solitary wasps has been limited due to their solitary traits.

Materials and methods

Sample preparation

The female wasps of *O. drewseni* were collected in Kyoto city (Japan). Few desired female wasps were collected in the field work on a hot and sunny summer day. Nine venom sacs were dissected and extracted with acetonitrile/water/trifluoroacetic acid 1/1/0.001 that formed a crude extract.

MALDI-TOF MS and ESI MS analysis

MALDI-TOF MS was acquired using a Voyager Elite (PerSeptive Biosystems, Foster City, CA) equipped with a delayed extraction source and 337 nm pulsed laser with α-CHCA as the matrix. Collision-induced dissociation (CID) and post source decay (PSD) analyses were performed with the Voyager Elite under the following conditions: collision gas (argon), accelerating voltage (20 kV). The ESI-TOF MS and MS/MS analyses were performed by a Q-TOF (Micromass, Manchester, UK) under the following conditions: ionization mode (positive), capillary voltage (1,800 V), cone voltage (50 V), collision gas (argon), collision energy (35 V).

HPLC analysis

The crude venom extract was purified by HPLC (Waters Alliance 2690 (Waters Milford, MA)) using a photodiode array detector under the following conditions: column, Tosoh TSKgel ODS 120T (4.6 mm i.d. × 250 mm); mobile phase (0.1% aq MeCN (0.1% TFA)—65% MeCN (0.1% TFA) in 40 min with linear gradient), flow rate (1 ml/min).

Peptide sequencing and synthesis

The amino acid sequence analysis was performed by automated Edman degradation using a PSQ-1 (Shimadzu, Kyoto, Japan). The amino acid analysis was performed by the AccQ Tag^{TM} derivatization (Waters, Milford, MA) followed by HPLC analysis. Peptides were synthesized using a Model 433-A (Applied Biosystems) based on the Fmoc strategy and purified on a C18-RP HPLC column under the conditions as described for the purification of peptides. Their retention times, molecular-related ions (*m/z*), and fragment patterns from the MS/MS analysis were identical to those of the natural peptides. The purity of synthetic peptides was confirmed by MALDI-TOF MS and MS/MS. The amino acid sequence homologies were analyzed by Swiss-Prot/TrEMBL (http://www.expasy.ch/) and PepBank (http://pepbank.mgh.harvard.edu/) database searches.

Hemolytic activity test

The hemolytic activities induced by the peptides were performed as described previously (Nagai et al. 2000). The synthetic peptides were employed for the hemolytic activity test. The synthetic peptides were incubated with 0.2 ml of a sheep blood cell solution (1% in 0.85% NaCl) for 1 h at 40°C. The solution was centrifuged at 2,000 rpm for 10 min. The optical density of the supernatant (0.1 ml) was measured at 550 nm on a Model 450 (BIO-RAD, Hercules, CA) microplate reader. The complete hemolysis was estimated using 500 μg/ml of saponin. The values indicate the average of thrice tests.

Results

A small amount of the crude venom extract (1/100 of the one venom sample) was subjected to the MALDI-TOF MS analysis. Two intensive $[M + H]^+$ ion peaks at *m/z* 1269.9 and 1552.9 in a range of 0–10,000 u were found in the peptide distribution map (Fig. 1). These molecules were found to be novel peptides based on a peptide database search. The crude venom extract was purified by HPLC to give two large peaks labeled P-1 and P-2 (Fig. 2). The molecular weight of P-1 (1,551.8) was calculated by an ESI-Q-TOF MS analysis giving $[M + 2H]^{2+}$ ion at *m/z* 776.9. The Edman degradation of P-1 provided twelve amino acid residues as GRILSFIKGLAE-X-Y (X, Y = unknown residue). The MALDI- PSD-TOF MS analysis of P-1 gave *b*-series fragment ions at *m/z* 1536.1, 1423.1, 1285.7, and 1156.6. Generation of an ion at *m/z* 1536.1 (elimination of ammonium ion (−17 u)) indicated that the C-terminal residue was amidated. Observations of the other fragment ions suggest that Ile or Leu was the putative candidate for the C-terminal residue, leading to a partial sequence from the unidentified "-X-Y" sequence that was estimated as -His-(Ile/Leu)-NH_2. The C-terminal amino acid residue was determined as Leu by the amino acid analysis of P-1. Thus, the full sequence of P-1 was assigned to be GRILSFIKAGLAEHL-NH_2.

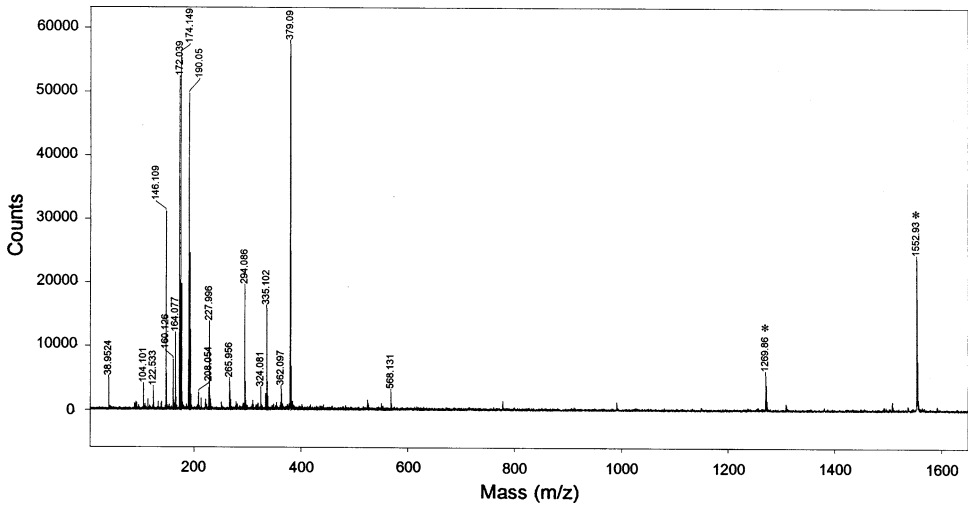

Fig. 1 MALDI-TOFMS spectrum of crude extract of venom sac from *O. drewseni drewseni*. An aliquot of the extract was placed on a MALDI stainless plate and the same volume of αCHCA, dissolved in 50% aq MeCN (0.1% TFA) was overlaid and dried. MALDI-TOF MS was used in the linear mode

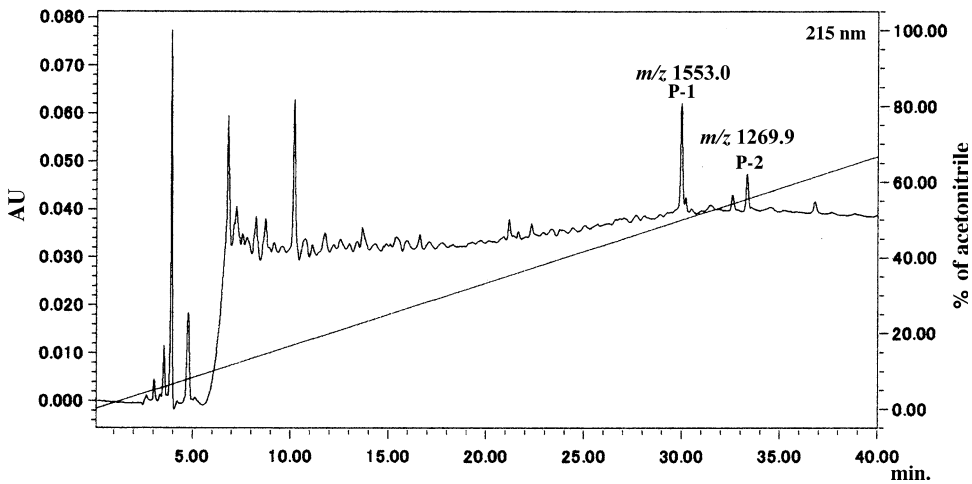

Fig. 2 HPLC chromatogram of venom sac extract. HPLC was performed using a Waters Alliance 2690 system with photodiode array detector. HPLC conditions: column, Tosoh TSKgel ODS 120T (4.6 i.d. × 250 mm), mobile phase (0.1% aq MeCN (0.1% TFA)—65% aq MeCN (0.1% TFA) in 40 min with linear gradient), flow rate (1 ml/min), Detector (UV at 215 nm)

ESI-TOF MS of P-2 showed a $[M + 2H]^{2+}$ ion at *m/z* 635.4 indicating its molecular weight as 1268.8. The MALDI-PSD-TOF MS of P-2 afforded a fragment ion at 1253.5 (−17 u), suggesting that the C-terminal of P-2 was amidated. The Edman method and MALDI-PSD-TOF MS analysis of P-2 suggested the partial sequence of P-2 as ILGIIT-X-LLK-Y-L-NH_2 (X and Y at positions 7 and 11 are unidentified residues). Three possible combinations, "Ala and Cys", "Cys and Ala", and "Ser and Ser", were speculated by the theoretical mass calculation of P-2. To clarify the possibilities, P-2 was subjected to MALDI-PSD-TOF MS to give *b*-series ion peaks at *m/z* 611.9 and 698.7. The difference (87.3 u) between these ion peaks was assigned as X^7 to be Ser. Thus, the sequence of P-2 was proven to be ILGIITSLLKSL-NH_2.

P-1 is a tetradecapeptide that consisted of hydrophobic amino acids residues with three basic amino acids. The sequence is found to be similar to that of mastoparan known as a typical biologically active peptide in the venom of social wasps. P-2 exhibited a high sequence homology to that of protonectin originally isolated from a Brazilian social wasp, *Protonectarina sylveirae*. Thus, we called P-1 Eumenine mastoparan-OD (EMP-OD) and P-2 Orancis-Protonectin. EMP-OD is the second mastoparan analog isolated from the venom of solitary wasps. Orancis-Protonectin is the first example of a protonectin analog isolated from the venom of solitary wasps.

Hemolytic activity is one of the pharmacological characteristics of mastoparan and its analogs isolated from the venom of the social wasps. We next examined and compared the hemolytic activities of EMP-OD, Orancis-Protonectin, and mastoparan. All the peptides induced hemolysis of the sheep blood cells at the concentration of 50 μM, respectively (Fig. 3). The hemolytic activities of EMP-OD and Orancis-Protonectin were found to be more potent than that of mastoparan.

Discussion

O. drewseni (Eumeninae) is known to be potter wasp (or mason wasp). The adult female wasp is a solitary predator

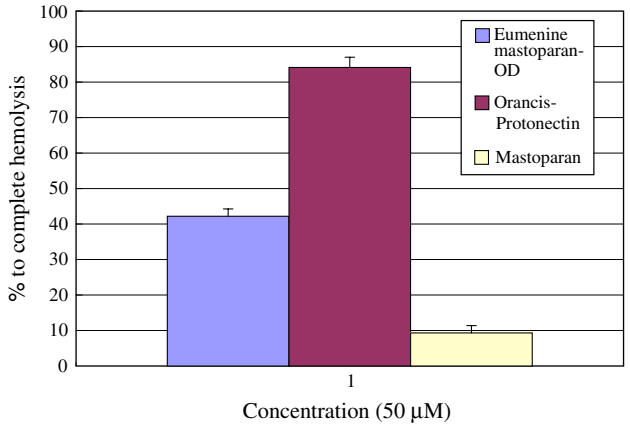

Fig. 3 Hemolytic activities of peptides. Sheep blood cell solution (0.2 ml, 1% in 0.85% NaCl) was incubated with peptides for 1 h at 40°C. The solution was centrifuged at 2,000 rpm for 10 min. The optical density of the supernatant (0.1 ml) was measured at 550 nm. The complete hemolysis was estimated with 500 μg/ml of saponin. *Values* indicate the averages of these tests. *Error bar* represents the standard deviation

of caterpillars and places the pray in the nest to serve as food for their larvae. *O. drewseni* is black and marked with strikingly contrasting patterns of yellow like hornets and paper wasps. Comparisons of their morphological characters (Carpenter 1982; Gullan and Cranston 2005; Grimaldi and Engel 2005). as well as a phylogenic analysis based on the data from the four nuclear gene fragments of these wasp families (Hines et al. 2007) indicated that the wasps of Eumeninae are the nearest relative to the social wasps, Polistinae and Vespinae. The wasps of Eumeninae have attracted much attention as a key group to search for the early evolution stages of eusocial behaviors.

In this study, we found mastoparan analogs (EMP-OD) and a protonectin analog (Oransis-protonectin) in the venom of *O. drewseni* by successful application of the analysis-guided approach to these limited amounts of venom samples. It is interesting to note that both peptide analogs were commonly found in the venom of the vespid social wasps. Our results showed that both are preserved in the venom of the solitary Vespid wasp, *O. drewseni*. This fact would provide an insight into the phylogeny of Vespinae, Polistinae, and Eumeninae from the view point of the venom peptide distribution.

Many mastoparan analogs have been isolated from the venom of hornets, yellow jackets, and paper wasps (Vespidae and Polistinae) (Fig. 4). These consisted of 14 amino acid residues in which the C-terminal residue was amidated. Mastoparan is rich in hydrophobic amino acids such as Leu, Ile, and Ala, and a few basic amino acids (Nakajima et al. 1984; Piek 1986). The sequence of EMP-OD is characterized by the presence of three different kinds of positively charged amino acids, Arg, Lys, and His at the 2, 8, and 13 positions, and the presence of hydrophilic Ser at the 5 position when compared to mastoparans isolated from the venom of Vespinae in which these Lys residues are usually located at positions 4, 11, and 12 in their sequences. The presence of the Lys residue at position 8 was previously observed in the mastoparan analogs isolated from the venom of Polistinae and EMP-AF isolated from the venom of *Anterhynchium flavomarginatur Mikado* (Eumeninae) (Sforca et al. 2004; Konno et al. 2000). Recently, it has been reported that eumenitin (*Eumenes rubronotatus*) (Konno et al. 2006) and decoralin (*Oreumenes decoratus*) (Konno et al. 2007) are linear cationic α-helical peptides. EMP-OD showed more potent hemolytic activities than that of mastoparan. The sequence of EMP-OD were partially similar to those of mastoparan B (MP-B) isolated from the black-bellied hornet (*Vespa basalis,* Vespinae) venom in terms of the presence of a cationic Lys residue at position 2 and a hydrophilic Ser residue at position 5 (Ho et al. 1996). A structure activity

Fig. 4 Mastoparans in the venom of Vespinae, Polistinae, and Eumeninae. Eumenie-mastoparan-OD was isolated in this work

Vespinae [Position] 1 2 3 4 5 6 7 8 9 10 11 12 13 14	name (origin)
Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2	Mastoparan (*Paravespula lewisii*)
Ile-Asn-Leu-Lys-Ala-Ile-Ala-Ala-Leu-Ala-Lys-Lys-Leu-Leu-NH_2	Mastoparan-M (*Vespa mandarinia*)
Ile-Asn-Trp-Lys-Gln-Ile-Ala-Ala-Met-Ala-Lys-Lys-Leu-Leu-NH_2	Mastoparan-X (*Vespa xanthoptera*)
Ile-Lys-Trp-Lys-Ala-Ile-Leu-Asp-Ala-Val-Lys-Lys-Val-Leu-NH_2	Mastoparan-A (*Vespa analis*)
Ile-Asn-Leu-Lys-Ala-Ile-Ala-Ala-Phe-Ala-Lys-Lys-Leu-Leu-NH_2	Mastoparan-T (*Vespa tropica*)
Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Val-Lys-Lys-Val-Leu-NH_2	Mastoparan-II (*Vespa orientalis*)
Ile-Asn-Leu-Lys-Ala-Leu-Leu-Ala-Val-Ala-Lys-Lys-Ile-Leu-NH_2	Mastoparan-C (*Vespa carbo*)
Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2	Mastoparan-B (*Vespa basalis*)
Polistinae [Position] 1 2 3 4 5 6 7 8 9 10 11 12 13 14	**name (origin)**
Val-Asp-Trp-Lys-Lys-Ile-Gly-Gln-His-Ile-Leu-Ser-Val-Leu-NH_2	Polistes mastoparan (*Polistes jadwigae*)
Ile-Asn-Trp-Ala-Lys-leu-Gly-Lys-leu-Ala-Leu-Glu-Val-Ile-NH_2	no name (*Parapolybia indica*)
Ile-Asn-Trp-Ser-Lys-leu-Leu-Ser-Met-Ala-Lys-Glu-Val-Ile-NH_2	no name (*Ropalidia sp.* (Papua New Guinea))
Ile-Asn-Trp-Ser-Lys-leu-Leu-Ser-Met-Ala-Lys-Glu-Val-Ile-NH_2	no name (*Ropalidia sp.* (Papua New Guinea))
Ile-Asn-Trp-Lys-Ala-Leu-Leu-Asp-Ala-Ala-Lys-Lys-Val-Leu-NH_2	Protonectarina mastoparan (*Protonectarina Sylveirae*)
Ile-Asn-Trp-Leu-Lys-Leu-Gly-Lys-Ala-Ile-Ile-Asp-Ala-Leu-NH_2	Agelaia-MP (*Agelaia pallipes pallipes*)
Ile-Asp-Trp-Lys-Lys-Leu-Leu-Asp-Ala-Ala-Trp-Gln-Ile-Leu-NH_2	Polybia-MPI (*Polybia paulista*)
Ile-Asn-Trp-Leu-Lys-Leu-Gly-Lys-Lys-Val-Ser-Ala-Ile-Leu-NH_2	Protopolybia MPI (*Protopolybia exigua*)
Ile-Asn-Trp-Lys-Ala-Ieu-Ile-Glu-Ala-Ala-Lys-Gln-Ala-Leu-NH_2	Protopolybia MPII (*Protopolybia exigua*)
Ile-Asn-Trp-Leu-Lys-Leu-Gly-Lys-Ala-Val-Ile-Asp-Ala-Leu-NH_2	Protopolybia MPIII (*Protopolybia exigua*)
Ile-Asn-Trp-Leu-Lys-Leu-Gly-Lys-Lys-Ile-Leu-Gly-Ala-Ile-NH_2	Polistes-mastoparan-R 1 [Pm-R1] (*Polistes rothneyi iwatai*)
Leu-Asn-Phe-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2	Polistes-mastoparan-R 2 [Pm-R2] (*Polistes rothneyi iwatai*)
Ile-Asn-Trp-Leu-Lys-Leu-Gly-Lys-Gln-Ile-Leu-Gly-Ala-Leu-NH_2	Polistes-mastoparan-R 3 [Pm-R3] (*Polistes rothneyi iwatai*)
Eumeninae [Position]	**name (origin)**
Ile-Asn-Leu-Leu-Lys-Ile-Ala-Lys-Gly-Ile-Ile-Lys-Ser-Leu-NH_2	Eumenine Mastoparan-AF (*Antherhynchium flavomarginatum micado*)
Gly-Arg-Ile-Leu-Ser-Phe-Ile-Lys-Gly-Leu-Ala-Glu-His-Leu-NH_2	**Eumenine mastoparan-OD (*Orancistrocerus drewseni drewseni*)**
Pentadeca peptide analog [Position]	**name (origin)**
Ile-Asn-Leu-Lys-Glu-Ile-Phe-Lys-Lys-Val-Ala-Ser-Leu-Leu-Thr	Eumenitin (*Eumenes rubronotatus*)

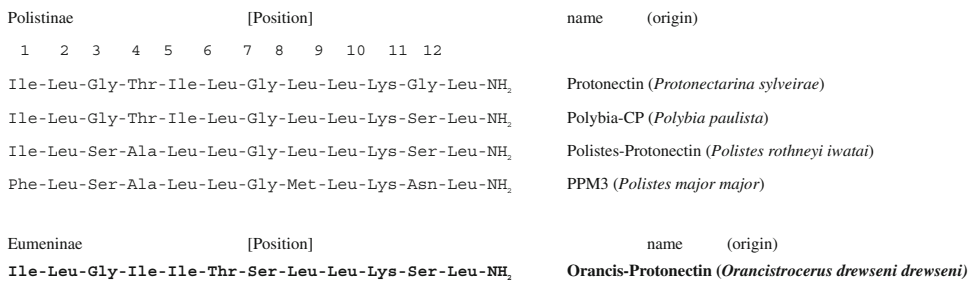

Fig. 5 Protonectins in the venom of Vespinae, Polistinae, and Eumeninae. Orancis-Protonectin was isolated in this work

relationship study of MP-B (LKLKSIVSWAKKVL-NH_2) revealed that replacing Lys^2 by asparagine in the MP-B sequence caused about a 90% decrease in the hemolytic activity at 30 μM of the peptide, while the same substitution at Lys^4 did not cause any significant change (Ho et al. 1996). This SAR study indicates the contribution of the Arg^2 in EMP-OD to the potent hemolytic activity.

Protonectin is a dodecapeptide amidated at the C-terminal and originally isolated from the Brazilian social wasps *Protonectarina sylveirae* (Dohtsu et al. 1993). Protonectin is rich in hydrophobic amino acid residues and exhibited hemolytic activities and was recently isolated from the venom of *Agelaia pallipes pallipes* (Mendes et al. 2004). Potonectin analogs were found in the venom of the social wasps *Polybia paulista* (polybia-CP) (Souza, 2005) and *Polistes rothneyi iwatai* (Polistes-Protonectin) (Murata et al. 2006) (Fig. 5). Orancis-Protonectin is the first example of the protonectin analog isolated from the venom of the vespid solitary wasp. It is interesting to note that the potency of the hemolytic activity of Orancis-Protonectin lies in the same order of magnitude as that of polistes-protonectin (50 μM, 97%) that exhibits a more potent hemolytic activity than that of mastoparan. These results would contribute to the design of biologically active peptides in this class.

Adult wasps of *O. drewseni* are known to be a predator of caterpillars (Piek 1986). The caterpillar is paralyzed for a long time but not killed. It is conceivable that the venom of *O. drewseni* plays an important role in this paralytic activity. Further studies investigating the role of these peptides in the long-term paralysis as well as extension of the MS based approach to the venom of other vespid solitary wasps are now underway in our laboratory.

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