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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, 5/06, 5/08, C07K 14/35, A61K 38/17, 39/35

A1

(11) International Publication Number: **WO 97/13854**

(43) International Publication Date: 17 April 1997 (17.04.97)

(21) International Application Number: PCT/AU96/00632

(22) International Filing Date: 9 October 1996 (09.10.96)

(30) Priority Data:
PN 5839 9 October 1995 (09.10.95) AU

(71) Applicant *(for all designated States except US)*: NORTHERN SYDNEY AREA HEALTH SERVICE [AU/AU]; Pacific Highway, St. Leonards, NSW 2065 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DONOVAN, Gregory [AU/AU]; 6 Woodcourt Road, Berowra Heights, NWS 2082 (AU). BALDO, Brian [AU/AU]; 14 Canisius Close, Pymble, NWS 2073 (AU).

(74) Agent: F B RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PILOSULIN 2

(57) Abstract

An isolated nucleic acid molecule including a nucleotide sequence encoding a polypeptide derived from ant venom and having biological activity of Pilosulin 2, wherein Pilosulin 2 is a polypeptide having the amino acid sequence from residues 49 to 75 shown in the Figure. An isolated polypeptide having biological activity of Pilosulin 2 and methods of lowering blood pressure in an animal using Pilosulin 2.

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                                                                  Met
                    G AAT TCC GTT GCT GTC GAC TTG AGA TTT AAT AAG CAT TAC AAG ATG
                                10                  25                  40

                                10                                      20
Lys Leu Ser Cys Leu Leu Leu Thr Leu Ala Ile Ile Phe Val Leu Thr Ile Val His Ala
AAA CTG TCG TGT TTG TTG TTG ACC CTT GCT ATA ATC TTC GTC TTA ACT ATT GTG CAT GCT
          55                  70                  85                  100

                                30                                      40
Pro Asn Val Glu Ala Lys Ala Leu Ala Asp Pro Glu Ser Asp Ala Val Gly Phe Ala Asp
CCC AAT GTG GAA GCG AAA GCA TTA GCC GAT CCG GAA TCC GAT GCA GTC GGT TTT GCC GAT
          115                 130                 145                 160

                                50                                      60
Ala Val Gly Glu Ala Asp Pro Ile Asp Trp Lys Lys Val Asp Trp Lys Lys Val Ser Lys
GCA GTT GGT GAG GCC GAT CCC ATC GAT TGG AAA AAA GTC GAT TGG AAA AAA GTA TCA AAG
          175                 190                 205                 220

                                70
Lys Thr Cys Lys Val Met Leu Lys Ala Cys Lys Phe Leu Gly
AAA ACG TGT AAA GTG ATG TTG AAA GCA TGC AAA TTT CTC GGA TAA GAG CGA CAA GAA TAA
          235                 250                 265                 280

TAT GAA GAT GAG TCT ACG AGG AAG ACA TAT ACA TGT GAT ACA AAG ATG TTG AAT ACA AGG
          295                 310                 325                 340

ATG CTG AAA TAT ATA TTC TTA ATC GAA ATA ATA AAA TGT ATT TGT AAA CAT ATA TTT GGG
          355                 370                 385                 400

AAT TAT ATA AAA GAA AAT AAA TAA TAG ACT TGC ACT ATC AAA AAA AAA AAA AAA AAA AAA
          415                 430                 445                 460

AAA AAA AAA AAA AAA AAA AAA GCG GCC GC
          475                 490
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Pilosulin 2

Technical Field

This invention relates to nucleic acid molecules encoding novel polypeptides derived from ant venom and uses of the polypeptides in methods of treating conditions in subjects.

Background Art

The sting of the Australian jumper ant *Myrmecia pilosula* causes severe IgE antibody-mediated reactions, including anaphylaxis in sensitised individuals. Blotting studies with sera from allergic individuals have shown that the venom contains two major immunologically defined groups. One group contains 5 bands having molecular masses between 5 and 8 kDa and the second group is represented by a single band at 3 kDa. One of the major allergens, Pilosulin 1, has already been cloned and nucleotide sequenced [1]. This allergen has a deduced amino acid sequence of 112 residues and IgE antibodies that recognise it bind to the 5-8 kDa polypeptide group of polypeptides identified in the electrophoretically separated venom [2]. Fifty percent of the IgE antibody binding can be attributed Pilosulin 1 in subjects that recognise all the allergenic components of this venom. Subsequent studies showed that Pilosulin 1 contained a single 14 amino acid residue IgE-binding determinant located between residues 93-106 which appeared to be invariant for all allergic subjects that recognised this allergen [3].

The present inventors have now cloned and characterised a second allergen that is immunologically cross-reactive with the 3kDa major IgE-binding band. This sequenced allergenic polypeptide has been named Pilosulin 2 and has been found to have useful biological activities.

Disclosure of Invention

In a first aspect, the present invention consists in an isolated nucleic acid molecule including a nucleotide sequence encoding a polypeptide having biological activity of Pilosulin 2.

Pilosulin 2 is the polypeptide having the amino acid sequence from residues 49 to 75 shown in Figure 1.

Preferably, the isolated nucleic acid molecule includes a nucleotide sequence substantially as shown in Figure 1, or a functionally equivalent nucleotide sequence thereof, or a sequence which hybridises to the nucleotide sequence of Figure 1, or a sequence which shows at least 60% homology with the nucleotide sequence of Figure 1. More preferably, the

nucleic acid molecule has at least 80% homology with the nucleotide sequence of Figure 1 and most preferably the nucleic acid molecule has at least 90% homology with that sequence.

As is stated above the present invention includes nucleic acid molecules which hybridise to the sequence shown in Figure 1. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the abbreviation of a buffer of 0.15 M NaCl, 0.015 M trisodium citrate. Three x SSC is three times as strong as SSC and so on.

As will be recognised by those skilled in the art, recombinant expression vectors suitable for transformation of a host cell including the nucleic acid molecule of the present invention operably linked to a regulatory sequence can be prepared. When host cells are transformed with such an expression vector the transformed cells can be used for preparing a Pilosulin 2 polypeptide which preferably has an amino acid sequence substantially as shown in Figure 1.

As used herein the term "functionally equivalent nucleotide sequence" is intended to cover minor variations in the Pilosulin 2 nucleotide sequence which, due to degeneracy in the DNA code, does not result in the molecule encoding a polypeptide having substantially different biological activity from the native Pilosulin 2 polypeptide. The encoded Pilosulin 2 polypeptide can have an altered amino acid sequence from the sequence shown in Figure 1 but retains substantially the same biological activity as the native Pilosulin 2 polypeptide. This may be achieved by various changes in the sequence, such as insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the biological activity of the encoded Pilosulin 2 polypeptide.

In a second aspect, the present invention consists in an isolated polypeptide having biological activity of Pilosulin 2 polypeptide. Preferably, the isolated polypeptide comprises an amino acid sequence from residues 49 to 75 as shown in Figure 1, a functionally equivalent amino acid sequence thereof, or an active portion thereof.

As used herein the term " functionally equivalent amino acid sequence " is intended to cover minor variations in the amino acid sequence described which results in a polypeptide having relative activity which is not

substantially less than that of the corresponding native polypeptide. Preferably, a polypeptide having an altered amino acid sequence from its sequence shown in Figure 1 has substantially the same or greater activity than that of the native polypeptide. This may be achieved by various changes in the sequence, such as insertions, deletions and substitutions.

Polypeptides including the amino acid sequence shown in Figure 1, or Pilosulin 2, or active portions thereof, or functionally equivalent amino acid sequences thereof, can be produced recombinantly or produced synthetically by standard methods known to the art. Furthermore, modifications of the polypeptides can be carried out by known techniques after production of the polypeptides.

It will also be appreciated that the polypeptides of the present invention may have at least one of the peptide bonds between the amino acids replaced by peptide bond mimics (mimetics) to form pseudopeptide analogues. Such mimics are well known to the art and include N-methyl isosteres, hydroxyl isosteres, reduced peptide bonds and retro-inverso-peptomimetics. Alterations of the peptides in this manner may reduce or eliminate their antigenicity so as to reduce the chance of stimulating the immune response in subjects when administered with the peptides over a prolonged period.

In a third aspect, the present invention consists in a method of lowering blood pressure in an animal comprising exposing the animal to an effective amount of a polypeptide according to the second aspect of the present invention such that the blood pressure is lowered.

In a preferred embodiment of the third aspect of the present invention, the animal is a human subject. In particular, the method of the present invention is suitable for lowering blood pressure in an individual with hypertension. It will be appreciated that Pilosulin 2 and/or active forms thereof may be used as a replacement of medications currently used to treat hypertension or in combination with such drugs.

The polypeptide can be administered at a concentration of at least 1 μg/kg body weight, preferably at least 10 μg/kg body weight. It will be appreciated, however, that the concentration required will vary on the animal to be treated, the extent of reduction in blood pressure required, and the activity of the polypeptide used.

In a fourth aspect, the present invention consists in a composition for use in lowering blood pressure in an animal comprising a polypeptide according to the second aspect of the present invention and a pharmaceutically acceptable diluent.

In a fifth aspect, the present invention consists in a method of inhibiting the growth of a cell comprising exposing the cell to an effective amount of a polypeptide according to the second aspect of the present invention.

The effective amount of the cytotoxic polypeptides according to the present invention will often depend on the cell type being inhibited. Concentrations of Pilosulin 2, for example, of approximately 5 μg/ml have been found to inhibit cells but lower concentrations of other polypeptides may also be suitable to inhibit certain cell types.

Preferably, the inhibition causes cell death.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and drawings.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of *Myr p* 2 and predicted amino acid sequence of Pilosulin 2;

Figure 2 shows the combined restriction and deletion map of pMp37 cDNA insert;

Figure 3 shows hydropathicity profiles of the derived amino acid sequence of the complete cDNA insert pMp37 where hydropathicity was calculated using two different algorithms, Kite and Doolittle [4] (A) and Hopp and Woods [5] (B);

Figure 4 shows the results of percent fall in blood pressure in laboratory rats verses Pilosulin 2 concentration;

Figure 5 shows the results of blood pressure in laboratory rats verses Pilosulin 2 concentration;

Figure 6 shows the results of percent change in pulse width in laboratory rats verses Pilosulin 2 concentration;

Figure 7 shows the results of percent change in heart rate in laboratory rats verses Pilosulin 2 concentration; and

Figure 8 shows the results of heart rate in laboratory rats verses Pilosulin 2 concentration.

Best Modes for Carrying Out the Invention

MATERIALS AND METHODS

Radioimmunoassay (RIA) for IgE antibodies to *M. pilosula* venom

Sera from *M. pilosula* venom-allergic individuals were tested for the presence of venom-specific IgE-antibodies using nitrocellulose (Bio-Rad Laboratories, Hercules, CA. USA) discs (6 mm discs, 0.45 μm pore size), impregnated with 2.5 μg of *M. pilosula* venom proteins/disc. as previously described **[6]**, or 20 μg of total λ or plasmid lysates (see below), in a RIA. Protein concentrations were determined using the Pierce BCA protein determination kit (Pierce, Rockford, IL, USA). Bound IgE antibodies were detected using ^{125}I-labelled anti-human-IgE antibody (Bioclone, Sydney, Australia). Test results are expressed as the percentage uptake of the total amount of anti-IgE antibody (30000 cpm per tube) used in the assay. Routine controls included an IgE-free control (human cord serum) and an IgE-positive control (a serum with IgE-antibodies to allergens other than ant venom).

Denaturation, reduction and alkylation of *M. pilosula* venom

M. pilosula venom sac extracts were prepared from ants collected in several locations in Victoria and New South Wales, Australia, as previously described [6].

Alkylation of reduced jumper ant venom was carried out using a modified method of Hollecker [7]. Venom protein (200 μg) in 100 μl of reduction buffer (10 mM Tris-HCl pH 8.0, 1 mM di-Na EDTA, 10 mM dithiothreitol (DTT), 8 M urea) was incubated for 30 min at 37°C. Eighty microgram (40 μl) of reduced venom was gently mixed with 10 μl of a 0.25 M iodoacetamide (Sigma, St. Louis, MO, USA) solution prepared in 0.25 M Tris-HCl pH 8.0. The solution was incubated at room temperature for 15 mins and stored on ice until required.

To ensure that reduction of disulphide bonds and alkylation of the thiol groups on the now exposed cysteine residues was occurring, samples were also prepared as above but in modified reduction buffer containing no DTT.

SDS-polyacrylamide gel electrophoresis and Western blotting of the venom allergens

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of jumper ant venom was carried out in a Sturdier SE400 (Hoefer Scientific Instruments, San Francisco, CA, USA using the *N*-tris(hydroxymethyl)methylglycine-SDS-

PAGE system [8] with a 16% total, 6% crosslinker separating gel containing 6 M urea as previously described [1,8]. Briefly, freeze dried venom samples were alkylated as described above and prepared in loading buffer (4% (w/v) SDS, 12% (v/v) glycerol, 50 mM Tris-HCl (pH 6.80.01% Serva Blue G) at 50 μg/ml protein concentration. The samples were heated (50°C for 10 min), cooled to room temperature and 300 μl (15 μg) of venom preparation was loaded across the full width of the stacking gel (4% T, 3% C) of a large vertical slab gel. Polypeptide low molecular weight calibration markers (Pharmacia, Uppsala, Sweden) were run on either side of the main well.

Separated venom proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher and Schull, Dassel, Germany, 0.1 μm pore size) as described previously [9,10]. The venom electroblots were blocked by incubation tor 1 h in 0.1 % Tween 20 in TBS (0.01 M Tris-HCl pH 8.0, 0.15 M NaCl) and then cut into 4 mm wide strips and probed with sera from ant venom-allergic individuals as has been previously described [10]. Venom polypeptides which bound IgE were detected using a ^{125}I-labelled anti-human- IgE anti body (Bioclone) and autoradiography [2]. The controls used in the RIA tests were routinely used in all protein blotting experiments. Molecular weight estimations of IgE-binding bands were calculated from the position of their relative mobility (R_f) value on the polypeptide markers molecular weight calibration curve.

Isolation and characterisation of Pilosulin 2 cDNA

A λgt11 Sfi-Not cDNA library was previously prepared from poly(A)$^+$ mRNA isolated from the abdomens of *M. pilosula* ants collected at Springwood, NSW, Australia [1].

Sera from *M. pilosula* venom-allergic individuals whose IgE bound to the higher molecular weight IgE-binding component (3kDa) of *M. pilosula* venom, were pooled and used at a 1:10 dilution in 0.005% (v/v) Tween 20, 0.5% (w/v) bovine serum albumin, 0.1% (w/v) sodium azide, 0.01 M Tris-HCl (pH 8.0) buffer to immunoscreen the cDNA library for IgE-binding clones. Immunoscreening of the cDNA library was carried out as previously described [1]. A modified method for the rapid immunological analysis of recombinant phage clones [11] as described previously [1] was used to immunocharacterise the isolated λgt11 clones.

RIA discs were prepared from the crude lysates of the induced lysogens of nominated IgE-binding clones in the bacterial strain Y1089 [12].

Individual sera from jumper ant venom-allergic subjects were incubated with these discs in IgE-RIAs (see above) to determine the frequency of recognition of the expressed IgE-binding clones. Control discs were prepared from lysates of expressed λgt11 Sfi-Not in the strain Y 1089 and used in all assays with expressed lysate discs.

Nucleotide sequencing of cDNA clones

The cDNA inserts of IgE-binding clones were subcloned into the vector pGEMEX-1 (Promega, Madison, WI, USA) and transformed into the *Escherichia coli* strain JM109. as previously described [1].

Sequencing of the coding and non-coding DNA strands was carried out using the T3 and SP6 dye-labelled primers of a dye primer cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on a Perkin Elmer Cetus thermal cycler. The sequencing reaction products were analysed using an Applied Biosystems 373A DNA Sequencer (Applied Biosystems) and the generated nucleotide sequences were analysed using DNASIS™ (Hitachi Software Engineering, Yokohama, Japan). Hydropathicity studies using the methods of Hopp and Woods [5] and Kyte and Doolittle [4], were performed using a public domain software package PROFILEGRAPH. Nucleotide and amino acid sequence homology searches were performed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service, accessed via the Australian National Genomic Information Service (ANGIS). Isoelectric point estimations were performed using the University of Wisconsin Genetics Computer Group's set of programs (GCG Programs) accessed through ANGIS.

Generation of unidirectional deletions in the cDNA

For the production of unidirectional deletion subclones, the DNA of interest was initially digested with two restriction enzymes to generate an Exonuclease III-resistant 3' overhang and either a 5' overhang or a blunt end that are both Exonuclease III-digestible [12]. The Pilosulin 2 clone pMp37, in the expression vector pGEMEX-I (Promega), contained no useful restriction sites for the deletion of the DNA insert's poly(A)$^+$ tail. Consequently, the DNA insert was directionally subcloned into another expression vector pGEM-7Zf(+) (Promega), through the *Eco*RI and *Hin*dIII restriction sites [12]. The pGEM-7Zf(+) subclone DNA was prepared using a scaled-up procedure of the mini-prep protocol [12]. The plasmid DNA was purified by CsCl-ethidium bromide ultracentrifugation as described previously [13]. Closed

circular plasmid DNA isolated from the CsCl gradient was extracted 5 times with an equal volume of H_2O-saturated 1-butanol and dialysed for 48 h at 4°C against several changes of distilled water and a final dialysis against several changes of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer. The purified pGEM-7Zf(+) plasmid DNA was digested with *Sac*I and *Hin*dIII as previously described [12], generating a 5' overhang at the poly(A)$^+$ tail of the insert DNA. Unidirectional deleted clones were generated thorough the *Hin*dIII Exonuclease II-susceptible restriction site as previously described [12] and these clones were nucleotide sequenced using the T7 and SP6 dye-labelled primers (Applied Biosystems) as described above.

Northern and Southern hybridisation

The materials and methods for Northern and Southern hybridisations of *M. pilosula* RNA and DNA have been described in detail elsewhere [1]. The probe used in these hybridisations was generated by a *Cla*I-*Not*I double-digest of the pGEMEX-I subclone and the insert recovered as previously described [1].

N-terminal analysis of venom polypeptides

M. pilosula venom was separated by Tricine-SDS-PAGE, as described above except that a high loading (200 micrograms) of reduced and alkylated venom was applied to the gels with polypeptide markers (Pharmacia) loaded in both end wells of the stacking gel. The electrophoretic transfer of the separated polypeptides to polyvinylidene difluoride (PVDF) membrane was carried out in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 11.0, 10% methanol as described by Matsudaira [14]. Bands corresponding to IgE-binding proteins of interest were identified by immunoprobing (as described above), of sample strips from the PVDF transfer with serum from subjects having a restricted pattern of recognition that identified either Pilosulin 1 or Pilosulin 2. The IgE-binding bands were identified on the remaining Coomassie Blue stained PVDF transfer [14] by alignment with autoradiographs of the probed strips, excised and N-terminal sequenced by the Peptide Laboratory, Baker Medical Research Institute, Prahran, Victoria using a Applied Biosystems 470A Gas Phase Sequenator.

Measurement of Blood Pressure and Heart Rate in Anaesthetised Rats.

Normotensive female Sprague Dawley rats (250-300g) were anaesthetised with a barbiturate (Inactin: 5-*sec*-butyl-5-ethyl-thiobarbitone sodium) at a dose of 100 mg/kg i.p. (injection volume 1 ml/kg). A 2 cm

incision was made in the ventral surface of the neck allowing access to the trachea, jugular veins and carotid arteries. The trachea is intubated to facilitate respiration. A jugular vein and a carotid artery were cannulated using 1 mm o.d. polythene tubing which is filled with saline containing 0.2 mU/ml heparin to prevent blood clotting. After surgery the rats were left to stabilise for 20 min before experimentation continued.

Drugs were infused or given as bolus injections (injection volume 1 ml/kg) via the jugular vein. Blood pressure was measured by attaching the carotid artery catheter to a Bell & Howell model 4-327 pressure transducer which itself is attached to a Neotrace pen recorder. Heart rate was electronically derived from the blood pressure pulse. Due to biological variation between blood pressures of different rats, groups of 6 rats were required to achieve satisfactory statistical data. The extent and duration of the pain experienced by the rats was minimal and was limited to the initial administration of anaesthetic. At the completion of the experiment the rats were killed painlessly by administration of a large overdose of anaesthetic.

Cytotoxicity studies

Flow cytometry was used to measure cell viability by their ability to exclude a fluorescent dye such as propidium iodide. EBV transformed B-cells were cultured to log-phase growth and venom and venom peptides were added and the cells incubated at room temperature for the required test period. Propidium iodide was added to the cells and incubated for 5 minutes. Cells were then analysed by flow cytometry and cells which took up propidium iodide (evidenced by red fluorescence) were non-viable.

RESULTS

SDS-PAGE and immunoblotting

Sera from a number of allergic subjects with RIA values (see Materials and Methods) of > 2% uptake of the ^{125}I anti-human-IgE antibody were used to probe 4 mm wide strips of nitrocellulose protein blots of *M. pilosula* venom separated by Tricine-SDS-PAGE. Protein blotting identified 5 IgE-binding bands between 5 and 8 kDa and a strong single band at 3 kDa. IgE-binding profiles were identified on these *M. pilosula* venom strips, using the individual sera from a number of ant venom-allergic subjects. Profiles obtained are representative serological IgE-binding patterns. The IgE-binding profiles commonly identified were either all six IgE-binding bands, the 5-8 kDa bands or the 3 kDa band.

The major allergen Pilosulin 1 isolated from the *M. pilosula* λgt11 Sfi-Not cDNA library expressed a polypeptide which was immunologically cross-reactive to IgE-binding bands between 5-8 kDa identified by Tricine-SDS-PAGE and protein blotting [2]. Thus, individual sera with IgE-binding profiles to *M. pilosula* venom which identified the higher molecular weight IgE-binding band of 3kDa were used to screen the same *M. pilosula* cDNA library for new IgE-binding clones.

Isolation and characterisation of cDNA clones encoding Pilosulin 2

Three cDNA clones were selected from the *M. pilosula* library by immunoscreening with a pool of 10 sera from venom-allergic individuals selected to recognise the 8.5 kDa molecular mass IgE-binding band. Three clones λMp37, λMp41 and λMp42 were plaque purified and immunocharacterised using the rapid method for the immunological analysis of recombinant phage clones [11]. The three expressed clones were used to adsorb IgE-antibodies from the diluted serum pool. The bound antibodies were eluted and used to probe nitrocellulose blots of *M. pilosula* venom separated by Tricine-SDS-PAGE. The three clones all of similar size, λMp37 (495 bp), λMp41 (465 bp) and λMp42 (485 bp), each expressed a polypeptide that bound IgE-antibodies which were crossreactive with the 8.5 and the diffuse 2-4 kDa bands in the native venom. An expressed control, λgt11 Sfi-Not, did not adsorb IgE from the serum pool and the eluted material did not bind to native venom.

The lysate from expressed λMp37 was used to prepare test discs for use in IgE-RIAs. In a study involving 57 subjects using the expressed λMp37 lysates, 35% of these subjects recognised this expressed clone. The cDNA insert of λMp37 was subcloned into the expression vector pGEMEX-1 for nucleotide sequencing. To ensure that the pGEMEX-1 subclone of λMp37 retained the IgE-binding characteristics of λMp37 the *E. coli* strain JM109DE3 was transformed and expressed. Lysates prepared from the expressed material were recognised in an IgE-RIA by all subjects who recognised the expressed λMp37 (results not shown). The inventors were therefore satisfied that nucleotide sequencing of the subclone would generate a deduced amino acid sequence that contained the IgE-binding determinants of λMp37.

Nucleotide sequence analysis of Pilosulin 2

Sequencing reactions of pMp37 carried out using the T3 dye-labelled primer revealed the presence of a *Cla*I restriction site in the insert DNA. This

*Cla*I site was used to prepare two subclones pMp37.1 and pMp37.2 (Fig. 2), for further sequencing of the pMp37 insert.

Sequencing of the non-coding strand using the SP6 dye-labelled primer through the poly(A)$^+$ tail of the insert cDNA in pMp37 and pMp37.2 produced very poor sequence data. As no unique restriction sites could be found near the poly(A)$^+$ tail, unidirectional deleted subclones of pMp37 were prepared in pGEM-7Zf(+) as described in Materials and Methods. The poly(A)$^+$ tail was deleted in these subclones using Exonuclease III after a double digestion with *Hin*dIII and *Sac*I restriction enzymes. Multiple subclones were generated by this method, but only three pMp37.3, pMp37.4 and pMp37.5 (Fig. 2), were selected for sequencing based on their size as determined by agarose gel electrophoresis (results not shown). Sequencing of these clones through the 3' tail of the cDNA insert generated useable sequence data for the completion of the sequencing of the pMp37 cDNA insert. Unidirectional deleted clone pMp37.3 (450 bp) contained only 5 adenosines of the poly(A)$^+$ tail of pMp37, whereas pMp37.5 (314 bp) and pMp37.4 (375 bp) contained none of the poly(A)$^+$ tail of pMp37 (Fig. 2).

Sequencing of the coding and non-coding strands of the various subclones of pMp37 was performed a number of times to generate overlapping sequence data in the 5' to 3' and the 3' to 5' directions for verification of the pMp37 nucleotide sequence. Sequence data generated were analysed using the DNA sequence input and analysis software DNASIS™ (Hitachi Software Engineering). The insert cDNA of pMp37 was verified as 495 base pairs in length with an open reading frame of 225 base pairs which translates an unprocessed polypeptide of 75 amino acids in length (Fig. 1).

A methionine residue identified at nucleotides 44-46 is followed by a sequence of 25 largely hydrophobic amino acid residues that correspond to a possible signal peptide leader sequence. A signal peptide cleavage site is predicted at amino acid residues 22-26 (Pro-Asn-Val-Glu-Ala) (nucleotides 107-121). Cleavage of this signal peptide at the alanine residue, nucleotides 119-121 would produce a polypeptide of 52 amino acids with a lysine as the first residue of this processed molecule (Fig. 1). This predicted processed form of the expressed pMp37 has a molecular mass of 5333 Da and a predicted isoelectric point of 8.65. A second possible signal peptide cleavage site Pro-Glu-Ser-Asp-Ala is present at residues 32-36 (nucleotides 136-150).

Cleavage between the Ala and Val at residues 36 and 37 would produce a polypeptide of molecular mass of 4336 and predicted pI value of 9.93.

M. pilosula venom was separated by Tricine-SDS PAGE and transferred to PVDF membrane in CAPS buffer pH 11.0 as described above. The membrane was stained with Coomassie Blue and the five 5-8 kDa and 3kDa IgE-binding bands corresponding to Pilosulin 1 and Pilosulin 2, respectively, were excised and N-terminal sequenced. Eight sequencing cycles were completed on the 3 kDa band, and the following sequence generated, Ile-AspTrp-Lys-Lys-Val-Asp-Trp. The isoleucine (Ile) residue at position 49 of the deduced amino acid sequence of Pilosulin 2 (nucleotide positions: 188-190) (Fig. 1), was thus identified as the first amino acid of the fully processed form of the encoded full-length allergenic molecule. This IgE-binding molecule, when expressed in its fully post-translationally processed form, is a polypeptide of 27 amino acids in length with a calculated molecular mass of 3208 Da and predicted pI value of 10.70. Eight sequencing cycles were completed on the five 5-8 kDa bands corresponding to Pilosulin 1 and the following sequence generated, Gly-Leu-Gly-Ser-Val-Phe-Gly-Arg for the 8 kDa band. The glycine residue at position 57 of the deduced amino acid sequence of Pilosulin 1, was identified as the first amino acid of the fully processed form of the encoded allergenic molecule. Pilosulin 1 when expressed in its fully processed form is a peptide of 56 amino acids in length with a calculated molecular mass of 6045 Da.

Northern and Southern hybridisations

The cDNA insert of the *ClaI* subclone pMp37.2 (Fig. 2), corresponding to the unique 307 bases of the clone pMp37, hybridised to a single mRNA band of approximately 500 bases in length. This would indicate that pMp37 with a cDNA insert of 495 bp as determined by nucleotide sequencing, contains the complete full-length cDNA of this new IgE-binding clone. The subclone pMp37.2 was used in these hybridisations as it lacked 5' sequence homology with Pilosulin 1 as was seen with the full length clone pMp37 (see below).

Multiple bands of restriction enzyme-digested genomic DNA were identified by hybridisation of the ^{32}P-labelled cDNA insert of pMp37.2 with an *M. pilosula* genomic DNA Southern blot. Eight restriction enzymes were used to digest the *M. pilosula* genomic DNA and none of these enzymes contained restriction sites within the pMp37.2 cDNA insert.

Hydropathicity analysis of Pilosulin 2

A highly hydrophobic domain was identified in the N-terminal 20 amino acids of Pilosulin 2 using both algorithms [4,5] (Fig. 3). Several potential antigenic domains: residues 22-25, 30-35, 47-64 (Kyte and Doolittle [4]) and residues 23-36, 42-64 (Hopp and Woods [5]) were also identified (Fig. 3).

Database searches

Comparison of the nucleotide and derived amino acid sequences of pMp37 against current DNA and protein databases using ANGIS, revealed a 62% homology with the other allergen of *M. pilosula* venom, Pilosulin 1.

Comparison of Pilosulin 1 and Pilosulin 2

The sequence homology between Pilosulin 1 and Pilosulin 2 revealed an overall homology of 62% due to localised regions of high homology. Identification of these highly homologous regions revealed a 94% homology between the first 47 amino acid residues of each polypeptide (nucleotides 33-184 of pMp37) (Fig. 2). Also a 91% homology between the final 171 base pairs of the 3' non-coding regions of both these allergens was identified. From amino acid residues 49 to 75 of the Pilosulin 2 sequence and from residues 68 to 112 of the Pilosulin 1 sequence, however, no homology exists between these two sequences.

Within the first 47 amino acid residues of both allergens, single base changes in the nucleotide sequences of both clones are present at residues 11,15, 20, 35, 43, 44 and a two base change is present at residue 28. Of these variations in DNA sequence, only changes at residues 28, 35 and 43 have resulted in a change in the amino acid sequence of the proteins. From amino acid residue 48 onwards in both sequences, however, the DNA and amino acid homologies vary greatly.

High sequence homology in the 3' non-coding regions of each sequence is re-established at nucleotide positions 346 in Pilosulin 2 and 420 in Pilosulin 1 by realignment of the two nucleotide sequences. Comparison of Southern blots of pMp37 with the previously published Pilosulin 1 blot, shows three genomic DNA bands in common. The remaining 15 bands on the pMp37 blot, however, are unique to this IgE-binding clone.

Blood pressure reduction in rats by Pilosulin 2

Myr p 2 is a 75 residue polypeptide encoded by a 495 base-pair sequence. The polypeptide undergoes post-translational processing to a 27 residue polypeptide (residues 49-75) called Pilosulin 2. When a synthetic peptide of Pilosulin 2 was administered via the tail veins of anaesthetised laboratory rats it caused a substantial fall in blood pressure which commenced at within 20 seconds at 20 μg/kilogram body weight of administration. At 50 μg/kilogram body weight blood pressure had fallen 40%. This was accompanied by a fall in heart rate which might be the basis of the fall in blood pressure. The results of rat studies are shown in Figures 4 to 8.

Cytotoxicity studies

The effects of whole ant venom, Pilosulin 2 and Rnase A on cells were studied using proliferating EBV transformed B-cells. After incubation, the cells were analysed by flow cytometry to determine viability. The results are shown in Table 1 where it can be seen that over a 5 hour period at room temperature, Pilosulin 2 at a concentration of 5 μg/ml caused some cell death. At higher concentrations (50 μg/ml), Pilosulin 2 killed greater than 50% of cells within 5 minutes. From these results it will be appreciated that Pilosulin 2 can be used as a cytotoxic agent to inhibit the growth of cells. The polypeptide or active fractions thereof may be suitable to inhibit or kill cells *in vitro* or *in vivo*.

Table 1. Log phase proliferating EBV-transformed B cells were incubated with different concentrations of whole venom, Pilosulin 2 and Rnase A for 5min and 5h at room temperature.

Percent Cell Death

Time	Control	Whole Venom (ug/ml)			Pilosulin 2 (ug/ml)			Rnase A (ug/ml)	
		50	5	0.5	50	5	0.5	50	5
5 min	4	84.3	13.4	4.5	65.4	6.1	5.0	5.4	5.2
5 h	9.7	87.0	19.9	10.6	73.6	13.7	12.5	9.9	11

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Claims

1. An isolated nucleic acid molecule including a nucleotide sequence encoding a polypeptide having biological activity of Pilosulin 2, wherein Pilosulin 2 being a polypeptide having the amino acid sequence from residues 49 to 75 shown in Figure 1.

2. An isolated nucleic acid molecule according to claim 1 including a nucleotide sequence substantially as shown in Figure 1, or a functionally equivalent nucleotide sequence thereof, or a sequence which hybridises to the nucleotide sequence of Figure 1, or a sequence which shows at least 60% homology with the nucleotide sequence of Figure 1.

3. An isolated nucleic acid molecule according to claim 1 having at least 80% homology with the nucleotide sequence of Figure 1.

4. An isolated nucleic acid molecule according to claim 3 having at least 90% homology with the nucleotide sequence of Figure 1.

5. A recombinant expression vector including a nucleic acid molecule according to any one of claims 1 to 4 operably linked to a regulatory sequence.

6. A host cell transformed with an expression vector according to claim 5.

7. An isolated polypeptide having biological activity of Pilosulin 2, wherein Pilosulin 2 being a polypeptide having the amino acid sequence from residues 49 to 75 shown in Figure 1.

8. An isolated polypeptide according to claim 7 comprising an amino acid sequence from residues 49 to 75 as shown in Figure 1, a functionally equivalent amino acid sequence thereof, or an active portion thereof.

9. An isolated polypeptide according to claim 8 comprising an amino acid sequence from residues 49 to 75 as shown in Figure 1.

10. An isolated polypeptide according to claim 8 or 9 wherein at least one of the peptide bonds between the amino acids is replaced by a peptide bond mimic (mimetic) to form a pseudopeptide analogue.

11. An isolated polypeptide according to claim 10 wherein the peptide bond mimic is selected from the group consisting of N-methyl isostere, hydroxyl isostere, reduced peptide bond, and retro-inverso-peptomimetic.

12. A method of reducing blood pressure in an animal comprising exposing the animal to an effective amount of a polypeptide having biological activity of Pilosulin 2 such that the blood pressure of the animal is reduced.

13. The method according to claim 12 wherein the polypeptide comprises an amino acid sequence from residues 49 to 75 as shown in Figure 1, a functionally equivalent amino acid sequence thereof, or an active portion thereof.

14. The method according to claim 13 wherein the polypeptide comprises an amino acid sequence from residues 49 to 75 as shown in Figure 1.

15. The method according to any one of claims 12 to 14 wherein at least one of the peptide bonds between the amino acids of the polypeptide is replaced by a peptide bond mimic (mimetic) to form a pseudopeptide analogue.

16. The method according to claim 15 wherein the peptide bond mimic is selected from the group consisting of N-methyl isostere, hydroxyl isostere, reduced peptide bond, and retro-inverso-peptomimetic.

17. The method according to any one of claims 12 to 16 wherein the animal is a human subject.

18. The method according to any one of claims 12 to 17 wherein the polypeptide is administered at a concentration of at least 1 μg/kg body weight.

19. The method according to claim 18 wherein the polypeptide is administered at a concentration of at least 10 μg/kg body weight.

20. A composition for use in lowering blood pressure in an animal comprising a polypeptide according to any one of claims 7 to 11 and a pharmaceutically acceptable diluent.

21. A method of inhibiting the growth of a cell comprising exposing the cell to an effective amount of a polypeptide according to any one of claims 7 to 11.

22. The method according to claim 21 wherein the inhibition causes death of the cell.

```
                                                                   Met
                  G AAT TCC GTT GCT GTC GAC TTG AGA TTT AAT AAG CAT TAC AAG ATG
                            10                  25                  40

                                    10                                  20
Lys Leu Ser Cys Leu Leu Leu Thr Leu Ala Ile Ile Phe Val Leu Thr Ile Val His Ala
AAA CTG TCG TGT TTG TTG TTG ACC CTT GCT ATA ATC TTC GTC TTA ACT ATT GTG CAT GCT
          55                  70                  85                  100

                                    30                                  40
Pro Asn Val Glu Ala Lys Ala Leu Ala Asp Pro Glu Ser Asp Ala Val Gly Phe Ala Asp
CCC AAT GTG GAA GCG AAA GCA TTA GCC GAT CCG GAA TCC GAT GCA GTC GGT TTT GCC GAT
          115                 130                 145                 160

                                    50                                  60
Ala Val Gly Glu Ala Asp Pro Ile Asp Trp Lys Lys Val Asp Trp Lys Lys Val Ser Lys
GCA GTT GGT GAG GCC GAT CCC ATC GAT TGG AAA AAA GTC GAT TGG AAA AAA GTA TCA AAG
          175                 190                 205                 220

                                    70
Lys Thr Cys Lys Val Met Leu Lys Ala Cys Lys Phe Leu Gly
AAA ACG TGT AAA GTG ATG TTG AAA GCA TGC AAA TTT CTC GGA TAA GAG CGA CAA GAA TAA
          235                 250                 265                 280

TAT GAA GAT GAG TCT ACG AGG AAG ACA TAT ACA TGT GAT ACA AAG ATG TTG AAT ACA AGG
          295                 310                 325                 340

ATG CTG AAA TAT ATA TTC TTA ATC GAA ATA ATA AAA TGT ATT TGT AAA CAT ATA TTT GGG
          355                 370                 385                 400

AAT TAT ATA AAA GAA AAT AAA TAA TAG ACT TGC ACT ATC AAA AAA AAA AAA AAA AAA AAA
          415                 430                 445                 460

AAA AAA AAA AAA AAA AAA AAA GCG GCC GC
          475                 490
```

Figure 1

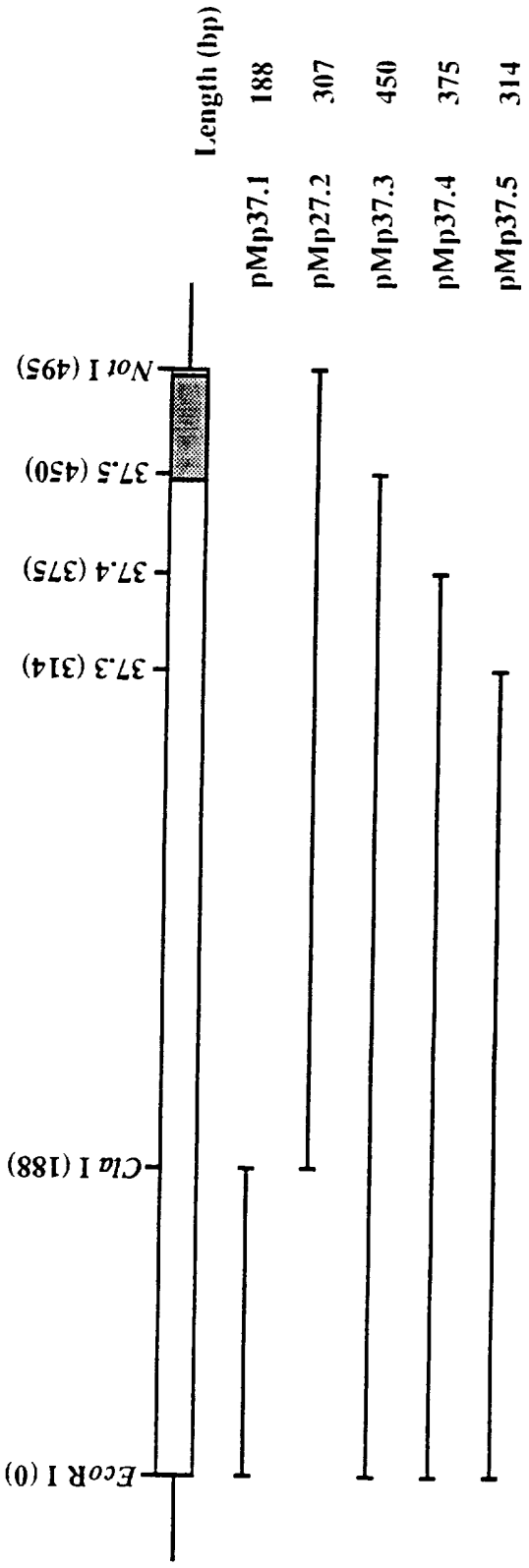
Length (bp)
pMp37.1 188
pMp27.2 307
pMp37.3 450
pMp37.4 375
pMp37.5 314
Not I (495)
37.5 (450)
37.4 (375)
37.3 (314)
Cla I (188)
EcoR I (0)

Figure 2

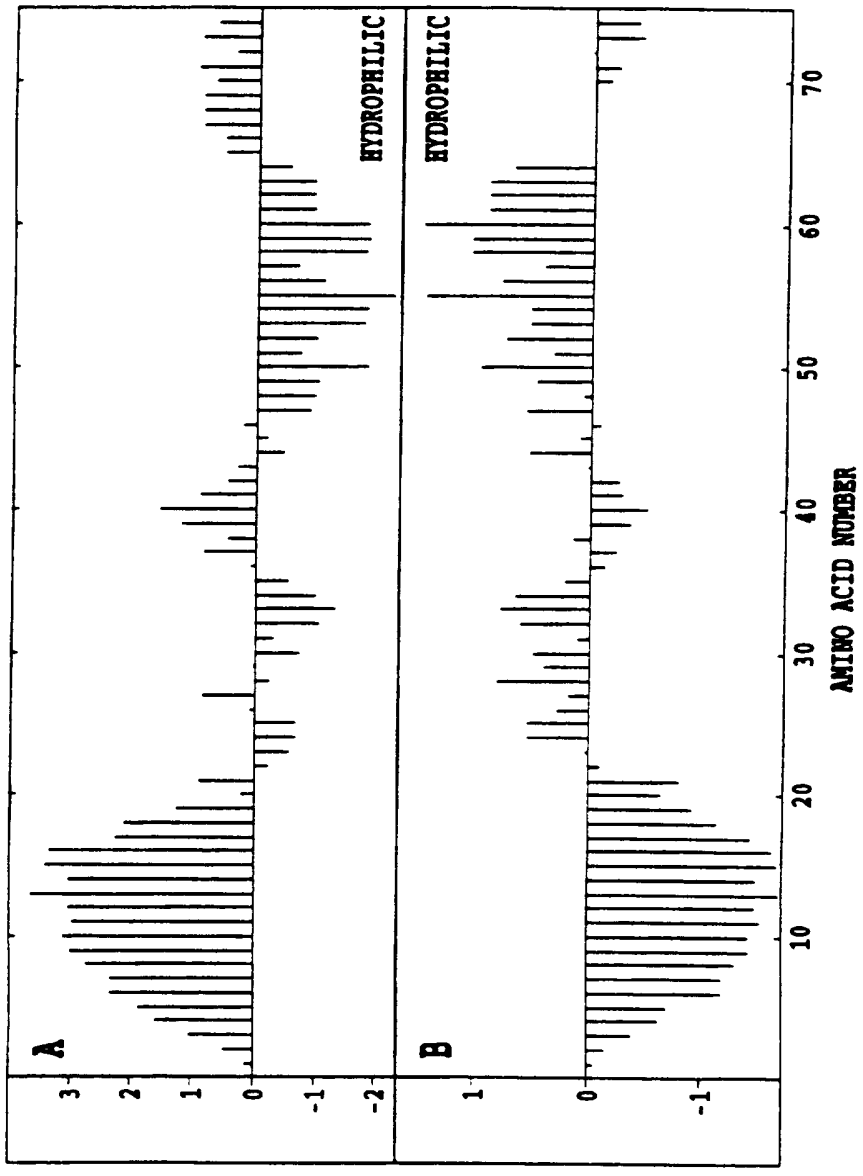
A
B
HYDROPHILIC
HYDROPHILIC
3
2
1
0
-1
-2
1
0
-1
10
20
30
40
50
60
70
AMINO ACID NUMBER

Figure 3

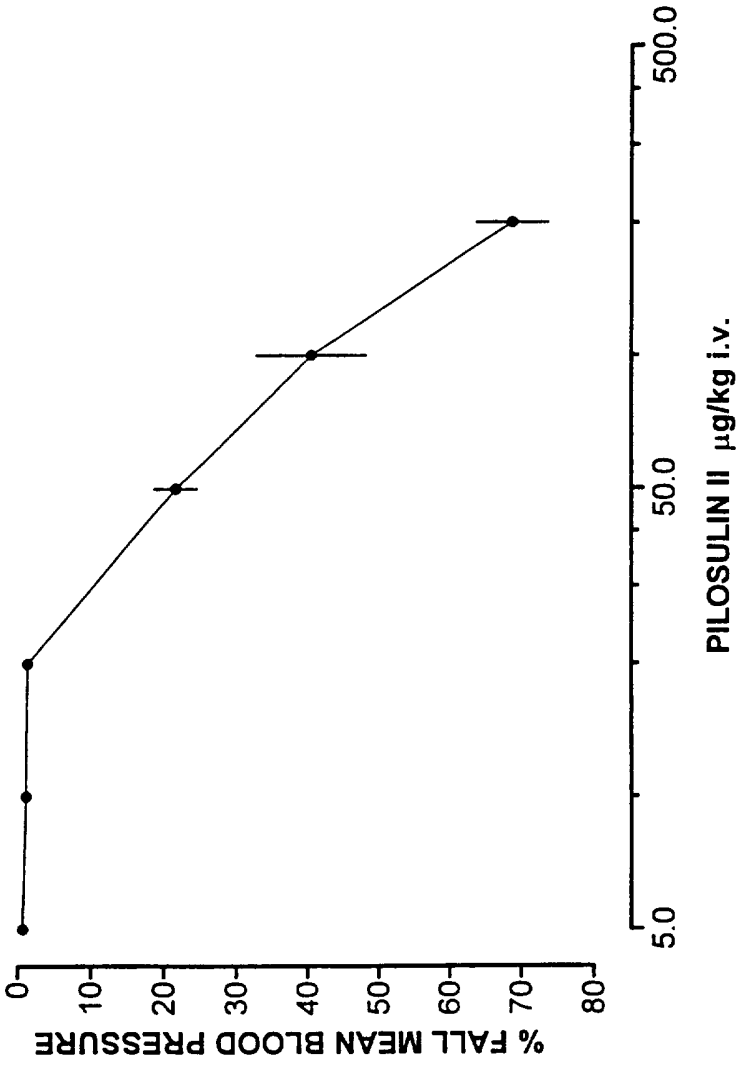
% FALL MEAN BLOOD PRESSURE
0
10
20
30
40
50
60
70
80
PILOSULIN II µg/kg i.v.
5.0
50.0
500.0

Figure 4

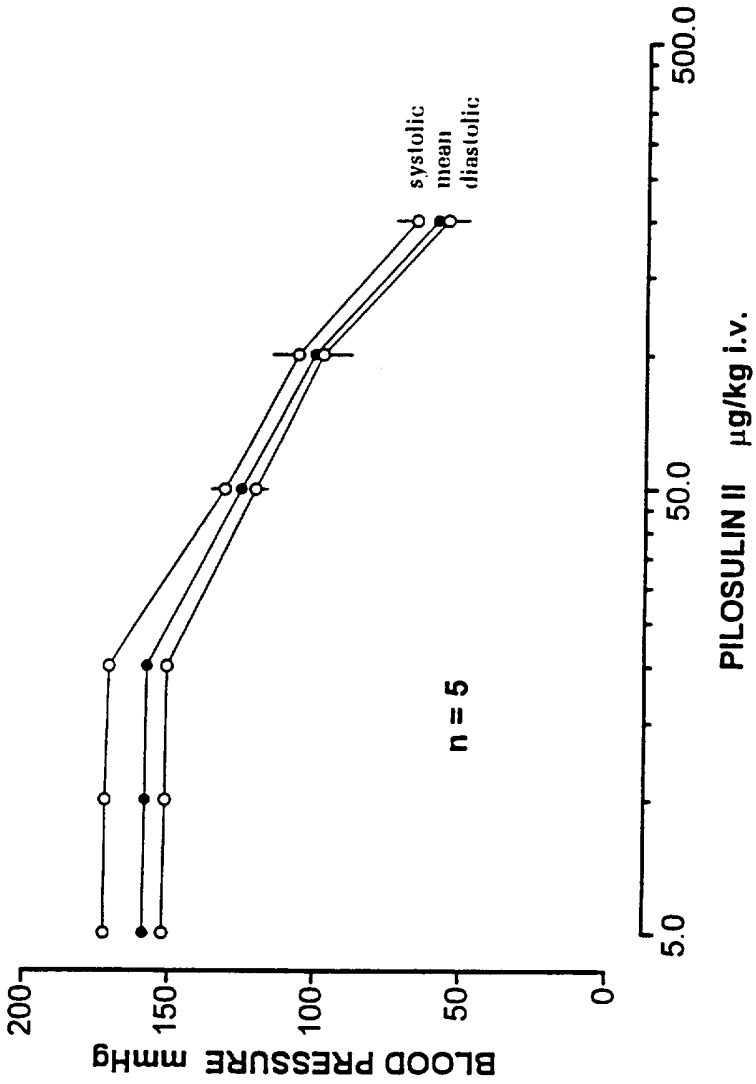
BLOOD PRESSURE mmHg
200
150
100
50
0
n = 5
systolic
mean
diastolic
5.0
50.0
500.0
PILOSULIN II µg/kg i.v.

Figure 5

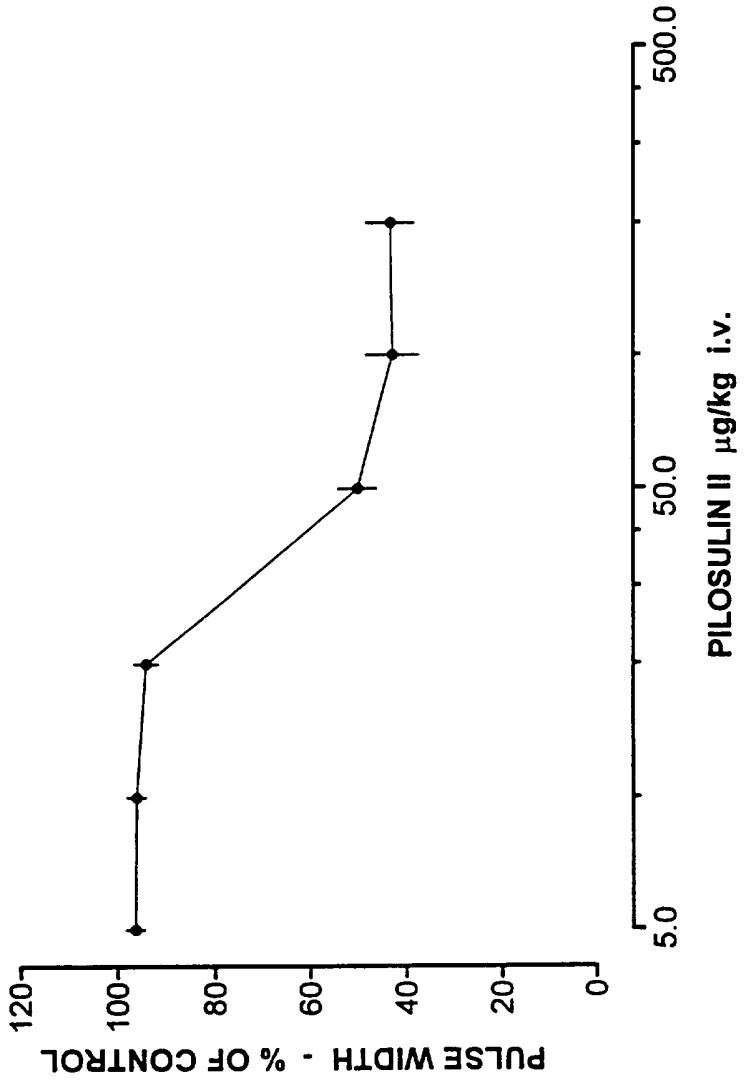
PULSE WIDTH - % OF CONTROL
120
100
80
60
40
20
0
5.0
50.0
500.0
PILOSULIN II µg/kg i.v.

Figure 6

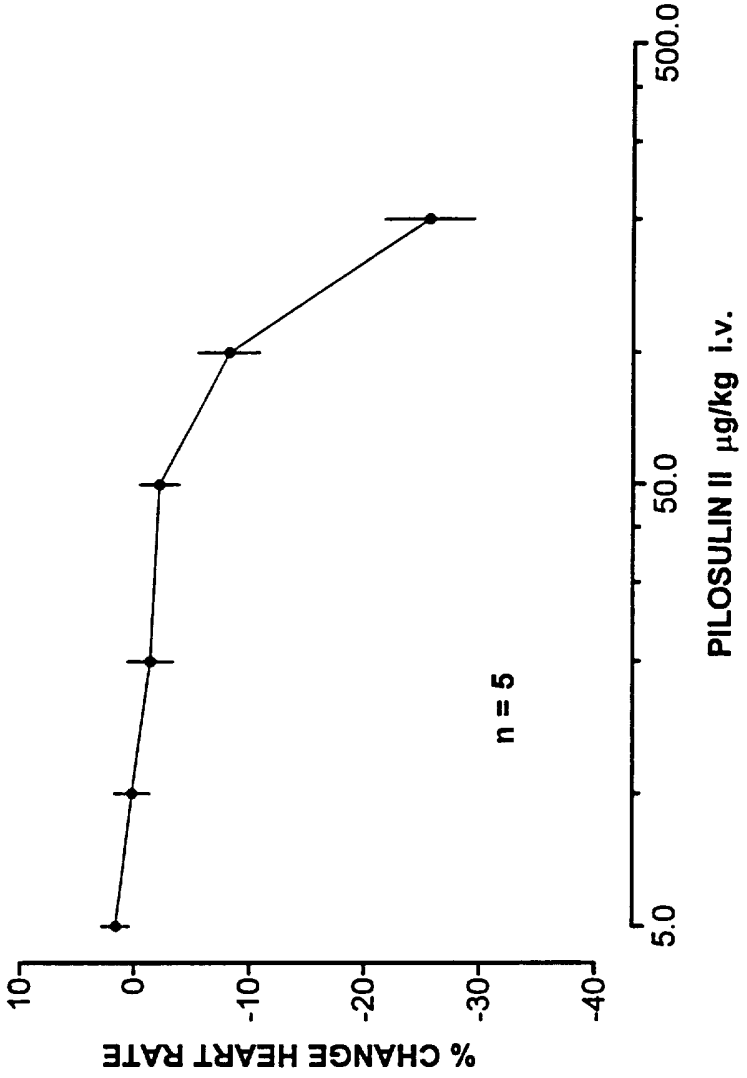
% CHANGE HEART RATE
10
0
-10
-20
-30
-40
n = 5
PILOSULIN II µg/kg i.v.
5.0
50.0
500.0

Figure 7

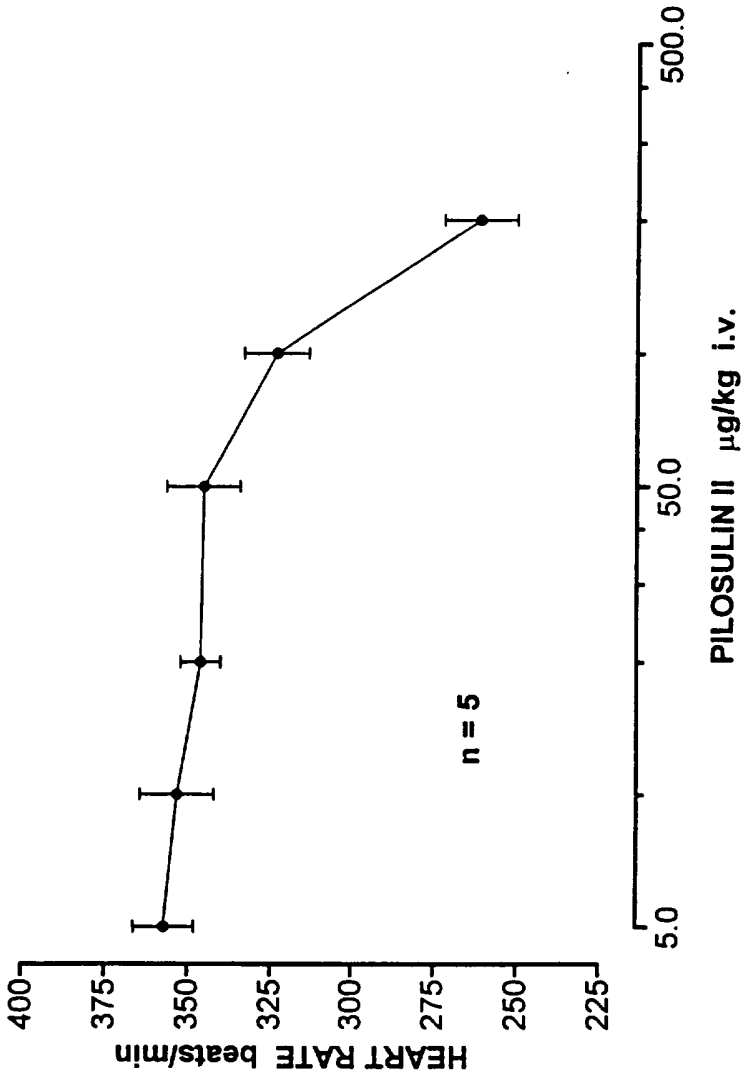
HEART RATE beats/min
400
375
350
325
300
275
250
225
n = 5
5.0
50.0
500.0
PILOSULIN II µg/kg i.v.

Figure 8

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00632

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/12, 5/06, 5/08; C07K 14/35; A61K 38/17, 39/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT and Chem Abstracts. See details in Electronic Database Box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPM, JAPIO

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent WPAT, USPM, JAPIO Keywords: ((ant# and venom#) or (MYR)) and (C12#IC or A61KIC or C07KIC)
and Chem Abs Keywords: Pilosul? or MYR P or jumper ant or fire ant or myrmecia venom#
Chem Abs: YKKVDYK|KVMLKACK/SQSP

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Biochimica et Biophysica Acta (1996), Street, M.D. et al., vol.1305: 87-97. "Molecular Cloning and Characterisation of the major allergen Myr p II from the venom of the jumper ant Myrmecia pilosula: Myr p I and Myr p II share a common protein leader sequence". See whole document	1-11
X	Electrophoresis, (1995), Donovan, G.R. et al., vol.16: 804-810. "Separation of jumper ant (Myrmecia pilosula) venom allergens: A novel group of highly basic proteins". See whole document	1-11

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 January 1997	22 JAN 1997
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer for JESSICA WYERS Telephone No.: (06) 283 2624